Innervation of dipteran eclosion muscles: ultrastructure, immunohistochemistry, physiology and death

J. A. MIYAN AND N. M. TYRER

Department of Biochemistry and Applied Molecular Biology, U.M.I.S.T., P.O. Box 88, Manchester M60 1QD, U.K.

SUMMARY

The thoracic eclosion muscles of flies die by cytotoxic attack under neural control. We have investigated the innervation, ultrastructure and immunohistochemistry of the ventral eclosion muscle of *Glossina*. Two neurons located in the thoracic ganglion innervate this muscle. One of these is immunoreactive for serotonin and does not provide motor innervation. It appears to terminate near the attachment of an immunocyte involved in the dismantling of the muscle. The neuromuscular junction has features that distinguish it from any other chemical junction. A narrow, 3 nm gap separates pre- and post-synaptic membranes and this apparently acts to limit diffusion into and out of the junction. The immunocyte may use neuromuscular innervation as a path-finder to all muscle fibres and may even receive direct input from this source. Neuromuscular transmission is probably chemical as decreasing temperature results in decreasing amplitude of the (graded) muscle potential.

1. INTRODUCTION

Holometabolous insects have special muscles to assist in their escape from the pupa (eclosion), after which they degenerate. Of these, the thoracic muscles are apparently destroyed as a result of a cytotoxic attack from a neurally controlled haemocyte (Miyan 1989*a*,*b*, 1990). The thoracic ventral longitudinal eclosion muscle (vlem) of *Glossina morsitans* is supplied by a nerve from the thoracic ganglion that innervates no other target. Electron microscopy shows just two neuron profiles in this nerve (Miyan 1990), but physiological studies indicate that the vlem is a single motor unit with no direct inhibitory innervation (Miyan 1991), so it seems that only one of these neurons innervates the muscle. We believe the other neuron activates a solitary immunocyte (Gupta 1985) involved in the destruction of the muscle after eclosion. The evidence for direct neural control of the immunocyte is that cutting the nerve stops process growth from the cell, degeneration ceases and the remaining muscle fibres survive (Miyan 1990).

One of the two vlem neurons is immunoreactive to serotonin, but electron dense vesicles seen along the length of one of the axons are not found at neuromuscular junctions. Furthermore, serotonin immunoreactive fibres terminate in the region of the muscle where the immunocyte is located, although to date we have been unable to find endings on the immunocyte itself. Synaptic contacts with the muscle have ultrastructural features that differ from conventional neuromuscular junctions. Their ultrastructure suggests that direct (probably electrical) as well as chemical transmission may be taking place. However, although cobalt does not inhibit transmission (Miyan 1991) this may reflect protection against diffusion of ions from the haemolymph into the junction rather than a non-chemical mechanism of transmission; the neuromuscular junctions are thus unusually protected from the bathing medium. It is important to establish the nature of the innervation to the vlem in order to address questions about the role of the two neurons and of the possibility of different modes of transmission in motor function and muscle cell death.

Here we give an account of the morphology of the neurons, immunohistochemistry of one of them, and details of the ultrastructure of terminals. We present physiological evidence that these neuromuscular junctions behave as chemical synapses and that the muscle's electrically excited response behaves as a junction potential rather than an action potential. A preliminary report on the unusual ultrastructure of the neuromuscular terminals has been published (Miyan & Preece 1992).

2. MATERIALS AND METHODS

Living pupae of *Glossina morsitans* were supplied by the Tsetse Research Laboratory of the University of Bristol. These were maintained at 24°C in a humid atmosphere until they eclosed approximately one month after the deposition date noted on their containers.

(*a*) *Neuron morphology*

Cobalt backfills of the vlem nerve were made to establish the number and location of neurons involved with this muscle in the thoracic ganglion complex. Because of the length and delicacy of the nerve, fills

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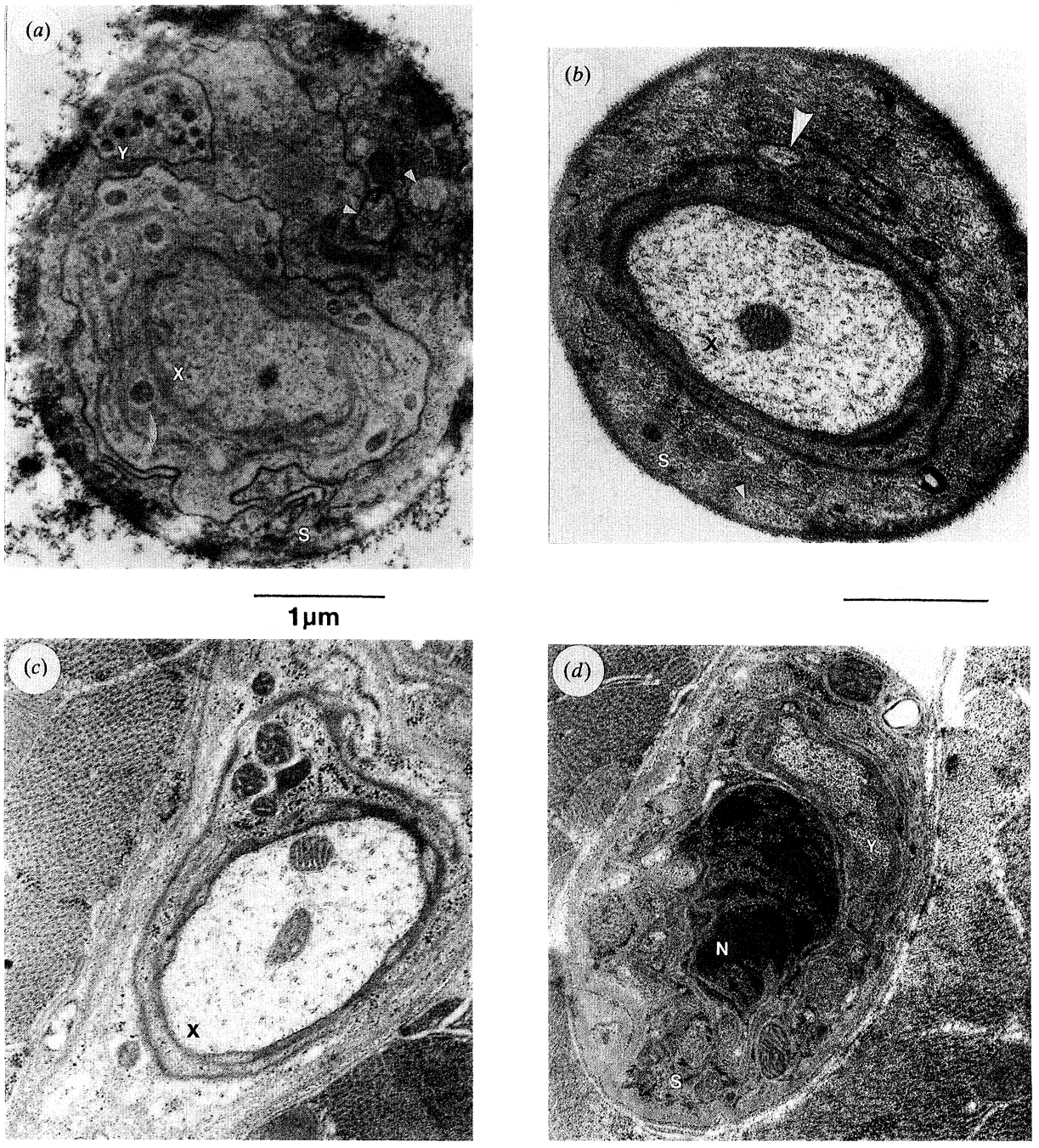

Figure 1. Transmission electron micrographs (TEM) of sections along the vlem nerve. (*a*) Two neuron profiles (X and Y) within a thick mesaxonal sheath (s). The small fibre (Y) contains numerous electron dense vesicles of varying diameters. The arrowheads indicate small diameter profiles that project for a short distance from the ganglion. (*b*) A subsequent section in which the fibre Y (large arrowhead) is greatly decreased in diameter and contains no vesicles. (*c*) The fibre X projects posteriorly along the vlem to innervate all muscle fibres while (*d*) the fibre Y projects anteriorly within a thick sheath and without any branching. N is a nucleus within the sheath, presumably of a glial cell.

were performed *in vivo* using the muscle itself, cut from its attachments, to manipulate the nerve (Altman & Tyrer 1980; Strausfeld *et al.* 1987). A Vaseline bowl was constructed under and around it using a finely drawn plastic pipette tip connected to a syringe full of pure white Vaseline. The muscle was positioned in a small depression on the Vaseline and a wall of Vaseline built around it to seal it and the nerve into the bowl. After the thorax was emptied of saline using a tissue wick, a 1.5% solution of cobalt was placed, as a drop on the end of a drawn pipette, into the Vaseline bowl. This was sealed in with more Vaseline, the chamber filled with saline to cover all tissues and then placed in a covered container in the refrigerator overnight. No success was obtained with higher concentrations of cobalt, nor with extended filling times. In all cases silver intensification was required to reveal filled neurons and was performed according to the method of Bacon & Altman (1977).

Backfills were also made using horseradish peroxi-

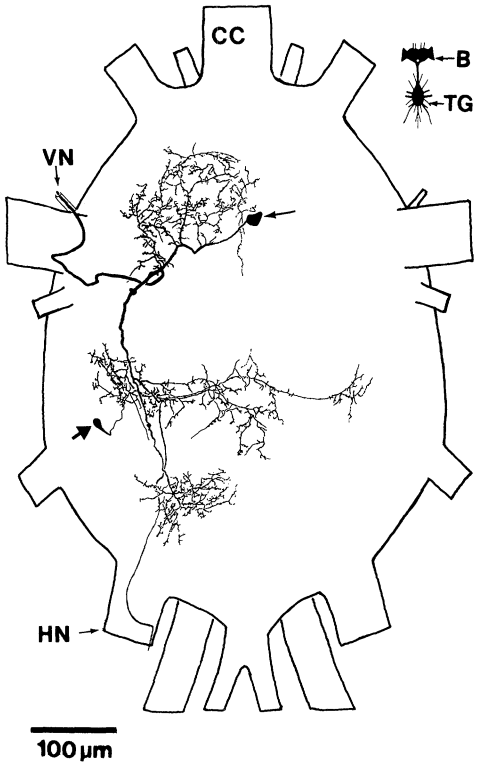

Figure 2. Camera lucida drawing of a cobalt chloride backfill of the vlem nerve. The outline is of the thoracic ganglion complex (TG) shown in relation to the brain (B) in the silhouette drawing. The two axons in the vlem nerve (VN) run so closely together that in whole mounts they appear as one but sections (see figure 3) clearly show two profiles. Within the ganglion two somata can be identified (arrows). These have overlapping processes and arborizations although the prothoracic components appear to be restricted to the anterior cell (long arrow). The posterior cell is distinguished by the possession of a second major process that projects out of the ganglion along the haltere nerve (HN) to innervate the posterior eclosion muscle. CC = neck connective.

dase for identification of neurons in ultrathin sections. Although 12–24 h fills were required to see neurons in wholemount preparations for the light microscope, fills between half and 2 h were found to be best for electron microscopy. Longer durations gave poor ultrastructural preservation and made it difficult to identify individual profiles unequivocally. Preparations were fixed in paraformaldehyde-glutaraldehyde in cacodylate buffer (0.1 M, pH 7.2) overnight. Thorough washing in phosphate buffer at pH 7.2 and 6.5 was followed by incubation in DAB 5 mg/10 ml phosphate buffer (pH 6.5) with 1% Triton X-100 for 1.5 h. This was sometimes enough to stain filled cells but was usually followed by a staining step in the same solution plus 2 ml/100 ml 0.3% H_2O_2. The reaction was stopped by immersion in fresh phosphate buffer and the preparations were processed for wholemount viewing or electron microscopy. Light micrographs were taken on a Reichert photostar IV using Kodak technical pan film or (for figure 5) by computer imaging using a video image capture board (Magnifeye Screen Machine).

(*b*) *Electron microscopy*

The thorax of newly eclosed flies was opened with a dorsal midline incision and the vlems exposed by pulling aside the major air sacs. The whole preparation was then flooded with a fixative consisting of 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) with 0.1 M sucrose added to ensure an isotonic solution. Fixation was overnight followed by direct transfer to 1% osmium tetroxide in 0.1 M cacodylate buffer containing 0.1 M sucrose for 2 h. Specimens were dehydrated in ethanol and embedded in Spurr's resin. Sections were cut on an LKB ultratome using glass or diamond knives and collected on 200 mesh copper grids. They were stained with uranyl acetate and lead citrate and viewed in a Hitachi H600 electron microscope equipped with a goniometer stage for specimen tilting.

(*c*) *Immunohistochemistry*

Wholemount staining was accomplished using the method of Schäfer (Bicker *et al.* 1985; Schäfer & Bicker 1986). Specimens were fixed in 4% paraformaldehyde overnight and then washed thoroughly in phosphate buffered saline (PBS) containing 1% BSA (Bovine Serum Albumin). Specimens were then dehydrated in ethanol and cleared in methyl salicylate to permeabilize the ganglionic sheath. Treatment in 0.5% H_2O_2 in 100% methanol for 10–30 min was found to decrease background staining (probably due to endogenous peroxidases). Rehydration was followed by immersion in PBT (PBS containing BSA and 0.8% Triton-X 100) for 3 h with three changes of solution. The remaining stages were all carried out at refrigerator temperature with the preparations kept continuously mixed on a slowly oscillating shaker table. This was important for successful staining. Immersion in a 1:1000 dilution of the primary antibody (Rabbit-anti-5HT, Seralab) in PBT containing 2% normal goat serum for 24–48 h gave consistent results. Following a thorough wash in PBT, specimens were placed in a 1:200 solution of biotinylated goat-anti-rabbit secondary antibody (Vector Laboratory) for 24–48 h, washed in PTW (PBS containing 0.1% Tween 20), and placed in freshly prepared avidin–biotin complex (Vector Laboratory ABC kit) for 12–24 hours. Washes in PTW were followed by staining in a 3,3′diaminobenzidene (DAB) solution containing 250 µg DAB and 0.1 µl 30% H_2O_2 per ml PTW. The reaction was stopped by immersion in fresh PTW. Preparations were then cleaned, dehydrated and cleared before mounting in Canada Balsam.

(*d*) *Electrophysiology*

Intracellular recordings were made from fibres of the ventral longitudinal eclosion muscle (vlem) according to the method already published (Miyan 1991). Electrical stimulation of the vlem nerve was accomplished by placing a bipolar electrode on the ganglion surface at the root of the nerve. Electrodes

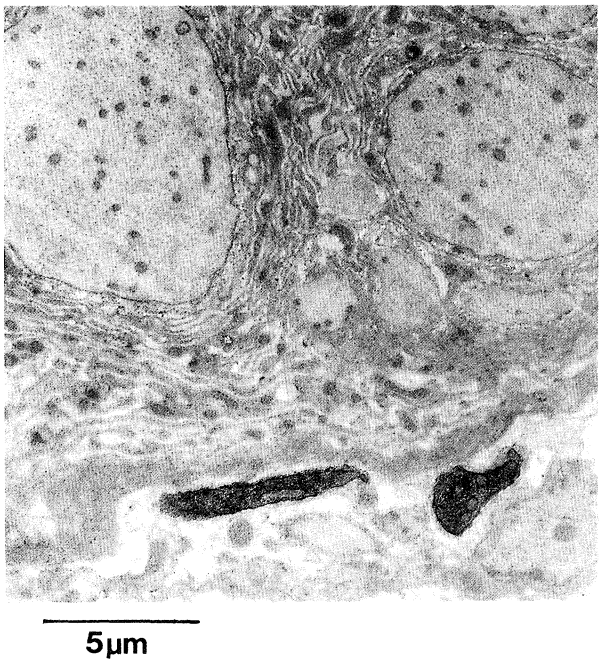

Figure 3. TEM of section through the side of the thoracic ganglion at the entry point of the vlem nerve filled with HRP. The reaction product is visible in two profiles at this point even though only one is distinguishable in whole-mount preparations of cobalt fills. Tissue shrinkage during cobalt processing probably brings the fibres closer together than the 2.5 μm separation observed in this section. This may explain the difficulty in resolving the two fibres when they run in parallel pathways both in the peripheral nerve and within the ganglion.

were fabricated from two lengths of lacquer-insulated Trimel wire twisted together and cut to a common point with a scalpel blade. Stimulus pulses were transmitted from a Grass stimulator through a stimulus isolation unit.

3. RESULTS

(*a*) *Vlem neurons*

Innervation of the ventral longitudinal eclosion muscle (vlem) is via a nerve from the thoracic ganglion complex that has no other peripheral target (Miyan 1989*a*). Sections of this nerve show the presence of two neuron profiles (Miyan 1990; figure 1). In serial sections, electron dense vesicles appear in the smaller of the two profiles at irregular intervals along the length of the nerve. At intervening regions the diameter of the fibre is greatly reduced (figure 1*b*). The second fibre has a constant diameter throughout the length of the nerve and has not been found to contain any electron dense vesicles in any of the sections examined. It has numerous mitochondria, neurofilaments and neurotubules, and has large numbers of electron lucent vesicles at its terminals on the muscle. One or two additional small diameter profiles have been observed at the proximal end of the vlem nerve but these do not project all the way to the muscle and do not backfill with cobalt. Near the muscle the two fibres separate, the larger fibre projects posteriorly and has numerous branches which innervate all the fibres of the muscle (figure 1*c*). The smaller fibre takes an anterior route within a thick mesaxonal sheath (figure 1*d*) and has not been observed to branch (see figure 9).

In over 20 fills, using both cobalt chloride and HRP, we have observed either one, or more usually two neurons filled within the thoracic ganglion (figure 2). We never observed more than two neurons in these preparations. The soma of one is located in the dorsal midline of the prothoracic neuromere. Extensive arborizations project from the main fibre, ventrally and laterally into the neuropilar region. The main fibre arcs from the soma laterally and posterially sending major arborizations medially into the mesothoracic and metathoracic components of the ganglion. In the mesothoracic region the branches cross the midline to terminate in contralateral areas. The second neuron soma is located laterally in the mesothoracic component. Its neurite connects with another curved process that has overlapping branches with the first neuron and also projects posteriorly into abdominal components of the fused thoracic ganglion complex. From the mesothoracic arborizations a second major (axonal) process projects a short distance along the haltere nerve before branching off towards the ipsilateral posterior dorso-ventral eclosion muscle.

One puzzling feature was a failure to distinguish two separate axons in the vlem nerve in these wholemount preparations. However, sections of the vlem nerve in preparations backfilled from the muscle with HRP, clearly reveal two axonal profiles (figure 3). It must be that the two axons run too close together to be resolved with the light microscope in wholemount preparations.

(*b*) *Immunohistochemistry*

Staining was carried out using antibodies to serotonin, arginine- and lysine-vasopressin, met-enkephalin, FMRF-amide and glutamate. Only serotonin-like immunoreactivity was found in fibres associated with the eclosion muscles. Here we describe the morphology of the one serotonin immunoreactive fibre to the vlem.

