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THE FORM AND DIMENSIONS OF THE GIANT SYNAPSE OF SQUIDS

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A study was made of the distal giant synapse, and of proximal synapses, in the stellate ganglion of the squid, *Loligo vulgaris*. For this purpose we injected iontophoretically dyes or cobalt ions into the pre- or postsynaptic axon. The intra-axonal movement of visible dyes was measured. Both presynaptic fibres, the main second order giant axon and the largest accessory axon, branched to make multiple synaptic contacts on the giant motor axons from near the perikarya down to near the exit of the stellar nerves from the ganglion. There were considerable individual variations in the branching patterns of the presynaptic giant axon and in the course and number of the postsynaptic giant axons. More than one accessory axon made contact with the largest motor axon.

Fine structural details of the synapse are presented. The size of the contact area made by the main presynaptic axon on the last postsynaptic axon of a medium-sized animal was estimated from low power electron micrographs. We measured and counted synaptic contacts, synaptic vesicles and mitochondria. The fine structure of proximal synapses was found to be very similar to that of the distal synapse.

Cobalt- or dye-injected ganglia showed that the perikarya of the axons which fuse to form the postsynaptic giant axons are located in diffuse and overlapping areas of the giant fibre lobe.

In freshly hatched larvae the giant synapse was well differentiated; a gradient of differentiation from brain to periphery was detectable.

The distal giant synapses of the oegopsid squid *Todarodes sagittatus* and of *Sepia officinalis* differed from the *Loligo* synapse. In *Todarodes* and *Sepia* collaterals and processes from both the presynaptic and the postsynaptic giant fibres are shown to meet in numerous contacts in the enlarged sheath surrounding the third order axon. In several respects the *Loligo* giant fibre system appears to represent in phylogenetical order the more evolved neuronal network.

1. Introduction

A synapse between an unmyelinated, large-diameter nerve fibre from the brain and a giant postsynaptic axon running to the muscles, in a flat, transparent and easily exposed ganglion appears to be an ideal preparation for physiological studies. Such a synapse, the giant synapse of squids, is one of the few neuro-neuronal synapses where it is possible to insert microelectrodes into both presynaptic and postsynaptic fibres (Bullock & Hagiwara 1957; Takeuchi & Takeuchi 1962). It is a chemical synapse that functions by quantal release of a transmitter substance whose identity still remains unknown (Miledi & Slater 1966; Miledi 1967, 1969). This synapse has been used for studies on the mechanism of transmitter release and the role played by various ions in this process (Katz & Miledi 1967, 1969, 1971; Miledi 1967, 1971, 1973; Kusano *et al.* 1967; Kusano 1970; Kusano *et al.* 1975; Llinas & Nicholson 1975; Llinas *et al.* 1976; Martin & Miledi 1978; Pumplin *et al.* 1981).

When we studied synaptic function at the giant synapse of the squid, on many occasions we found it necessary to have some information on the position and extent of the presynaptic terminal, on the movement of substances in pre- or postsynaptic axons, and on other synaptic contacts on the giant motor fibre. For this purpose we injected into the axons visible dyes, labelled amino acids (Heuser & Miledi 1970), or cobalt ions. In the present paper we report some of the results obtained during summers between 1967 and 1972. We also identified the proximal synapse by injecting cobalt ions into the accessory axon, and studied its fine structure. In the past only the giant synapse from myopsid squids has been used. Therefore, we compared the structure of this synapse with that of an oegopsid and a sepiid species.

After the description by Young (1939) of the giant fibre system of the squid, several papers have contributed to our knowledge of the construction of this synapse. Staub (1954) used microinjection of dyes to follow the third order axons to their origin. Hama (1962) reported fine structural details such as width of synaptic cleft, diameter of synaptic vesicles and size of synaptic contacts in the Japanese squid *Doryteuphis bleekeri*. Castejon & Villegas (1964) reinvestigated the giant synapse with electron microscopy. Young (1973) counted and measured the processes of the distal synapse. We described fine structural details of the giant synapse (Martin & Miledi 1975), reviewed data on the connections of the giant fibre system (Martin 1977), and more recently Pumplin & Reese (1978) studied membrane ultrastructure by using the freeze-fracture technique and electron microscopy.

2. MATERIALS AND METHODS

2.1. *Animals*

A giant fibre system is found in the three large groups of decapod cephalopods: the teuthoid suborders Myopsida and Oegopsida and the sepioid family Sepiidae. Before our work stellate ganglia have been studied almost exclusively in myopsids, for example, the Atlantic *Loligo pealii*, the Mediterranean *Loligo vulgaris*, and species of *Sepioteuthis* and *Doryteuphis*. Here we studied ganglia from one species of each major group: the myopsid *Loligo vulgaris*, the oegopsid *Todarodes sagittatus* and the sepiid *Sepia officinalis*. We used small- to medium-sized *Loligo* of 4–18 cm mantle length, medium-sized *Todarodes* of 14–18 cm mantle length and *Sepia* of 10–12 cm mantle length. The stellate ganglia of freshly hatched *Loligo vulgaris* were also examined.

2.2. *Terminology*

The single elements of the whole giant fibre system have been described by Young (1939). The number of stellar nerves and postsynaptic (third order, III) giant motor fibres varies in different individuals from 8 to 12. We therefore called the last and longest (also thickest) giant axon 'L' and the successively more cephalic and shorter (also thinner) giant axons 'L-1', 'L-2', etc. (Miledi 1967). The 'g.f.2a' of Young (1939) is here called also the main presynaptic axon or second order axon (II). First order neurons and axons are in the brain and are not considered here. By 'giant synapse', unless otherwise stated, we mean the distal synapse of the last branch of the second order axon with the last (L) third order giant axon. Similar giant synapses occur also on L-1, L-2, etc. In addition to the giant synapses the giant motor axons receive innervation from two or more axons that are called accessory. These axons enter the stellate ganglion in the same preganglionic nerve, the mantle connective, as the main presynaptic axon. The smaller contacts made by the accessory axons are termed proximal synapses. Many of these elements are evident in the living preparation of figure 1, plate 1.

2.3. *Electrophysiological methods*

The mantle–ganglion preparation (figure 1), or the isolated stellate ganglion, were used as described previously (Miledi 1967, 1972; Katz & Miledi 1967). The preparation was continuously perfused with oxygenated, natural or artificial seawater, usually containing tetrodotoxin (0.6 μM) to abolish nerve impulses. To reduce the changes in fluid level that occur during perfusion, the fluid was sucked up through a tube immersed deeply into the chamber. The immersed end of the suction tube was sealed and the tube had a small outflow hole at the desired level. In this way contact with the fluid is maintained during suction and the level remains fairly constant. The preparation was usually kept at about 10 °C.

Postsynaptic and presynaptic giant axons were injected with dyes by iontophoresis through a microelectrode. In the experiments of 1967 various dyes were used, including Fast Green–FCF (Gurr); water soluble fluorescein (Gurr), methylene blue, and several Procion dyes (I.C.I.), mostly Procion Rubine (suggested to us by Dr J. Alvarez and Dr E. J. Furshpan). More recently, cobalt ions were injected essentially as described by Pitman *et al.* (1972).

The dye microelectrodes were filled first with distilled water by boiling under reduced pressure and the water was then replaced by filtered aqueous solutions (1–10 %) of the dyes. For cobalt injections the micropipettes were filled directly by boiling in 200 mM $CoCl_2$ or $Co(NO_3)_2$. In general, dye-filled pipettes were suitable for intracellular recordings, while cobalt-filled pipettes were often troublesome, because their tips developed high resistances and positive potentials soon after impaling the axons. This sometimes made it difficult to tell when the electrode was properly inside the axon.

It is relatively easy to load small nerve cells, but loading giant axons is more problematic. Sometimes the jet of dye ejected by single pulses of 10 ms duration was visible under the dissecting microscope; at other times large current pulses flowed through the pipette without releasing any dye. This problem was even greater with the cobalt pipettes, because there was no way of checking the ejection of cobalt visually and one could not tell immediately whether sufficient cobalt ions were being injected.

The usual procedure was to insert first a microelectrode which was used to monitor the membrane potential of the axon. Then the dye- or cobalt-pipette was inserted and current pulses of about 100 ms duration were passed through it to release the marker and also to monitor the input resistance of the axon. The pulses were applied at frequencies of 1–5 s^{-1} for periods ranging from 10 min to several hours, depending on the degree of loading required. The intensity of the pulses varied from about 10^{-8} A to about 5×10^{-6} A. Sometimes the currents were low because of limitations in the pipette; but in presynaptic axons weak currents were usually used to avoid damage to the terminals by hyperpolarizing pulses, or by overstimulation with depolarizing cobalt ion pulses.

After injecting the dyes drawings were made of the living preparation as viewed through the dissecting microscope, or photographs were taken (figure 1). On a few occasions the dye-injected preparations were also examined after aldehyde fixation. In the case of the cobalt injections, once this was ended, the ganglia were transferred into a dish with 10 ml of seawater to which seven drops of a solution of 20 % $(NH_4)_2S$ in water were added. After 30 min the ganglia were fixed in fixative II (see below) for 2–4 h and then cleared with increasing concentrations of glycerol. From these preparations photographs were taken through a dissecting microscope usually with incident light. If the ganglion was to be used for electron microscopy, after precipitation of CoS for 20 min, it was rinsed for a few minutes in several changes of seawater and transferred to fixative IV (see below) for 30 min, then cut into small blocks and fixed for a further 2 h.

2.4. *Fixatives and fixation techniques for electron microscopy*

We tried different fixatives. Consistently useful blocks were obtained with osmium fixatives I and IV. However, specimens of *Todarodes* for unknown reasons, showed better preservation when fixed with aldehydes (fixatives II and III).

Fixative I: OsO_4, 0.04 M; NaCl, 0.17 M; sucrose, 0.35 M; Na-cacodylate, 0.1 M; in seawater diluted 1:1 with H_2O, pH 7.4. For perfusion.

Fixative II: Paraformaldehyde 1 M; glutaraldehyde, 0.1 M; sucrose, 0.2 M; NaCl, 0.3 M; $CaCl_2$, 0.018 M; Na-cacodylate, 0.1 M; in H_2O, pH 7.4.

Fixative III: Glutaraldehyde, 0.35 M in seawater; pH adjusted to 7.8 by NaOH.

Fixative IV: as fixative I, but OsO_4, 0.08 M. For immersion.

For vascular perfusion, the animal was anaesthetized in 0.1 M urethane in seawater. Complete anaesthesia was reached after less than 1 min. A hole was cut into the body from the dorsal side at the level of the systemic heart. A plastic tube was inserted into the cephalic aorta near the systemic heart, the blood was rinsed by fast perfusion of about 10 ml seawater containing 3.7 mM procaine, and 15–30 ml of fixative were passed at a flow rate of about 3 ml min^{-1}. The perfusion-fixed stellate ganglia were dissected, cut into slices and immersed in fixative for 2–4 h. Aldehyde-fixed ganglia were then washed and postfixed in fixative IV for 2 h. After this many blocks were impregnated for 30 min at room temperature with saturated uranyl acetate in pure ethanol. The blocks were dehydrated in ethanol and 1,2-epoxypropane and embedded in Durcupan or ERL (Spurr 1969). The giant synapse was located in semithin sections with the light microscope. Thin sections were stained with lead citrate.

2.5. *Perfusion with fixatives containing high concentrations of* Mg^{2+} *and* Ca^{2+}

The squids were left undisturbed in a large round tank for at least 5 h after they were brought in from the sea. They were then taken from the tank very gently in a large net and placed by hand in seawater with 0.09 M urethane. It was possible to anaesthetize a squid that had not produced a single escape jet during handling (animals that had ejected ink were not used). The animal was then perfused with an oxygenated isotonic solution of 0.34 M $MgCl_2 . 6H_2O$; HEPES buffer, 0.01 M; NaOH, 0.01 M; and urethane 0.1 M, pH 6.6, for 5 min, followed by 15 ml of this solution with 0.04 M OsO_4 instead of urethane. Urethane has been found to block transmitter release in the squid synapse (R. Miledi, unpublished). The time between anaesthesia and the beginning of perfusion was about 4 min.

2.6. *Incubation of ganglia in artificial seawater*

To assess the effect of incubation in artificial seawater for longer times (under 12 h), as is frequently necessary when electrophysiological experiments are carried out, we left two isolated ganglia in oxygenated artificial seawater at 13 °C for 12 and 15.5 h (NaCl, 0.58 M; 804 ml; $MgCl_2 . 6H_2O$, 0.37 M, 146 ml; $CaCl_2$, 0.39 M, 28 ml; KCl, 0.58 M, 18 ml; Tris-maleate buffer 0.005 M, pH 6.0). These ganglia were fixed by immersion in fixative IV.

3. Results

3.1. *Innervation of the giant motor axons*

The muscles in the mantle of the squid are innervated by two systems of motor axons that originate in the stellate ganglion (Young 1938). One of these systems consists of many small axons, while the other is made up of about 10 giant motor axons (figure 1) that are activated synchronously, to impart fast jet propulsion to the squid. These giant motor axons receive all their input through the preganglionic nerve, which contains several giant nerve fibres. In his original account Young (1939) noted that the giant motor axons receive innervation from one main and one accessory presynaptic giant fibre. However, Miledi & Slater (1966) noted that two to four preganglionic fibres establish synaptic connections with the giant motor axon.

Figure 2 illustrates intracellular potentials recorded simultaneously from the postsynaptic axon (top traces) and from the main presynaptic axon (lower traces) in a stellate ganglion of the squid *Loligo vulgaris*. With a weak stimulus to the preganglionic nerve, only a subthreshold synaptic potential was recorded from the postsynaptic axon (*a*). Increasing the intensity of the stimulus gave rise to an earlier and larger synaptic potential, that triggered an action potential in the motor axon; but still no impulse was initiated in the main presynaptic axon (*b*). A slight

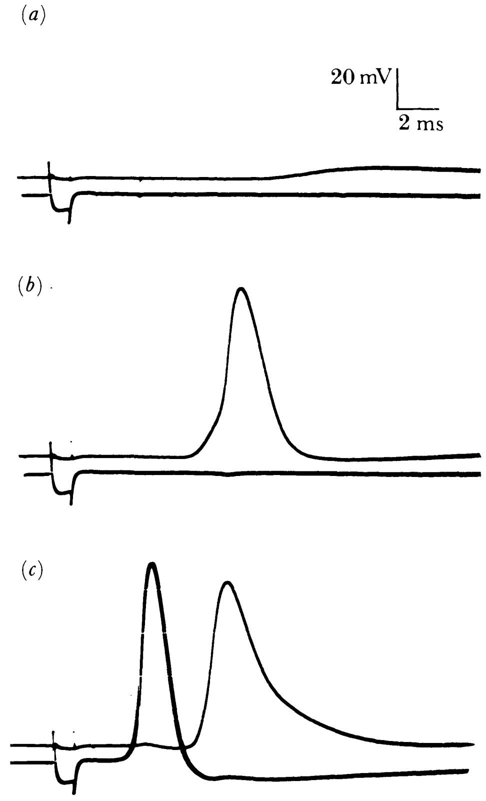

Figure 2. Preganglionic input to the giant motor axon in a stellate ganglion of *Loligo*. Intracellular membrane potentials recorded from the postsynaptic axon (top traces) and from the main presynaptic axon (lower traces). The preganglionic nerve was stimulated with pulses of increasing intensity (*a*–*c*). Postsynaptic potentials in (*a*) and (*b*) were evoked by impulses in two accessory presynaptic axons. In (*c*) the main presynaptic axon was additionally activated.

further increase in stimulus intensity triggered an impulse in the main pre-fibre and this generated a larger synaptic potential, that overlasted the action potential of the postsynaptic axon (*c*). Thus, this particular motor axon received inputs from, at least, the main pre-fibre and two accessory fibres. Occasionally, three accessory axons were found to synapse with the giant motor axon, and perhaps more will be discovered when a more extensive study is made.

The distribution of main and accessory presynaptic fibres is described below. Suffice to say here that sometimes we were able to identify, in the live preparation, the main and two accessory pre-fibres. In those cases the accessory fibres followed a similar route. Therefore, in this paper we shall simply say accessory fibre, without specifying if more than one accessory axons were present in the ganglion.

3.2. *Injection of dyes into giant axons*

Soon after starting to study the giant synapse of the squid, we found it desirable to establish better the course and distribution of the giant axons. In some squid, especially young ones, the stellate ganglion is transparent enough that some of the giant axons can be seen through most of their course. Usually, however, the branches of origin of the postsynaptic axons are not seen. To obviate this difficulty, and also to study the movement of substances introduced into the axons, various dyes were injected into the axons by iontophoresis through a micropipette. A large number of dyes were used, and some of these are already mentioned in the section on methods.

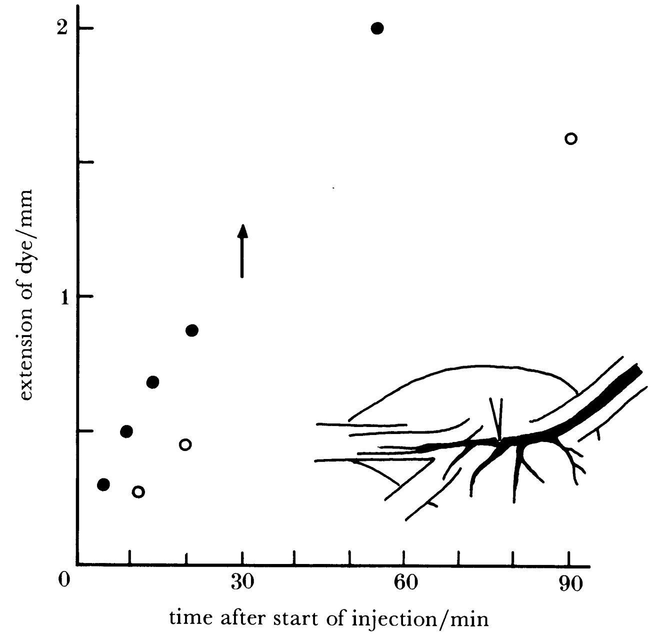

FIGURE 3. Intra-axonal movement of procion rubine dye in the main presynaptic axon (dots) and the L-4 postsynaptic axon (circles). In the plot for the pre-axon the first four dots denote downstream movement of the dye, while that at 55 min indicates upstream movement. The arrow marks the end of injection into the pre-axon. For the postsynaptic axon the three circles denote downstream movement of the dye. Stellate ganglion of *Loligo*, mantle length 67 mm. Diameter of pre-axon was 50 μm and of L-4 80 μm. Temperature 9.5 °C.

Soon after starting the iontophoretic pulses of dye, and sometimes even after a single pulse, a small spot of dye was clearly visible, inside the axon, around the dye pipette. As the injection continued the spot grew and the dye filled progressively more of the axon. Usually the extension of dye was symmetrical but occasionally the dye diffused slightly faster downstream, that is, away from the cell bodies. As expected, the length of axon over which the dye could be visually detected extended more rapidly in the smaller axons.

At the level of resolution used in these experiments, there was no obvious difference in the rate of movement of dye in pre- or postsynaptic axons (figure 3). However, different dyes clearly moved at different rates and, for instance, methylene blue moved slower than fluorescein. Figure 4 shows the movement of fluorescein and Procion Rubine injected into two giant motor axons in the same stellate ganglion. The figure shows the length of axon filled with dye, plotted against the time after the start of the injection. It should be noted, however, that the 'edge' of the dye-filled region is difficult to determine precisely and was simply monitored visually. Thus, it seems likely that a faster rate of movement will be measured when better methods of detection are used.

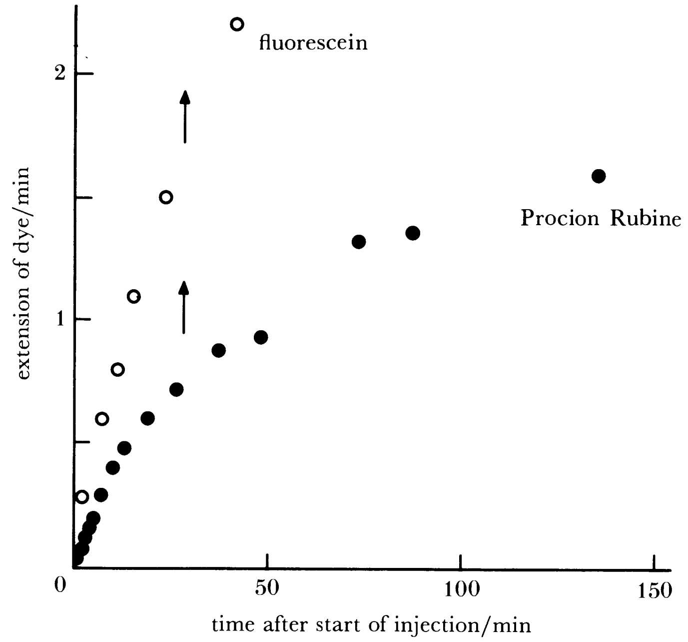

FIGURE 4. Intra-axonal movement of fluorescein and Procion Rubine in a stellate ganglion of *Loligo*. Mantle length 56 mm. Ordinates indicate total length of axon filled with dye. Circles: fluorescein into L-1, diameter 100 μm, temperature 9 °C. Dots: Procion Rubine into L-2, diameter 75 μm, temperature 8.5 °C. The arrows indicate the time at which the injections were stopped. The concentration of Procion Rubine near the site of injection was about 10 mg ml^{-1} and the limit of detection at the edge of the injection was about 0.1 mg ml^{-1}.

Several other features emerge from these experiments, only some of which will be briefly mentioned here. The movement of injected dye along the axon depends on several factors, including the rate of supply of dye and its permeability across the axon membrane. It appears that fluorescein diffuses in the axon faster than Procion Rubine and Fast Green because the latter dyes bind more strongly to intra-axonic material, and diffuse much more slowly than in free solution. Also, fluorescein comes out more rapidly from the axon so the end result is that both Procion Rubine and Fast Green yield nicer and brighter preparations that remain stained for many hours (figure 1). When compared with small capillaries filled with dyes and placed along the injected axons, these appeared as if the dye concentration had reached about 5 mM for fluorescein, and 10 mg ml^{-1} for Procion-Rubine-injected axons.

Dye injections rendered the axons and their branches, and sometimes even the cells of origin, clearly visible. This allowed us to study the patterns of ramification of pre- and postsynaptic axons (see below). Moreover, in some cases dye-filled branches from one giant motor axon were seen making anastomosis with the neighbouring motor axon. Therefore, the electrical coupling that is known to occur between the motor axons (Miledi 1967), is due, at least partly, to direct continuity between some of their branches.