A single fibre is labelled in the vlem nerve (figure 4) which must be one of the two axons previously seen, although, we have so far been unable to follow the stained fibre past the entry point of the nerve into the vlem itself. Staining in the nerve is punctate along its length and this correlates well with the irregular occurrence of dense vesicles in one of the fibres observed in electron microscope sections. In the flesh fly, *Sarcophaga bullata*, it is possible to follow this single fibre to its endings in a localized region in the posterior end of the vlem (figure 5). It seems unlikely that these are motor fibres since: (i) they are associated with only a few muscle fibres; (ii) they do not run the length of those few fibres they do contact; and (iii) electron dense vesicles are never seen at neuromuscular junctions. It is tempting to extrapolate from the results with *Sarcophaga* and *Glossina* and conclude

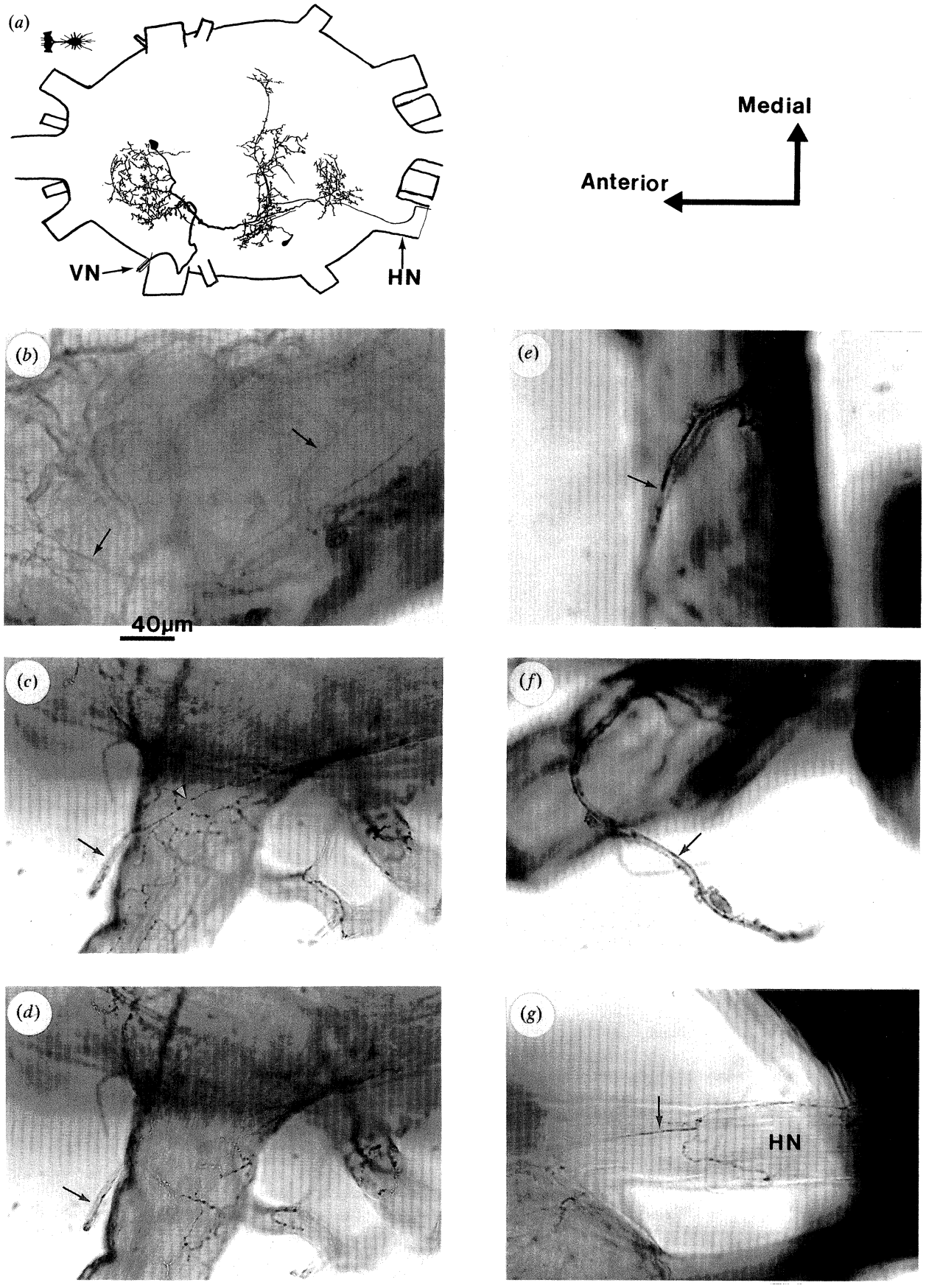

Figure 4. Light micrographs of serotonin-like immunoreactive fibres in wholemount preparations of the thoracic ganglion stained with a serotonin antibody. (*a*) Camera lucida drawing of cobalt filled vlem neurons within the ganglion. (*b*) Arcing fibre along the lateral edges of the ganglion with a similar path to vlem neurons (compare with (*a*)). (*c,d,e,f*) A single immunoreactive fibre projects out along the vlem nerve (VN in (*a*)). This has been observed in all preparations (three examples shown) and has a blebby appearance consistent with the changes in diameter and vesicle content observed for the smaller of the vlem neurons along the nerve. (*g*) Immunoreactive fibre (arrow) projecting along the haltere nerve (HN).

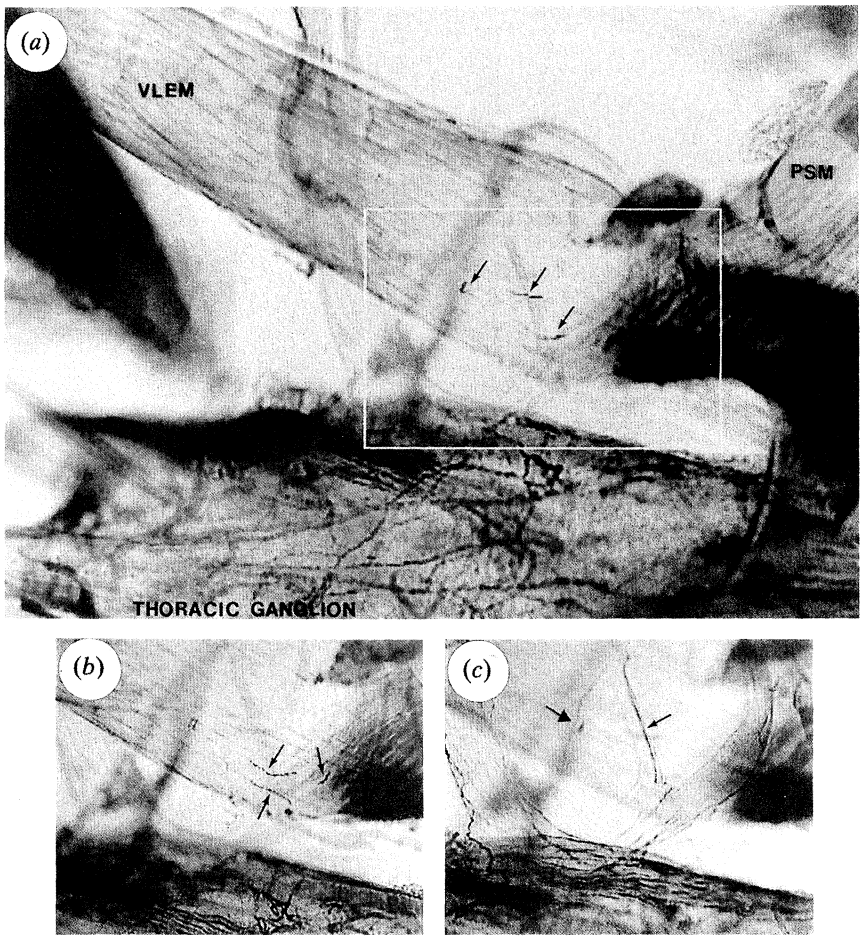

Figure 5. Wholemount preparation of the vlem of *Sarcophaga* showing 5HT-immunoreactive fibres (arrows) in the posterior end of the muscle (*a*,*b*) and a single immunoreactive fibre in one of the nerves innervating the muscle (*c*). Immunoreactivity was not observed in any other of the innervating nerves. Note that the 5HT-like fibres are restricted to only a few muscle fibres making it unlikely that they are motor.

that these fibres target the cytotoxic cell involved in vlem dismantling, but its location has not yet been determined for *Sarcophaga*. Centrally, in both *Glossina* and *Sarcophaga*, immunoreactive fibres with the characteristic vlem neuron sweeping arc pathway suggest a vlem neuron was labelled (figure 4*a*).

EM staining has been used as a means of discriminating between biogenic amine containing fibres, that have densely stained vesicles, and those containing other transmitter substances that usually have electron lucent vesicles (Knight 1977). It seems reasonable to conclude that the smaller of the two fibres observed in EM sections is the same fibre as that labelled with the 5HT antibody. 5HT immunoreactivity was also found in a single fibre projecting along the haltere nerve (figure 4*g*) similar to the fibre filled with cobalt via the vlem nerve. If this is indeed the same fibre then it is safe to conclude that the serotonin immunoreactive neuron is the posterior of the two filled with cobalt via the vlem nerve.

(*c*) *Ultrastructure of terminals*

Comparison was made between neuromuscular junctions on the vlem at the time of eclosion and those on the pleurosternal and neck muscles which are typical adult skeletal muscles. The neuromuscular junctions on typical muscles have all the characteristics of classical chemical synapses (figure 6). Electron lucent vesicles are associated with a pre-synaptic dense body that connects with the pre-synaptic membrane. There is a clear separation of about 15 nm between pre- and post-synaptic membranes and a post-synaptic dense bar lies opposite to the pre-synaptic structures. In addition, there are periodic densities extending between the pre and post-synaptic membranes which have been termed septate desmosomes (Osborne 1967). In favourable sections these appear to be formed from short lengths of unit membrane (see figure 4 in Osborne 1967; figure 8).

Although the neuromuscular junction in the vlem contains electron lucent vesicles and a pre-synaptic dense body in common with chemical synapses, it also has features that distinguish it from any other junction (figure 7). First, there is no clear separation between pre and post-synaptic membranes; second, the periodic densities seen in transverse sections are, in reality, a plate of dense material within the pre-synaptic membrane into which fingers of cytoplasm extend at regular intervals (figure 7*c–f*). Although these fingers of cytoplasm apparently form bridges between the pre and post-synaptic structures, physiological studies (see

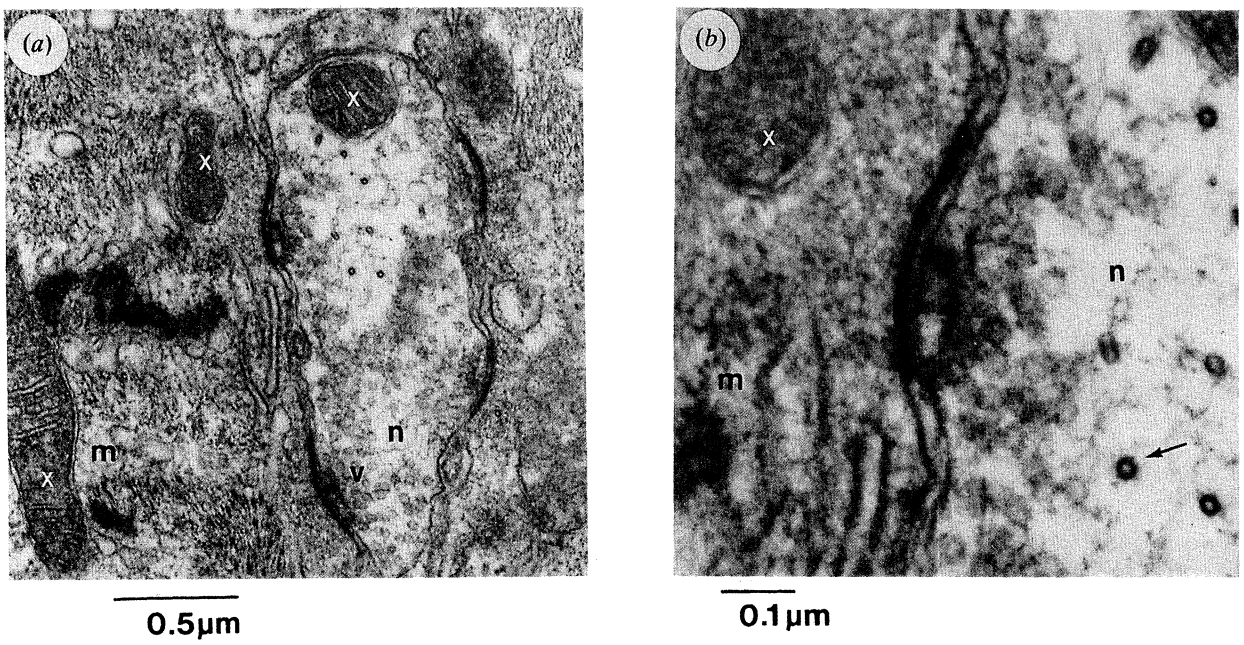

Figure 6. TEM of sections through the pleurosternal muscle of *Glossina* (psm in figure 5) showing a neural process (n) making a typical synapse on muscle fibres (m). Four synaptic sites can be seen with characteristic pre- and post-synaptic features and electron lucent vesicles (v). One of these is shown at higher power in (*b*). The synaptic density is associated with the post-synaptic membrane and a pre-synaptic dense body can be seen in the neuron as well as neural tubules (arrow). X are mitochondria.

below) suggest that this is a true chemical synapse, although it is possible that other modes of transmission (e.g. electrical) also occur.

The electron dense region of the junction with the eclosion muscle is a protrusion of the pre-synaptic terminal of some 30 nm which connects with the muscle. In most sections through these junctional structures, no cleft between pre-synaptic protrusion and post-synaptic cell membrane is visible. Using the goniometer stage, it is possible sometimes to reveal a post-synaptic membrane by tilting the section (figure 7*d*,*e*), but this is closely apposed to the pre-synaptic protrusion with a separation of no more than 3 nm.

If the sections are tilted to reveal the surface of the plate, regularly spaced electron lucent regions are seen which we interpret as pre-synaptic cytoplasmic fingers. (figure 7*f*). In transverse section the regular spacing of the fingers gives the illusion of a series of 30 nm particles quite different from the septate desmosomes characteristic of typical neuromuscular junctions (Osborne 1967). Furthermore, rather than spanning a synaptic gap these densities are clearly pre-synaptic extensions. The three types of neuromuscular junctions are compared in figure 8. The neuromuscular junction of these eclosion muscles is also different to that in other muscles that die at this time, by exposure to adult hormones. These have been studied most extensively in moths and have junctions resembling normal skeletal muscle synapses (Rheuben 1992).

Following the onset of degeneration there is little change in the appearance of these junctional structures until late in the dismantling of the muscle. Then the junctional densities are no longer seen as separate and are clearly not connected to the post-synaptic membrane (figure 9). The separation between membranes is still less than 5 nm in over 20 junctions examined. Electron lucent vesicles are still observed in the neuron terminals although they are less well defined. No other changes in terminal structure have been found. We found no changes in the structure of the vlem nerve along its entire length throughout the dismantling of the muscle fibres. This lack of change can be compared to the swelling of glial processes and invasion of the outer regions of motor nerves by phagocytic cells observed in moth metamorphosing muscles (Rheuben 1992).

(*d*) *Neuro-immune cell contacts*

Sections through the immunocyte have been observed in numerous preparations but no clear neuronal association has been seen. HRP fills of the vlem nerve reveal a single fibre that has its ending on the muscle close to, and possibly on a cell that is not muscular, is distinct from fat cells and contains granules similar to those of immunocytes (figure 10). Electron microscopy produces the occasional sections in which there is a very close association between neuromuscular terminal and immunocyte (figure 11), but no section has yet revealed an unequivocal destination of the second, serotonin-immunoreactive neuron. Serial sections beginning in the vlem nerve will have to be examined in order to follow this fibre to its endings. In the section shown in figure 11, a fine process from the nerve terminal extends towards the immunocyte suggesting possible interaction between motor neuron and immunocyte (see 'x' in figure 13).

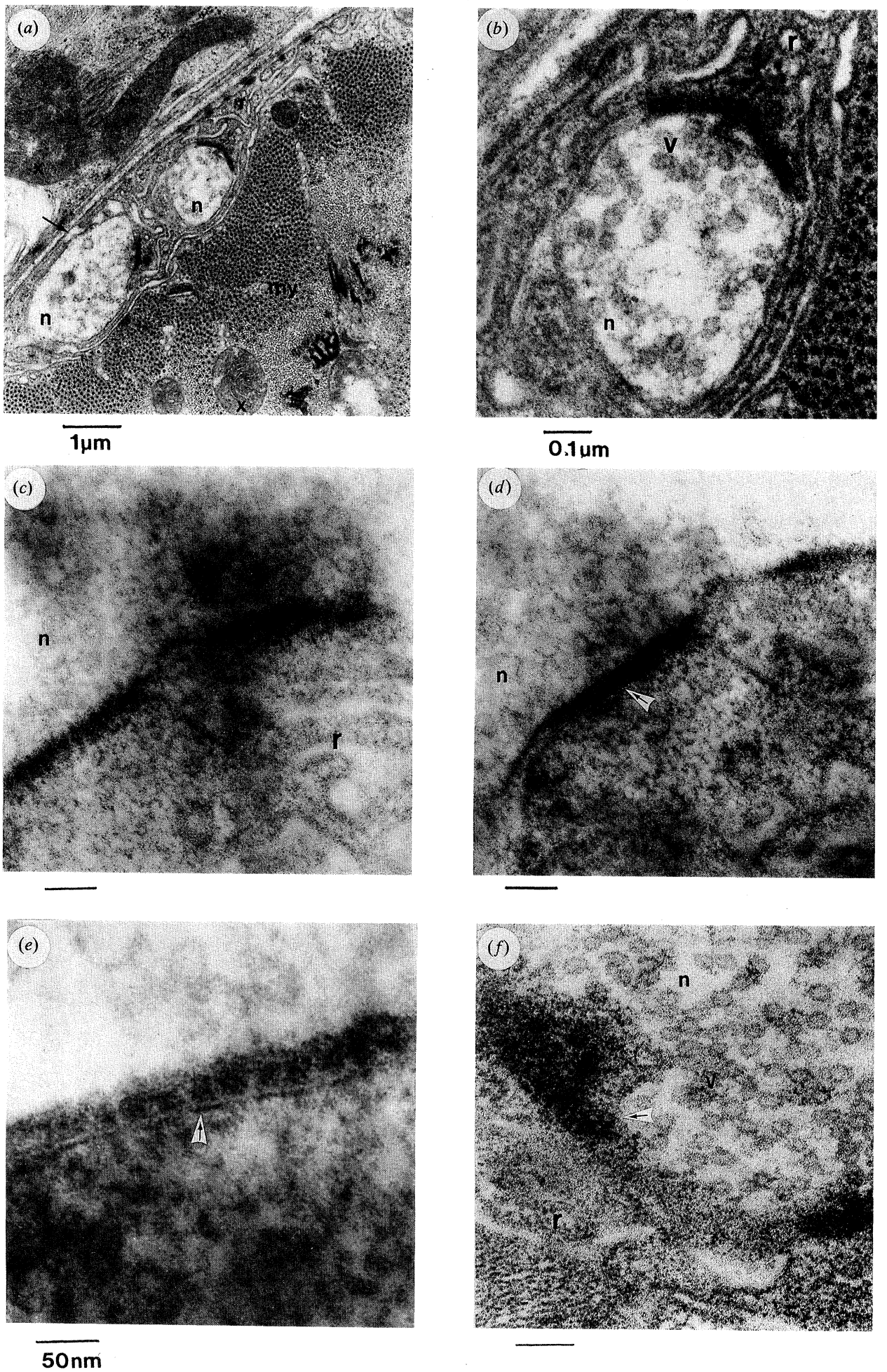

Figure 7. For description see opposite.

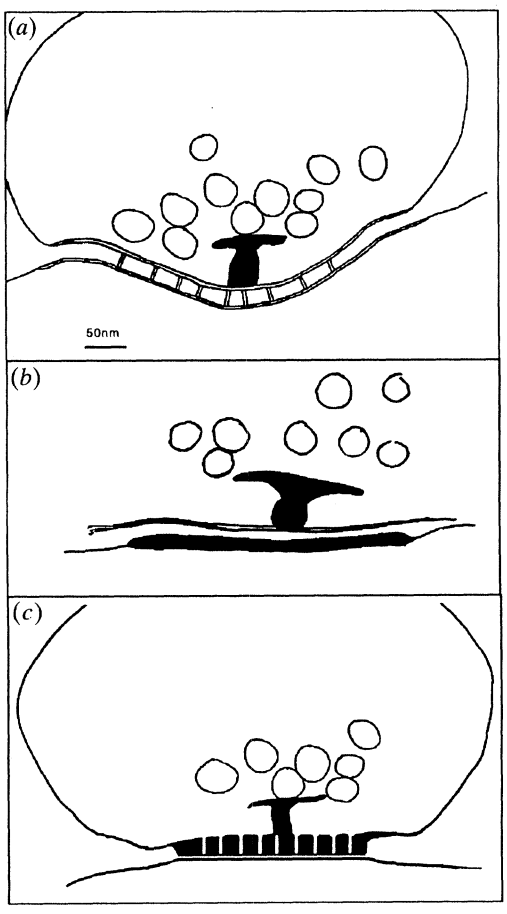

Figure 8. Diagrammatic representations of the three types of synaptic structure previously described in flies. (*a*,*b*) Junctions commonly interpreted as chemical synapses ((*a*) traced from Osborne 1967; (*b*) psm junction). (*c*) The vlem junction is shown with its distinctive pre-synaptic dense plate protruding from the nerve terminal. The fingers of cytoplasm that penetrate it lie very close to the post-synaptic membrane. This close apposition apparently forms an effective barrier to permeability for significant periods of exposure to changing external ions and may also limit diffusion of transmitters from the junction.

(*e*) *Electrophysiology*

Electron microscopic observations detailed above led us to question the mode of transmission at the vlem neuromuscular junction. Previous physiological studies had already demonstrated that neuromuscular transmission was not blocked by long periods of exposure to calcium free, or cobalt chloride solutions (Miyan 1991). From the ultrastructure, it was possible that an efficient diffusion barrier was present at the vlem junction. We sought to test the mode of transmission by intracellular recording from vlem while cooling the preparation (Nicholls & Purves 1972). The experimental chamber was mounted on three Peltier-type cooling devices (RS Components) connected in series to a low voltage, high current power supply. Water circulation chambers were attached to the hot faces of the Peltier cells and a minimal flow of water set up from a cold water tap. Temperature of the saline close to the preparation was monitored by a thermometer with a remote probe (RS Components).

Figure 12 shows the effect of decreasing temperature on the vlem muscle potential. No increase in amplitude is observed and no sudden changes in amplitude are found that might indicate the presence of some threshold phenomenon. After an initial plateau in which no change is observed, the potential shows a gradual decrease in amplitude down to zero (figure 12*a*). Examination of the potential reveals an increasing rise time that also follows decreasing temperature as well as a small increase in latency of the post-synaptic potential (figure 12*b*). The vlem muscle potential thus appears to behave as a simple junction potential with no all-or-none component. Dr W. M. Heitler (personal communication) has found that electrical junctions in the crayfish show an increase in post-synaptic potential amplitude as the temperature drops while Nicholls & Purves (1972) found no effect upon electrical transmission in the leech but a similar effect to that we have described for chemical transmission. The vlem response thus appears to indicate the presence of a chemical junction (see also discussion).

4. DISCUSSION

Insect nerve–muscle junctions bear all the characteristics conventionally associated with chemical synapses (see Osborne 1967, 1970, 1972). Membrane bound vesicles are associated with pre-synaptic densities and a definite separation exists between pre- and post-synaptic membranes. Many invertebrate junctions have periodic densities between pre- and post-synaptic membranes that resemble septate desmosomes found in vertebrate epithelial tissue. Although they may

Figure 7. TEMs through neuromuscular junctions on the vlem of *Glossina* observed within an hour of eclosion. (*a*) Two terminals (n) are seen with a characteristic appearance. Dense synaptic zones connect the nerve and muscle fibre and the synaptic region is surrounded by a subsynaptic reticulum (r) apparently formed by invaginations of the muscle membrane. Transmission must occur through this structure as it clearly isolates the terminals from the main muscle mass (my). This is more clearly seen in the higher power view (*b*) in which a pre-synaptic dense body and electron lucent vesicles (v) are also evident. The arrow in (*a*) points to a region where the basement membrane is apparently the only barrier between nerve membrane and external medium. (*c*,*d*) Two views of another junction at tilt angles of 27° to each other. Fingers of cytoplasm extend into the pre-synaptic dense plate and are separated by a gap of 3 nm from the post-synaptic membrane (arrow in (*d*)). This is seen at high magnification in (*e*). (*f*) A section tilted to view the neural face of the pre-synaptic density which shows the dense material is regularly perforated by electron lucent passages (arrow). Scale bars for (*c*,*d*,*f*) are as for (*b*).

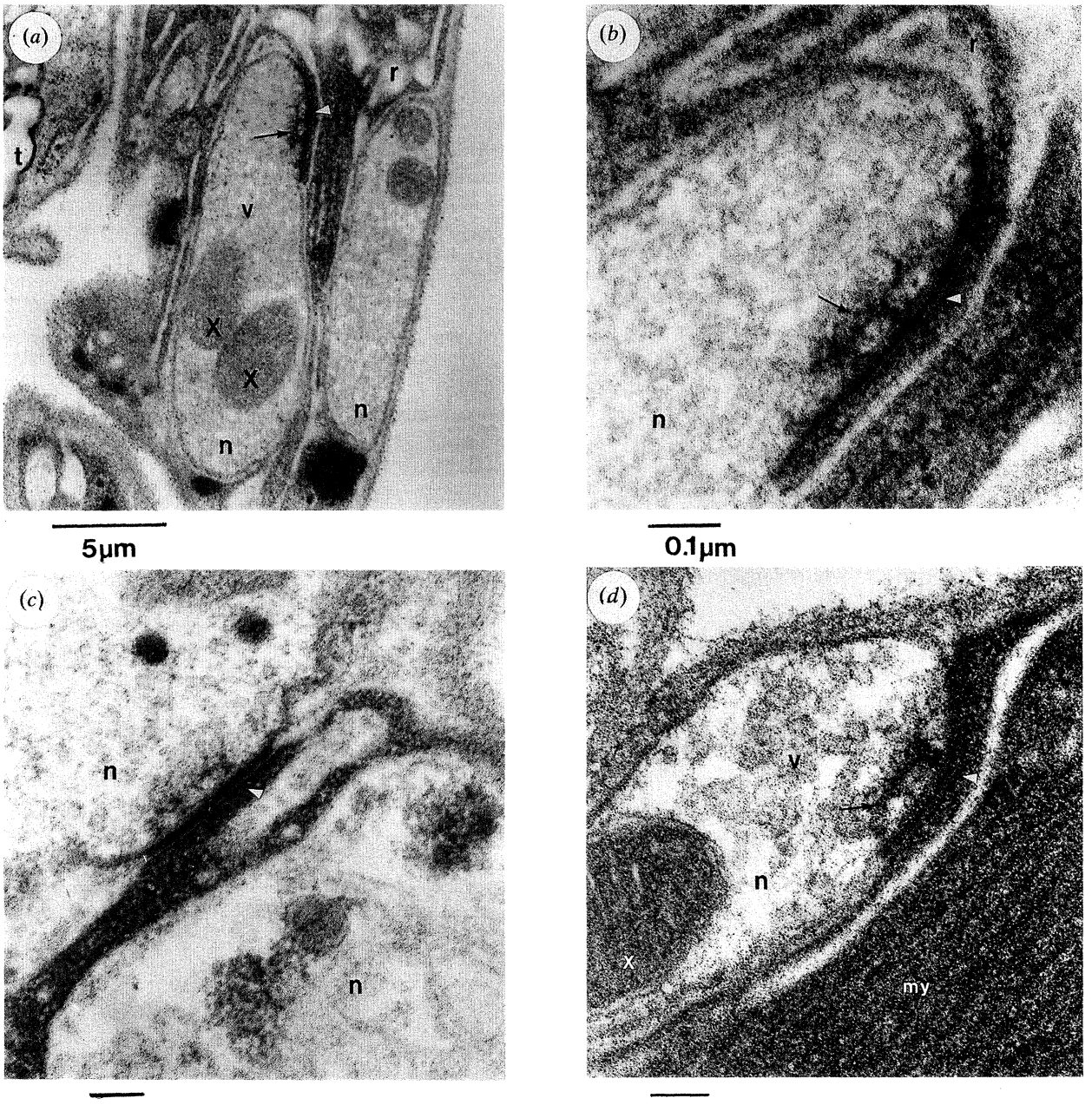

Figure 9. TEM of sections through the vlem terminal 46 h after onset of degeneration of the muscle. Junctional densities are still present (arrow) but the pre-synaptic plate no longer protrudes and perforation by the cytoplasmic fingers can only be seen in occasional sections (*d*). The gap between pre- and post-synaptic cells is still 3 nm and is now more clearly visible and seen in all sections (white arrowheads). Electron lucent vesicles are present in the terminals (v) and the subsynaptic reticulum (r) persists. Scale bars for (*c*) and (*d*) as for (*b*).

have similar structural functions, it has been postulated that the neuromuscular septate junction additionally serves to limit lateral diffusion of transmitter released into the synaptic cleft and to limit to some extent the invasion of haemolymph ions into the junction (Osborne 1967, 1970, 1972). The synaptic cleft varies in width between species, ranging from 25 nm in locust and stick insects (body wall muscles) to 5 nm in *Tenebrio* flight muscles (Osborne 1970; Smith 1960, cited in Osborne 1972). The post-synaptic membrane of many insect neuromuscular junctions is characterized by a sub-synaptic reticulum (Osborne 1967) in which the muscle membrane has a great number of invaginations. Unlike the situation in vertebrate nerve–muscle junctions, this reticulum does not constitute the post-synaptic site as it is formed from membrane lateral to the junctional densities. The junction region itself is formed by straight, parallel pre- and post-synaptic membranes. Osborne (1972) speculates that the subsynaptic reticulum connects to the transverse tubular system and serves to amplify electrical changes at the junction for transmission into the muscle. However, he points out that motor neurons can make contacts with the same

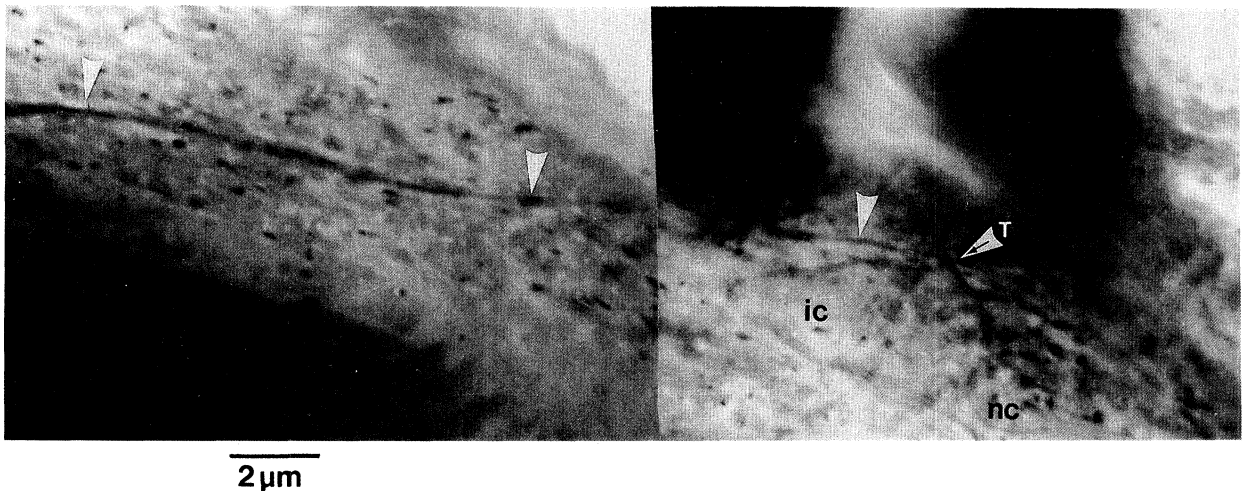

Figure 10. Light micrograph (phase contrast) of the anterior end of the vlem in wholemount showing an HRP-filled nerve fibre (white arrowheads) lying along the surface of a muscle fibre and ending (T) adjacent to a cell (ic) that is attached to the muscle surface which we interpret as an immunocyte identified by its characteristic ultrastructure (see figure 11). nc, nucleus of the immunocyte. The nerve fibre passes through some connective tissue and out of the focal plane near the attached cell.

muscle fibre in which a subsynaptic reticulum is not present at all junctions. The precise function of this striking structural feature is unknown.

The structure of the neuromuscular junction observed in the thoracic eclosion muscles has features which distinguish it from any other chemical junction including those on other muscles that die during metamorphosis (see Rheuben 1992). The dense plate with its numerous, regularly arranged fingers of cytoplasm, protrudes from the pre-synaptic cell, greatly reducing the distance between pre- and post-synaptic membrane. The purpose of this close apposition could be to exclude the external medium. The unusually close glial investment that appears to cover the terminals may further enhance exclusivity (although in one or two sections (for example see figure 7*a*) only the basement membrane appears to separate the nerve terminal from the external medium). In physiological studies, Miyan (1991) has shown that eclosion muscle terminals are apparently impermeable to cobalt ions as synaptic transmission is not affected by 2 mM cobalt in the external solution. This distinguishes these terminals from other insect junctions which have been shown to be permeable to ions such as lanthanum (Osborne 1967; Lane & Treherne 1972). Also it appears that pharmacological agents cannot access the junction (Miyan, unpublished) which necessitates the testing of the mode of transmission by other means such as cooling. These experiments suggest that the junctions are probably chemical and that the post-synaptic response is not of the all-or-none type, having instead characteristics similar to simple junction potentials.

The question now arises as to why these junctions are so heavily protected when other muscles in the animal have nerve terminals that are open to the external medium. We have proposed three possible pathways for initiation of cell death in these muscles (figure 13).

1. Direct innervation of the immunocyte is responsible for its activation and subsequent cytotoxicity. Motor innervation is not involved in cell death.
2. Transmitter or co-transmitter release from motor terminals following cessation of motor activity causes changes in muscle membrane properties that direct attack from the immunocyte which is activated by its own innervation.
3. Changes in muscle membrane properties induced by cessation of motor activity, release of co-transmitter, or by another innervation (in which case the immunocyte is not innervated) is sufficient to direct a cytotoxic attack.

In the latter two pathways it would make sense to protect the nerve–muscle junction in order to restrict the effect to the muscle concerned. Many surviving muscles are in close proximity to the eclosion muscles and any leakage of transmitter could result in more widespread cytotoxicity. The fact that the neuromuscular junction is so heavily protected could well be related in some way to the cell dismantling/death process. Similarly, immunocyte processes are found near to neuromuscular junctions with indications of possible direct interactions suggesting at least a path-finding role for them. The exact nature of this involvement has yet to be elucidated. Changes in the antigenicity of cell membranes is bound to be a factor in directing immunological activity and neuromuscular transmission could have a role in the process. Differential sensitivity to cytotoxic substances is also a factor that exists in cells and forms the basis of many therapeutic strategies; the apparent protection against transmitter leakage suggests the presence of some more general effector in the fly.

We have screened a number of potential transmitter substances using both immunohistochemistry and physiological studies. We have identified serotonin-like immunoreactivity in one of the fibres innervating

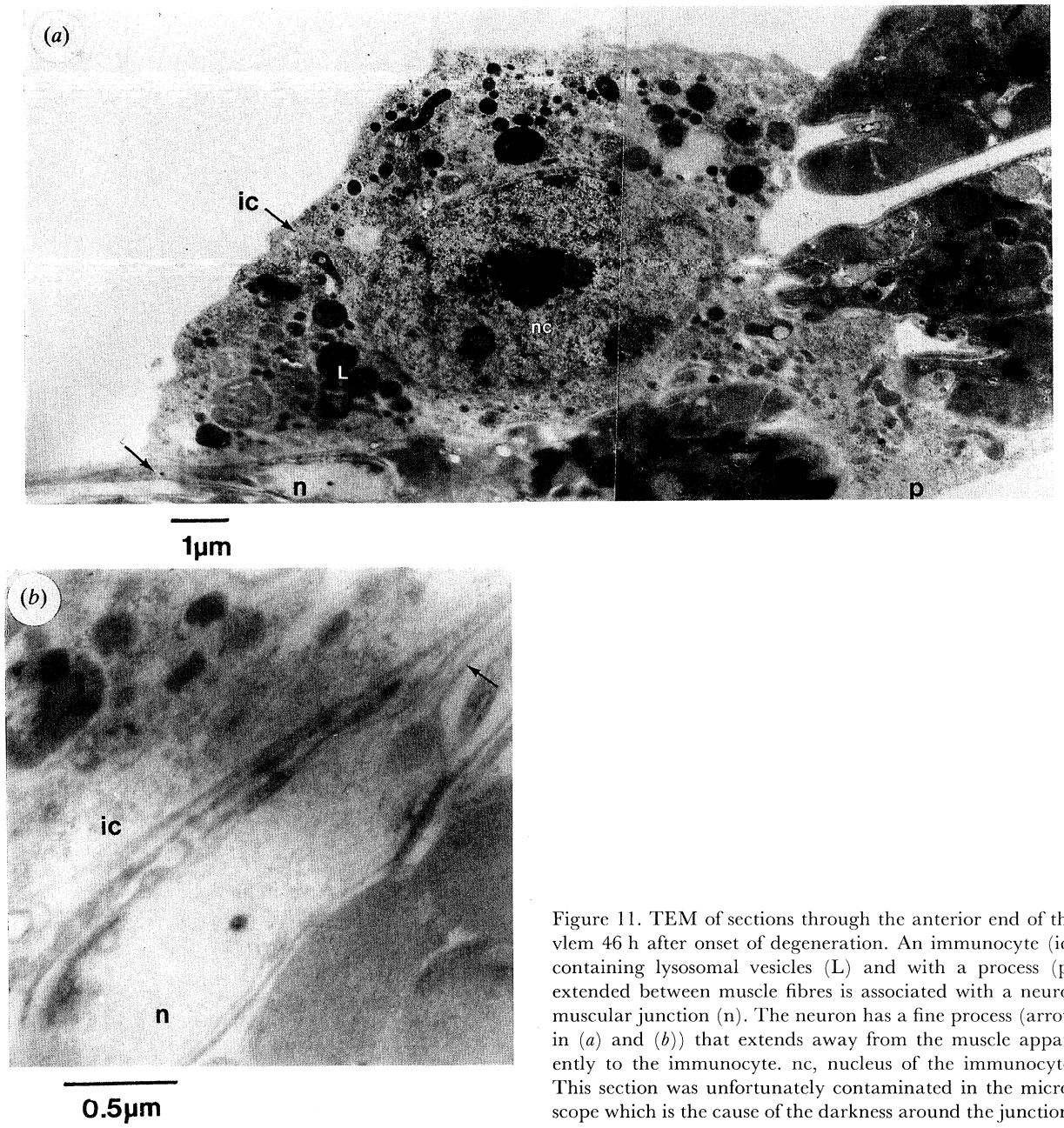

Figure 11. TEM of sections through the anterior end of the vlem 46 h after onset of degeneration. An immunocyte (ic) containing lysosomal vesicles (L) and with a process (p) extended between muscle fibres is associated with a neuromuscular junction (n). The neuron has a fine process (arrow in (*a*) and (*b*)) that extends away from the muscle apparently to the immunocyte. nc, nucleus of the immunocyte. This section was unfortunately contaminated in the microscope which is the cause of the darkness around the junction.

the vlem. Application of serotonin has no electrophysiological effect although it certainly modulates muscle twitch tension (Bothe & Miyan 1992). This modulation is likely to be mediated via activation of adenylate cyclase rather then directly through the neuromuscular junction (Evans 1984; Evans & Myers 1986) as Bothe & Miyan (1992, 1993) found no effect on the junction potential even though a 600% increase in twitch tension occurred with bath applied serotonin at 1 μM. Tumour necrosis factor (TNF) is a cytotoxic cytokine that is known to activate programmed cell death in target tissues in vertebrates. This cytokine has also been identified in invertebrate species although its mode of delivery and action have not been fully investigated. As a strong candidate for the activator of the programmed dismantling observed in the eclosion muscles it will be investigated.

Direct innervation of immunocytes in vertebrates has been postulated and much anatomical evidence has been collected for neural inputs to lymphoid tissues distinct from sympathetic innervation of blood vessels (Ader *et al.* 1991). Control pathways between the central neural and immune system are now well established and it seems only a matter of time before a direct neural–immune interaction is identified and characterized. The system identified in the fly, with just one immunocyte, could present an opportunity for detailed investigation of a connection between a specific neuron and immune cell.

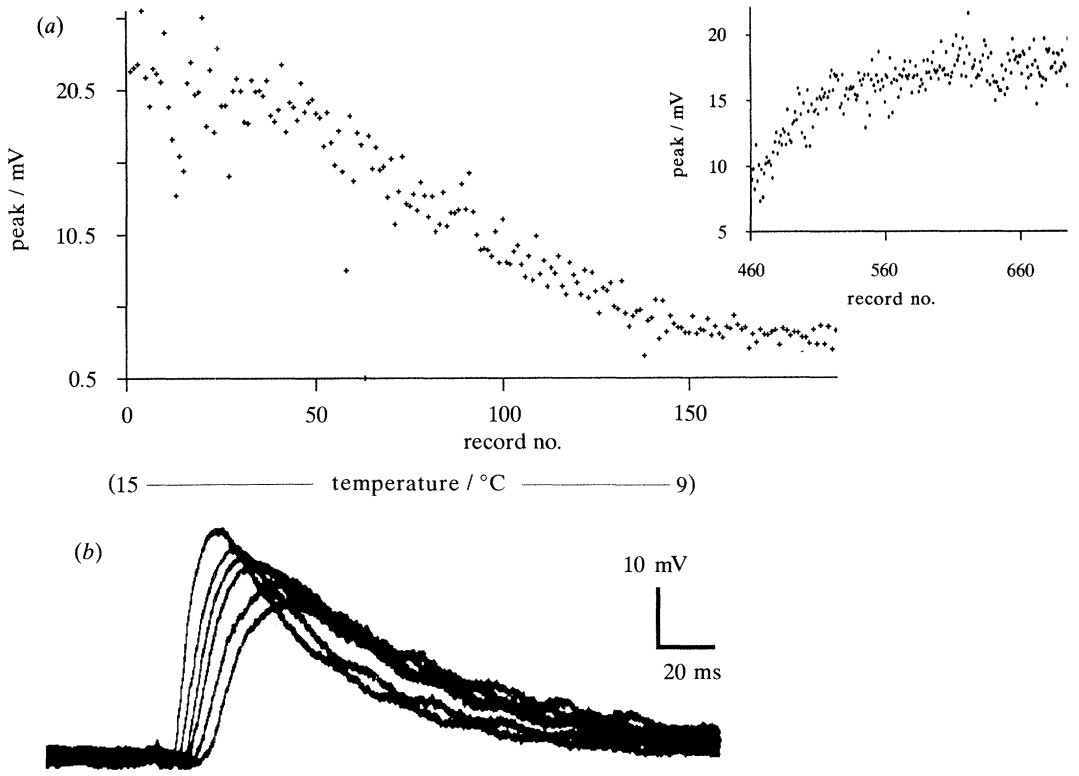

Figure 12. (*a*) Plot of nerve-evoked vlem muscle potential against time and temperature. As the temperature is decreased the muscle potential decreases in amplitude with no sudden drop that might indicate the presence of an all or none threshold. The inset shows recovery of amplitude when the preparation is brought back to room temperature. (*b*) Actual recorded potentials from an experiment that also show increasing rise times as temperature drops and a small increase in latency of the post-synaptic potential.