3.3. *The distal giant synapse of Loligo vulgaris*

3.3.1. *The ramification pattern of the second order giant axon in the stellate ganglion*

The second order giant fibre which usually is the thickest axon in the mantle connective (figure 1), conducts impulses from the palliovisceral lobe of the brain to the 8–12 third order giant axons in the stellate ganglion that innervate the mantle muscles via the stellar nerves. To reach all the postsynaptic axons the main presynaptic fibre pushes out branches like fingers of a hand (figures 5–10, plate 2; figure 11). These branches run parallel to the postsynaptic axons over considerable distances that in fresh or injected preparations can be measured through the stereomicroscope (table 1). Along the two parallel fibres are a large number of synaptic contacts.

It is evident from the cobalt- and dye-injected preparations that the branching pattern of the presynaptic fibre varies considerably from one animal to the other (cf. figure 11), and even

TABLE 1. THE LENGTH† OF THE DISTAL GIANT SYNAPSE AND THE DIAMETERS‡ OF THE SECOND AND THIRD ORDER GIANT AXONS

mantle length of specimens	length of synapse on L	length of other synapses	largest diameter of pre-fibre at synapse	diameter of postfibre at synapse
mm	mm	mm	µm	µm
43	—	L-2:0.5	—	—
45	0.6–0.7	—	28	100
47	0.67	—	*ca.* 50	—
55	0.78	—	—	—
60	—	L-1: 0.5, 0.7	—	—
60	0.5	—	—	
67	1.0	L-3: 0.7	50	—
70	0.9	—	—	—
70	1.2	—	—	—
72	—	—	30	110
75	0.7	—	80	—
76	0.9	—	—	—
80	1.0	—	80	165
80	—	—	90	—
100	0.75	—	75	—
150	—	—	88	300
160	1.0	—	*ca.* 100	—

† Measured from fresh or injected specimens.

‡ Determined in fixed specimens (fixative IV) by measuring the areas of the axons from 300- to 500-times-enlarged cross sections with a planimeter and by calculating the diameters of circles with these areas.

between the two ganglia of the same animal. The number of branches derived from the main trunk may vary from three (figure 11*l*) to nine (figure 11*i*). One primary ramification may form secondary, tertiary or quaternary branches (figure 11*c*). Figure 11*h*, *m* show branches that run backwards over a more proximal branch. Frequently the branches lie on top of the postsynaptic axons, that is, towards the surface of the ganglion, or laterally, but sometimes a branch may bend around the third order axon and proceed below it (figure 12*a*). We also found a specimen with two presynaptic branches on the main postsynaptic axon (figure 11*e*). Figure 12 shows several second order axons each with a long branch running parallel to the postsynaptic fibre and a short branch contacting a more central part of the same fibre. These twin distal synapses were found in 7 out of 14 ganglia.

Some variations may be observed also in the course of the postsynaptic axons. There was a ganglion in which the last stellar nerve did not have a giant axon; in this specimen the largest

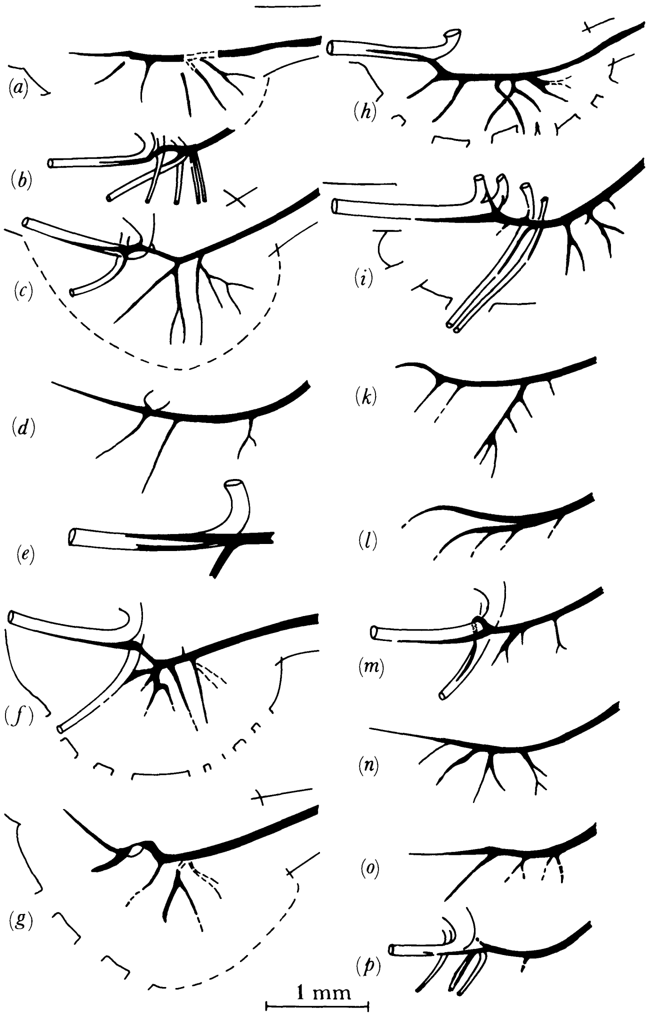

FIGURE 11. Ramification pattern of second order giant axons in the stellate ganglion of 15 different specimens of *Loligo vulgaris*. The drawings of axons (*a*), (*c*), (*d*), (*f*), (*g*) are taken from photographic negatives and are to scale (× 40): The other axons are freehand drawings and are not to scale. The axons were injected *in vivo* either with dyes or with cobalt ions. In several axons the marker had not diffused into all branches. This fact explains why there are often fewer branches than third order axons; also the branches may be longer than shown. In case (*i*) the giant axon is absent in the penultimate stellar nerve (L-1), and in L-2 there are two giant axons.

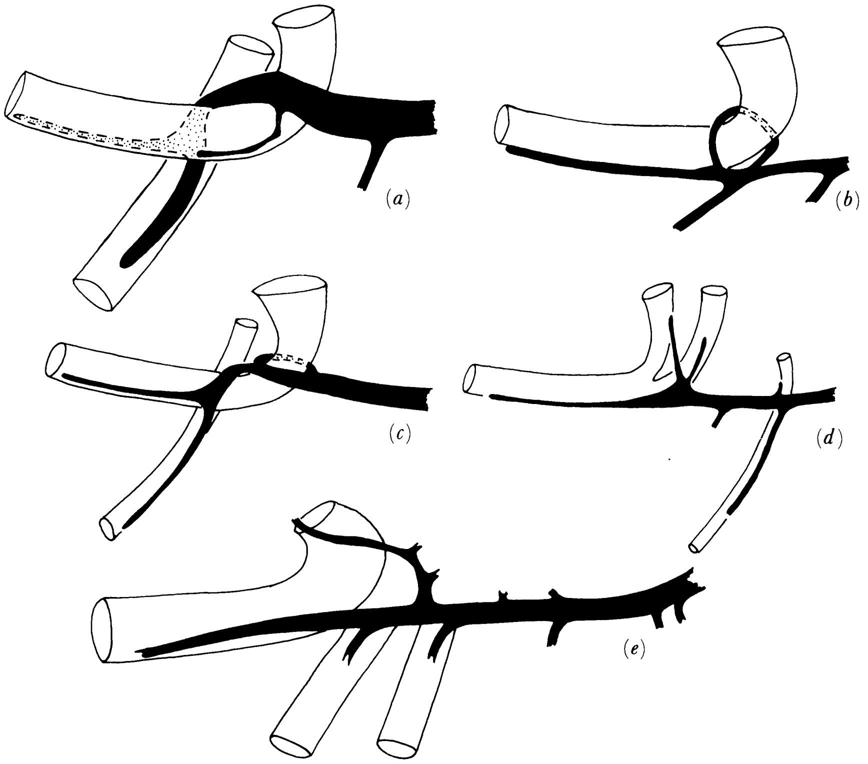

FIGURE 12. Enlargements showing details of the last giant synapse from the ganglia in figure 11*c*, *d*, *g*, *i*. Two or more branches of the presynaptic fibre (black) terminate on the same postsynaptic fibre at two different levels. Not to scale.

postsynaptic axon was L-1. In another ganglion the last axon was present, but it was smaller than L-1.

The branching pattern of the second order axon was found to be already fully developed in the freshly hatched larva (body length about 5 mm; figures 9 and 10). The second order axon was often thicker than the third order axon at this developmental stage.

3.3.2. *Dimensions of the distal giant synapse*

The data reported below were taken from low power electron micrographs (× 5000) of transverse and longitudinal sections. The transverse sections were cut at nine consecutive levels, 125 μm apart, extending from the bend of the main postsynaptic axon to the tip of its presynaptic fibre. The longitudinal sections were cut through the whole length of the third largest synapse (L-2). The specimens of *Loligo vulgaris* had mantle lengths of 18, 15 and 4 cm, and were fixed by perfusion with fixative I. Details of one of these synapses are shown in figures 17, 21, 22 and 25.

We estimated the length of the largest of these synapses by counting the number of 2 μm thick sections that were discarded between two levels plus an estimated length of 5 μm used up for the preparation of thin sections at each level. The estimated length was 1125 μm, a figure that agrees well with measurements of dye-injected preparations (table 1).

The presynaptic fibres taper progressively towards their tips (table 2), although in some instances abrupt transitions in diameter were observed. The presynaptic terminal of a specimen

Table 2. Summary of measurements and counts from the distal giant synapse of a specimen of *Loligo vulgaris* of 18 cm mantle length, taken from low power electron micrographs

(columns 1–9: the values at nine cross-sectional levels, 125 μm apart, from the proximal to the distal end of the synapse.)

	1	2	3	4	5	6	7	8	9
Circumference of the presynaptic fibre/μm	279	227	207	184	168	156	149	114	75
segment of prefibre with synaptic contacts (figure 13, A)/μm	145	132	124	106	89	62	54	37	—
number of synaptic contacts per cross section	24	28	21	25	19	16	5	3	—

estimated length of the synapse	1125 μm
estimated area containing synaptic contacts	81562 μm^2
estimated total number of synaptic contacts	14965
mean width of synaptic contacts (figure 13, B)	
in cross sections	1.25 μm ± 0.51 μm (s.d.)
in longitudinal sections	1.10 μm ± 0.28 μm (s.d.)
mean area of the single synaptic contacts	1.09 μm^2
estimated total area of synaptic contacts	16312 μm^2
length of processes (figure 13, C)	4–17 μm
thickness of sheath between pre- and post-synaptic axon (figure 13, D)	
average	5.8 μm
minimum	4 μm
maximum	14 μm

of 15 cm mantle length had a cross-sectional area of about 6000 μm^2 for the first 300 μm length of the synapse (measured with a planimeter on light micrographs), decreasing regularly to zero over the next 700 μm. The sum of the volumes of a cylinder of 300 μm length and a cone of 700 μm length, both with a base area of 6000 μm^2 is 3.2×10^6 μm^3 (= 3.2 nl). Similar calculations for the largest terminal in a smaller specimen (4.5 cm mantle length) resulted in a volume of 0.34×10^6 μm^3, while the second largest terminal (L-1) in a specimen of 4 cm mantle length had an estimated volume of 8×10^4 μm^3. Third order axons at the synapse are cylindrical with a nearly circular base. Their diameters are reported in table 1.

As shown schematically in figure 13 processes arising from the postsynaptic axon penetrate the sheath and end on the membrane of the presynaptic axon (figures 14, 16, plate 3; 21 and 22, plate 4). While this is the normal situation, in one out of about 40 specimens we found a long collateral of the presynaptic fibre that crossed the sheath and formed synaptic contacts with short processes of the postsynaptic axon.

Synaptic contacts were distributed over more than half the circumference of the pre-fibre (figures 14, 15 and 16, table 2) as the longest processes reach well up on the two sides. The synaptic segment (A in figure 13) of the presynaptic axon, spread out in a plane, approximates an equilateral triangle with a base of 145 μm, a height of 1125 μm, and an area of 81562 μm^2. In the nine segments examined the synaptic contacts covered about 20 % of the segmental width (sum of B, figure 13; table 2). Therefore, we estimate the area of synaptic contacts to be 16312 μm^2 (which is 20 % of 81562 μm^2).

The cross-sectional width of synaptic contacts ranged from 0.6 to 4 μm (mean 1.25 ± 0.51 μm s.d.). In longitudinal sections 44 synaptic contacts ranged in width from 0.45 to 1.64 μm

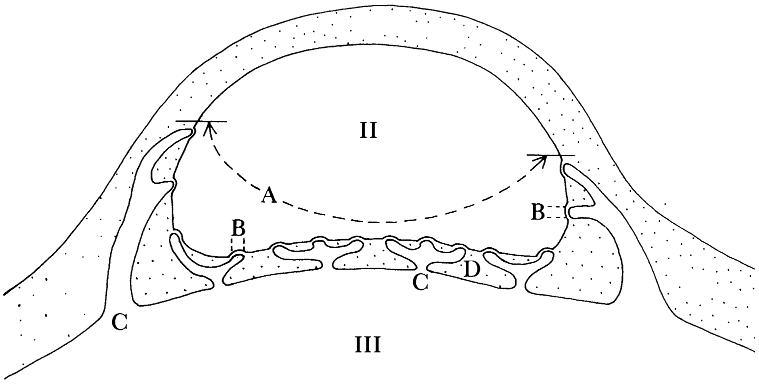

FIGURE 13. Schematic drawing of a cross-cut distal giant synapse. II, Presynaptic; III, postsynaptic axon; A, segment of the terminal with synaptic contacts; B, width of a synaptic contact; C, processes of the postsynaptic axon; D, the sheath of the two giant axons.

(average 1.10 ± 0.28 μm s.d.). Therefore, we estimate an average area per contact of 1.09 μm^2. Dividing the total area of synaptic contacts (16312 μm^2) by the mean area per contact (1.09 μm^2) results in a total number of 14965 synaptic contacts in this synapse.

A more direct estimate of the number of processes is possible from cobalt-injected postsynaptic axons, as that shown in figures 18, 19 and 20. An area of about 900 μm length shows denser black spots which appear as stars at higher magnification. Towards the bend of the axon and on its edge there are tree-like processes of up to 17 μm length. It is very likely that these stellate structures are the cobalt-filled processes of the postsynaptic fibre. The actual synaptic contacts are too small to be visible, and what is seen are the initial processes with some of their larger ramifications.

Young (1939) found an increase in the size of synaptic contacts and more abundant branching of the processes towards the distal end of the giant synapse. One of our specimens also showed very numerous contacts at the distal end (figure 16), but in another specimen the contacts appeared enlarged and were very rare at the end of the terminal. The density of contacts in particular areas appears therefore to vary greatly from specimen to specimen.

3.3.3. *Vesicles on the presynaptic side*

Typical synaptic vesicles of the squid synapse are electron-transparent, round and measure 54.4 ± 5.2 nm (s.d.) in diameter (Frösch & Martin 1972; figures 23–26, plate 5). So are the vesicles of the proximal synapses, and the vesicles of the giant synapse in larval squids, *Todarodes* and *Sepia*. The vesicles in a population are very homogeneous except that about 3 % are much larger (figures 23, 24 and 26). These large vesicles have an average diameter of 132 ± 37 nm (s.d.), often contain dense material, and are not in apposition to the synaptic membrane.

Usually, on both the pre- and postsynaptic sides, we observed some coated vesicles in areas covered by glia processes (figure 23). In freshly perfused specimens there were about three coated vesicles per 100 normal vesicles. Coated vesicles were often seen fused with the axon membrane forming a large opening to the cleft (figure 28, plate 6). They appeared to be more numerous near the end of the terminal.

Coated vesicles were much more numerous in synapses that had been incubated for 12–15 h in seawater. In these synapses we counted 23 coated per 100 normal vesicles. Often coated vesicles were lined up at the membranes of the terminal. The presynaptic area was also filled with patches of cytonet, the isolated coat material (figures 27 and 28; cf. Gray 1973). In these synapses the membranes of the presynaptic terminal, of postsynaptic processes and of glial cells showed deep invaginations, as if excessive membrane material had formed both on the pre- and on the postsynaptic side (figure 27).

DESCRIPTION OF PLATE 3

FIGURES 14–17. Light micrographs of the cross-cut second order axon (II) in synaptic contact with the third order axon (III) at the beginning (figures 14, 15 and 17) and at the end (figure 16) of the synapse. Figure 14 shows, on both sides of the presynaptic fibre, two very large postsynaptic processes (*), and many fine processes in the sheath between the two axons (arrow). At the end of the synapse (figure 16) the processes appear to be more numerous than further up. In Figure 17 axon L is contacted by two presynaptic fibres (arrows), the upper fibre in subsequent sections fused with the lower profile. Figures 14–16: plastic sections with toluidine blue staining; figure 17, interference contrast.

FIGURES 18–20. Photographs of a whole mount of a cobalt-injected third order axon. The presynaptic fibre and rest of the ganglion are not injected and practically invisible. The synaptic area is marked by numerous CoS-filled processes arising from the postsynaptic axon (dense spots in figures 18 and 20). Figure 19 shows large processes (arrows) in lateral view at the bend (be.) of the axon in the area of fusion (fu.).

DESCRIPTION OF PLATE 5

FIGURES 23–26. Enlargements of individual synaptic contacts. The postsynaptic processes (pr.) often contain as many vesicles as the presynaptic areas (figures 23 and 26). Coated vesicles are also found close to the membranes (figures 23 and 24, arrows). When synapses were fixed in isotonic magnesium the presynaptic vesicles were often more numerous, and the vesicles in the processes were seen near the postsynaptic membrane (figure 26). The large arrow (figure 26) points to one of the very large dense-cored vesicles.

DESCRIPTION OF PLATE 6

FIGURES 27 AND 28. Synaptic areas of giant synapses from ganglia incubated in a seawater bath for 12 and 15 h. Note folding of the membranes, and the many coated vesicles (arrows), some in fusion with the presynaptic and postsynaptic (figure 27) membranes. Patches of osmiophilic material (* figure 28) which presumably form the vesicular coats (cytonets) are abundant.

FIGURES 29 AND 30. Two sections showing details of large post-synaptic processes (pr.) with masses of vesicular profiles and mitochondria. The larger vesicles contain inhomogeneous osmiophilic material.

DESCRIPTION OF PLATE 7

FIGURES 31 AND 32. Branched filamentous material (fil.), bundles of microtubules (arrows) and vesicular organelles in the axoplasm of a longitudinally cut postsynaptic axon of an osmium-fixed specimen.

FIGURE 33. Phase contrast micrograph of an obliquely cut synapse. The pores in the synaptic area (s.a.) presumably are the postsynaptic processes in the sheath. Note the dense patches in the post-, but not in the presynaptic axon (arrows).

FIGURE 34. Electron micrograph of one of the dense patches (see figure 33) in the postsynaptic axoplasm. They consist of granular osmiophilic material and mitochondria.

FIGURE 35. Finger-like extensions of the perinuclear area of a sheath cell (sh.) into the postsynaptic axon. Similar extensions were also found in the presynaptic fibre. cl., Collagen in extracellular layers of the sheath.

DESCRIPTION OF PLATE 8

FIGURES 36–39. The giant synapse in the freshly hatched larva of *Loligo vulgaris*. The mantle connective (m.c.) contains one giant axon (figure 36, light micrograph). In the synaptic area the presynaptic fibre is about as thick as the postsynaptic axon (figure 37). Synaptic contacts are well differentiated (figure 38). Synaptic vesicles often appeared fused (figure 39). sh., Sheath cell processes.

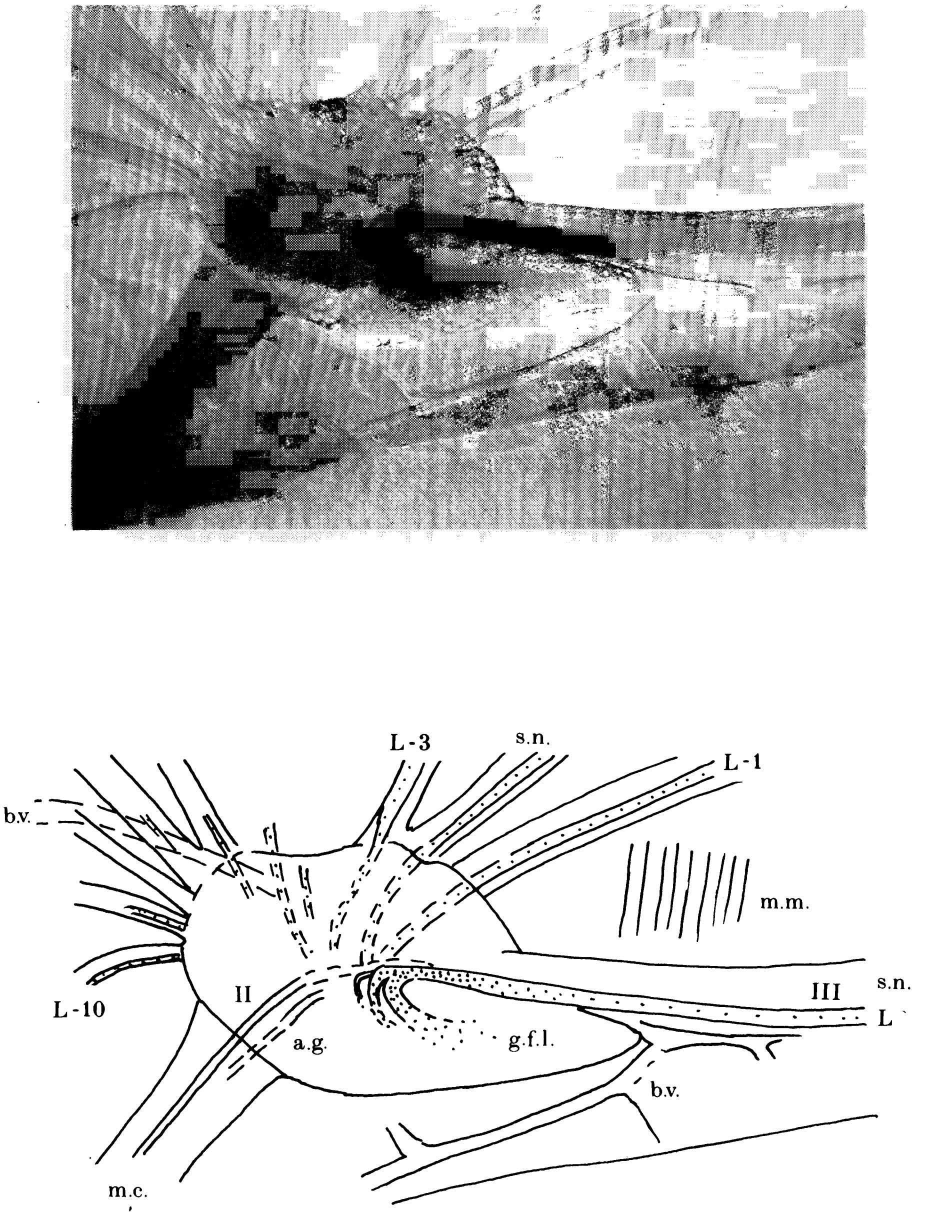

FIGURE 1. A living preparation of the stellate ganglion of the squid, *Loligo vulgaris*, as seen on the mantle muscle (m.m.) in transmitted light, during injection of Fast Green into the main (L) postsynaptic giant fibre (III). In the mantle connective (m.c.) the second order giant axon (II) and an accessory axon (a.g.) are detectable. The third order axons originate by fusion of a large number of axons the perikarya of which are accumulated in the giant fibre lobe (g.f.l.). Each stellar nerve (s.n.) includes a giant fibre in decreasing thicknesses (L, L-1, L-3, L-10). The ganglion is supplied by large blood vessels (b.v.).