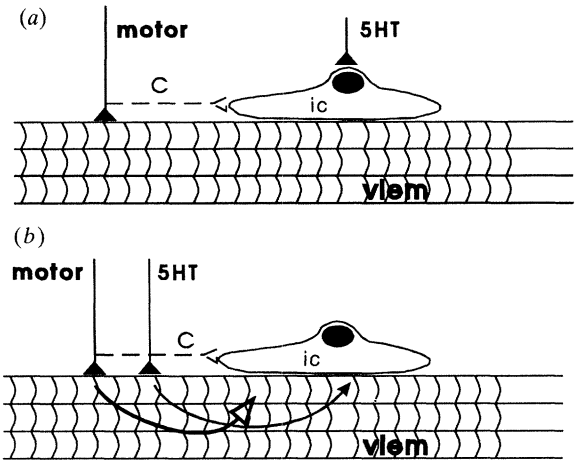

Figure 13. Proposed models for activation of the immunocyte and muscle degeneration. The simplest model (*a*) involves separate motor innervation and innervation to the immunocyte (ic). We have assumed that the 5HT-immunreactive fibre is the non-motor pathway. The immunocyte is switched into an autoimmune mode directed specifically and only at the vlem. The dotted pathway marked 'C' indicates a possible direct motorneuron influence on the immunocyte (see figure 11). In an alternative model (*b*) either (or both) of two mechanisms is proposed. No direct innervation of the immunocyte occurs (except possibly via the motor collateral 'C'); cessation of motor transmission is followed by release of a second transmitter (which may have been released during motor activity as a co-transmitter) either from the motor nerve or from the second non-motor neuron. Changes elicited in the muscle fibres direct the attack of the immunocyte. In none of the models have we included any humoral influence which cannot be discounted.

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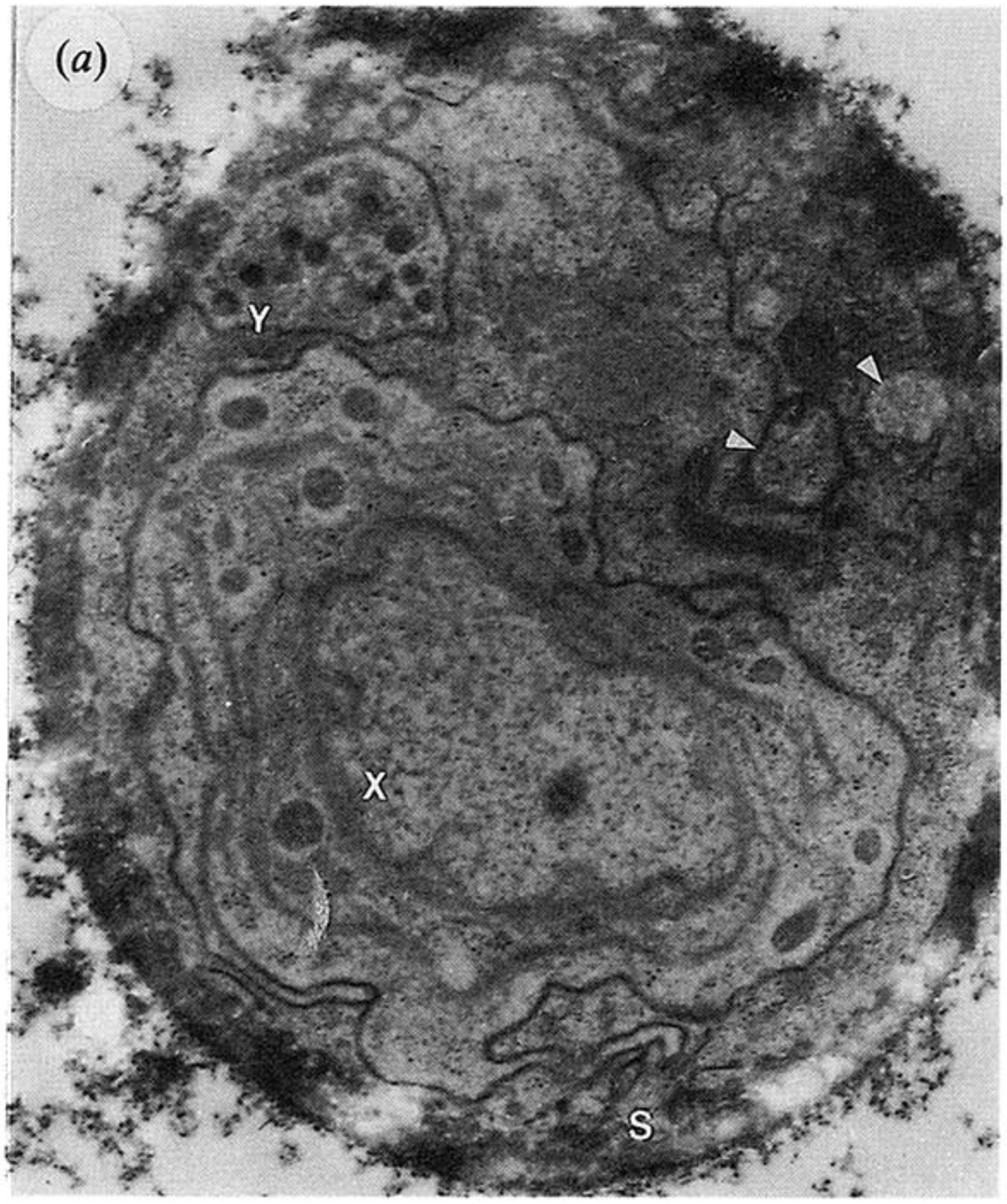

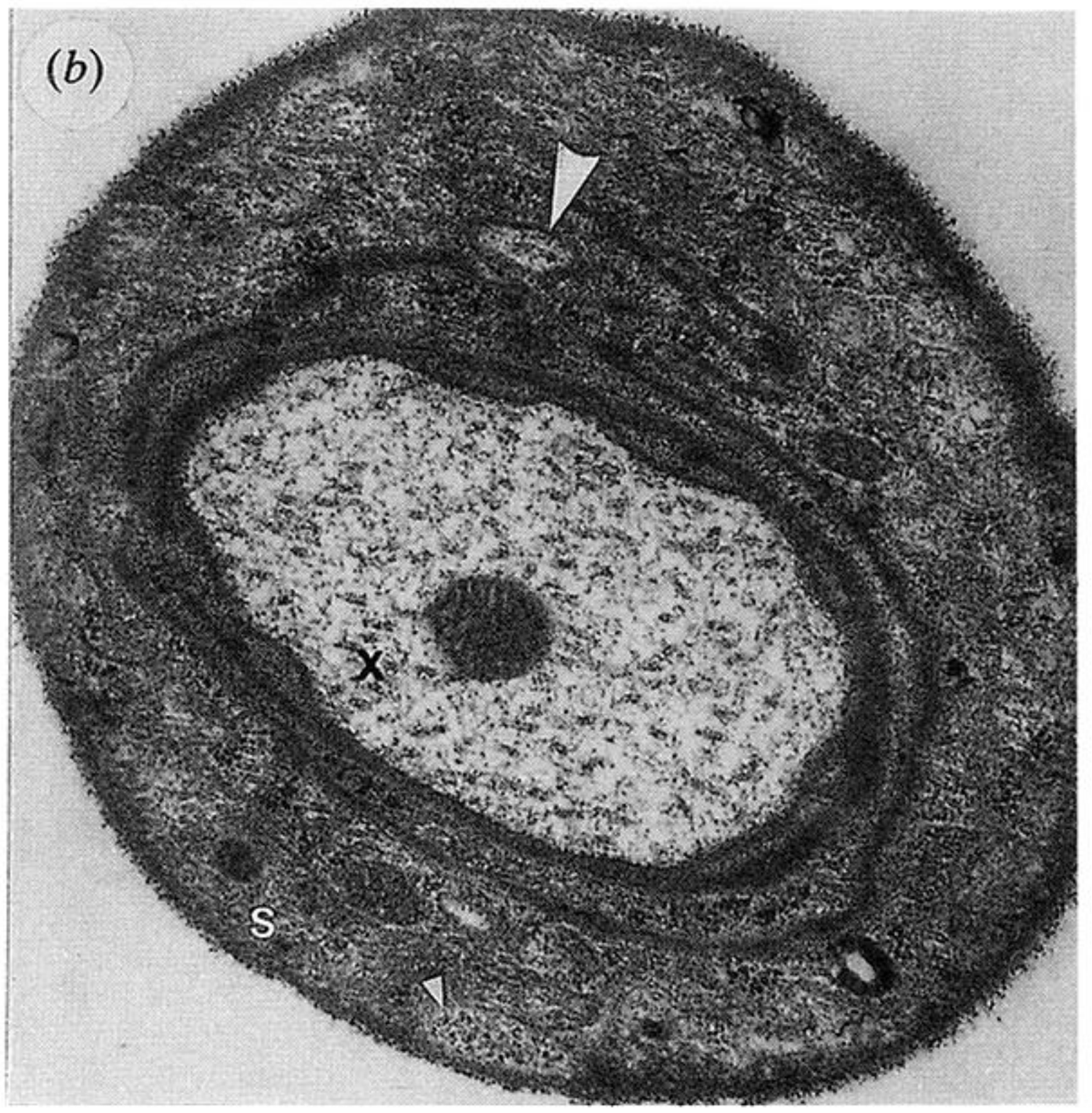

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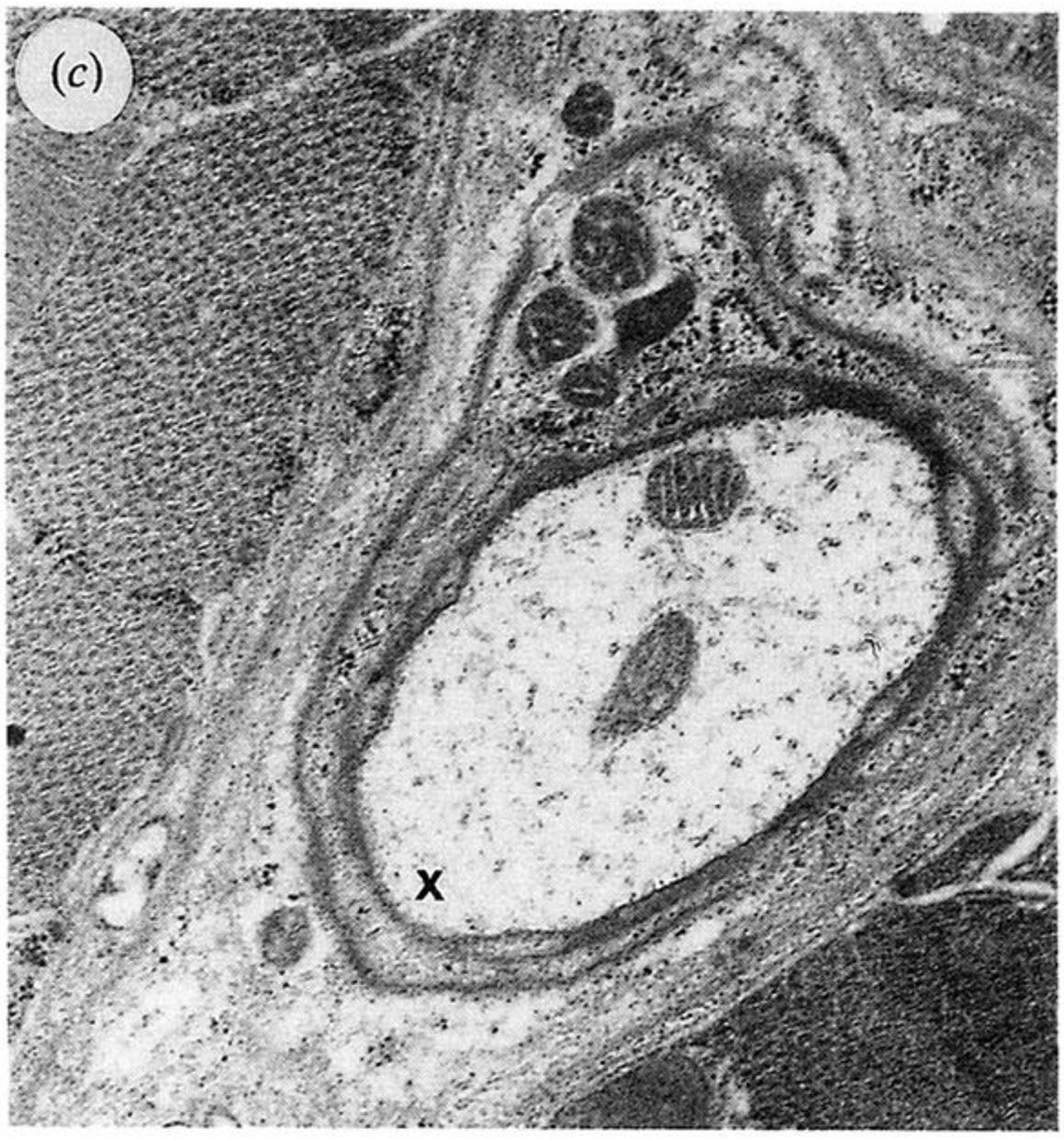


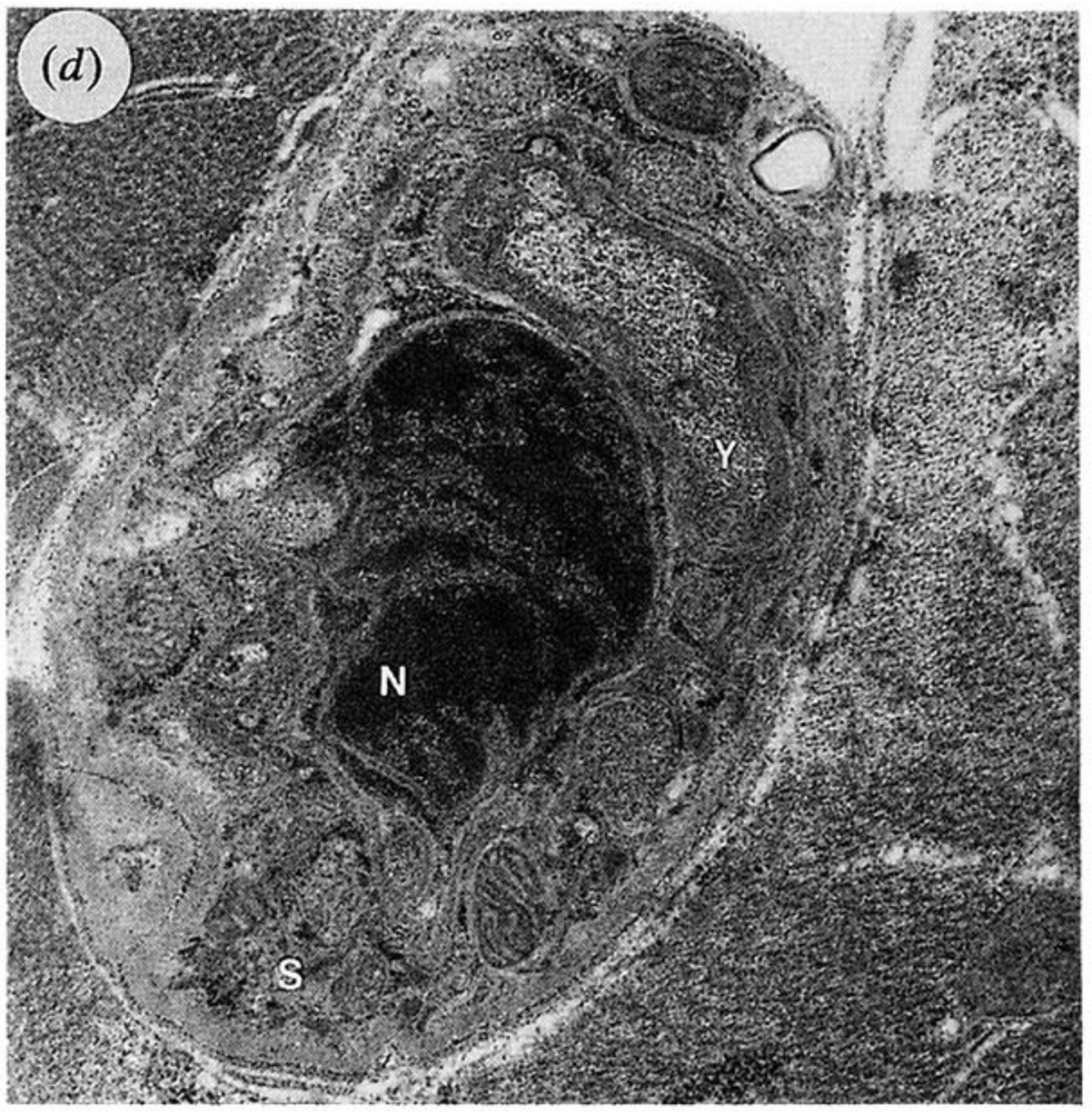


Figure 1. Transmission electron micrographs (TEM) of sections along the vlem nerve. (*a*) Two neuron profiles (X and Y) within a thick mesaxonal sheath (s). The small fibre (Y) contains numerous electron dense vesicles of varying diameters. The arrowheads indicate small diameter profiles that project for a short distance from the ganglion. (*b*) A subsequent section in which the fibre Y (large arrowhead) is greatly decreased in diameter and contains no vesicles. (*c*) The fibre X projects posteriorly along the vlem to innervate all muscle fibres while (*d*) the fibre Y projects anteriorly within a thick sheath and without any branching. N is a nucleus within the sheath, presumably of a glial cell.