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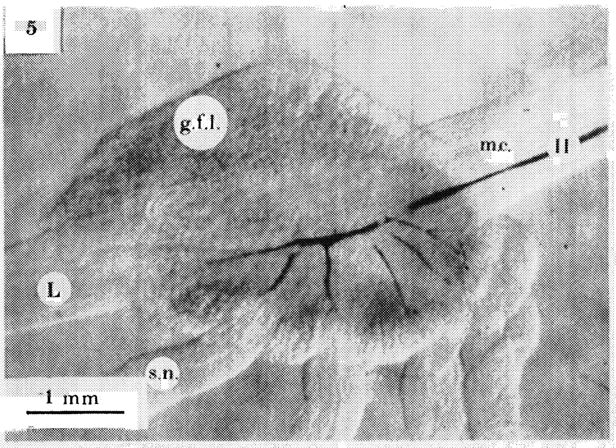

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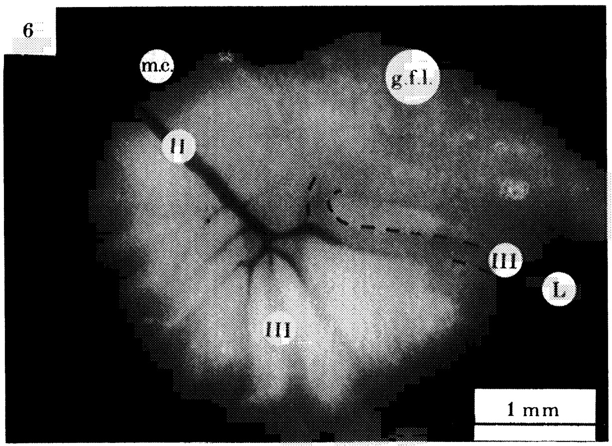

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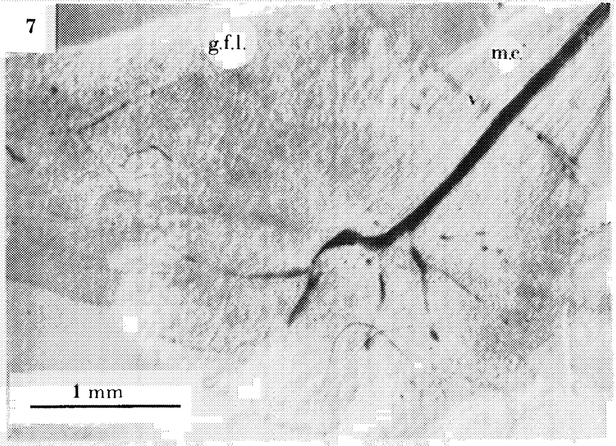

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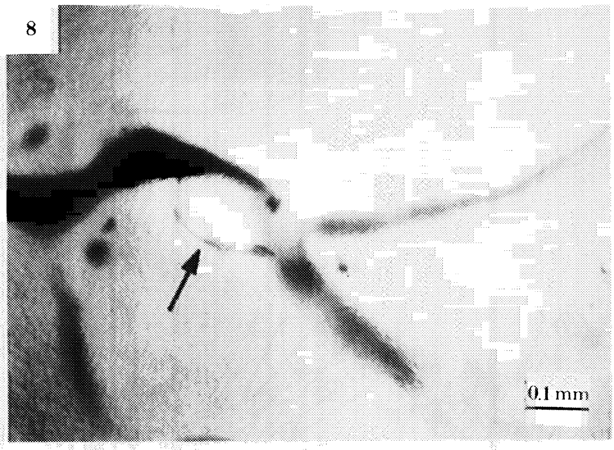

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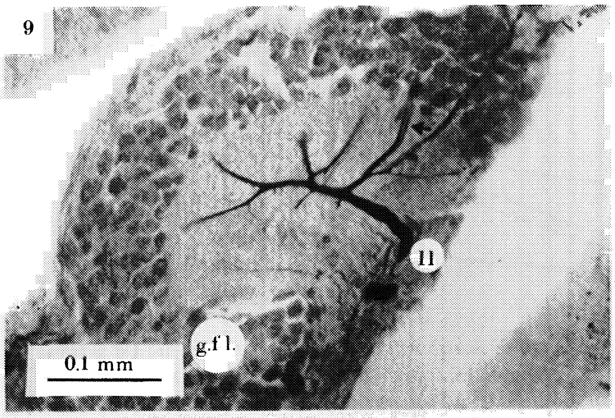

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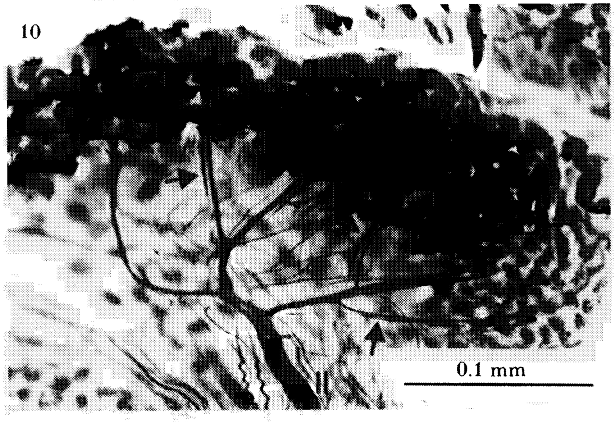
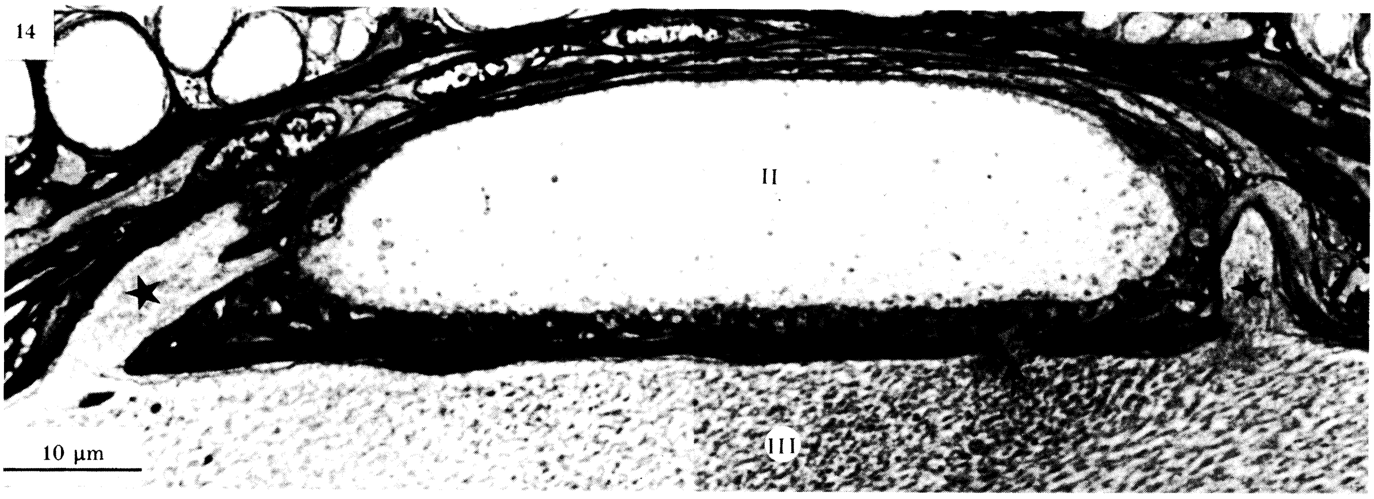
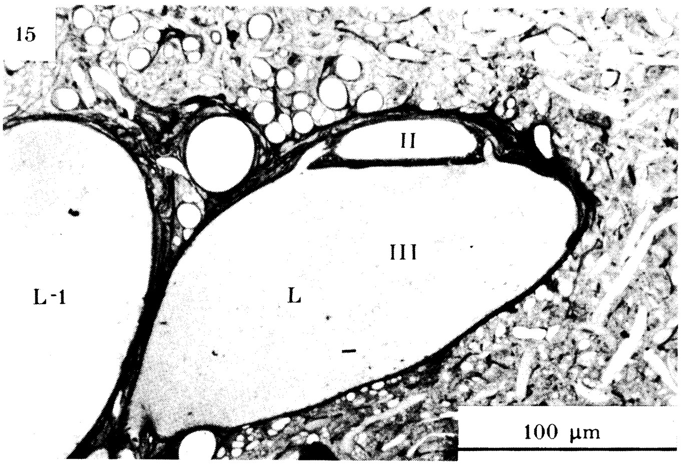
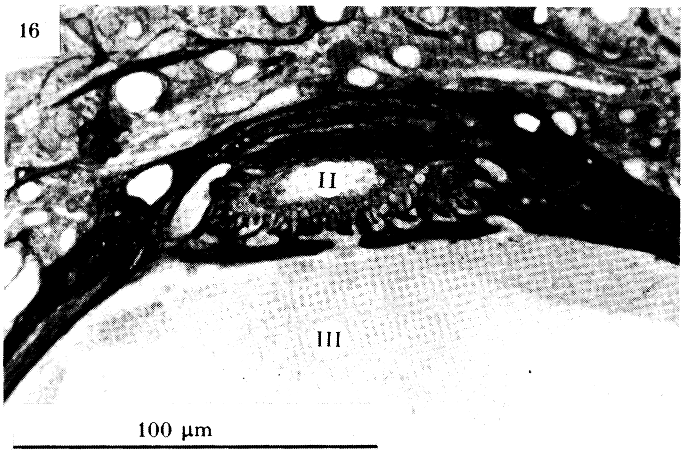


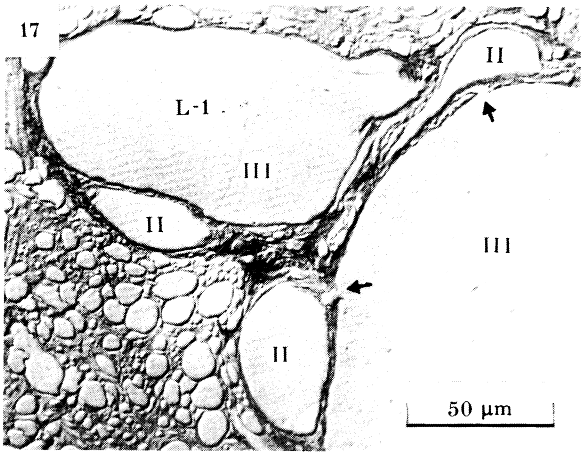
FIGURES 5–8. Stellate ganglia of *Loligo vulgaris* with cobalt injected second order (II) giant axons. In figure 6 several third (III) order axons are visible. Figure 8 is an enlarged part of figure 7: a small branch (arrow) with varicosities surrounds the third order axon in its bend (see also figure 12*a*). Abbreviations: g.f.l., giant fibre lobe; m.c., mantle connective; s.n., stellar nerve; L, last third order axon.

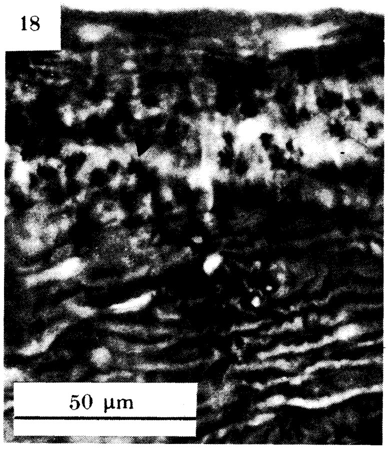
FIGURES 9 AND 10. Horizontal sections through stellate ganglia of freshly hatched larvae of *Loligo vulgaris*, stained with Bodian's method. The second order axons (II) are very large when compared with the third order axons (arrows).

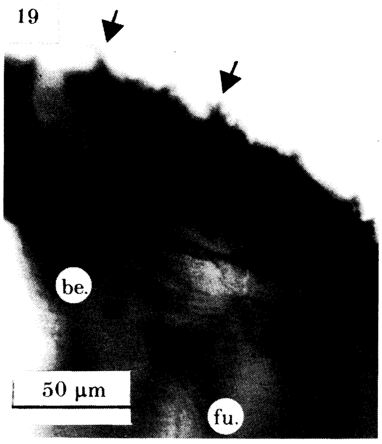


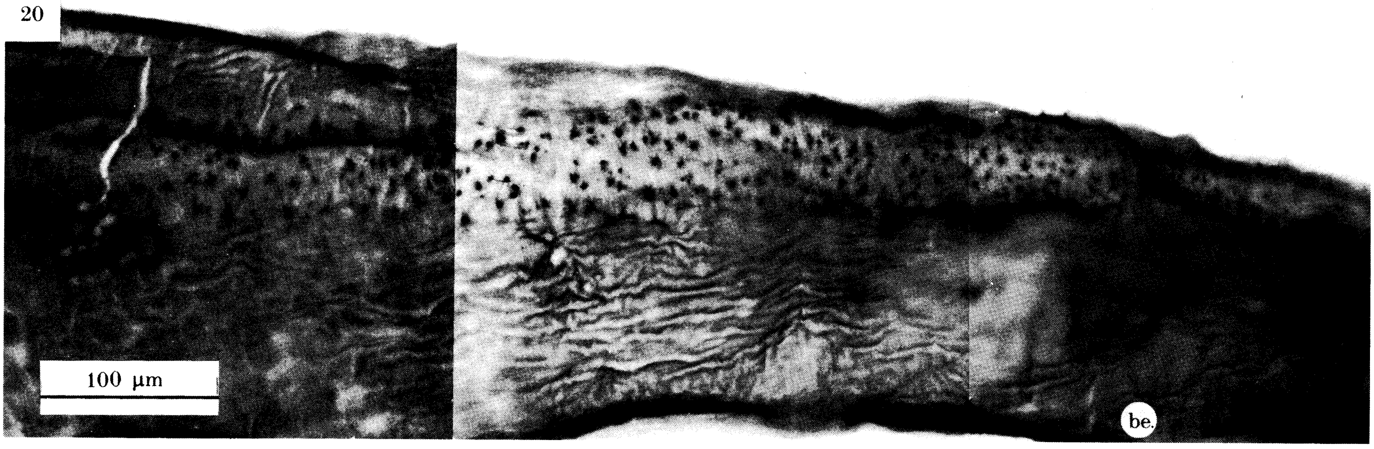












FIGURES 14–20. For description see p. 368.

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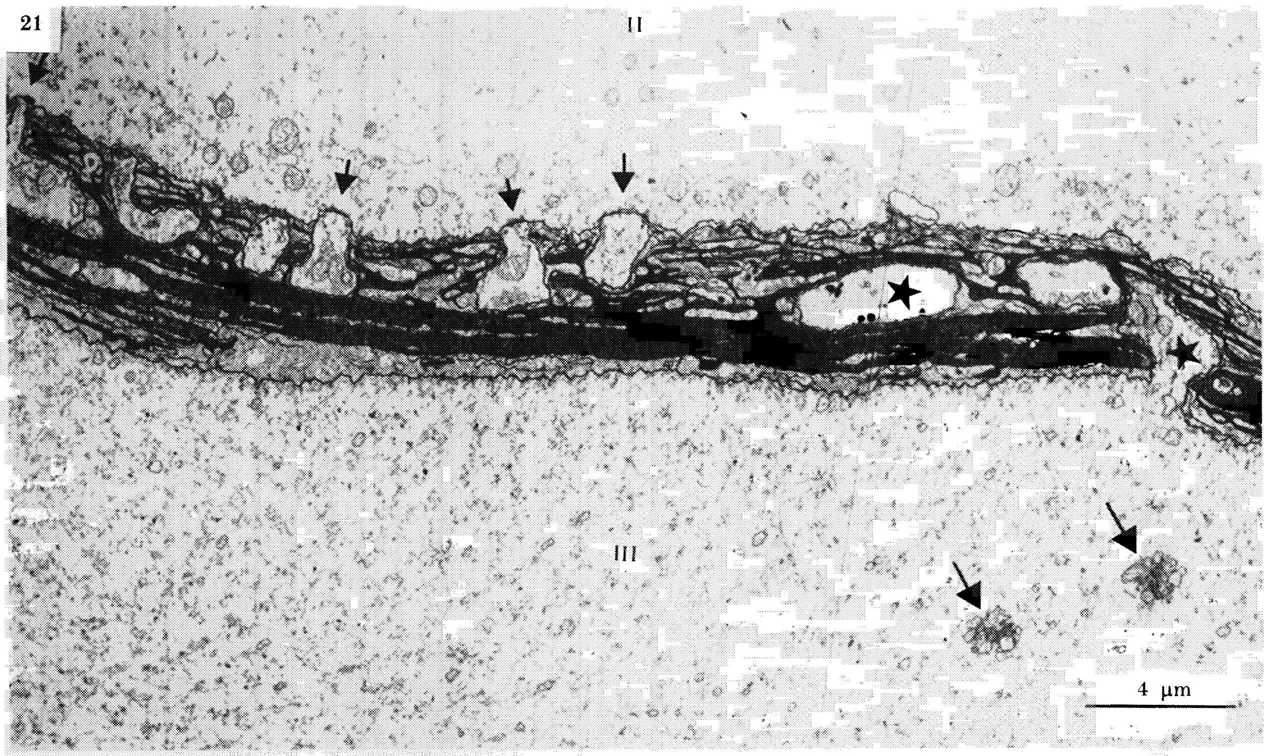

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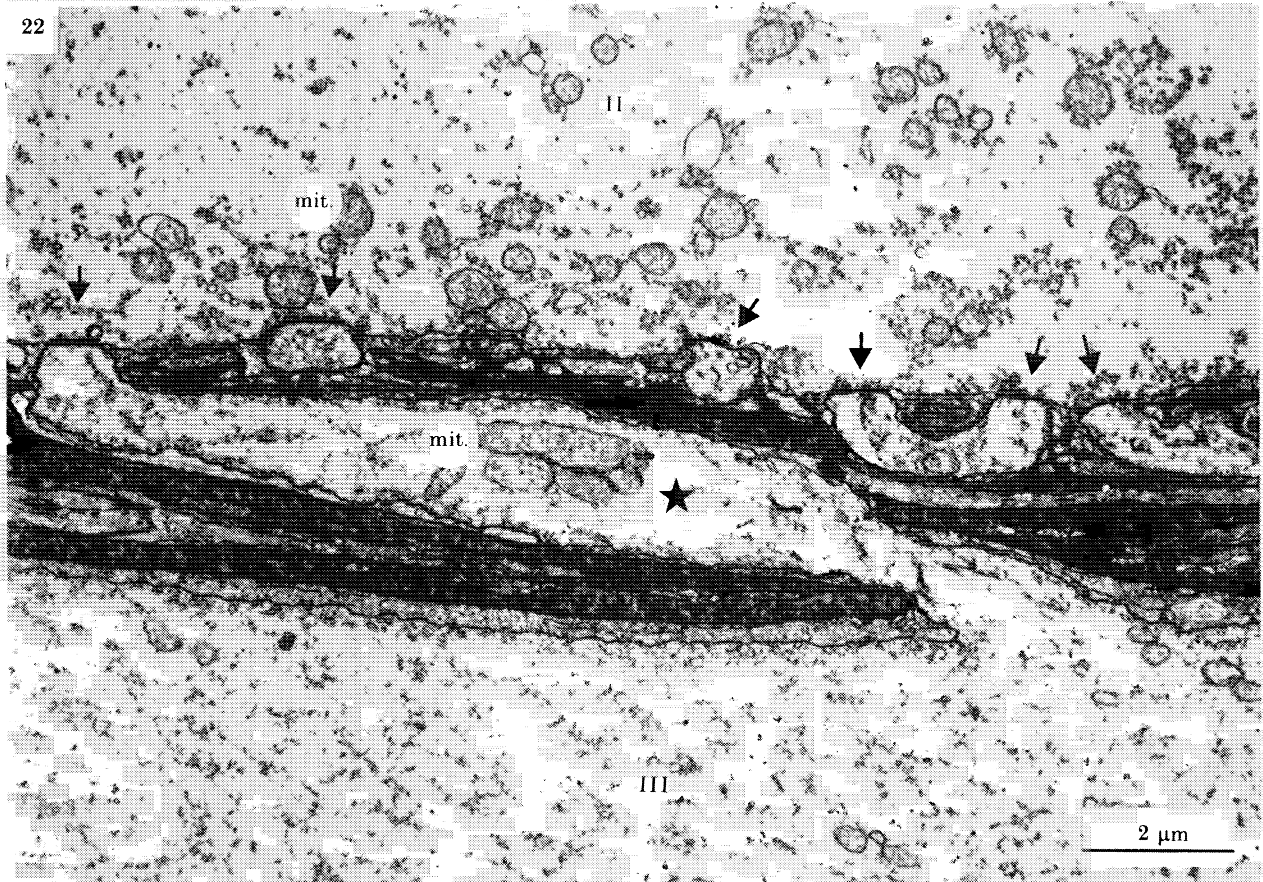

FIGURE 21. Electron micrograph of an area of the cross-cut presynaptic (II) and postsynaptic (III) giant axons with synaptic contacts (short arrows) and processes (*). The sheath between the axons is composed of extracellular material (black layers) and glial cell processes (grey layers). The axoplasm of the presynaptic fibre is less dense, owing to scarcity of filamentous and tubular material. There are two clusters of granular material and mitochondria in the postsynaptic axon (long arrows).

FIGURE 22. A process of the postsynaptic axon (III) penetrates the sheath and ends in synaptic contacts (arrows) on the presynaptic axon (II). The mitochondria in II and in the processes of III (*) are larger and much more numerous than those in the bulk of the third order axon (III).

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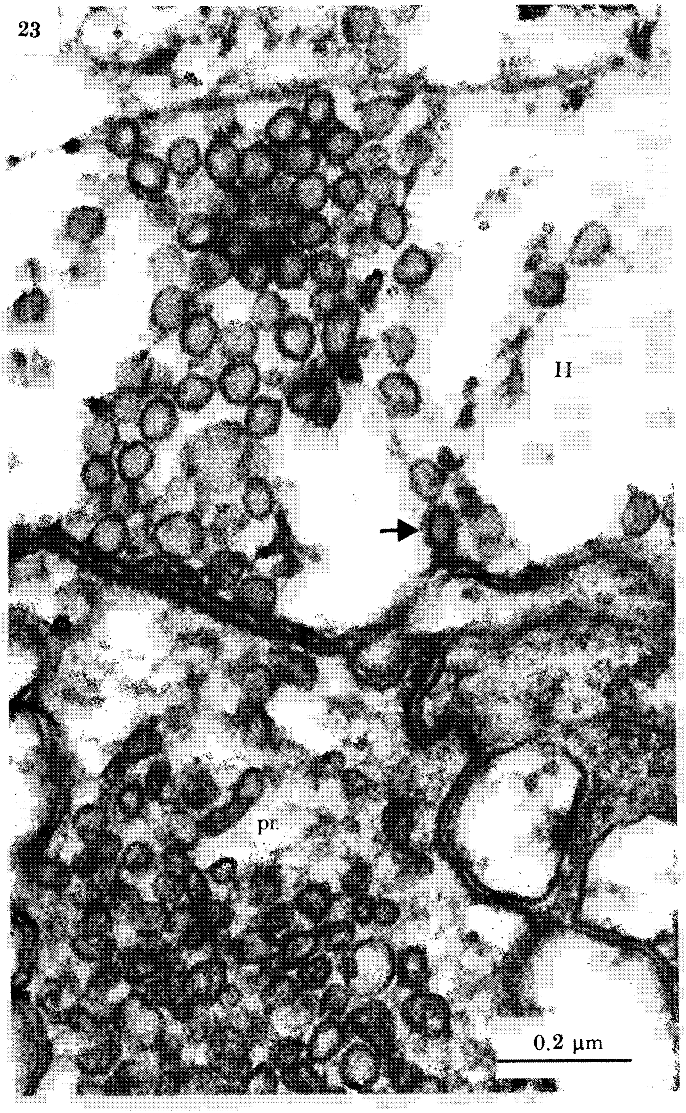

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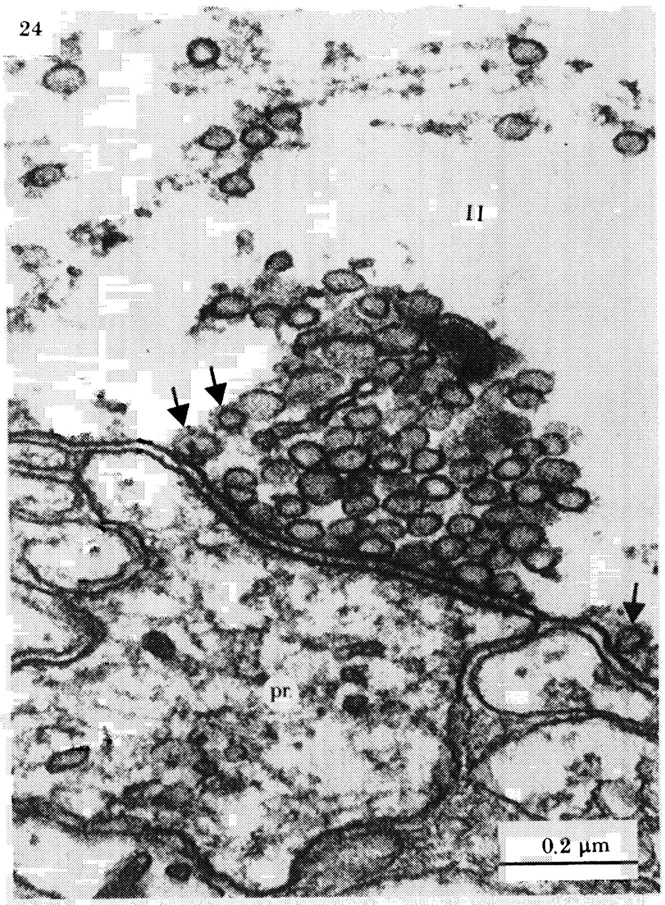

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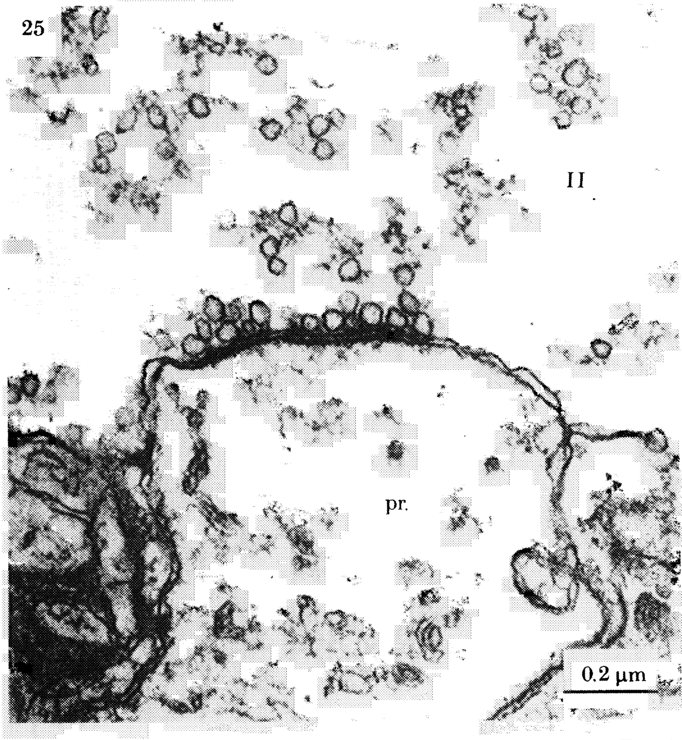

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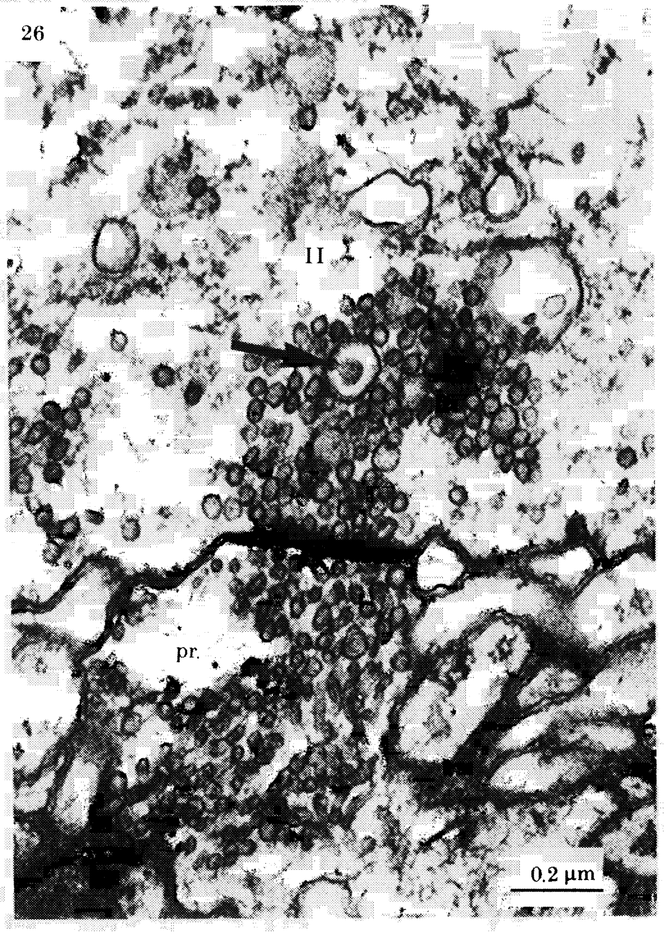

FIGURES 23–26. For description see p. 368

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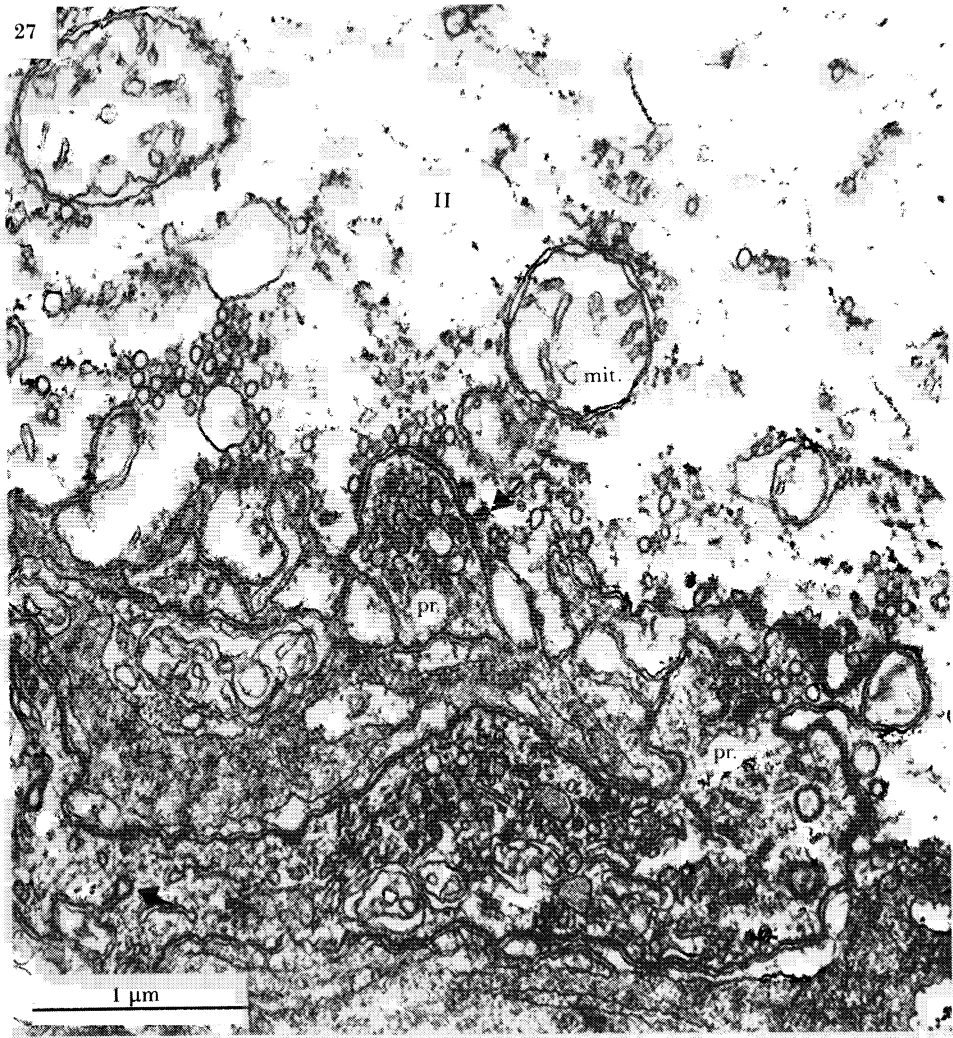

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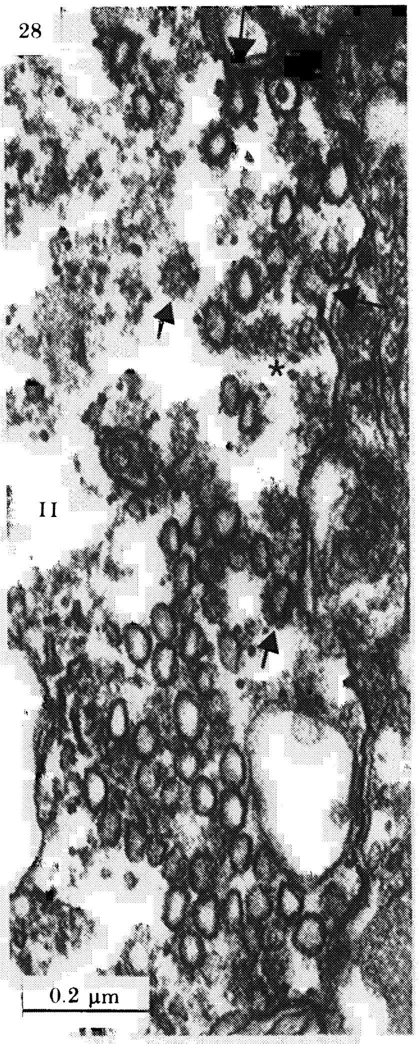

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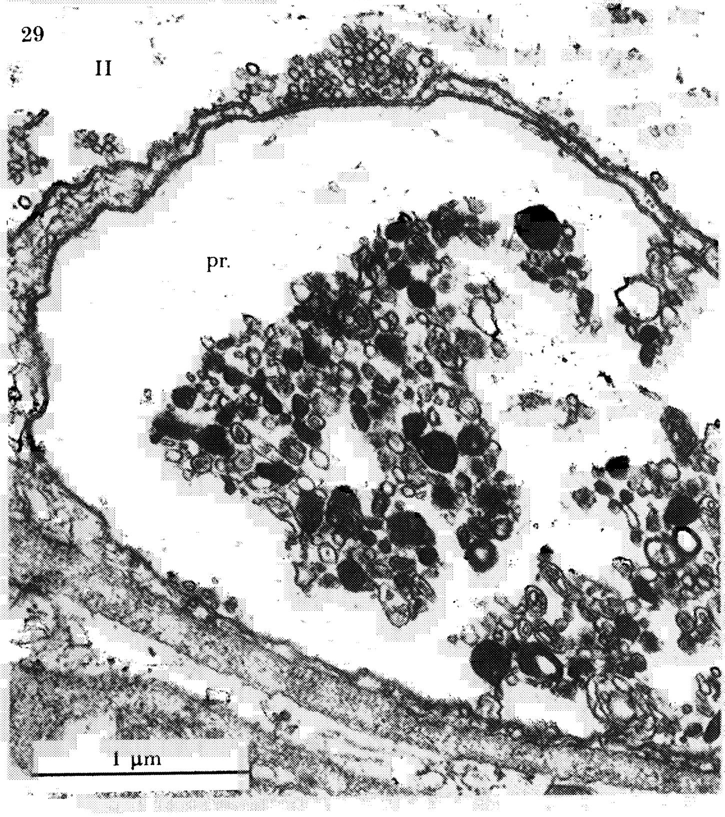

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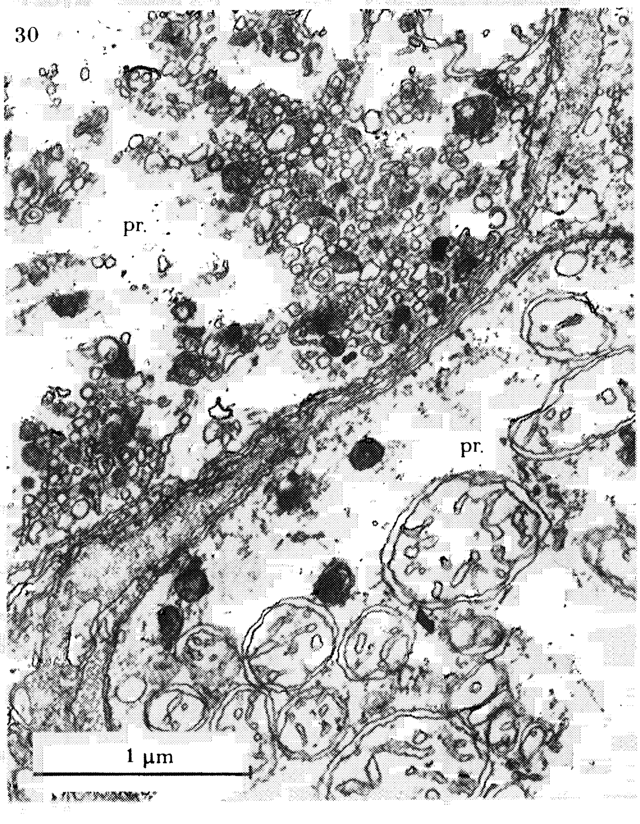

FIGURES 27–30. For description see p. 368

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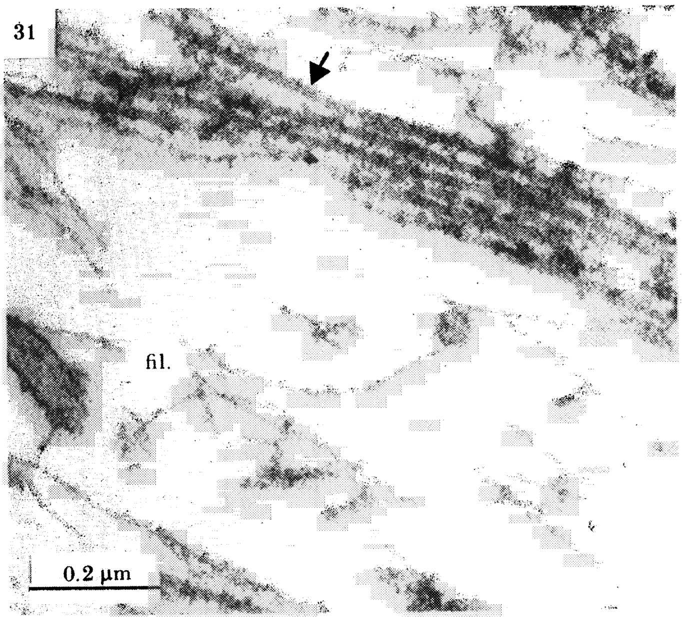

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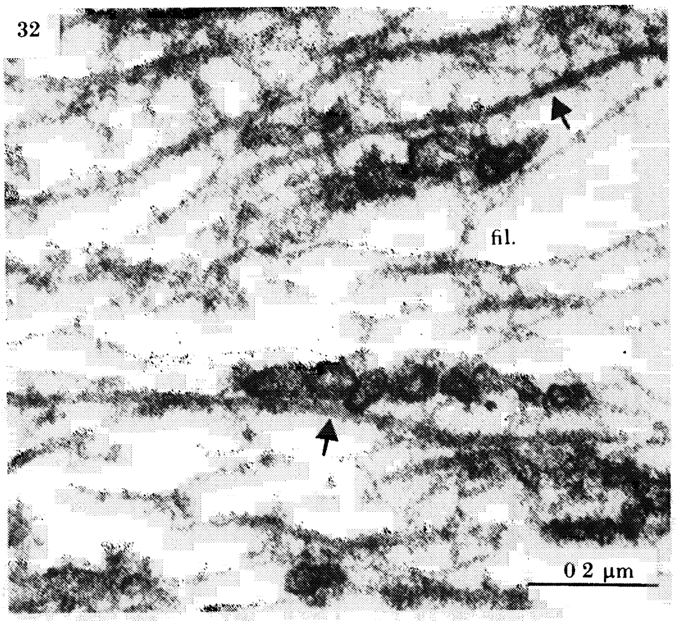

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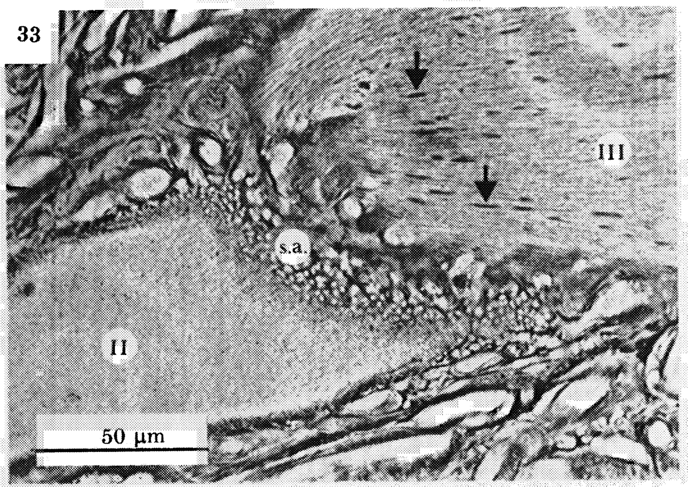

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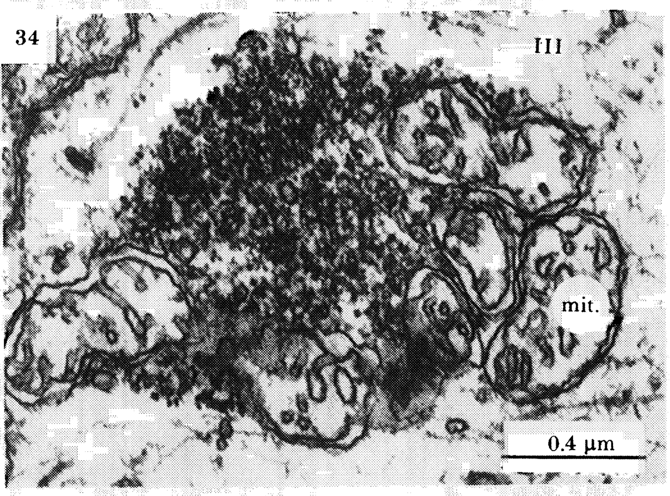