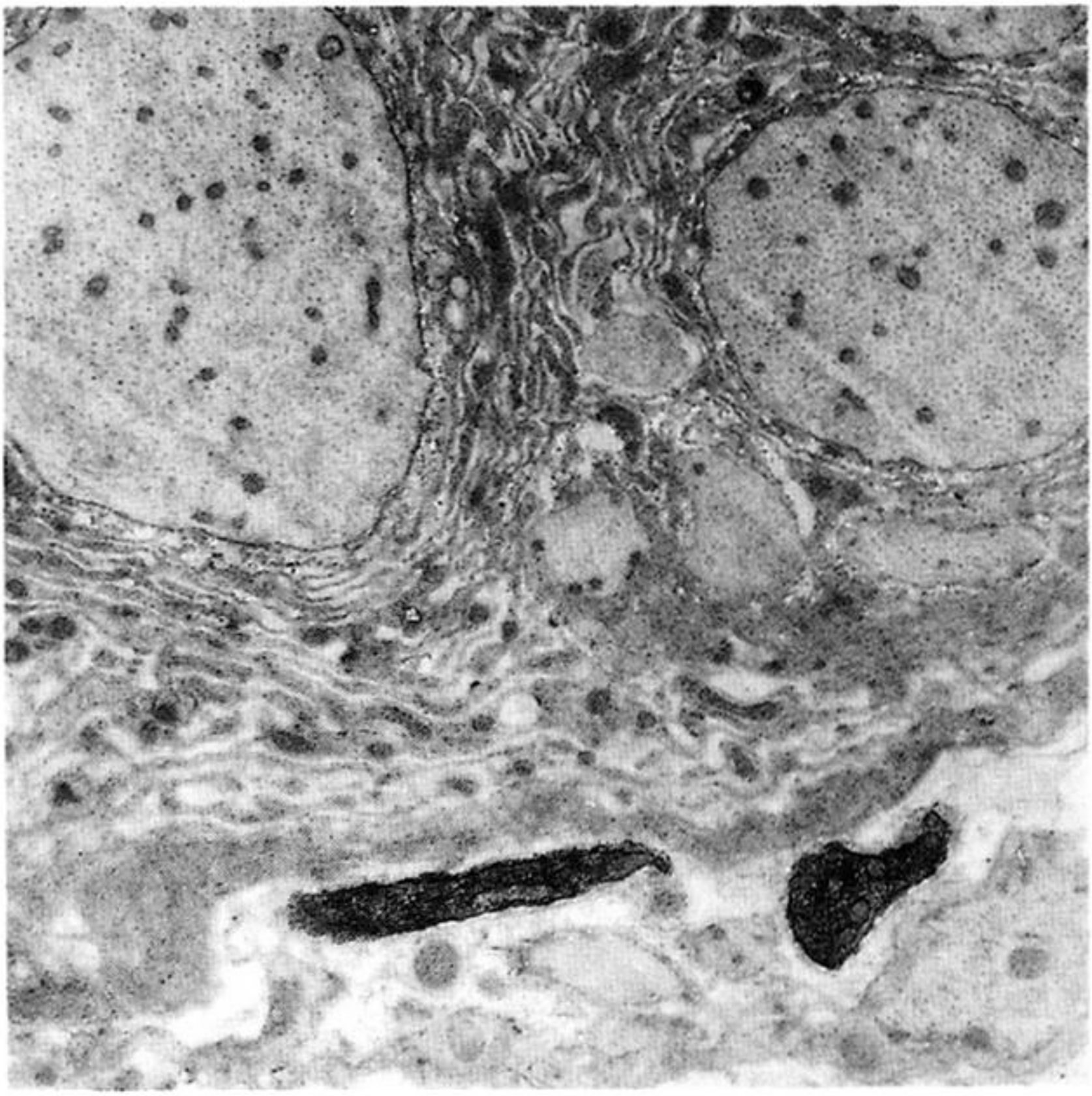
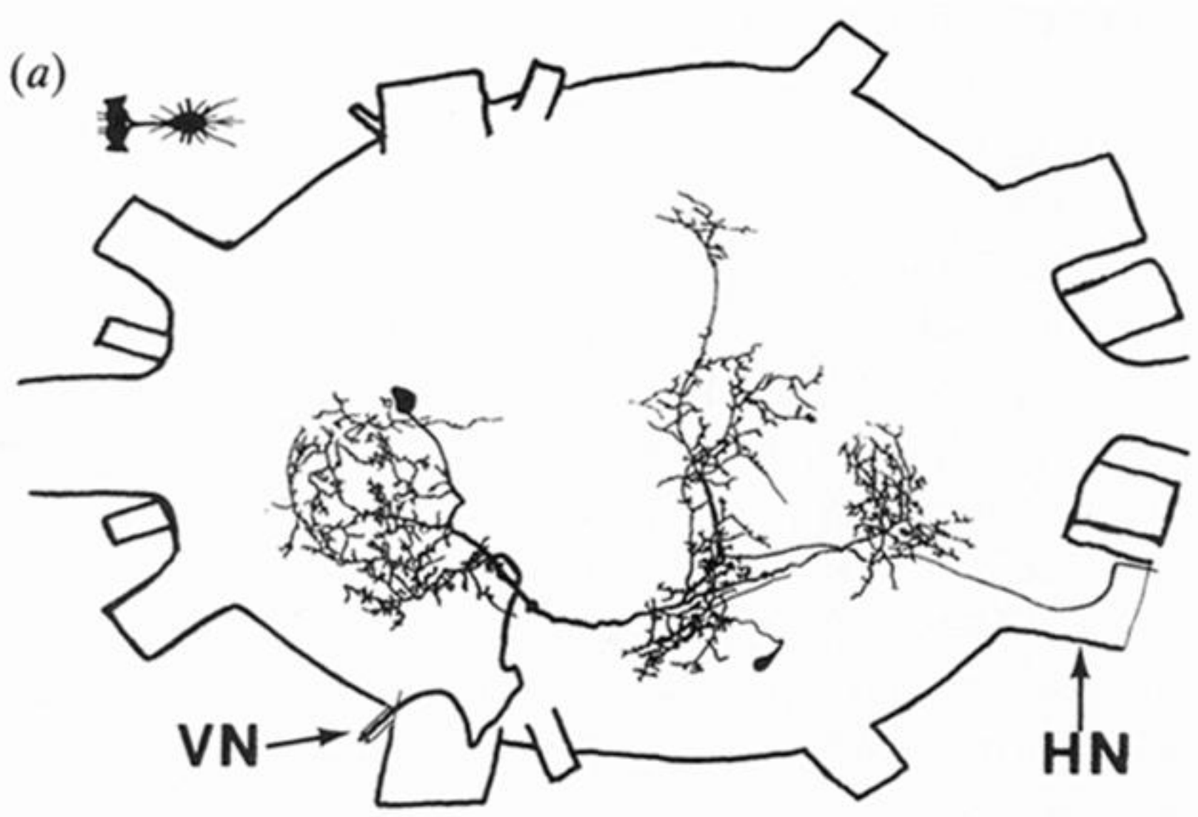
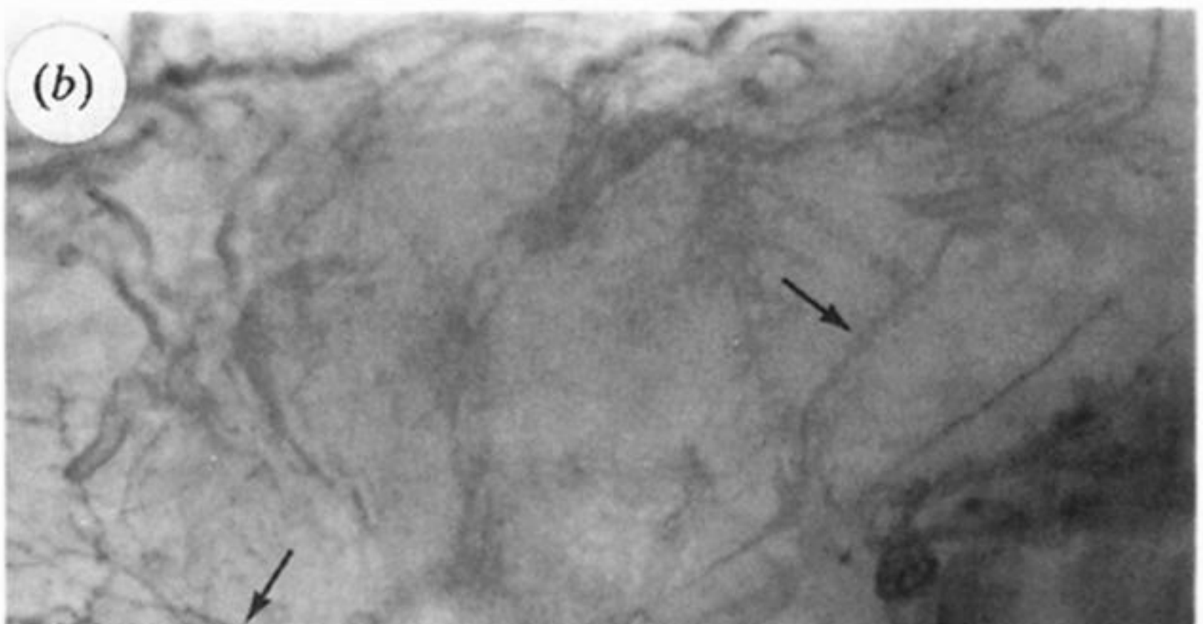
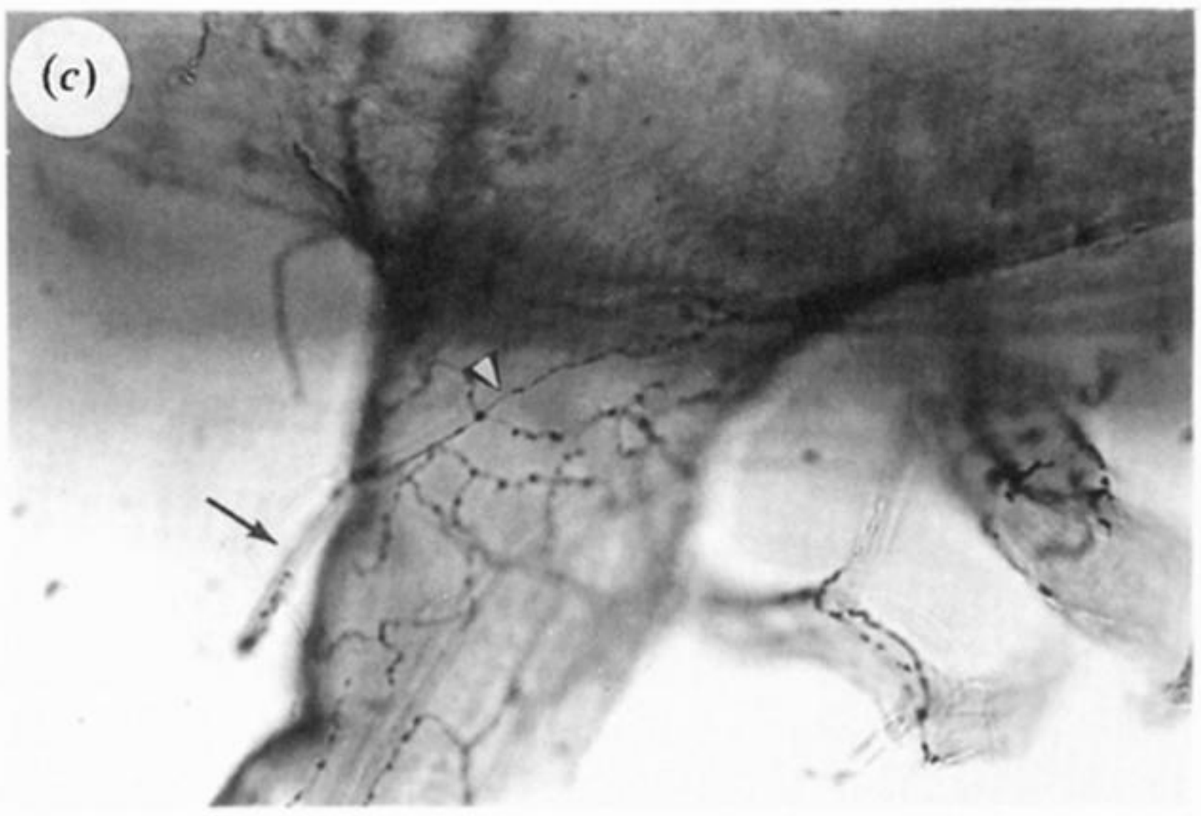
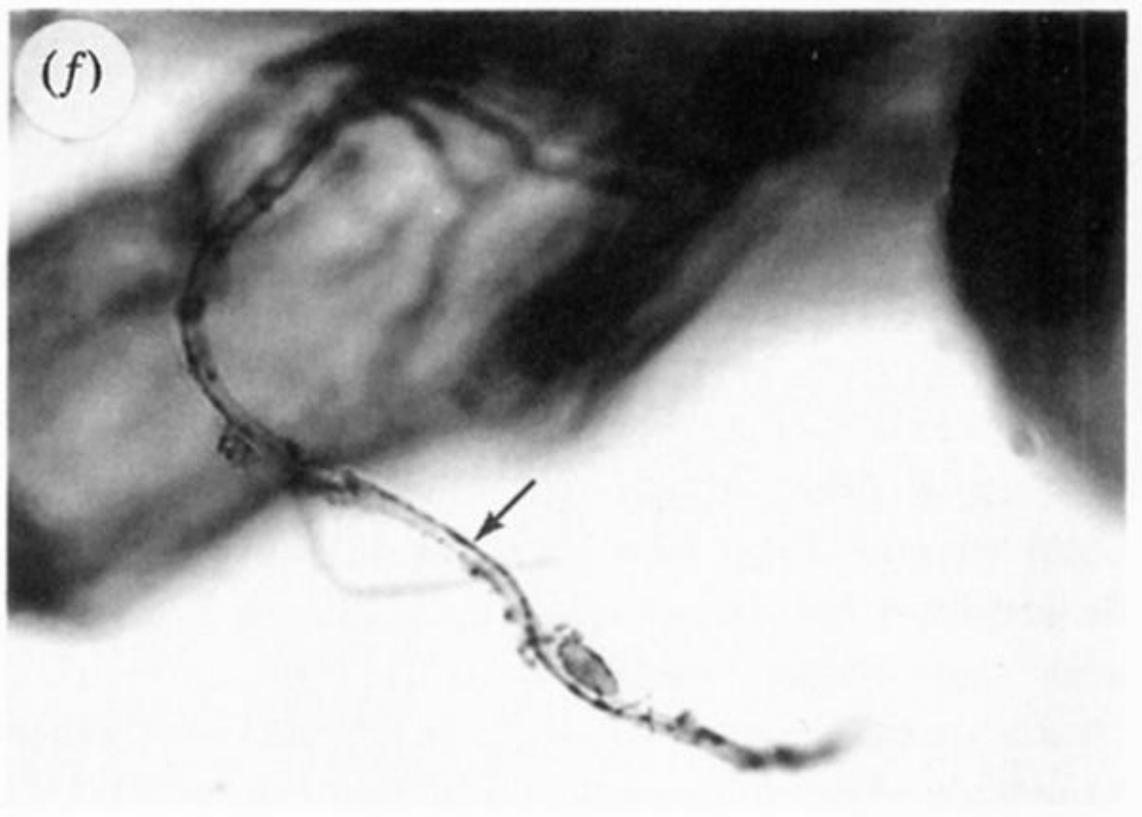


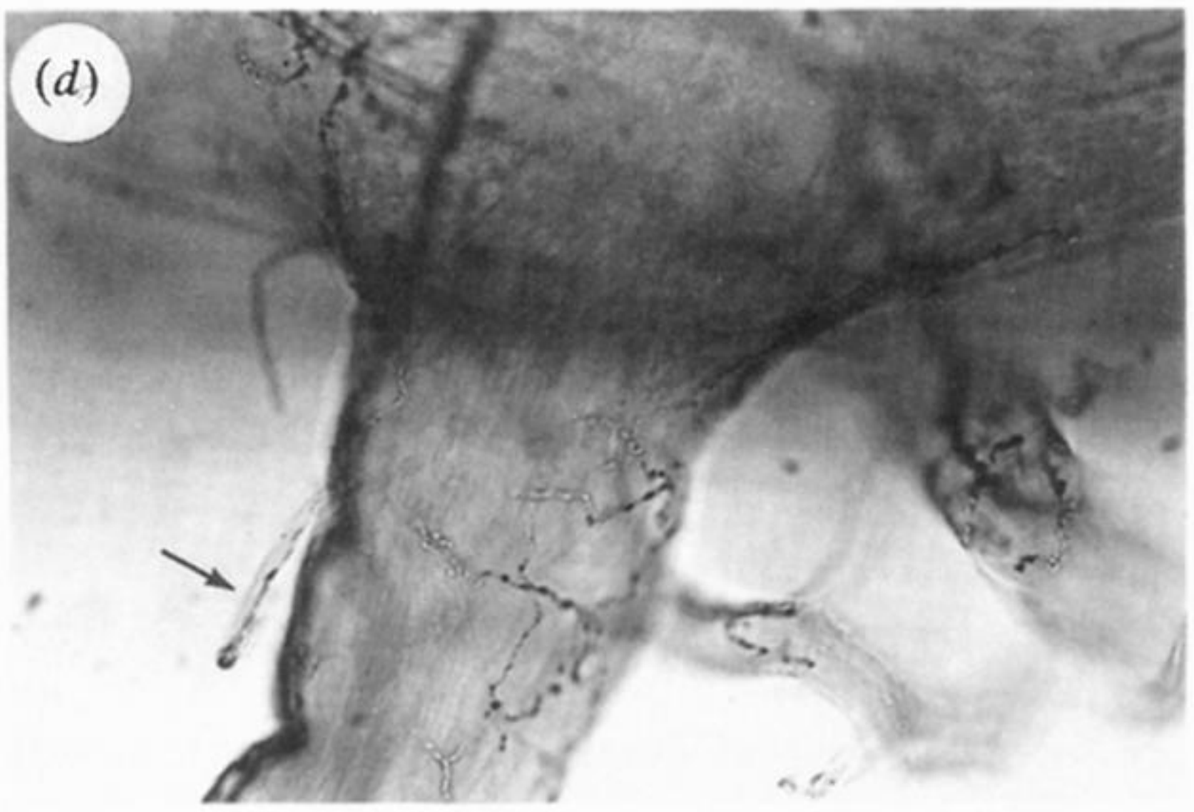
Figure 3. TEM of section through the side of the thoracic ganglion at the entry point of the vlem nerve filled with HRP. The reaction product is visible in two profiles at this point even though only one is distinguishable in whole-mount preparations of cobalt fills. Tissue shrinkage during cobalt processing probably brings the fibres closer together than the 2.5 µm separation observed in this section. This may explain the difficulty in resolving the two fibres when they run in parallel pathways both in the peripheral nerve and within the ganglion.











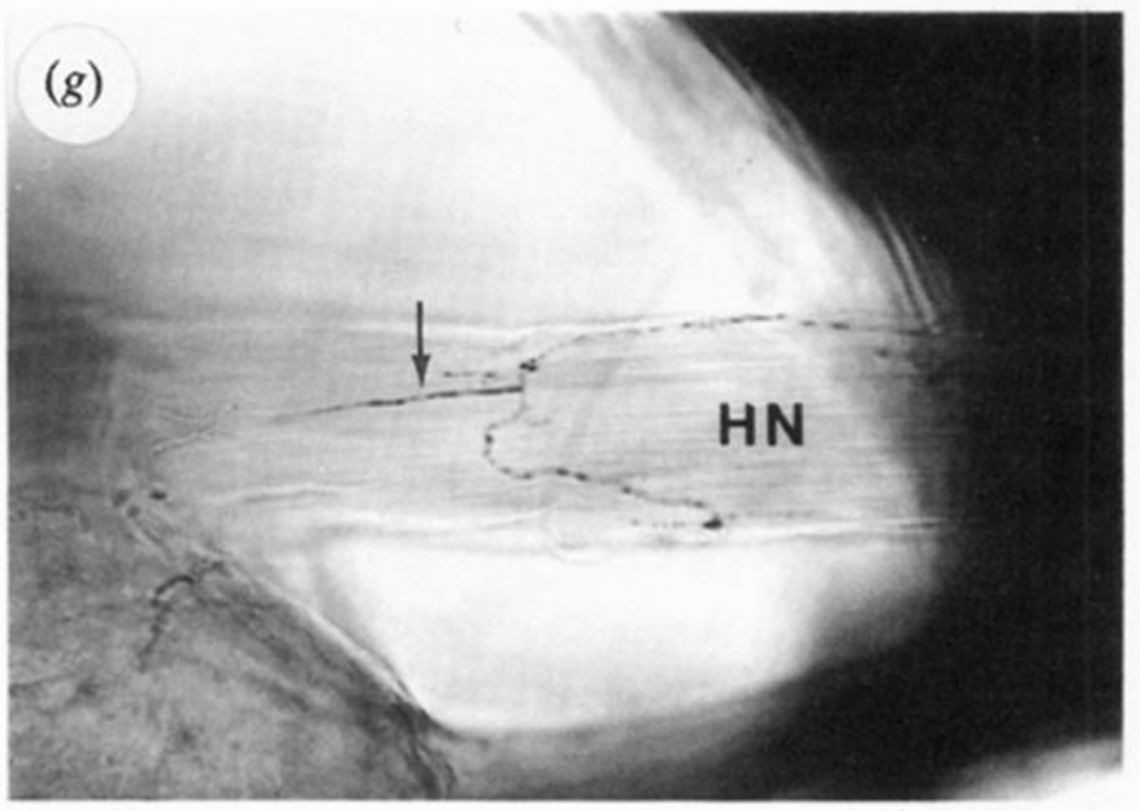


Figure 4. Light micrographs of serotonin-like immunoreactive fibres in wholemount preparations of the thoracic ganglion stained with a serotonin antibody. (*a*) Camera lucida drawing of cobalt filled vlem neurons within the ganglion. (*b*) Arcing fibre along the lateral edges of the ganglion with a similar path to vlem neurons (compare with (*a*)). (*c,d,e,f*) A single immunoreactive fibre projects out along the vlem nerve (VN in (*a*)). This has been observed in all preparations (three examples shown) and has a blebby appearance consistent with the changes in diameter and vesicle content observed for the smaller of the vlem neurons along the nerve. (*g*) Immunoreactive fibre (arrow) projecting along the haltere nerve (HN).

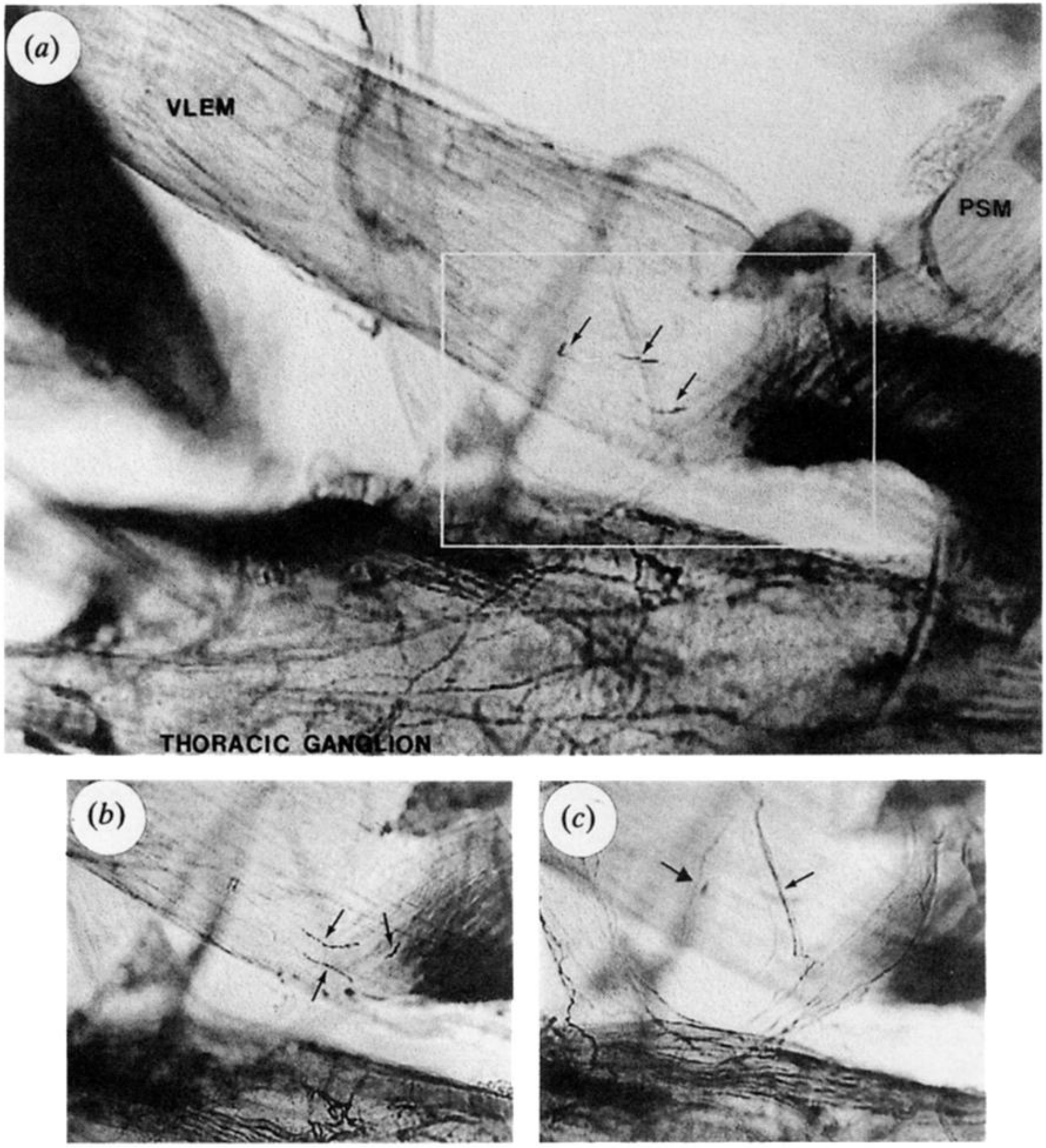
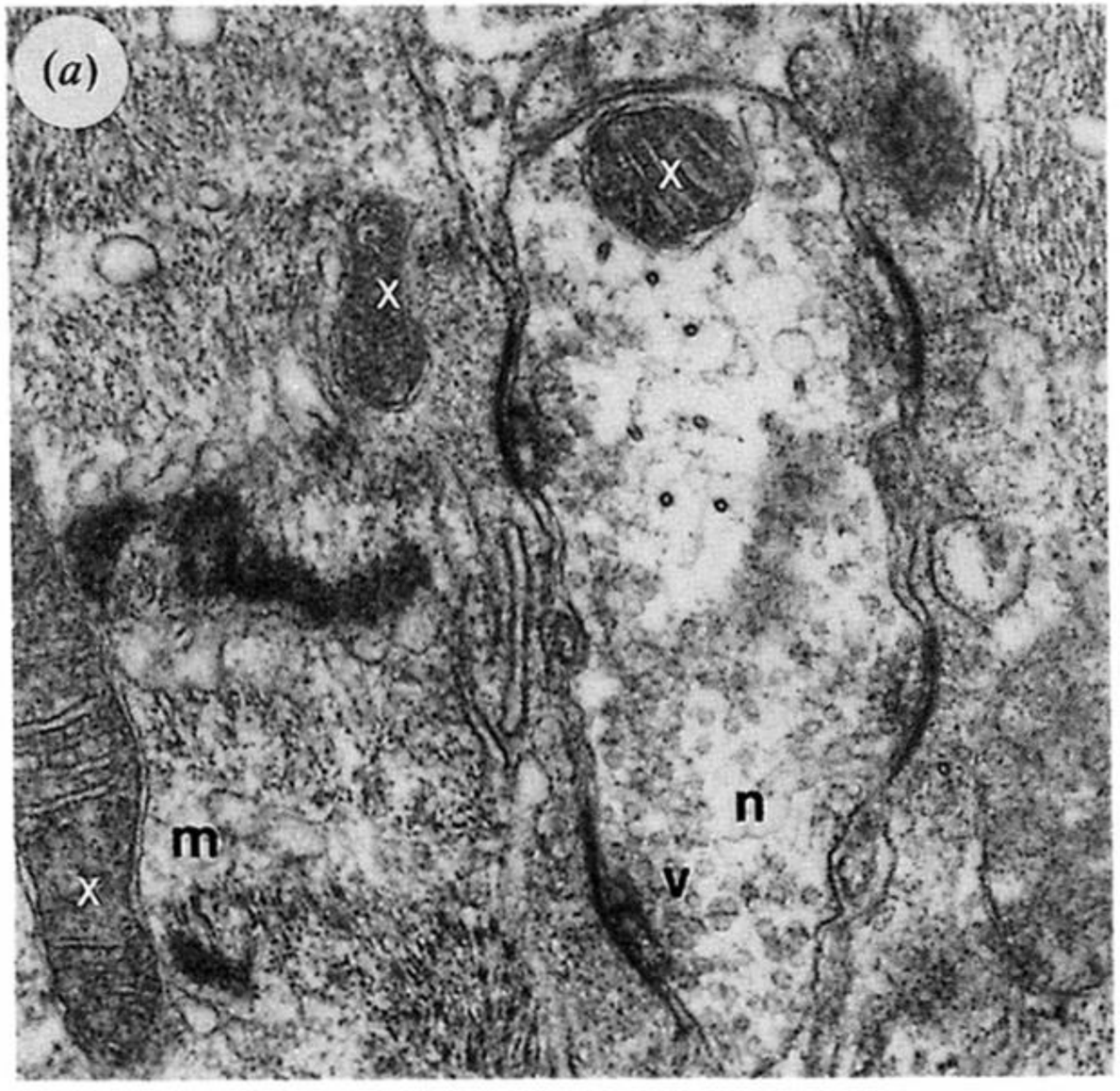


Figure 5. Wholemount preparation of the vlem of *Sarcophaga* showing 5HT-immunoreactive fibres (arrows) in the posterior end of the muscle (*a*,*b*) and a single immunoreactive fibre in one of the nerves innervating the muscle (*c*). Immunoreactivity was not observed in any other of the innervating nerves. Note that the 5HT-like fibres are restricted to only a few muscle fibres making it unlikely that they are motor.



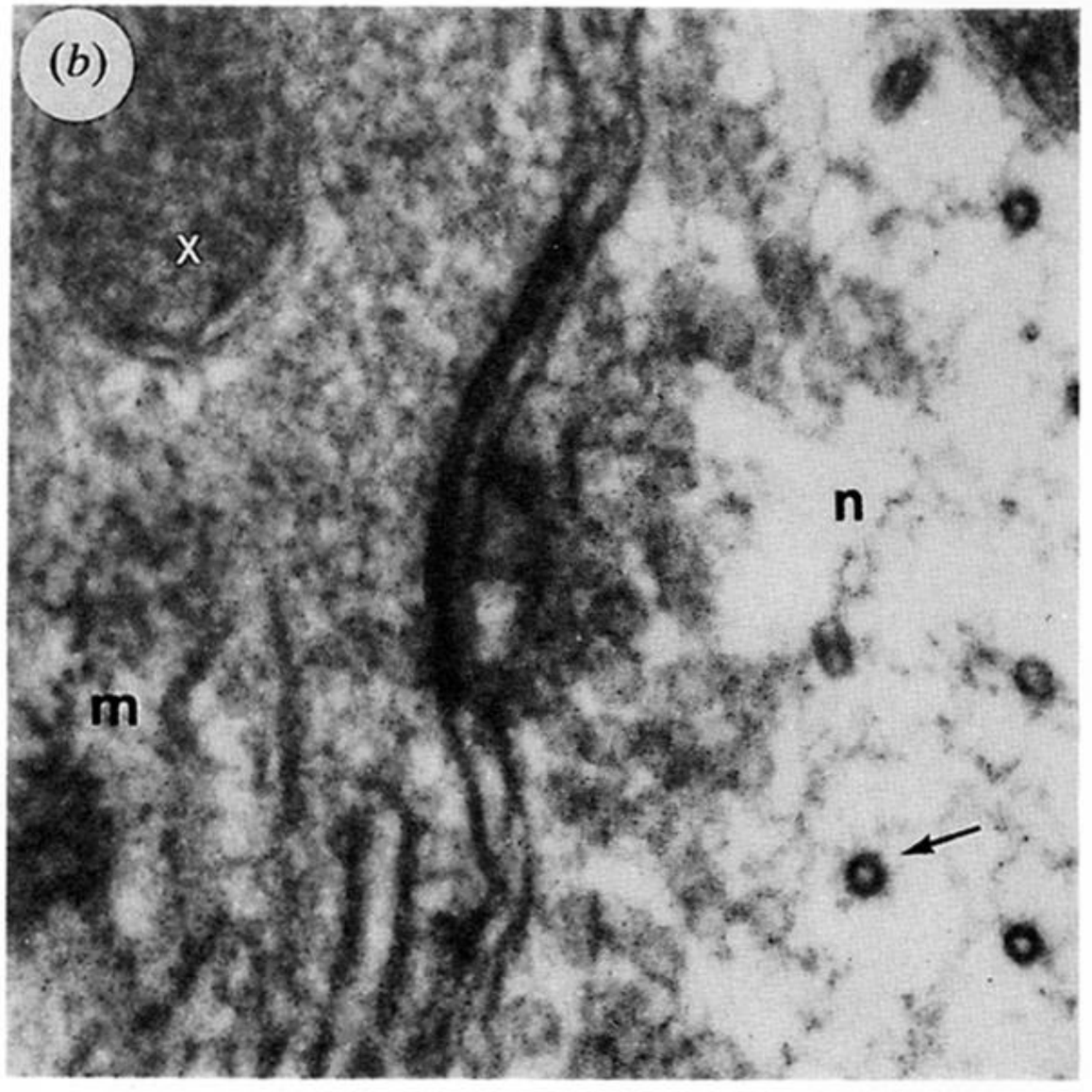
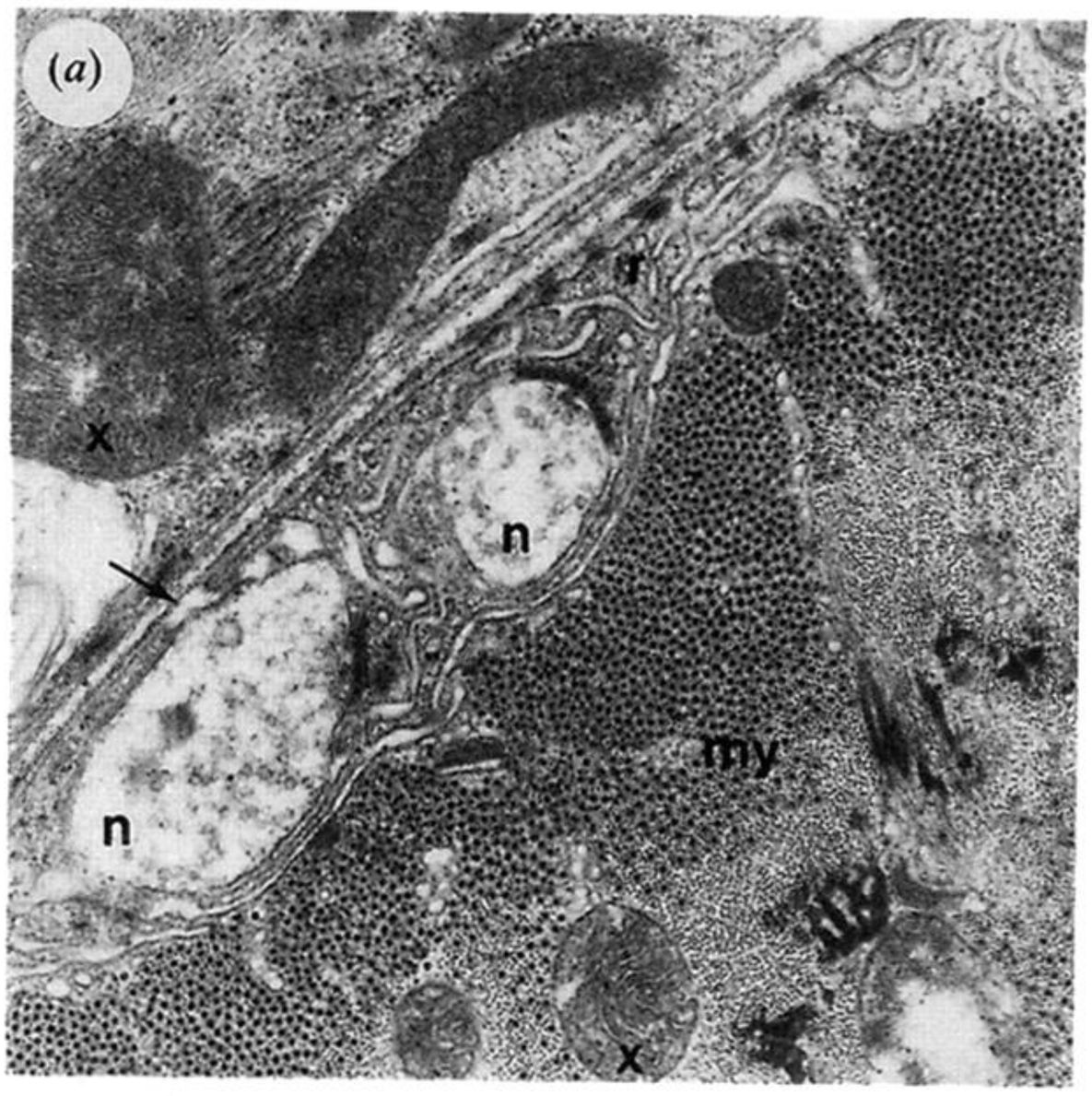
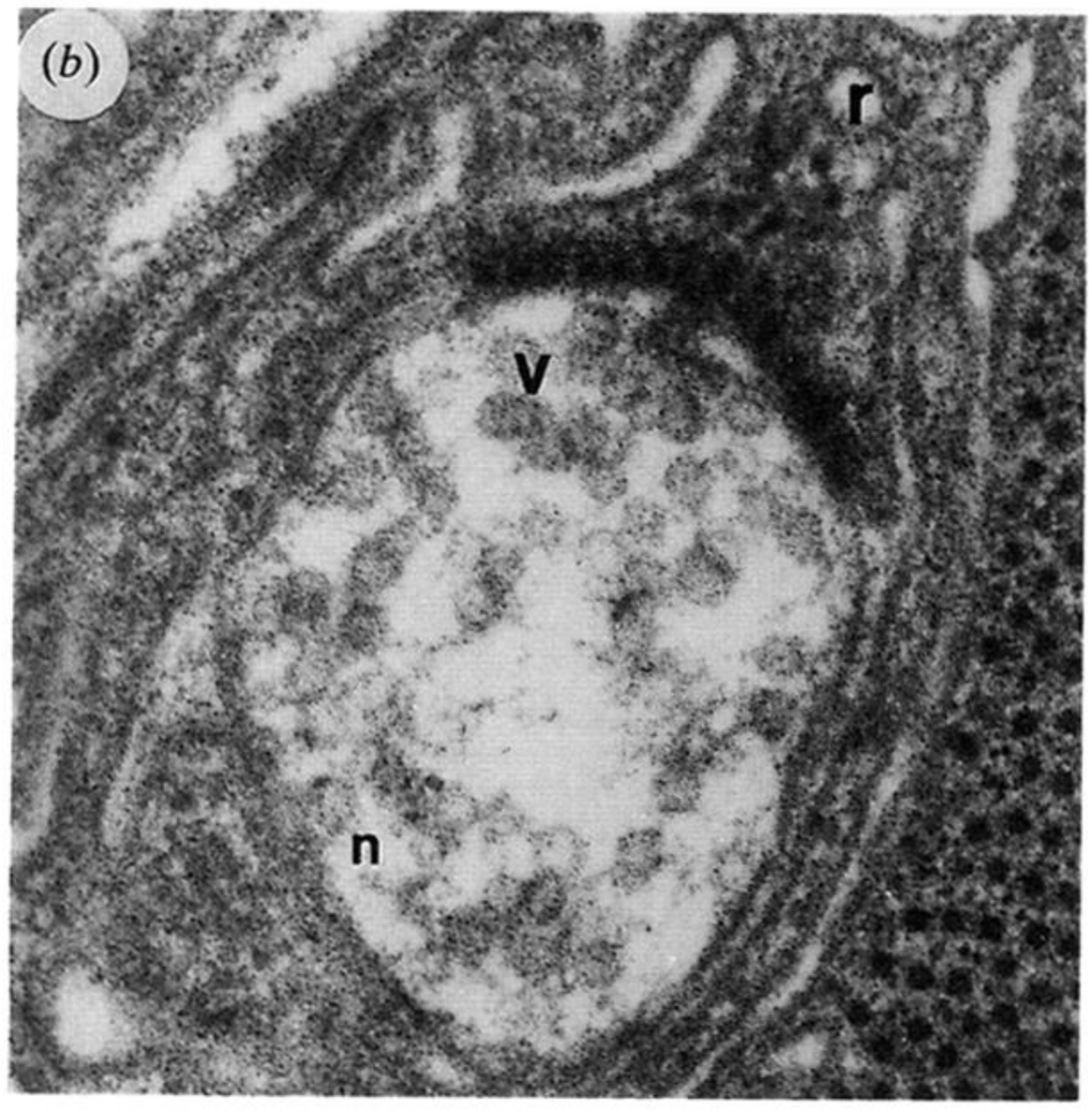
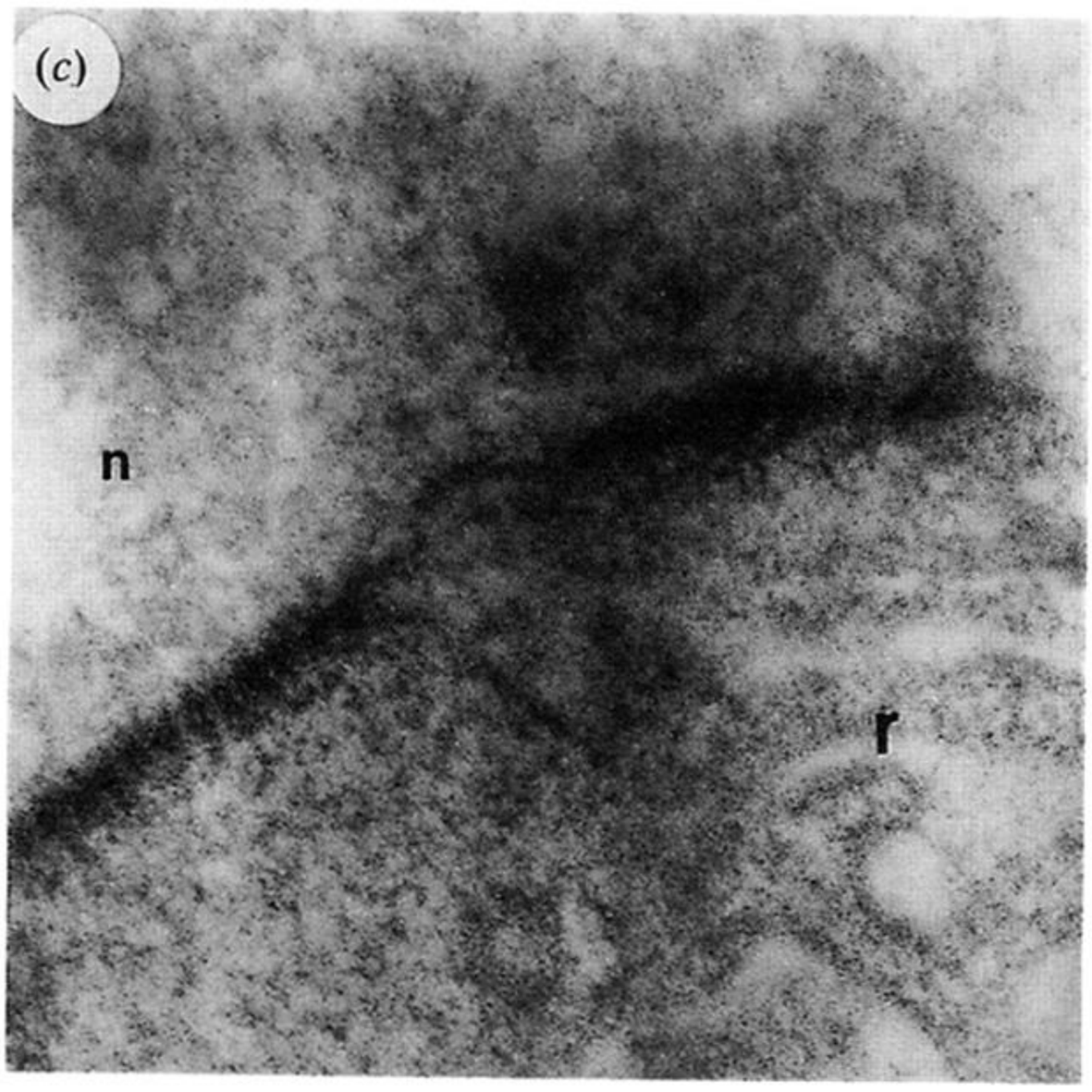
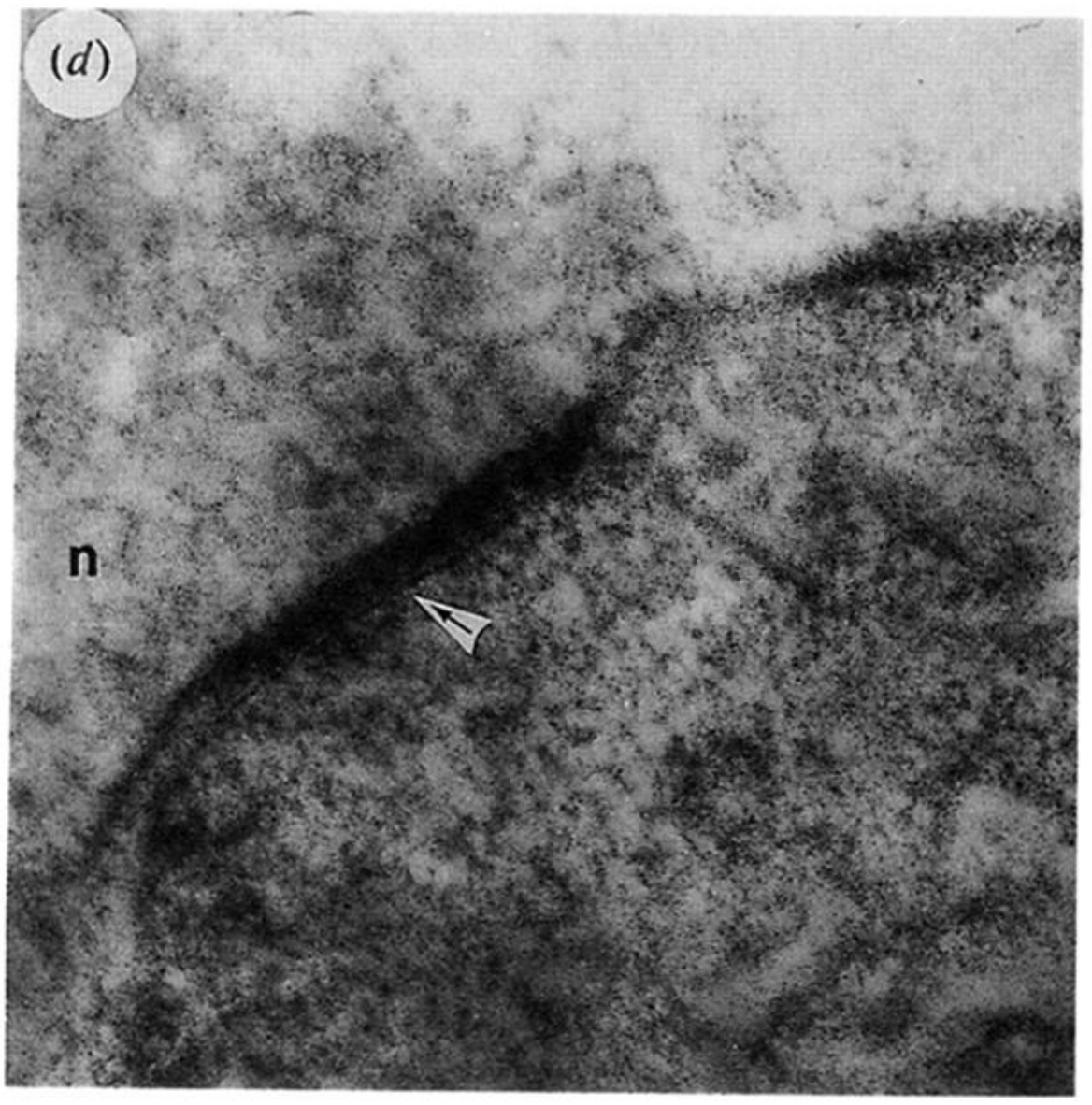