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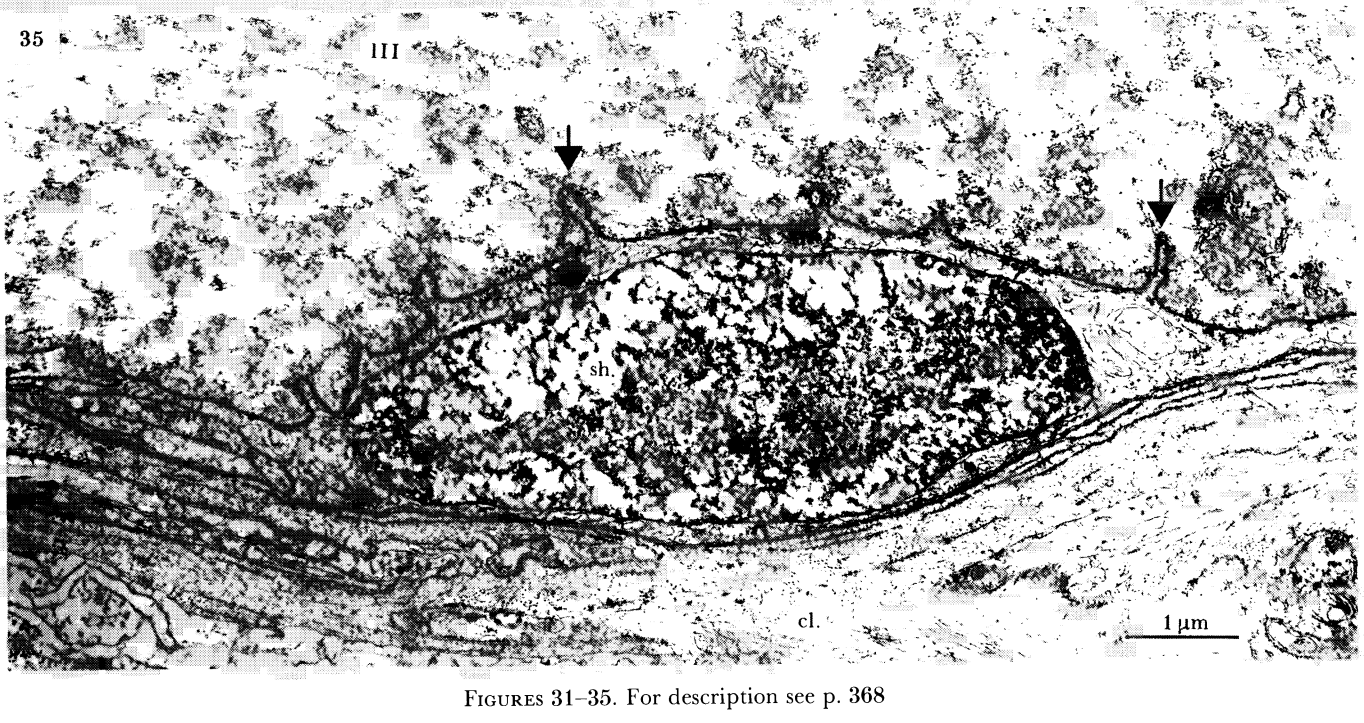
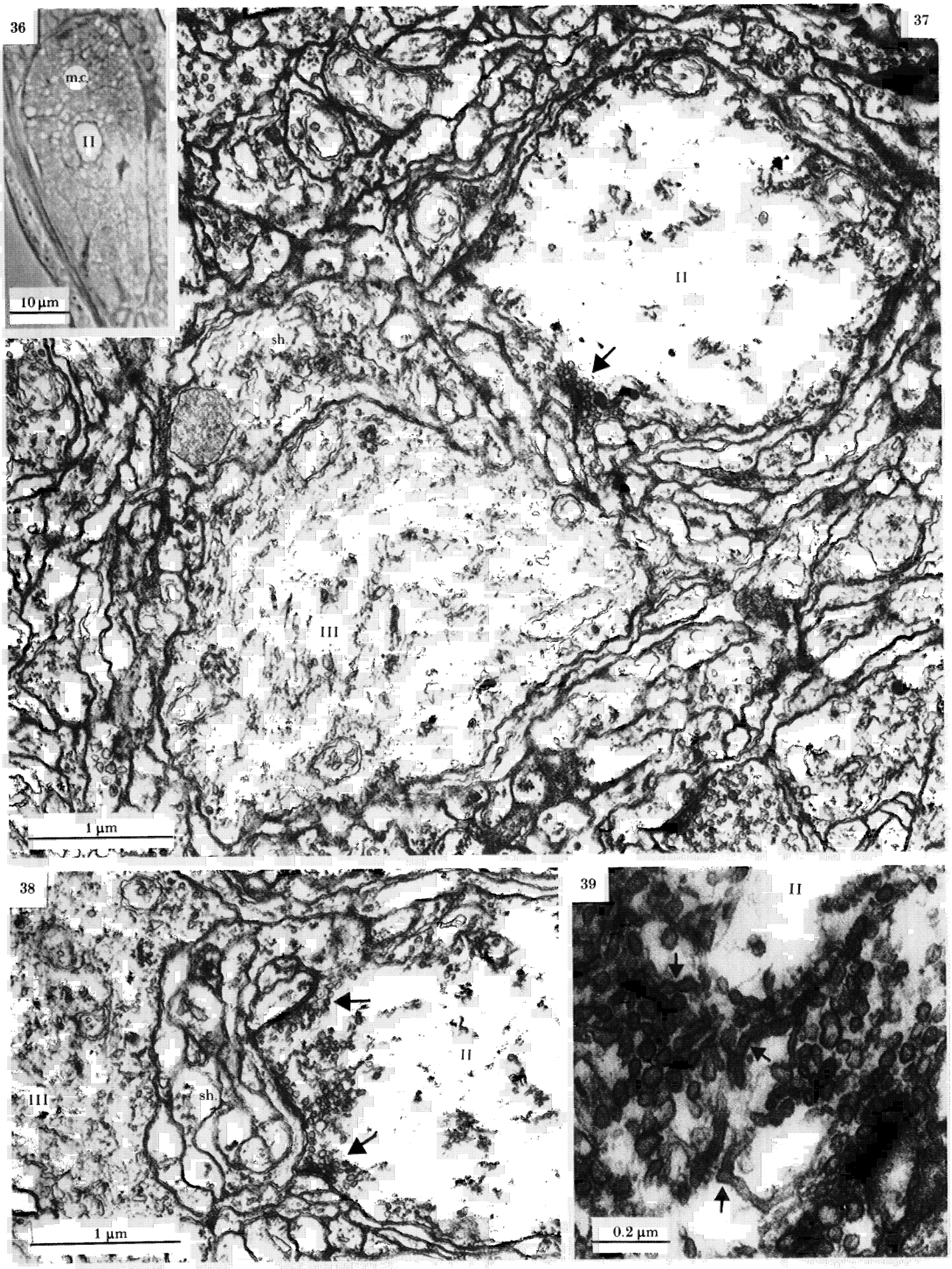
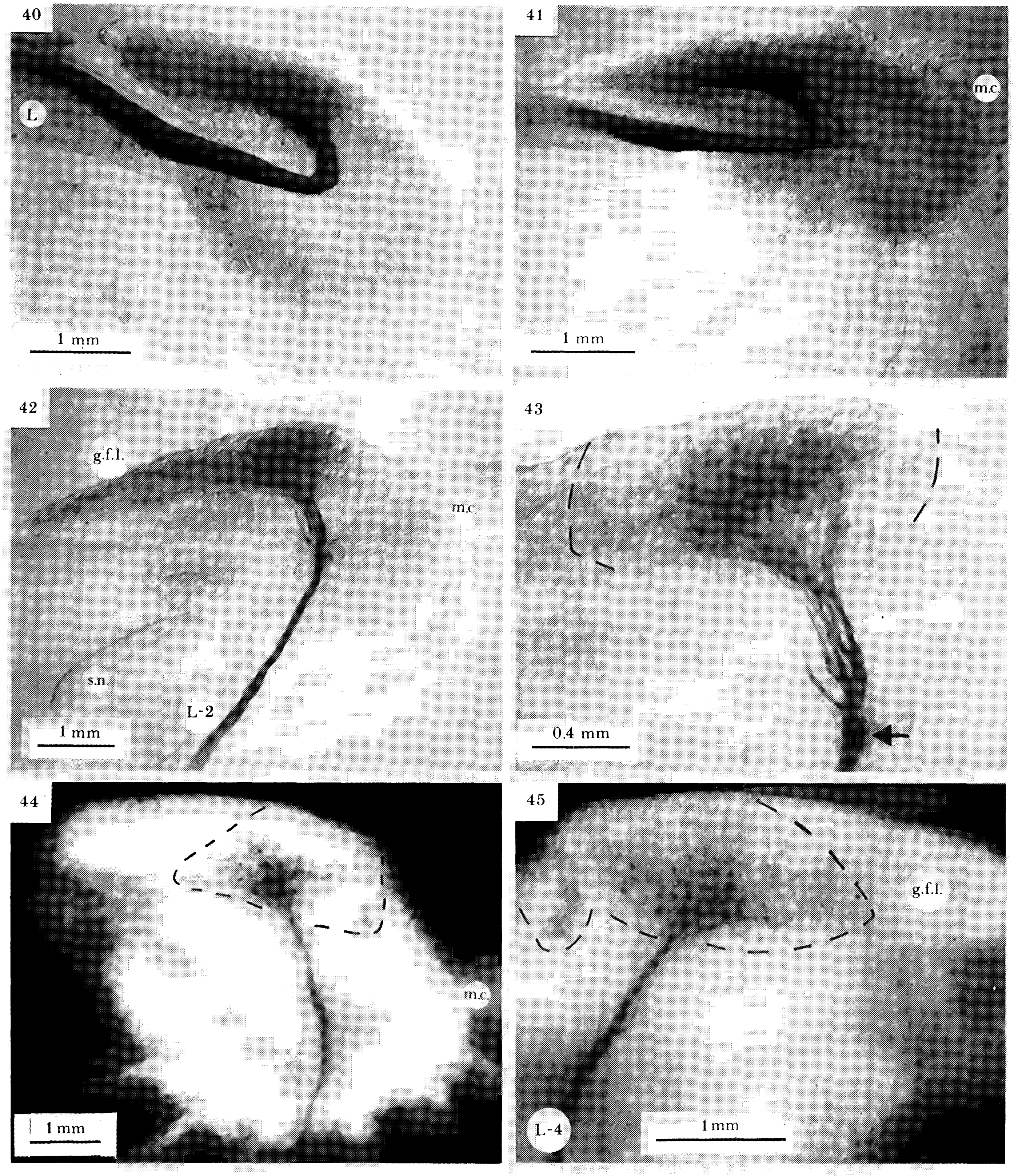


FIGURES 31–35. For description see p. 368



FIGURES 36–39. For description see p. 368



FIGURES 40–45. Cobalt injected third order giant axons. Abbreviations: g.f.l., giant fibre lobe; m.c., mantle connective; s.n., stellar nerve. Figures 40 and 41 show the largest axons (L) in two small squids of 7 cm mantle length, figures 42 and 43 the third largest axon (L-2) in a squid of 8 cm mantle length, figures 44 and 45 the fifth largest axon (L-4) of a squid of 18 cm mantle length, seen from the dorsal (figure 44) and from the ventral side of the ganglion (figure 45).

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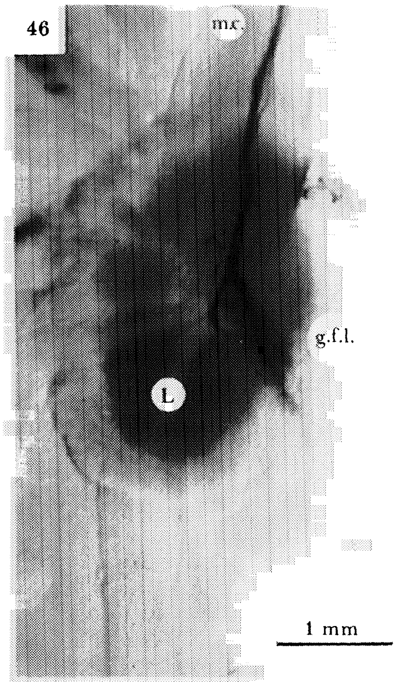

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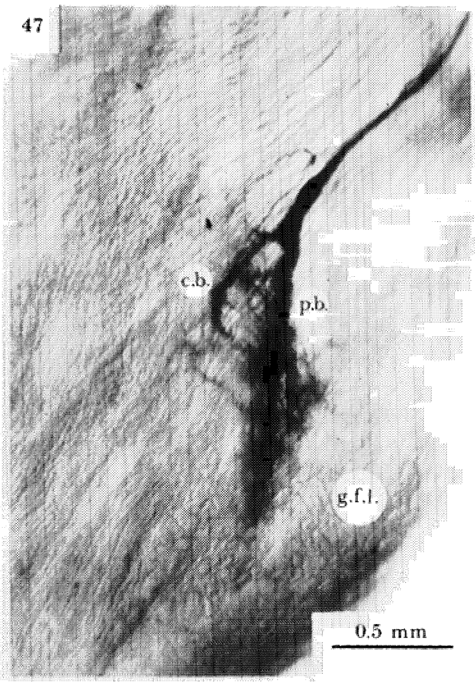

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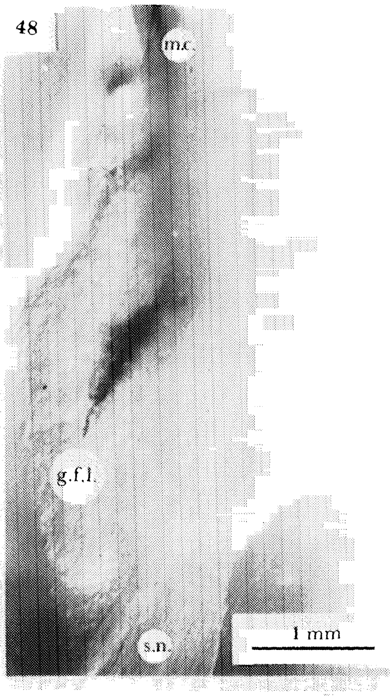

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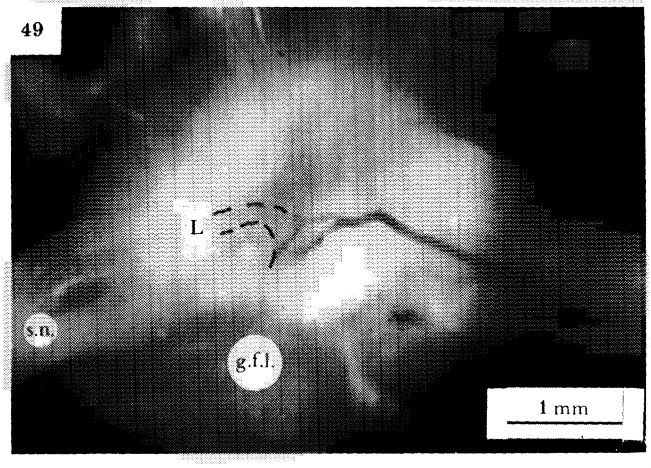

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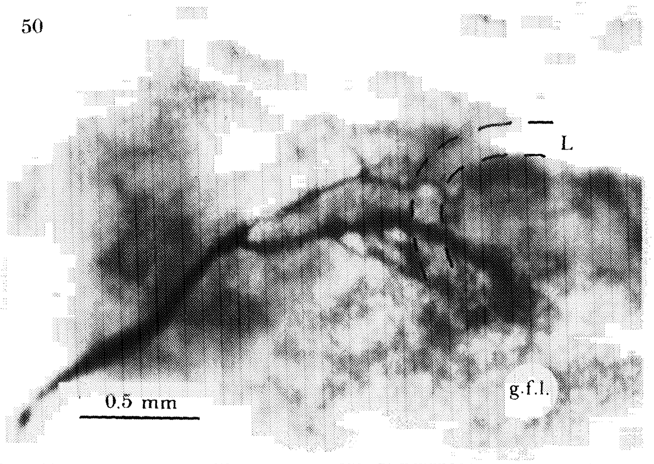

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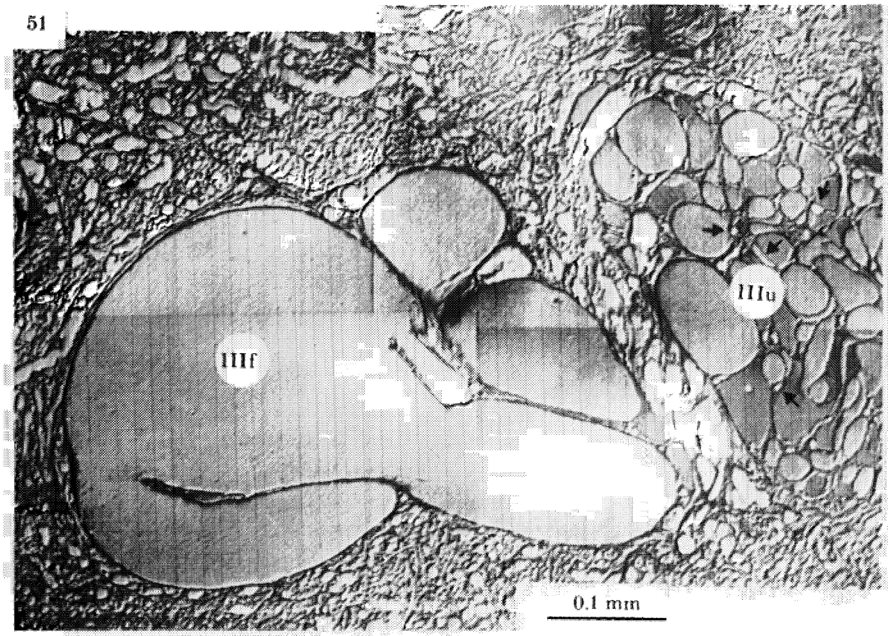

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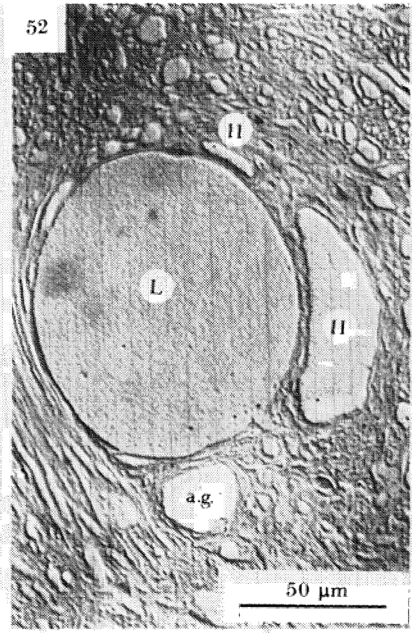
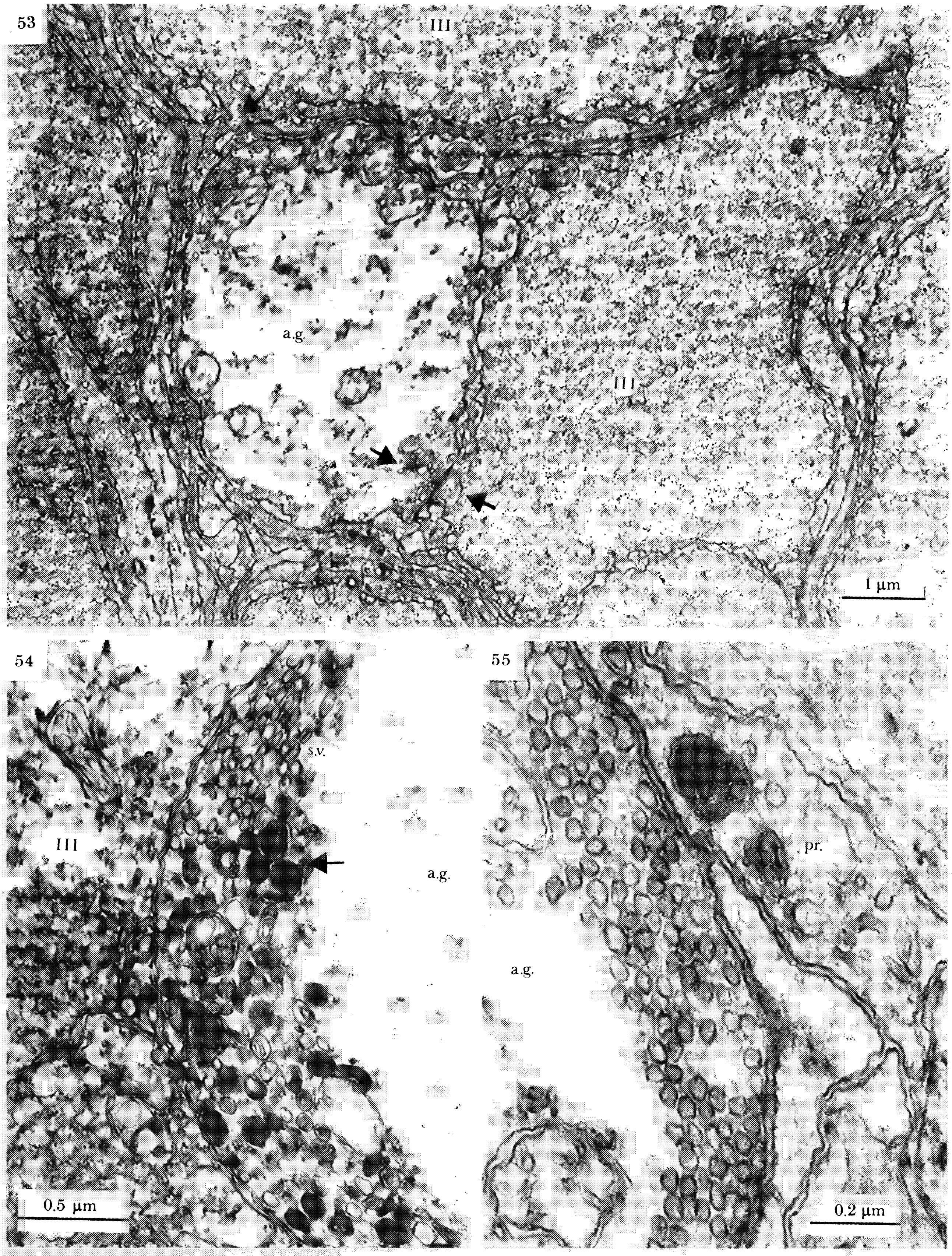
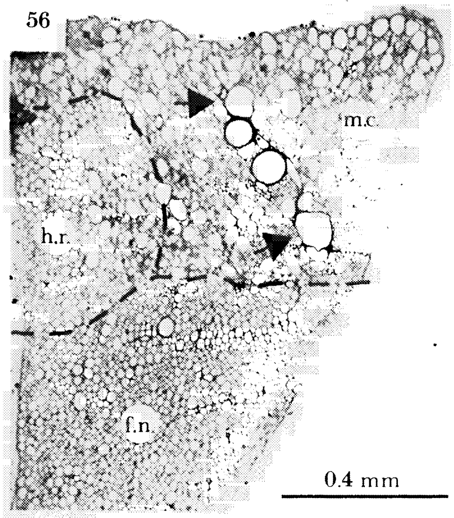
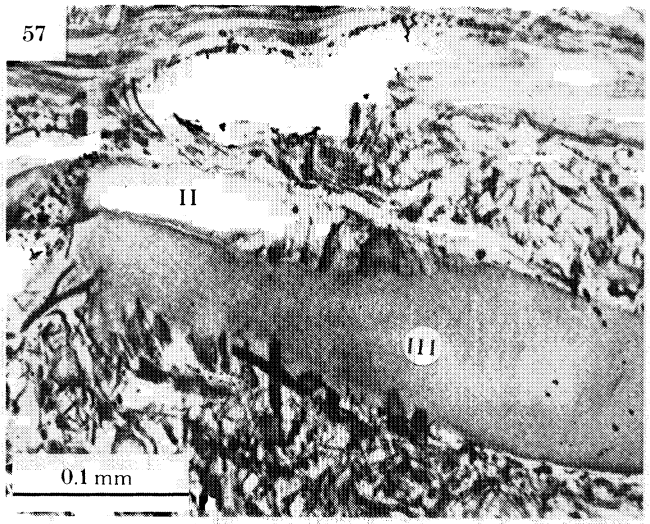


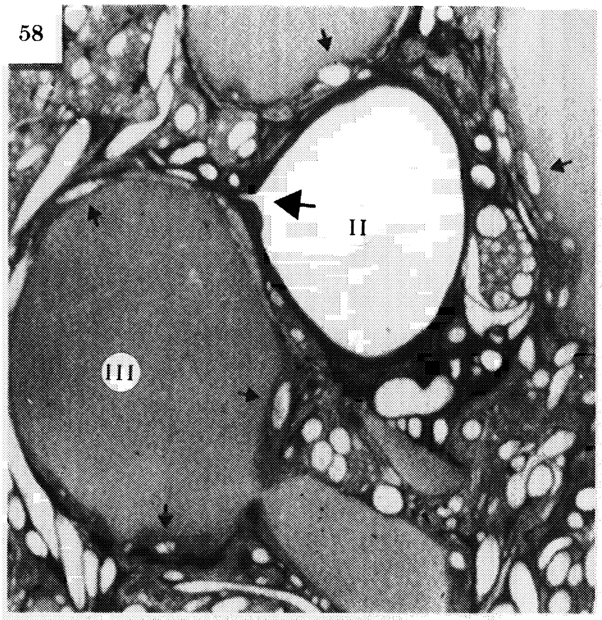
FIGURES 46–52. For description see p. 369.



FIGURES 53–55. Electron micrographs of synaptic contacts between accessory axons (a.g.) and unfused tributary branches of third order axons (III) in the neuropil of the giant fibre lobe. In figure 53 an accessory axon is in contact with two third order axons (arrows). The accessory axon in figure 54 was injected with cobalt. The dark precipitate is included in membrane-bound organelles (mitochondria?) in a group of synaptic vesicles (s.v.). Figure 55 shows an enlargement of a synaptic contact between an accessory axon and a postsynaptic process (pr.).







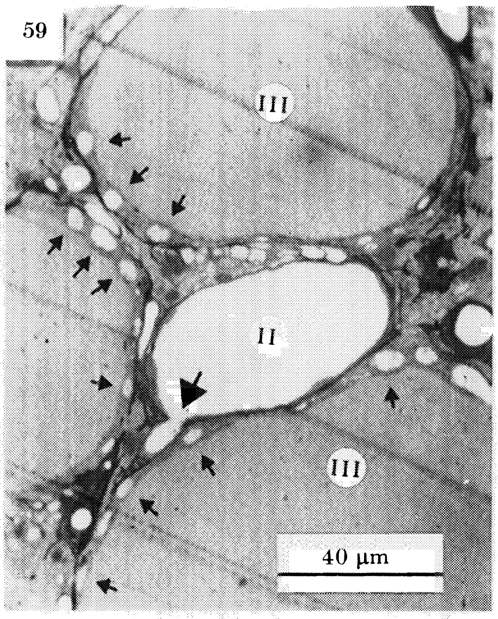


FIGURE 56. Cross-cut pallial nerve of *Todarodes* with three different nerves: the mantle connective (m.c.), the posterior head retractor (h.r.), and the fin nerve (f.n.). The mantle connective contains two giant axons (arrows), between these two profiles there are two cross-cut blood vessels (checked by electron microscopy). Toluidine blue.

FIGURE 57. Longitudinally cut second and third order axons in the stellate ganglion of *Todarodes*. Bodian-stained paraffin section.

FIGURES 58 AND 59. Groups of dark postsynaptic (III) and light presynaptic (II) axons in the stellate ganglion of *Todarodes*. Small branches bud off from the second order axons (large arrows) and run into the sheath of the third order axons (small arrows).

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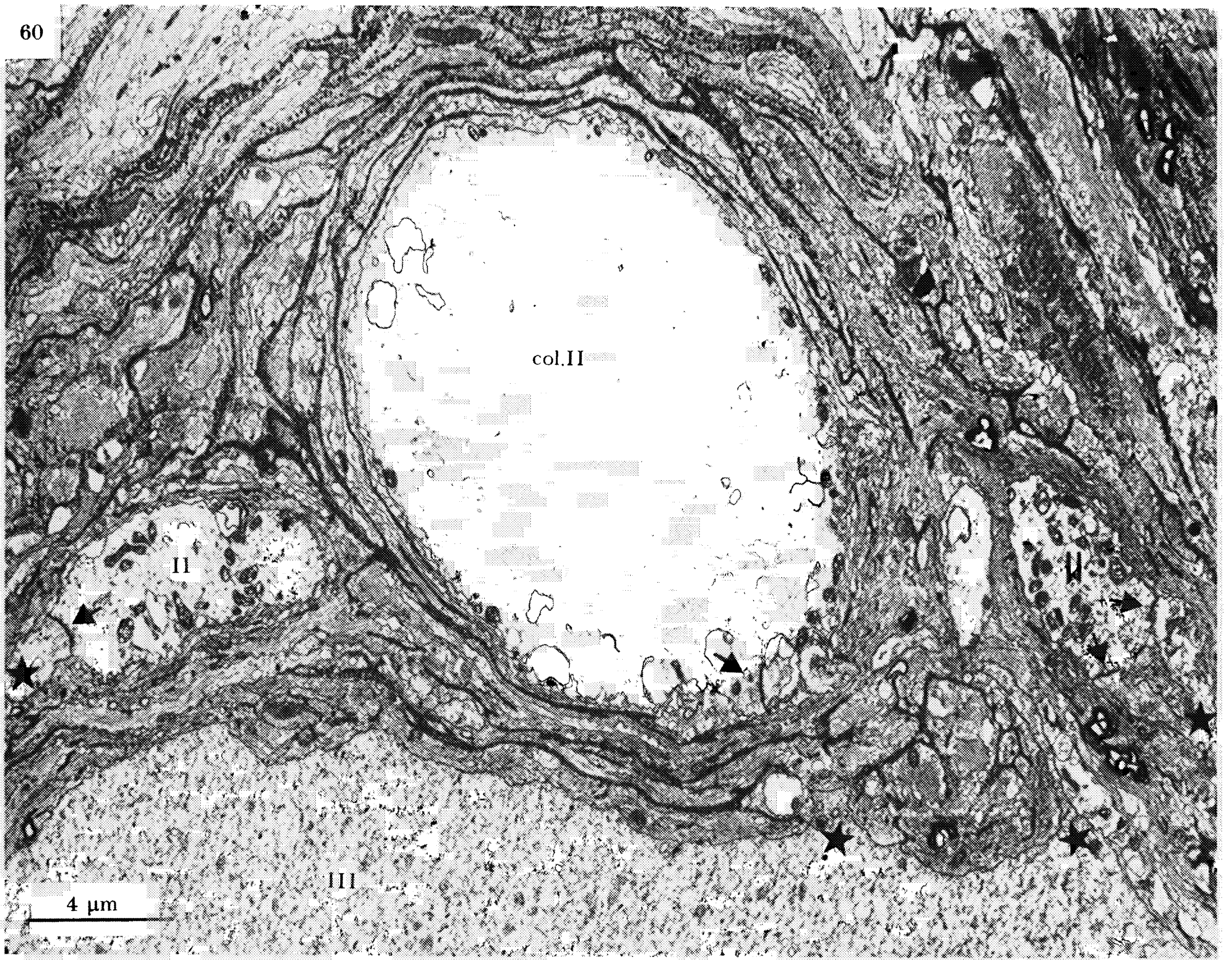

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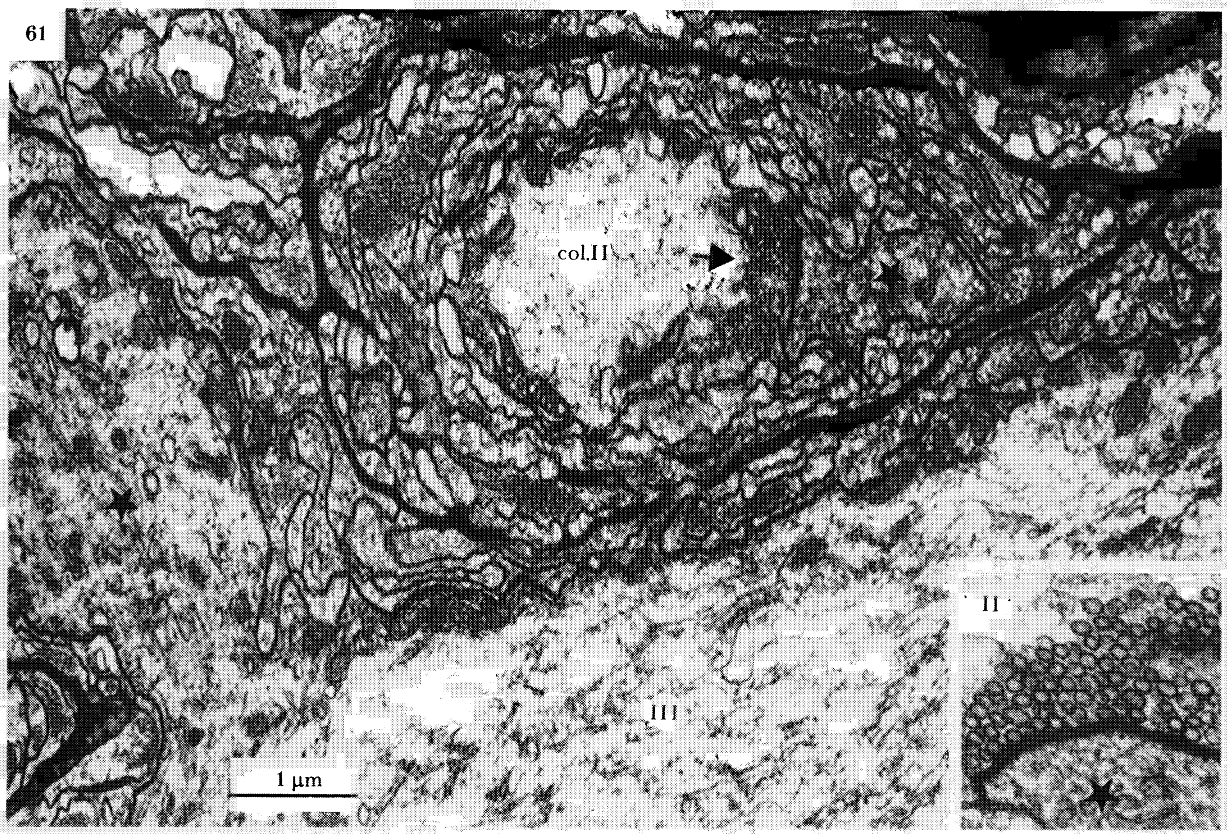

FIGURES 60 AND 61. Electron micrographs of the giant synapse of *Todarodes*. In the sheath of the third order axon (III) there are several collaterals of the second order axon (col.II) that are in synaptic contact (arrows) with processes (*) from the third order axon. Inset: a synaptic contact.

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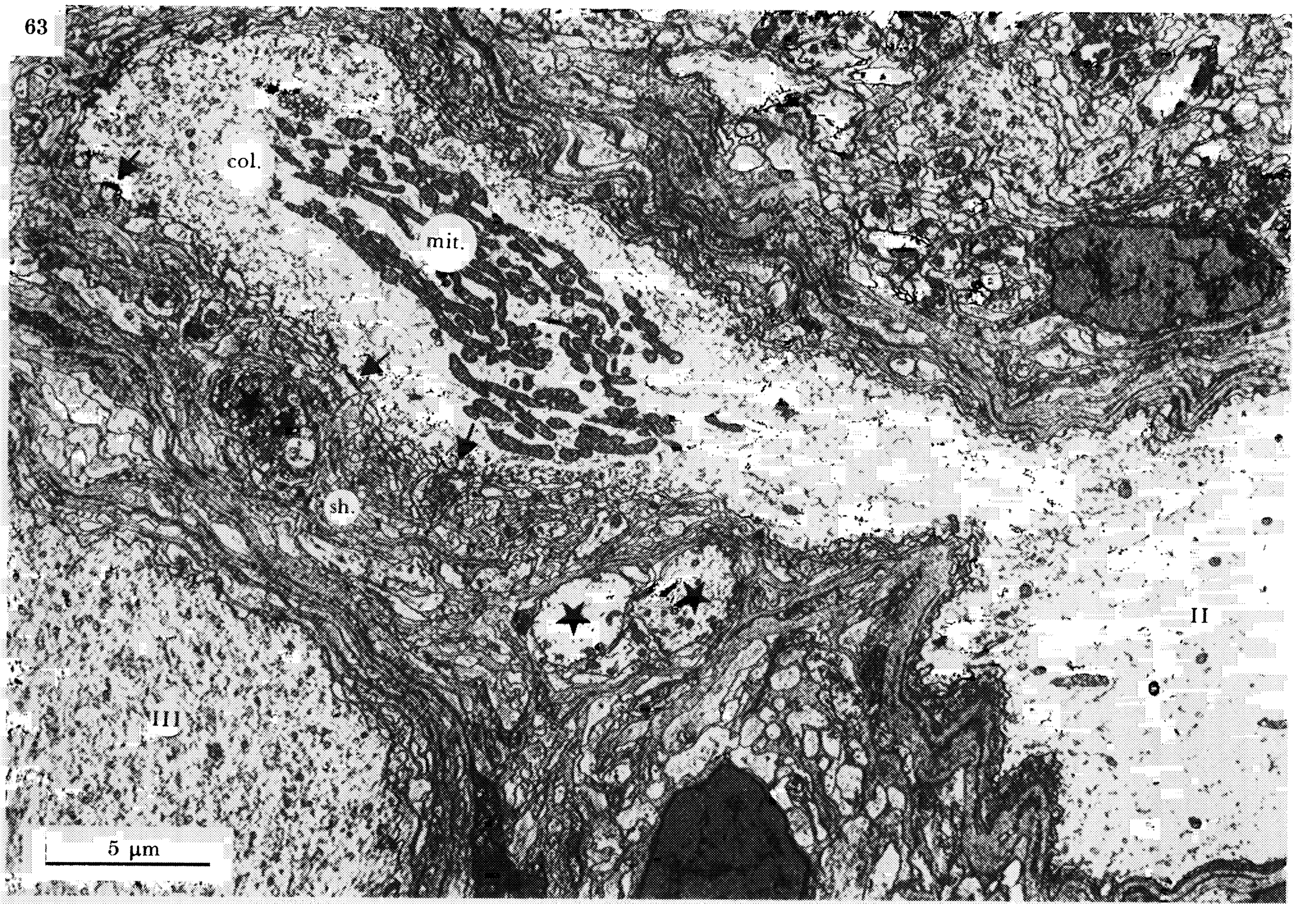

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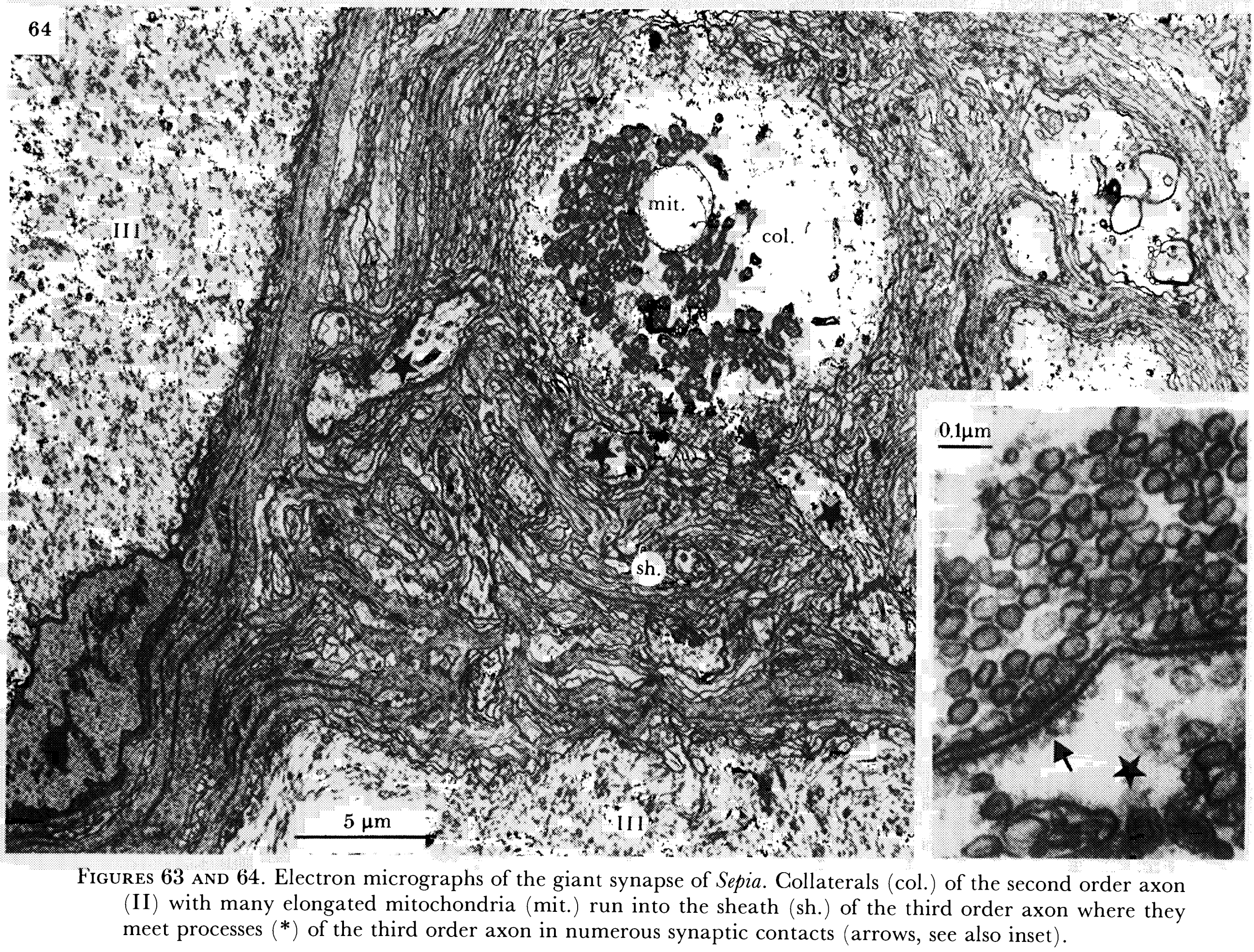

FIGURES 63 AND 64. Electron micrographs of the giant synapse of *Sepia*. Collaterals (col.) of the second order axon (II) with many elongated mitochondria (mit.) run into the sheath (sh.) of the third order axon where they meet processes (*) of the third order axon in numerous synaptic contacts (arrows, see also inset).

3.3.4. *The number of synaptic vesicles*

Synaptic vesicles usually lie in dense clusters above the presynaptic membrane (figures 21–26), while they are rare or absent between synaptic contacts. Even when considering only the areas of synaptic contact, where vesicles are accumulated, the number of vesicles varied considerably from specimen to specimen. In one animal (18 cm mantle length) we counted an average of 28 vesicles per micrometre length of synaptic contact. This figure was higher when we perfused squids, after taking care to avoid maximal mantle contractions, with a fixative containing urethane and Mg-ions in isotonic concentration (figure 26). The average numbers of vesicles per micrometre length of contact in three specimens were: 74, 61 and 58 vesicles. However, a fourth squid handled in the same way showed only 19 vesicles per micrometre length of contact, and an animal perfused with fixative I to which calcium ions had been added had more vesicles than any (an average number of 102 vesicles per micrometre length of contact).

The mean numbers per contact at different levels from proximal to distal were 20, 24, 18, 36, 33, 21, 44 in one synapse, and 31, 37, 76, 40, 39, 19, 24, 102, 44 in another synapse. So there were no obvious accumulations of vesicles along the whole synapse. Assuming a section thickness of 70 nm the vesicles cover an area of 0.07 μm^2. By extrapolation on to the total area of synaptic contacts (16312 μm^2) we estimate a total of 6.5×10^6 in one animal, or 2.4×10^7 synaptic vesicles in another animal (18 or 15 cm mantle length).

3.3.5. *Mitochondria at the giant synapse*

The space occupied in the axoplasm by the small oval mitochondria increases from proximal to distal along the presynaptic terminal. At the beginning of the terminal mitochondria cover 1.13 % and at the end 5.72 % of the cross-sectional area (measured at four levels with an electronic image analyser). The increase is approximately linear. We presume that the increasing number of mitochondria along the terminal is due to the flow of these organelles, and consequent accumulation at the blind end of the presynaptic axon. In fact the mitochondria are oriented in the terminal with their longer axis in the longitudinal direction.

The average largest diameter of mitochondria in cross sections of the pre-axon was 0.89 ± 0.22 µm (s.d.), and in longitudinal sections 1.0 ± 0.45 µm (s.d.). They were smaller in the postsynaptic axon than in the presynaptic axon. The number of mitochondria was smaller in the postsynaptic axon (1.03 % of the cross-sectional area covered by mitochondria) than in the presynaptic axon. There were no obvious accumulations of mitochondria at synaptic

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Description of plate 10

Figures 46–50. Cobalt injected accessory giant axons. Abbreviations: g.f.l., giant fibre lobe; L, largest postsynaptic axon; m.c., mantle connective; s.n., stellar nerve; c.b., central branch; p.b., peripheral branch of the accessory axon. Figure 46 was taken during precipitation of CoS: third order axons are still visible. Figures 47 and 48 are from the same preparation after fixation and clearing, seen from the ventral (figure 47) and from the dorsal side (figure 48).

Figure 51. Interference contrast micrograph of a plastic section in the area of fusion of the postsynaptic axon. On the left side the axons are fused or in the process of fusion (IIIf), on the right side axons run unfused in a thick bundle (IIIu). The arrows point to clear small axonal profiles, presumably branches of the accessory fibre that contact the unfused third order axons.

Figure 52. Cross-section of a third order axon with two branches of the same second order axon (II) and the central branch of the accessory axon (a.g.).

contacts on the presynaptic side. However, the largest number of mitochondria was found in some of the postsynaptic processes where they formed clusters which covered up to 9.58% of the axoplasmic area in the process (figure 30).

3.3.6. *Fine structural details*

The synaptic membranes. The synaptic cleft in the contacts of the distal giant synapse (figures 23 and 24) appeared to be smaller than usually reported for chemical synapses. We measured 13 contacts in a synapse at 48 different points at a magnification of × 300000, after having tilted the sections with a goniometric device to perpendicular orientation. Both pre- and postsynaptic membranes were about 7.5 nm thick, and the very regular gap between them measured about 12 nm. At the cytoplasmic surfaces floccular material was attached to the membranes, and in the cleft there was also some diffuse dense material.

Invaginations formed by the membrane of the presynaptic axon together with the opposite glia cell membrane were frequently encountered at the terminals (figure 35, plate 7). Giant axons as well as small axons had these invaginations.