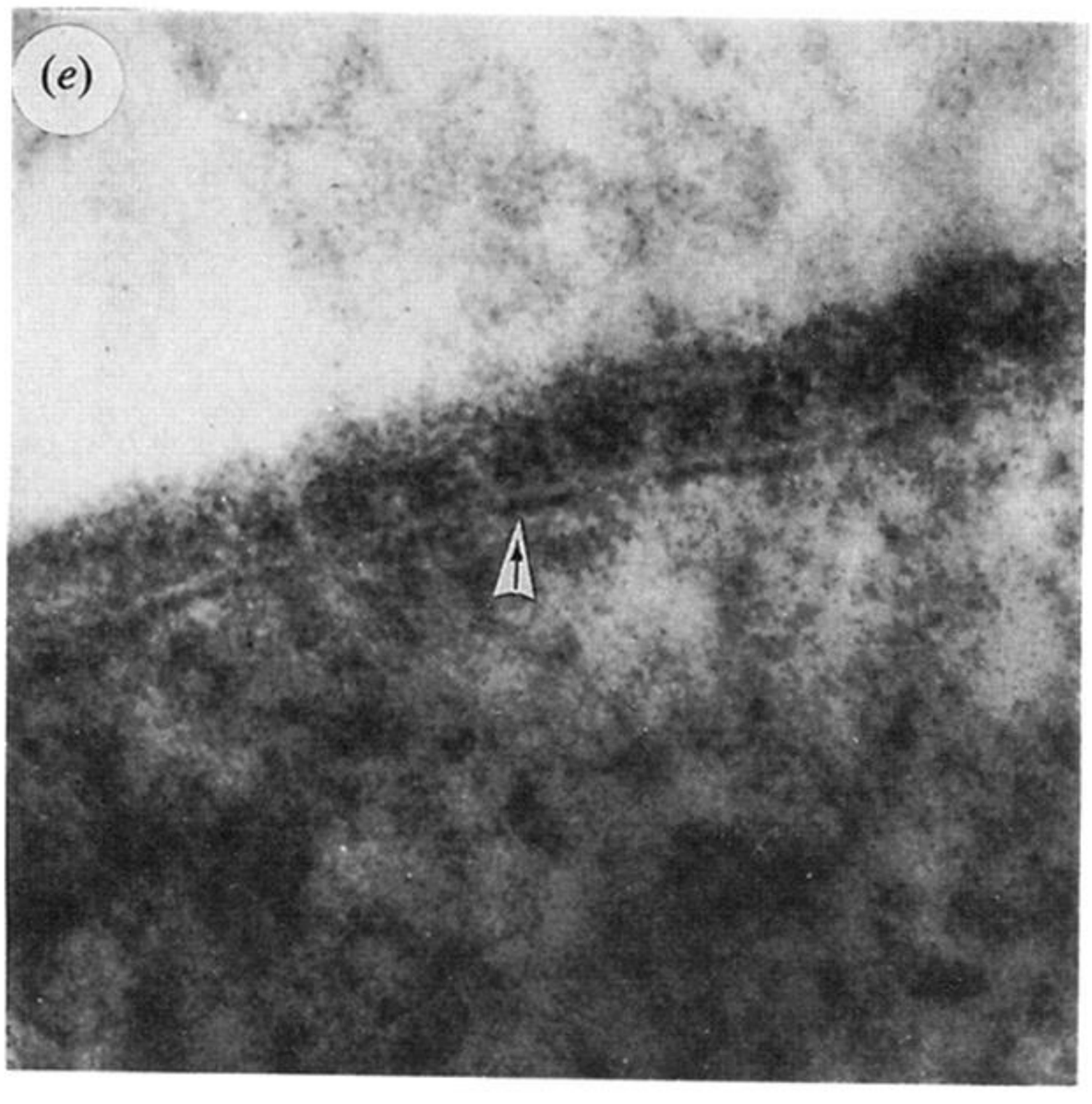
Figure 6. TEM of sections through the pleurosternal muscle of *Glossina* (psm in figure 5) showing a neural process (n) making a typical synapse on muscle fibres (m). Four synaptic sites can be seen with characteristic pre- and post-synaptic features and electron lucent vesicles (v). One of these is shown at higher power in (*b*). The synaptic density is associated with the post-synaptic membrane and a pre-synaptic dense body can be seen in the neuron as well as neural tubules (arrow). X are mitochondria.











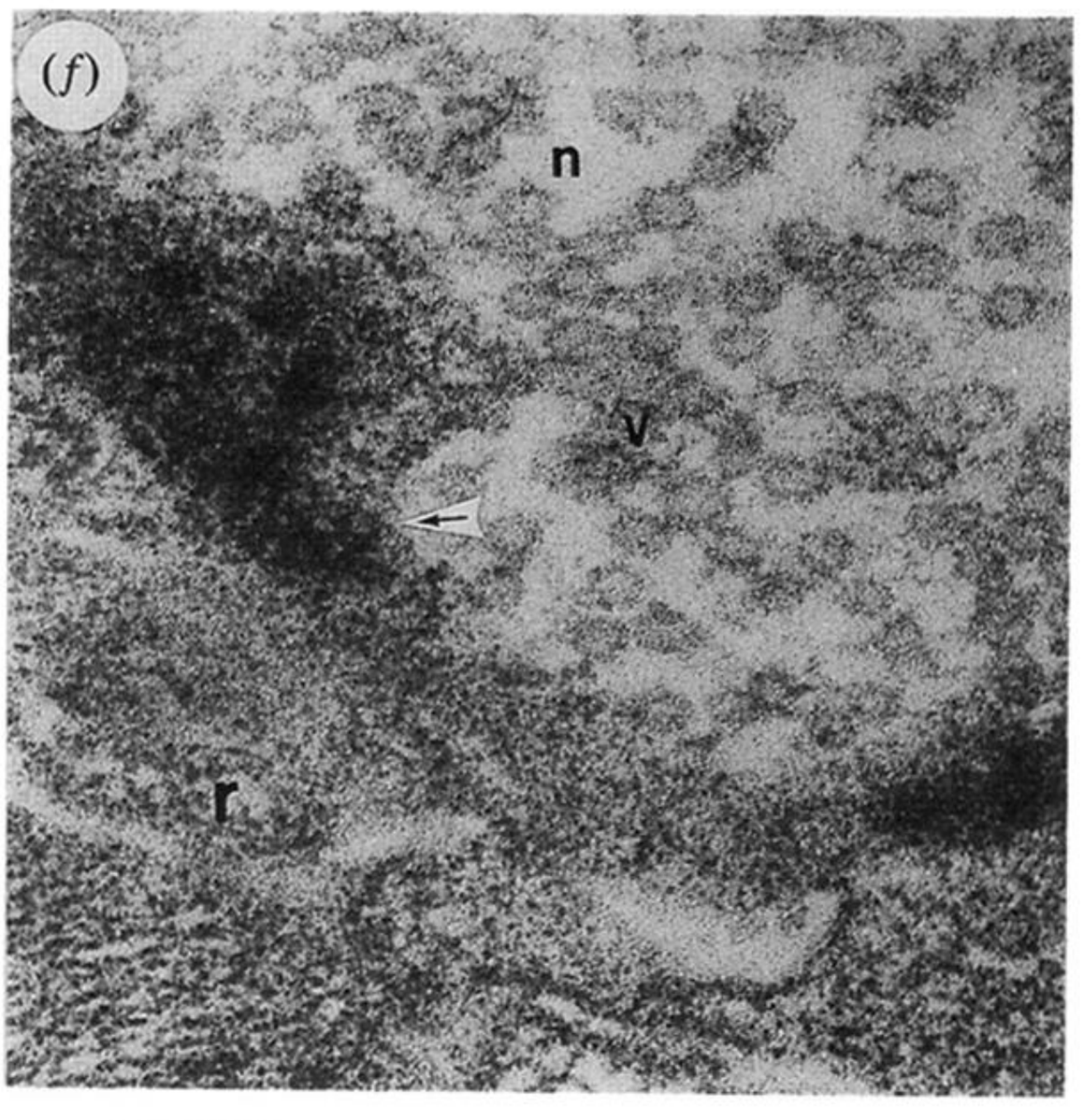
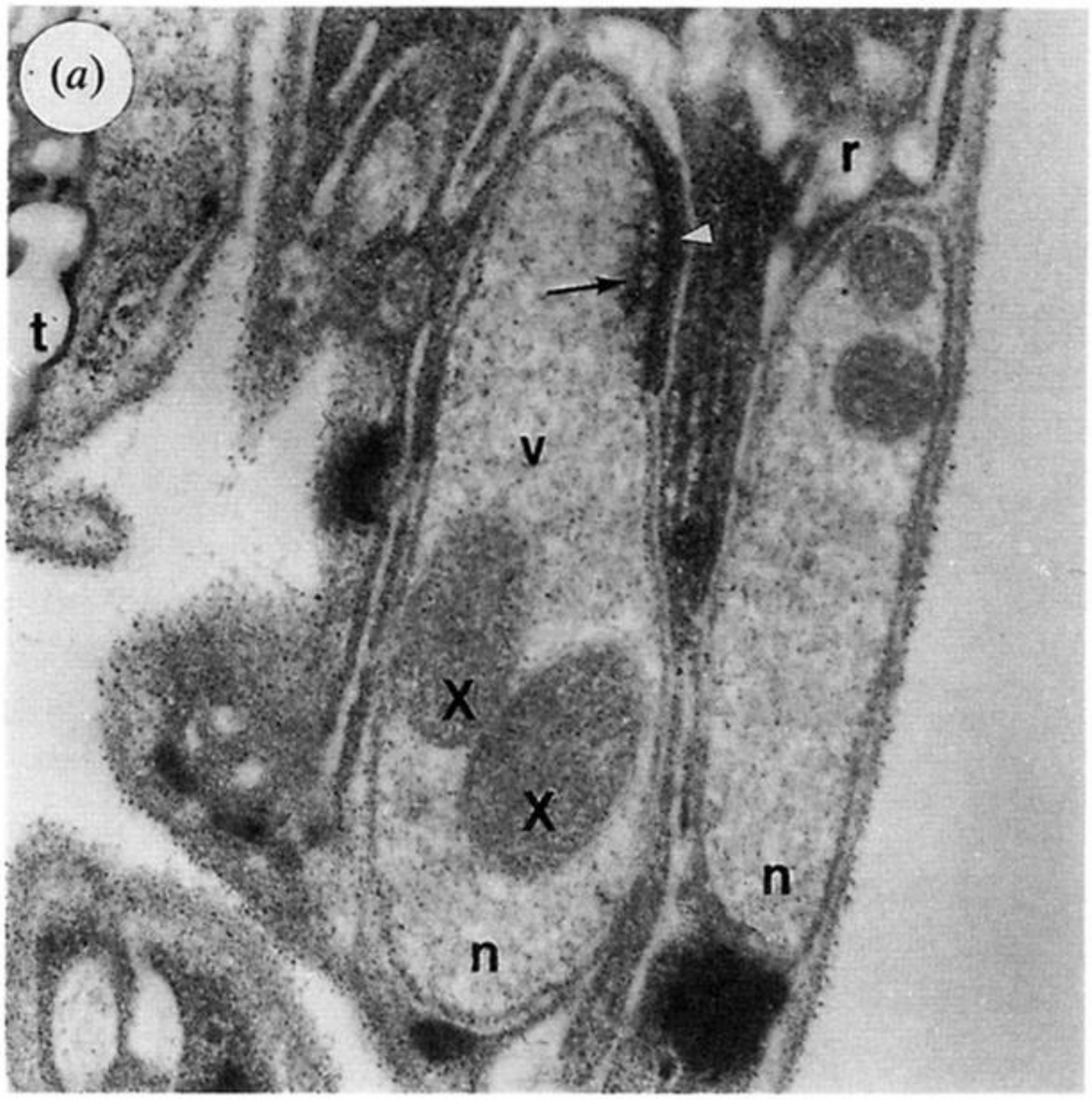
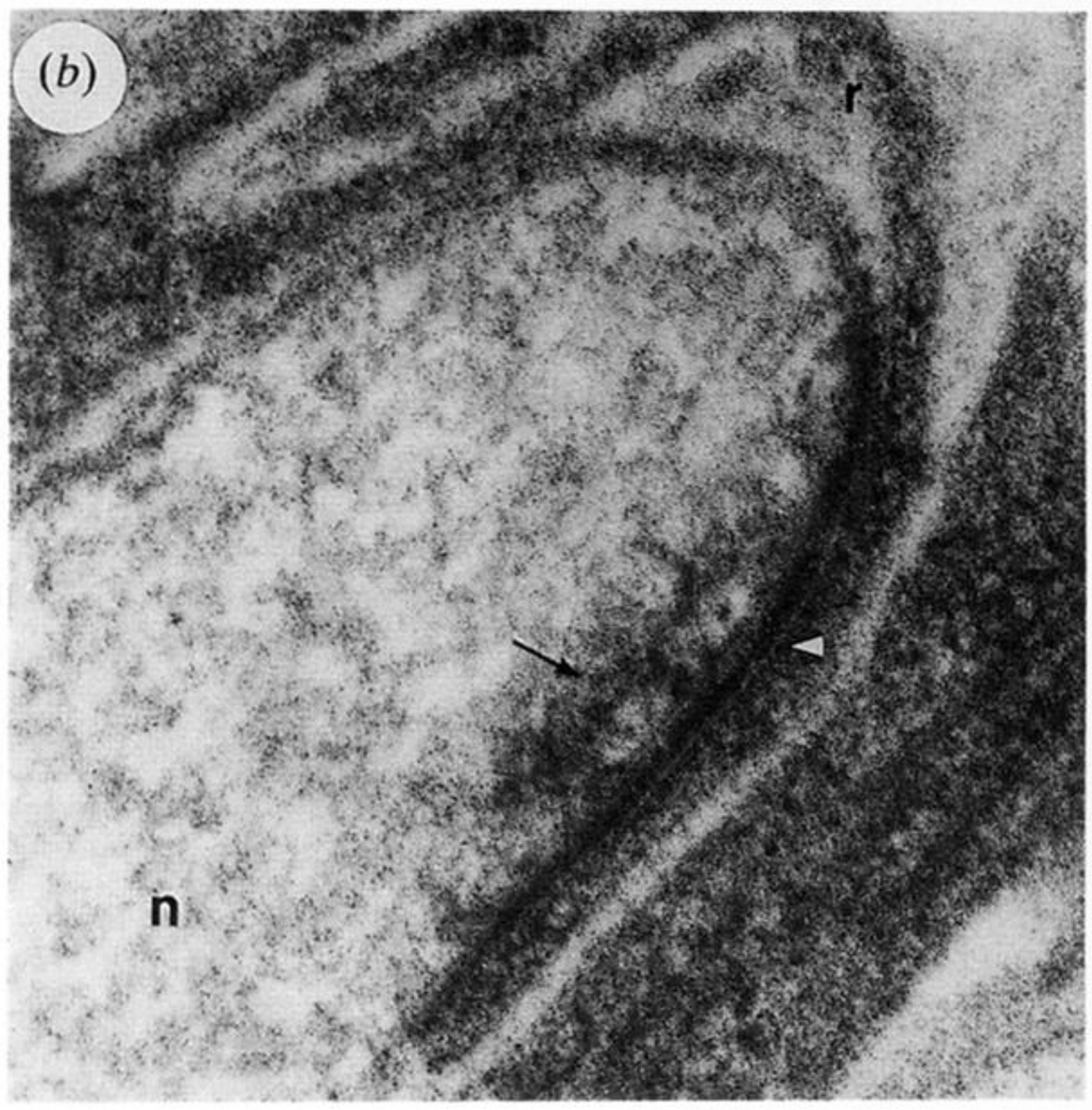
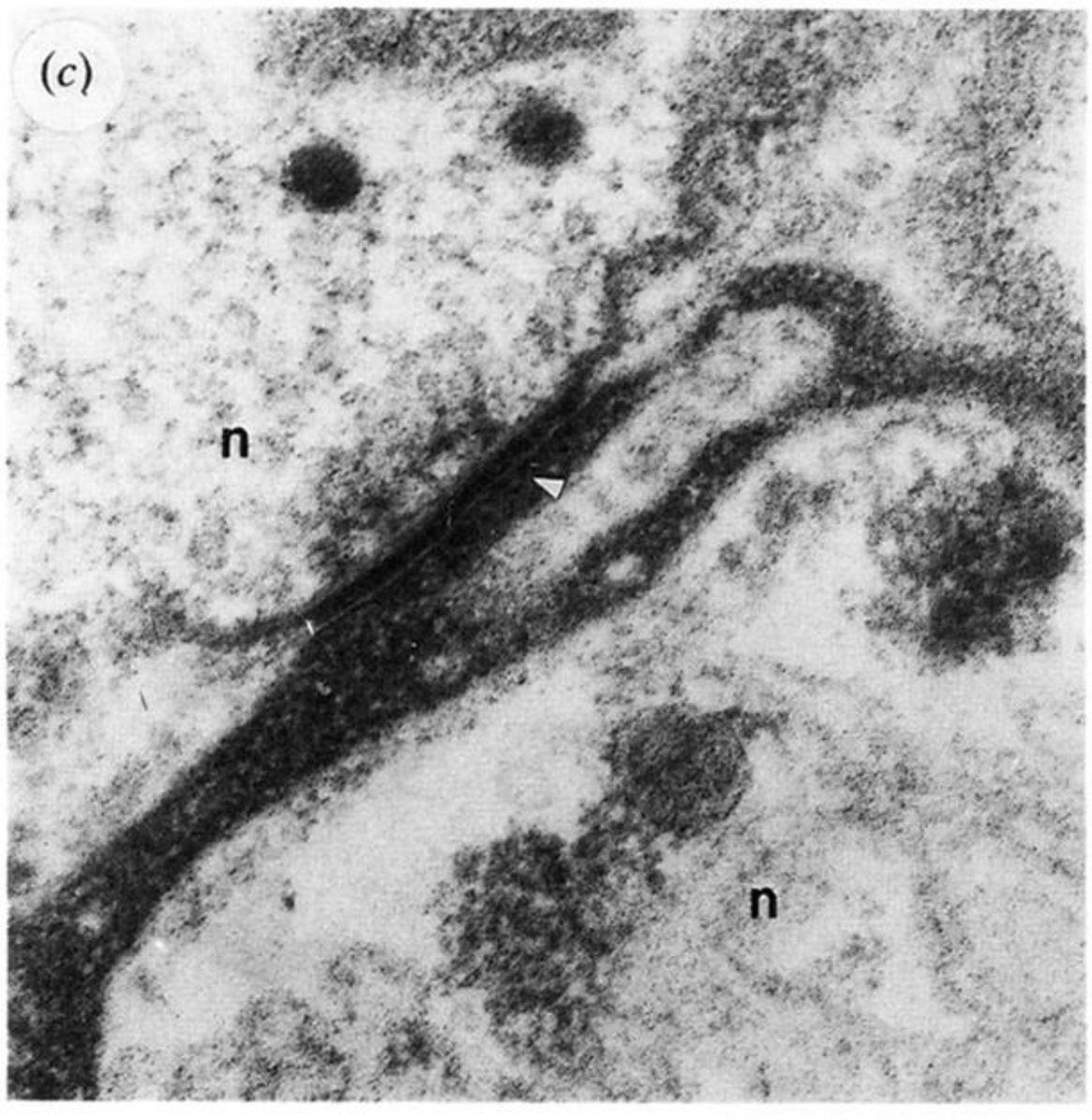


Figure 7. For description see opposite.







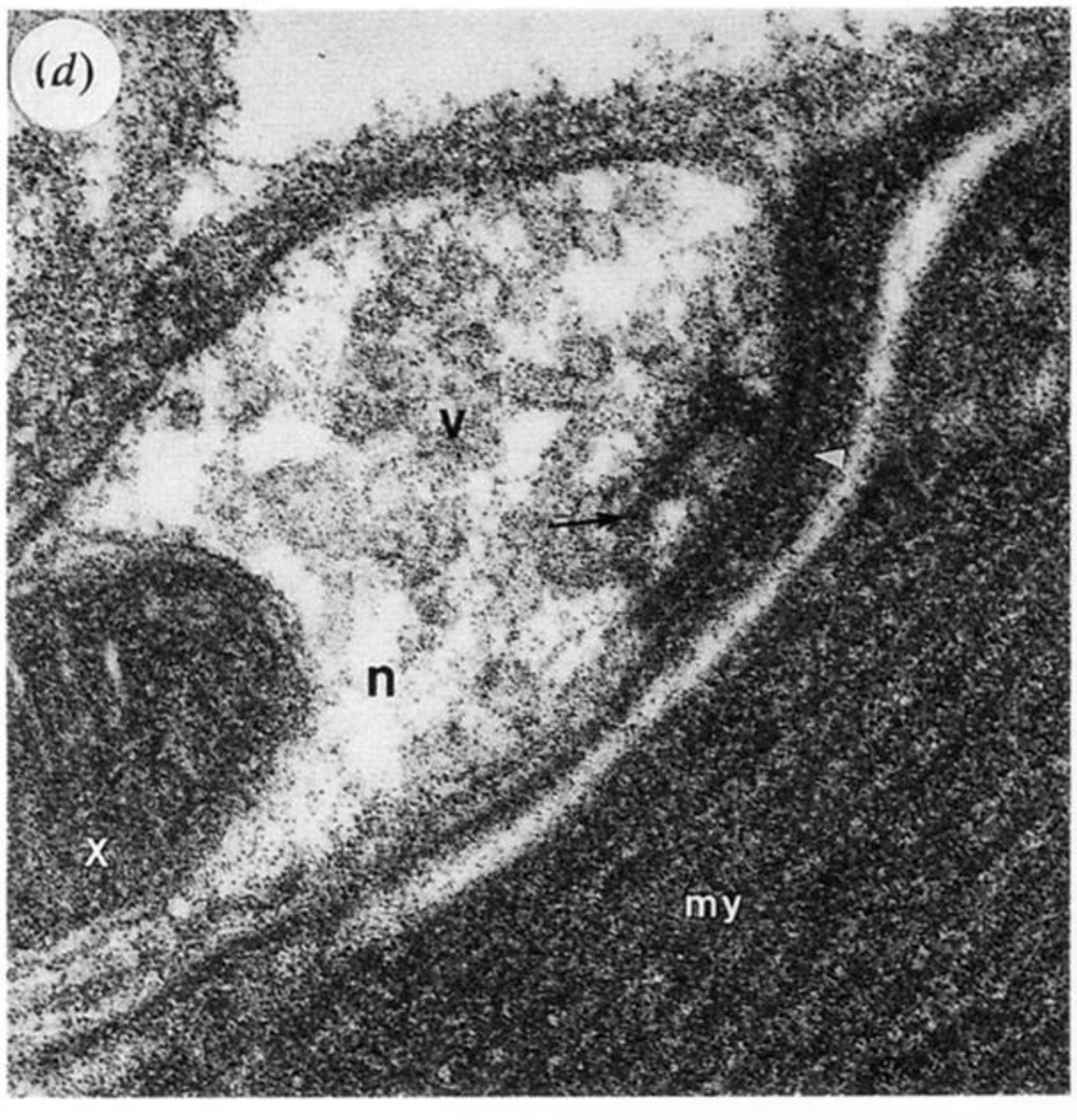


Figure 9. TEM of sections through the vlem terminal 46 h after onset of degeneration of the muscle. Junctional densities are still present (arrow) but the pre-synaptic plate no longer protrudes and perforation by the cytoplasmic fingers can only be seen in occasional sections (*d*). The gap between pre- and post-synaptic cells is still 3 nm and is now more clearly visible and seen in all sections (white arrowheads). Electron lucent vesicles are present in the terminals (v) and the subsynaptic reticulum (r) persists. Scale bars for (*c*) and (*d*) as for (*b*).

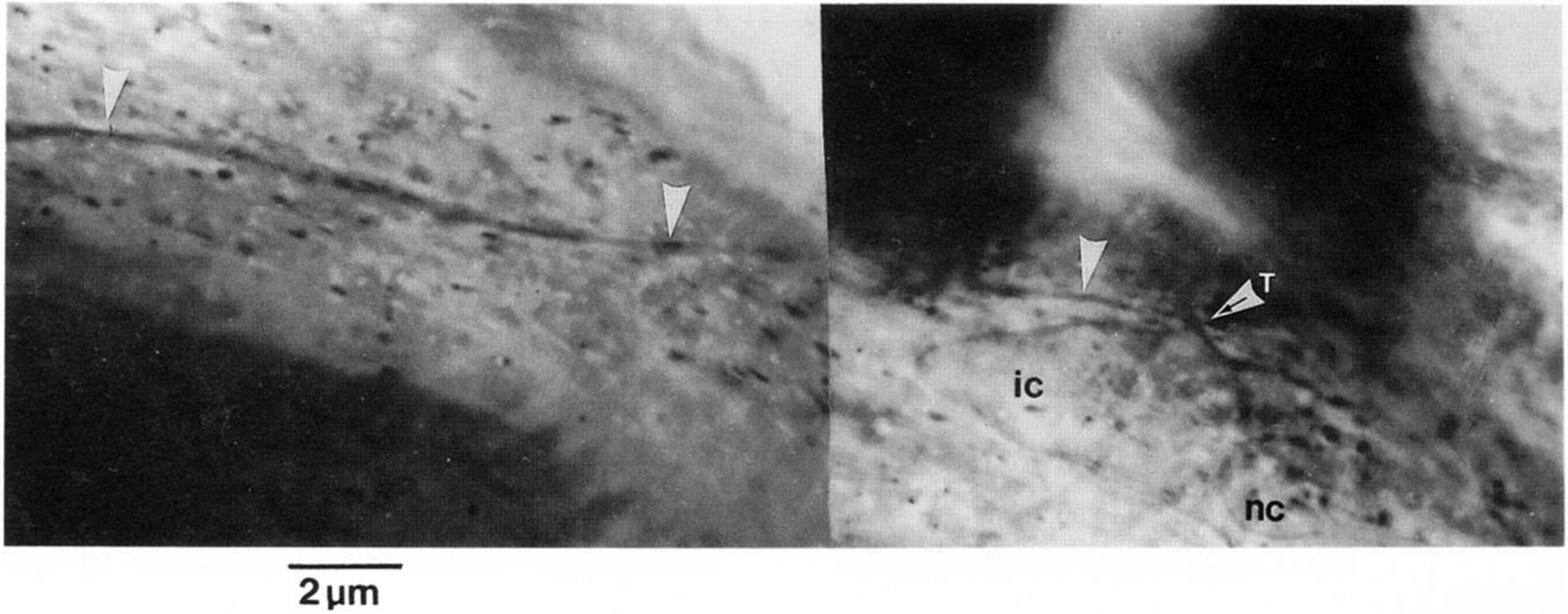
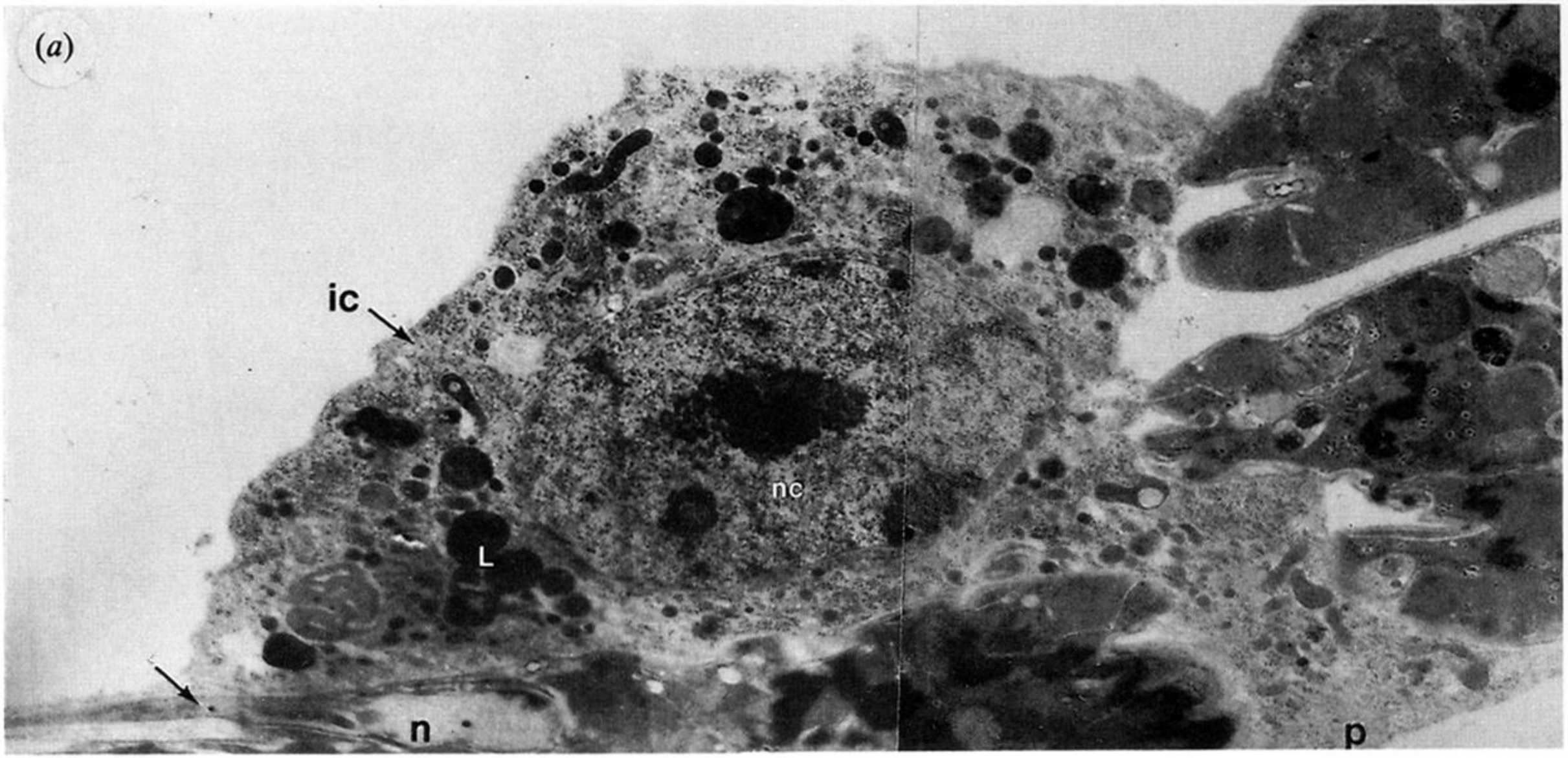


Figure 10. Light micrograph (phase contrast) of the anterior end of the vlem in wholemount showing an HRP-filled nerve fibre (white arrowheads) lying along the surface of a muscle fibre and ending (T) adjacent to a cell (ic) that is attached to the muscle surface which we interpret as an immunocyte identified by its characteristic ultrastructure (see figure 11). nc, nucleus of the immunocyte. The nerve fibre passes through some connective tissue and out of the focal plane near the attached cell.



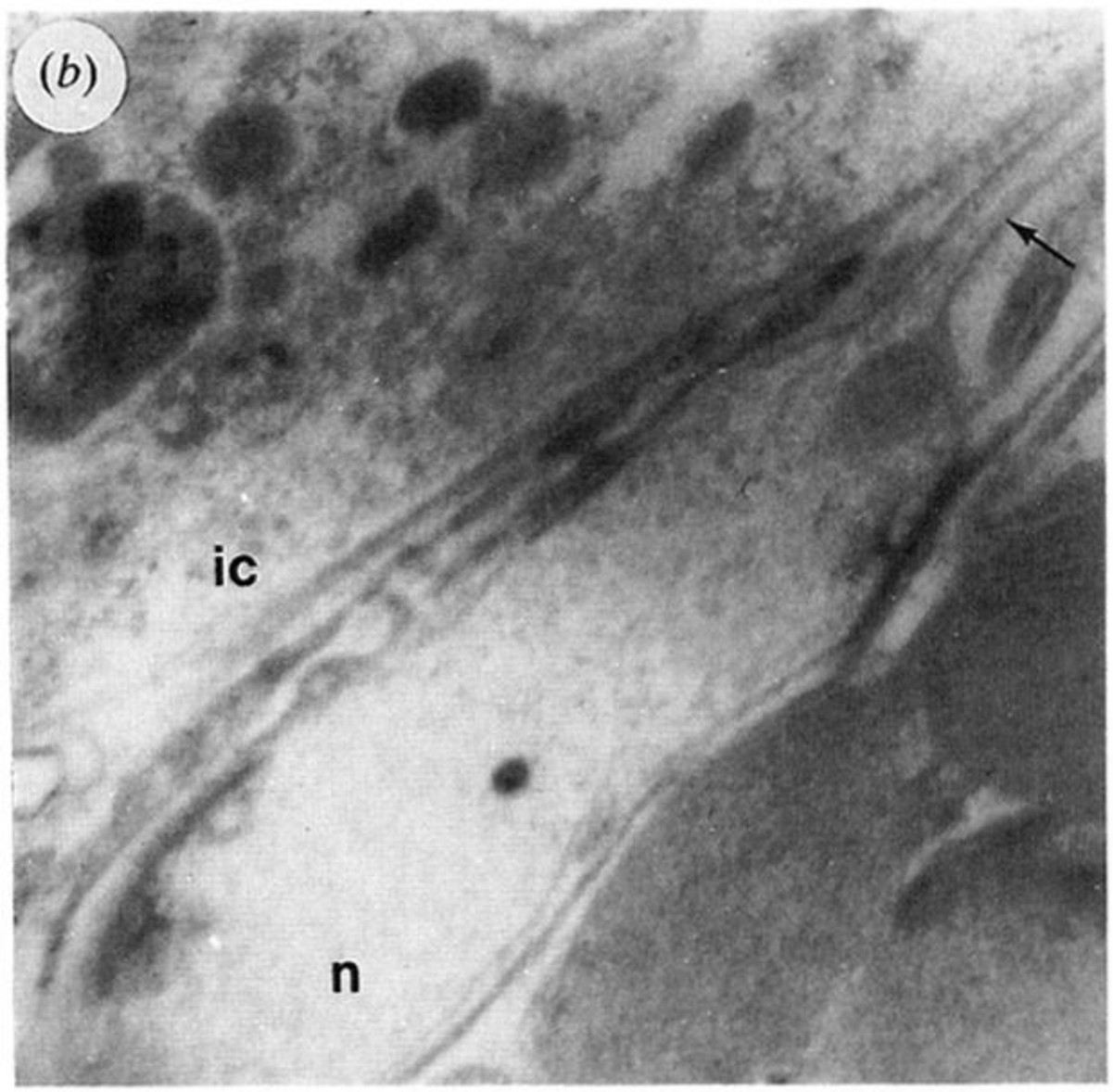


Figure 11. TEM of sections through the anterior end of the vlem 46 h after onset of degeneration. An immunocyte (ic) containing lysosomal vesicles (L) and with a process (p) extended between muscle fibres is associated with a neuromuscular junction (n). The neuron has a fine process (arrow in (*a*) and (*b*)) that extends away from the muscle apparently to the immunocyte. nc, nucleus of the immunocyte. This section was unfortunately contaminated in the microscope which is the cause of the darkness around the junction.