Axoplasmic organelles. Both the pre- and the postsynaptic axons were filled with a diffuse network of branched filaments that in longitudinal sections were oriented more or less along the length of the axon (figures 31 and 32). Especially at the point of ramification they tended to precipitate osmium in small spots. In cross sections the filaments spread irregularly in all directions. They were usually 7.5 nm thick, but the diameters varied very much. The impression of a periodical substructure in the filaments was often obtained.

The axoplasm of the postsynaptic axon was always denser than that of the presynaptic terminal, due to the presence of bundles of straight unbranched rods (microtubules?) with a diameter of 17 nm (figures 31 and 32). They were arranged in longitudinal direction, appeared to be compact, not tubular, and were thinner than the microtubules shown by Davison & Huneeus (1970) after application of special methods.

Other organelles in the axoplasm of both pre- and postsynaptic giant nerve fibres were vesicular profiles and cisterns (figure 32). Cisternal structures occurred frequently at the end of the terminals. The presence of lamellae in presynaptic complexes has already been described in a separate paper (Martin & Miledi 1975).

The fine structure of postsynaptic processes. Structural details of postsynaptic processes give the impression that this is a very active area in the giant synapse. Most of the processes contained a number of vesicular inclusions which were often more numerous than the vesicles on the presynaptic side of the contact (figures 23, 26, 27, 29 and 30). Usually these vesicles were at some distance from the postsynaptic membrane, but sometimes they were in contact with the diffuse undercoating of this membrane (figure 26). The vesicles had external diameters ranging from 31 to 209 nm and their profiles were much more heteromorphous than those of presynaptic vesicles. Vesicles that were larger than 80 nm usually had a dense core or contained vesicular inclusions similar to multivesicular bodies. Often the processes also contained groups of mitochondria that were larger than those in the postsynaptic axons (figures 22 and 30). In ganglia that were incubated for several hours in seawater many coated vesicles had formed also in the postsynaptic processes (figure 27).

3.3.7. *The state of differentiation of the giant synapse in the freshly hatched larva*

In the brain of freshly hatched squids (about 6 mm total length), the first and second order giant neurons and their axons were well developed (Martin 1965, 1969). When we followed the mantle connective from the brain to the stellate ganglion in semithin sections we found one relatively large axon profile (figure 36, plate 8). In the ganglion this fibre made contact with the fused third order axons. An accessory axon was not distinguishable by size from other nerve fibres of the connective.

In the giant synapse the more electron-lucent presynaptic axon had a diameter of about 2.7 μm and was similar in size to the denser postsynaptic axon (figures 10 and 37). The two axons were ensheathed by glial processes and the extracellular material, which in the adult is so voluminous (figures 21 and 22) was absent. Synaptic processes from both the pre- and the postsynaptic axon reached the membrane of the opposite axon (figures 37 and 38). The vesicles in the presynaptic axon were similar in average diameter to those in adults, but often had irregular shapes. Tubules with constrictions suggested that vesicles were either in the process of being formed by splitting off from tubes, or were fusing (figure 39).

3.4. *The localization of the cell bodies of the third order giant axons*

Each of the 8–12 third order giant axons is formed by fusion of many smaller axons and, in *Loligo*, the cell bodies of these axons lie in the giant fibre lobe of the stellate ganglion (Young 1939).

To locate the cell bodies that give rise to specific postsynaptic giant axons we injected fibre L, the thickest and hindmost third order axon, fibre L-2, the third largest, and fibre L-4, the fifth largest axon. In the two smaller axons it was comparatively easy to fill with cobalt longer tracts of the axons, including their cell bodies. To fill the largest axon up to its cell bodies was more difficult with the limited ion flow obtainable from the iontophoretic pipettes used.

The fusing axons of fibre L were found to arise from practically the whole length of the giant fibre lobe (figures 40 and 41, plate 9). The cell bodies of L-2 (figures 42 and 43) and L-4 (figures 44 and 45) were dispersed in wide overlapping areas in the anterior half of the lobe. Even the relatively small axon L-4 appeared to be formed by fusion of many axons arising from not less than 100 neurons.

3.5. *The ramification of the accessory axon in the stellate ganglion and the fine structure of the proximal synapse*

Accessory giant axons of unknown origin join the stellate ganglion by way of the mantle connective (cf. figure 2). These axons can be nearly as thick as the main presynaptic giant axon. The two fibres usually run close to each other until they reach the ganglionic neuropil. The accessory axon ends close to the cell bodies of the third order axons, the synaptic terminals being interspersed among the unfused axons near the giant fibre lobe (Young 1939). Like the distal synapses of the second order giant axon the proximal synapses of the accessory axon are excitatory (Bryant 1959).

Cobalt injections revealed that large accessory axons divide in the stellate ganglion into two main branches (figures 46, 47, 49 and 50, plate 10). The more centrally situated shorter branch makes contacts with the largest third order axon at the site of fusion in its bend. In one specimen this branch followed and contacted the main postsynaptic axon (L) for at least 300 μm of its length, parallel to the main presynaptic axon (figure 52). The other branch runs towards the giant fibre lobe where it ramifies into a larger number of fine collaterals that follow upstream the bundles of unfused third order axons (figure 47).

In these bundles we found many synaptic contacts between clear and dense axon profiles (figure 53, plate 11). The dense axons were more frequent and obviously represented the fusing third order axons. The clear profiles, containing clusters of synaptic vesicles in the contact areas were presynaptic (figures 53, 54 and 55). That the clear profiles belonged to the accessory axon was demonstrated by processing cobalt-injected accessory axons for electron microscopy. In these preparations the clear profiles contained electron opaque inclusions (figure 54) that were mixed with the synaptic vesicles. These membrane-bound inclusions resembled mitochondria, however, they were considerably smaller than mitochondria in uninjected axons (figure 54).

In the synaptic areas short processes of the third order axon penetrated the thin sheath and ended in specialized synaptic contacts at the membrane of the accessory axon (figures 53 and 55). The synaptic vesicles were electron-transparent and measured 51.5 ± 3.9 nm in diameter. They were not different from synaptic vesicles of the main presynaptic axon. Also, accessory axon profiles without a sheath were observed in direct contact with the membrane of the third order axon.

3.6. *The distal giant synapse of Todarodes sagittatus*

To our knowledge, the structure and function of the giant synapse of species belonging to the suborder Oegopsida has not been studied. The Mediterranean oegopsid *Todarodes sagittatus* has a well developed giant fibre system. In paraffin sections of small specimens (*ca.* 16 cm mantle length) the third order giant axons measured up to 200 μm in diameter at the exit from the stellate ganglion, the second order giant fibre in the mantle connective and ganglionic neuropil was less than 100 μm thick (figure 56, plate 12). As in *Sepia* (cf. Young 1939) the cell bodies of the syncytial third order axons lie in the dorsal wall of the stellate ganglion; there is no distinct giant fibre lobe.

In the mantle connective, as in *Loligo*, there were two large axons (figure 56). Presumably, these were the second order axon and an accessory axon. In the neuropil of the stellate ganglion clear second order axons were seen running parallel to dense third order axons (figures 57, 58 and 59).

At the level of the synaptic contacts the giant synapse of *Todarodes* was clearly different from that of *Loligo* (figure 62). The processes of the postsynaptic axon did not contact directly the presynaptic axon, but ended on a system of collaterals (2–20 μm diameter) from the presynaptic fibre, within the enlarged sheath between the two axons. The numerous clear collaterals of the presynaptic fibre are shown in figures 58 and 59. In the sheath of one postsynaptic axon there were up to ten fine presynaptic collaterals. Each of these presynaptic collaterals was surrounded by processes from the postsynaptic fibre that established synaptic contacts from all sides (figures 60 and 61, plate 13). This complex system of processes from the postsynaptic fibre, collaterals from the presynaptic fibre and large numbers of synaptic contacts, often covered most of the circumference of the postsynaptic axons. The synaptic contacts were embedded in several layers of glial processes and extracellular material (figures 60 and 61).

The presynaptic collateral contained clusters of electron-clear synaptic vesicles that measured 52.0 ± 3.2 nm (s.d.) in diameter. The cleft between the two synaptic membranes was about 10 nm wide (figure 61).

3.7. *The distal giant synapse of Sepia officinalis*

The giant synapse in *Sepia* is comparable to that in *Todarodes* and also differs from the *Loligo* synapse. The second order axon runs parallel to the third order axons and gives off collaterals, several micrometres thick, that penetrate the enlarged sheaths of the third order axons.

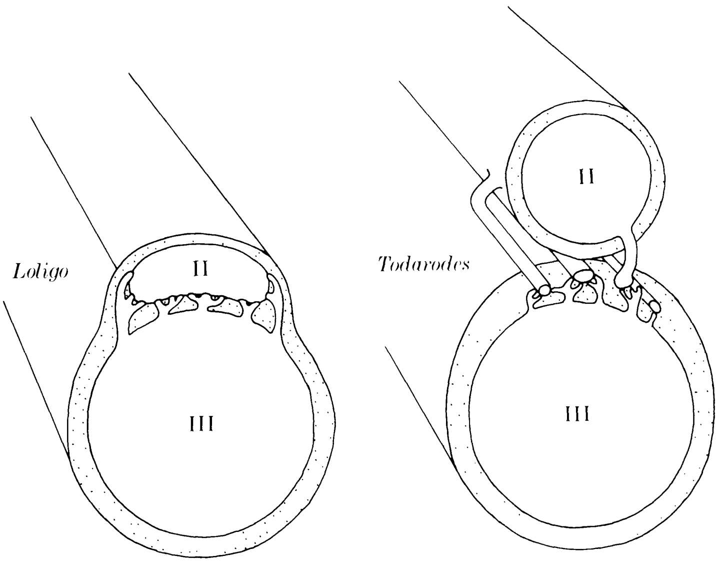

FIGURE 62. Schematic drawings of the giant synapses of *Loligo* and *Todarodes*. In *Todarodes* collaterals of the second order axon make synapses with processes of the third order axon in numerous synaptic contacts.

Numerous small processes from the third order axons contact these collaterals from different sides, forming synaptic junctions. In the presynaptic collaterals there were electron-transparent vesicles of 55.4 ± 4.3 nm (s.d.) diameter and masses of elongated mitochondria (figures 63 and 64, plate 14).

There appeared to be several second order axons. Young (1939, 1972) found about ten large fibres of up to 100 μm diameter in the mantle connective. On entering the neuropil of the stellate ganglion they were seen to branch and to run to the third order fibres.

As in *Todarodes*, there is no giant fibre lobe in the stellate ganglion of *Sepia*. The syncytial third order axons arise by fusion of numerous axons from neurons in the dorsal wall of the stellate ganglion.

4. DISCUSSION

The system of giant nerve fibres in decapod cephalopods triggers nearly synchronous contractions of the mantle producing strong jets of water for fast escape, or attack reactions (Trueman & Packard 1968; Packard 1972). The giant synapse in the stellate ganglion is the site at which impulses from the brain arrive via the second order giant axon and are transmitted to the giant motor axons that innervate the mantle muscles. In the brain these impulses are generated by two very large cells, the first order giant cells that collect signals from the sense organs and conduct them bilaterally to the second order axons (Young 1939).

The very large single elements of the giant synapse present in a flat and transparent ganglion that is easily removed from the underlying musculature, offer unique possibilities for electrophysiological and biochemical work. The largest presynaptic terminals are thick enough to place several electrodes. The synaptic contact area in medium-sized animals extends over a length of about 1 mm, and both the pre- and the postsynaptic fibre can be injected with ions, dyes or isotopes by using electric pulses or pressure.

Not every ganglion is suitable for a particular experimental design because of marked individual variations in the ramification pattern of the presynaptic fibre or in the course of the postsynaptic axons. The terminals may pass below the postsynaptic fibre or, because of multiple branching, be inconvenient for placing electrodes. It appears that during embryonic development the second order fibre does not follow a rigid pattern in its course and ramification. At hatching this fibre is relatively very large, about as thick as the postsynaptic axons and much thicker than the accessory axon. The two first order giant cells are also well differentiated at hatching (Martin 1965; Martin & Rungger 1966). So there appears to be a gradient of differentiation from brain to periphery, and it may be that the ramification pattern of the outgrowing second order axon is determined by its first contact with the third order axons in the stellate ganglion.

Multiple synaptic contacts of the presynaptic second order axon, and of the accessory axons, with the largest third order axon have been observed in this study. The main branch of the second order fibre forms the most extensive contact area, the distal synapse. In one out of two animals this presynaptic fibre had a smaller branch that made further contacts more centrally, that is closer to the area of fusion and to the bend of the motor fibre. The accessory axon also had two main branches, a central branch that contacted the motor fibre in the area of fusion and a tree-like extension with many fine contacts to the numerous unfused axons near their cell bodies in the giant fibre lobe. The largest motor axon may therefore have as many as four contact areas in its course from the giant fibre lobe to the exit from the ganglion. The more proximal contacts are under control of the accessory axons and the more distal parts under that of the second order giant axon. The origin of the accessory axon in the brain is unclear. The fine structure of the proximal and distal synapses was very similar.

Since both the main and the accessory presynaptic axons are capable of triggering impulses in the postsynaptic axon (Bryant 1959; Miledi & Slater 1967) there is some redundancy in the system, but the safety of transmission is greatly increased. At present we do not know if all accessory axons make contact with all postsynaptic axons and more work is needed on this.

The localization of the cell somata of the third order axons in the giant fibre lobe in our preparations differed considerably from a map that has been drawn previously based on dye-injected ganglia of *Loligo pealii* (Staub 1954). In contrast to this map the axons that formed the largest motor fibre were seen converging from nearly the whole length of the giant fibre lobe. Also the population of neurons forming the third largest axon in our material were situated in a much less circumscribed area of the anterior giant fibre lobe. So the nascent axons of the different motor axons before their fusion appear to proceed without a circumscribed arrangement. Miledi (1967) showed that there are electrical connections between the postsynaptic axons, and there may therefore be areas, presumably close to the giant fibre lobe, in which impulses in the motor fibres are exchanged and coordinated.

A number of characteristic organelles in the giant synapse deserve attention. Postsynaptic processes very often contained heterogeneous vesicular structures. In some processes, especially after fixation in presence of high concentrations of magnesium ions, the vesicles were apposed to the postsynaptic membrane. Moreover, the occurrence of coated vesicles in the postsynaptic processes indicated dynamic events. Clusters of postsynaptic as well as presynaptic vesicles have also been observed in synaptic contacts on the first order giant axon in the brain (Gervasio *et al.* 1971), and in the chiasm of the first order axons of the oegopsid *Ilex coindetti* (Martin 1969). Since the squid synapse is unidirectional and transmits signals by the release of transmitter quanta (Miledi 1967), the presence of these vesicles on the postsynaptic side is surprising. However, we have previously shown that some substances are transported retrogradely from the post- to the presynaptic fibre (Martin & Miledi 1978), and it may be that the postsynaptic vesicles are involved in these processes of transfer of materials between the axons. On the other hand it is obvious from dye and cobalt injections that there are no large communication channels between pre- and postsynaptic axons, or vice versa, through which these substances could flow.

Other subcellular structures are the previously described complexes of lamellae in the presynaptic area (Martin & Miledi 1975), and patches of osmiophilic material that in size and staining characteristics resemble ribosomes and are associated with mitochondria in the postsynaptic fibre. This material as well as the filamentous and tubular proteins of the squid axoplasm need further examination. In our osmium-fixed specimens filaments and tubules differed in structure compared with the microtubules isolated by Davison & Huneeus (1971) from the axoplasm of *Dosidicus*. The masses of axoplasmic proteins in the postsynaptic axon at light microscopical enlargements may appear in a helical conformation (Metuzaals & Izzard 1969). There is a gradient of axoplasmic density from the giant fibre lobe to the peripheral course.

While in *Loligo* processes arise from the postsynaptic axon and contact directly the membrane of the presynaptic terminal, in the two other species examined a complicated system of collaterals from the presynaptic axon and processes from the motor fibre were found to form a large number of synaptic contacts embedded into the sheath between the two axons. In view of the different structure of the synapses it seems that for many types of experiments the giant synapse of *Loligo* is better than that of *Todarodes* or *Sepia*, because in the latter species the presence of the presynaptic processes introduces additional complicating features.

Distinct differences between the three squid species were also seen in the more central parts of the system within the brain, especially in the chiasm of the first order axons (Martin 1969). The *Loligo* chiasm is unusual in that the crossing first order axons are completely fused forming an axoplasmic bridge (Young 1939), while the *Sepia* and *Todarodes* chiasms are formed by individual axons which synapse in the decussation (Martin 1969). If the maintenance of separate individual nerve fibres that communicate by specialized contacts is the more archaic arrangement, the *Loligo* system appears to represent the phylogenetically derived condition. The distal giant synapse of *Loligo* without presynaptic collaterals is presumably also the more evolved synaptic structure. Moreover, the cell bodies that form the giant motor axons in *Loligo*, in contrast to the other two species, have become concentrated in a special lobe, the giant fibre lobe. Myopsid squids appear to possess, in phylogenetic order, the more specialized and derived network of giant fibres. In any case, the *Loligo* synapse is probably the most convenient preparation for experimental work.

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Abbreviations used in figures

a.g.	accessory giant axon
be.	bend of largest postsynaptic axon
b.v.	blood vessel
c.b.	central branch of accessory giant axon
col.	collaterals
cl.	collagen
fil.	filaments
f.n.	fin nerve
g.f.l.	giant fibre lobe
h.r.	posterior heat retractor
L	last postsynaptic (third order, motor) giant axon last stellar nerve
L-1	penultimate postsynaptic giant axon
L-3	fourth largest postsynaptic giant axon
m.c.	mantle connective
mit.	mitochondria
m.m.	mantle muscles
p.b.	peripheral branch of the accessory giant axon
pr.	postsynaptic process
s.a.	synaptic area
sh.	sheath cell processes, nucleus
s.n.	stellar nerve
s.v.	synaptic vesicles
II	second order (presynaptic) giant axon
III	third order (postsynaptic, motor) giant axon

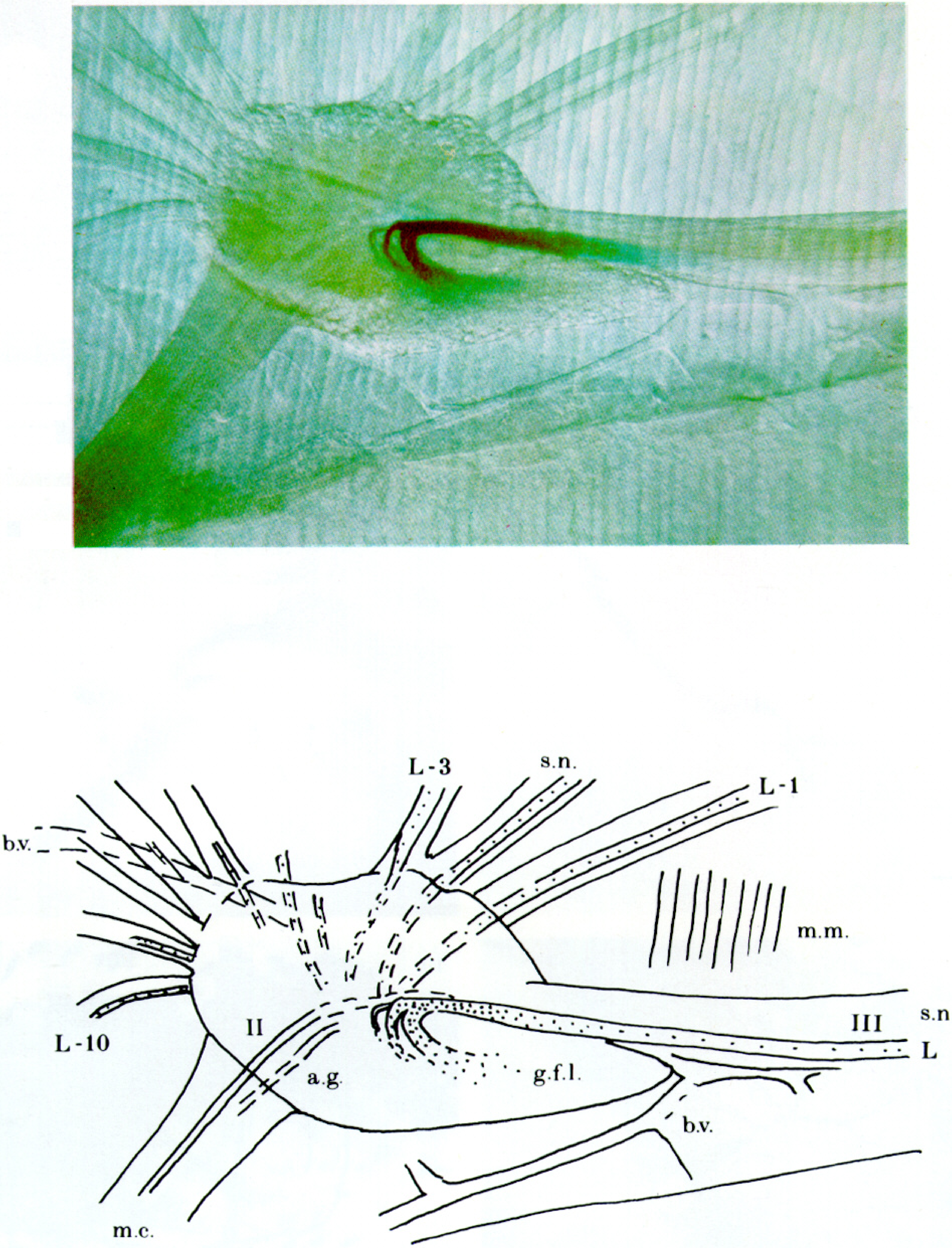
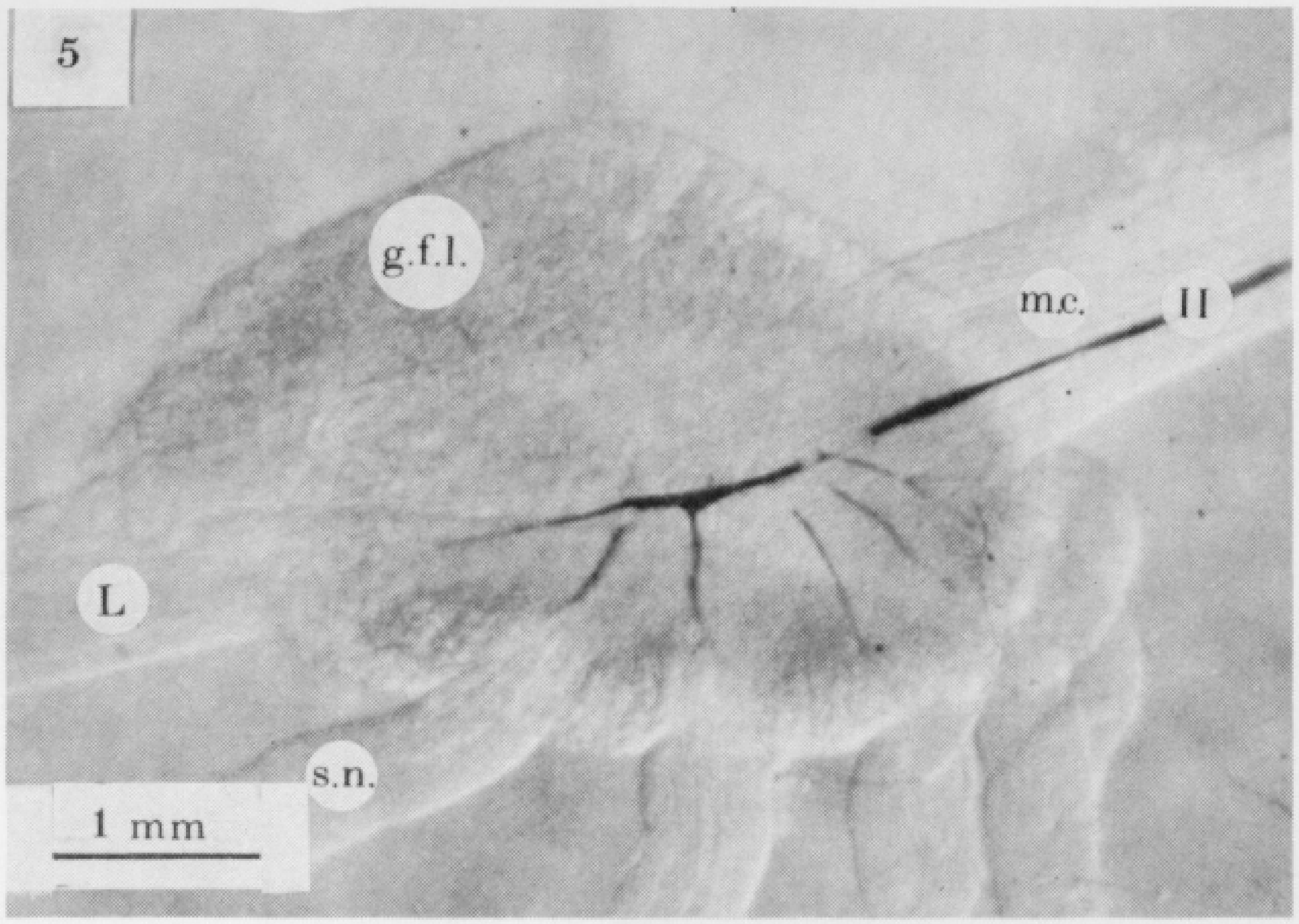
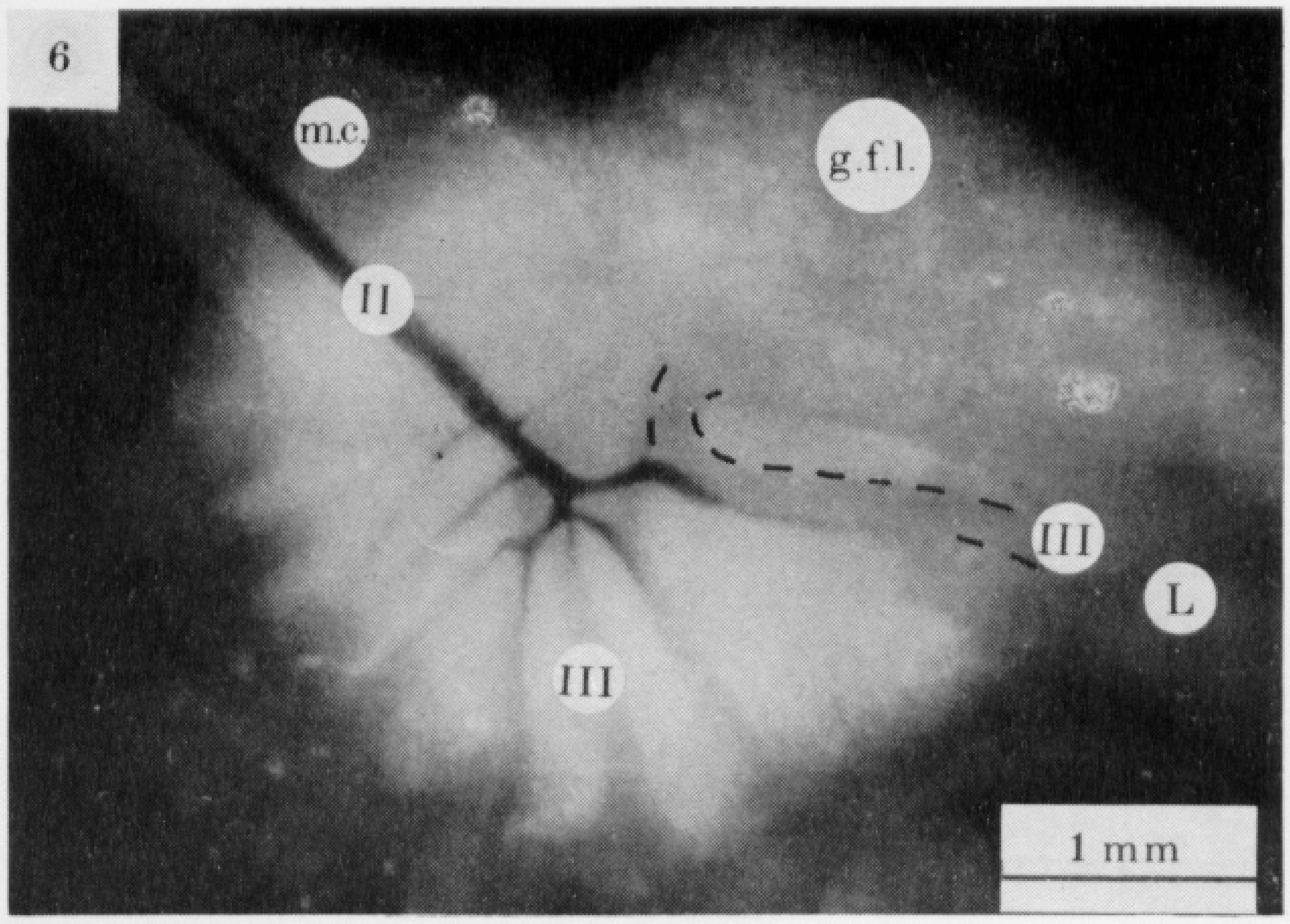
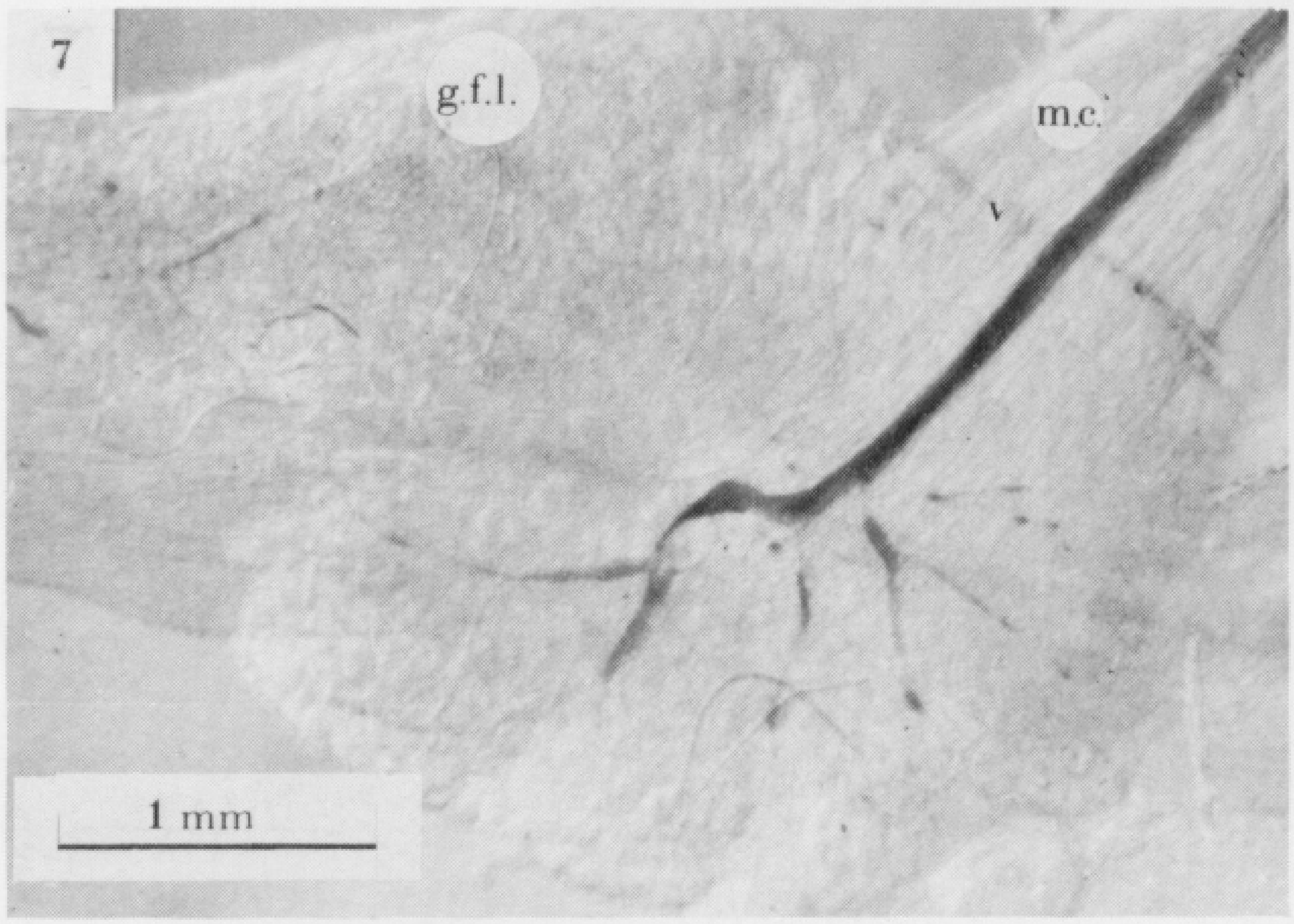
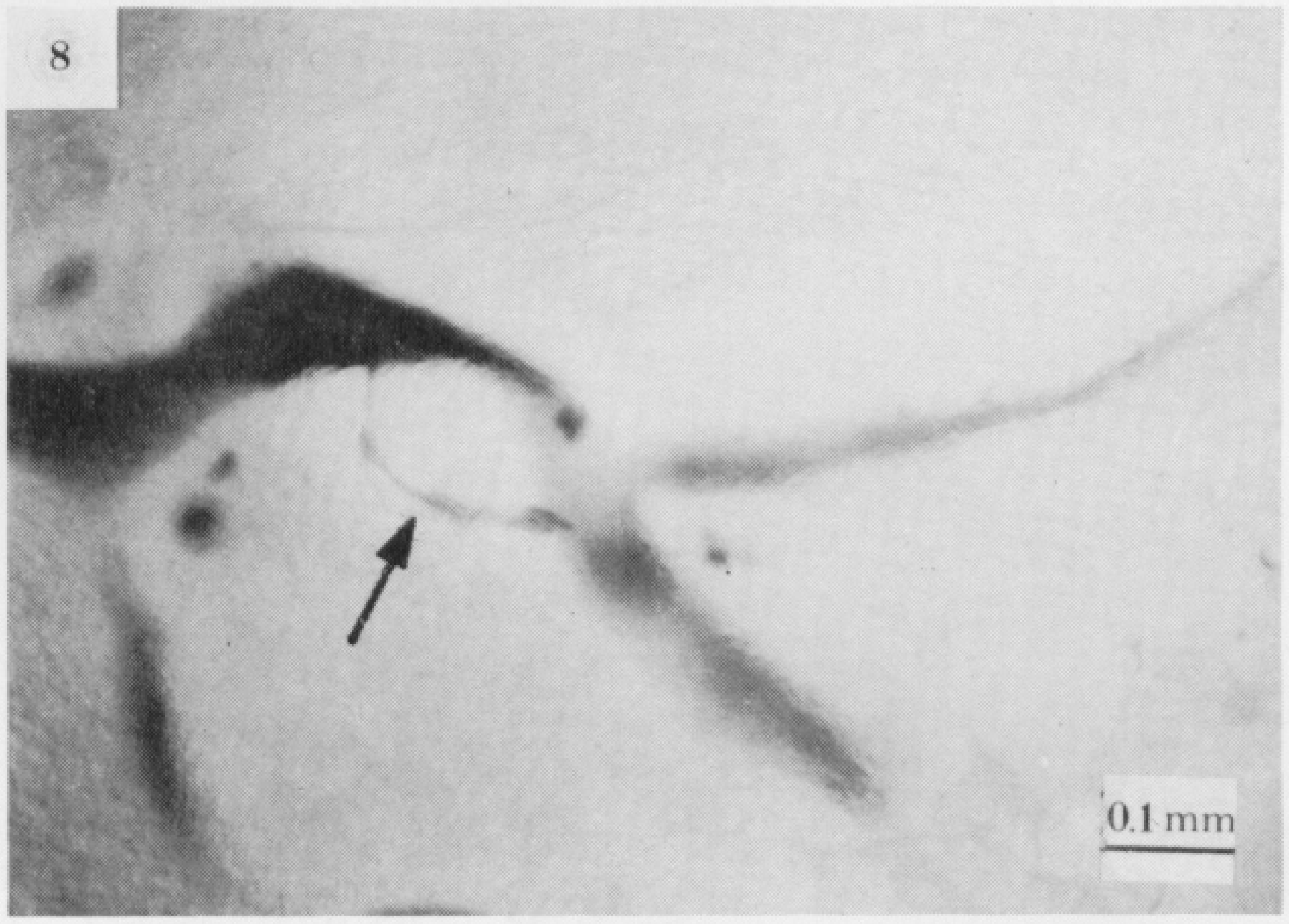


FIGURE 1. A living preparation of the stellate ganglion of the squid, *Loligo vulgaris*, as seen on the mantle muscle (m.m.) in transmitted light, during injection of Fast Green into the main (L) postsynaptic giant fibre (III). In the mantle connective (m.c.) the second order giant axon (II) and an accessory axon (a.g.) are detectable. The third order axons originate by fusion of a large number of axons the perikarya of which are accumulated in the giant fibre lobe (g.f.l.). Each stellar nerve (s.n.) includes a giant fibre in decreasing thicknesses (L, L-1, L-3, L-10). The ganglion is supplied by large blood vessels (b.v.).

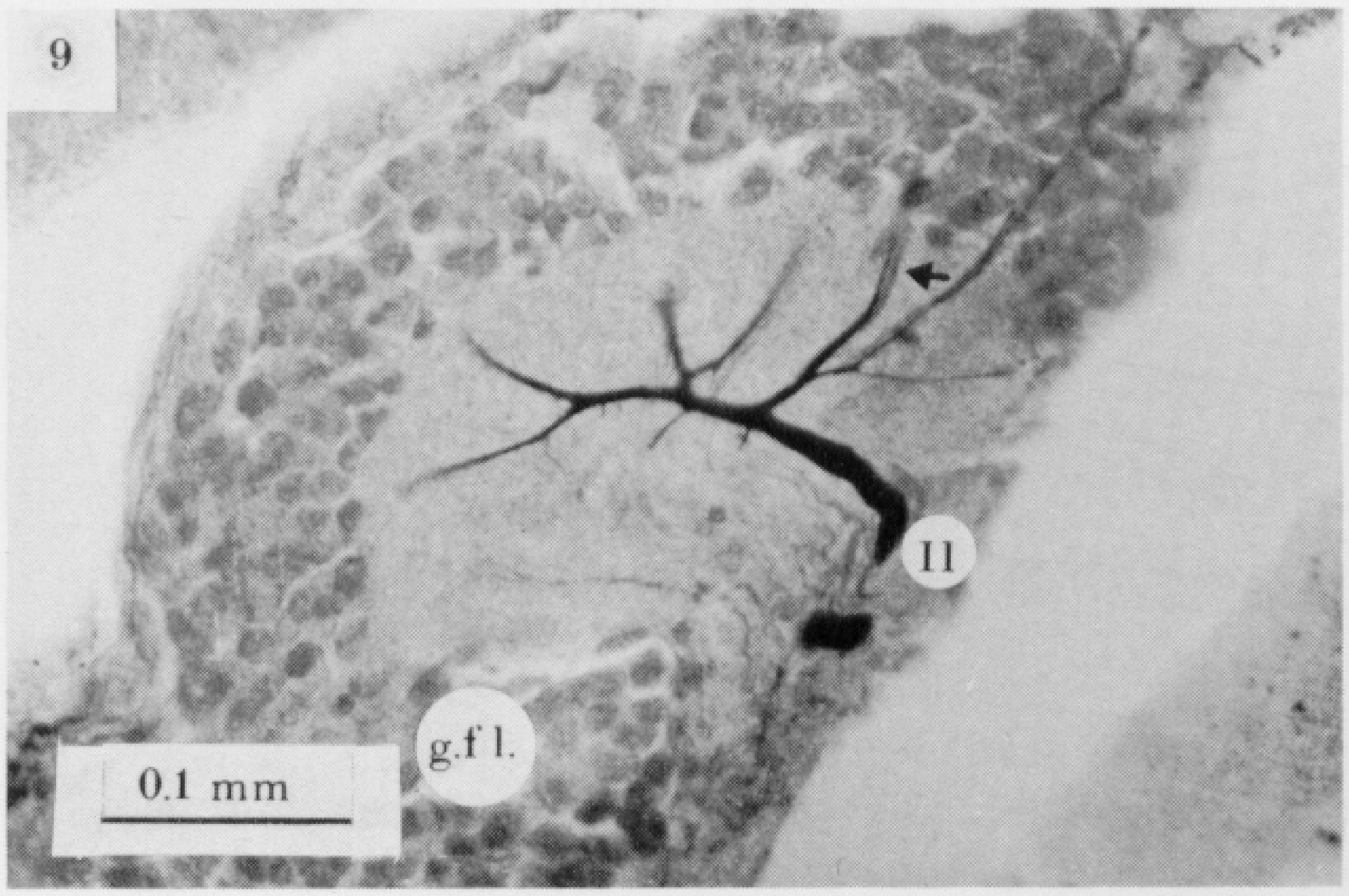


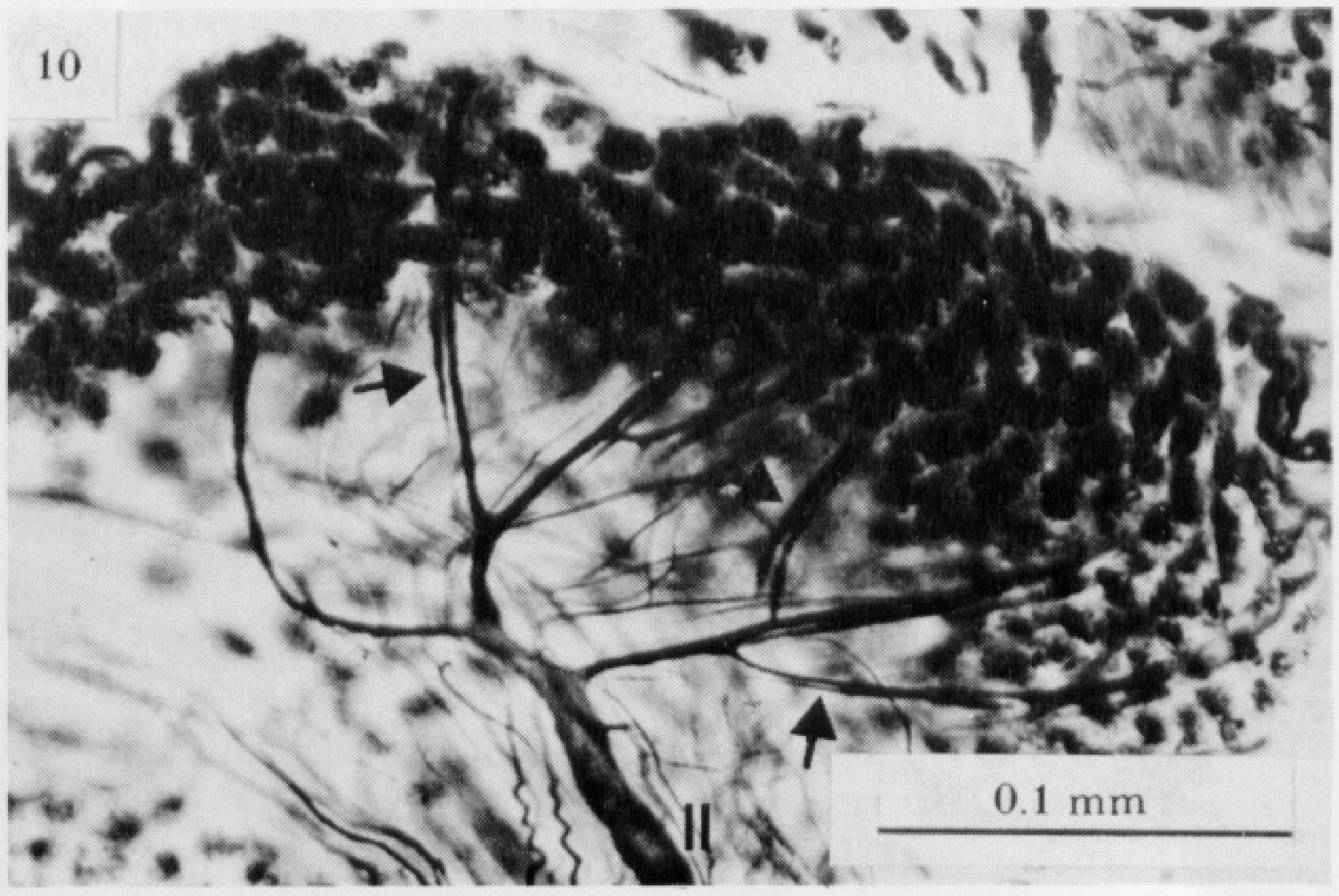






FIGURES 5–8. Stellate ganglia of *Loligo vulgaris* with cobalt injected second order (II) giant axons. In figure 6 several third (III) order axons are visible. Figure 8 is an enlarged part of figure 7: a small branch (arrow) with varicosities surrounds the third order axon in its bend (see also figure 12*a*). Abbreviations: g.f.l., giant fibre lobe; m.c., mantle connective; s.n., stellar nerve; L, last third order axon.





FIGURES 9 AND 10. Horizontal sections through stellate ganglia of freshly hatched larvae of *Loligo vulgaris*, stained with Bodian's method. The second order axons (II) are very large when compared with the third order axons (arrows).

Figures 14–20. For description see p. 368.

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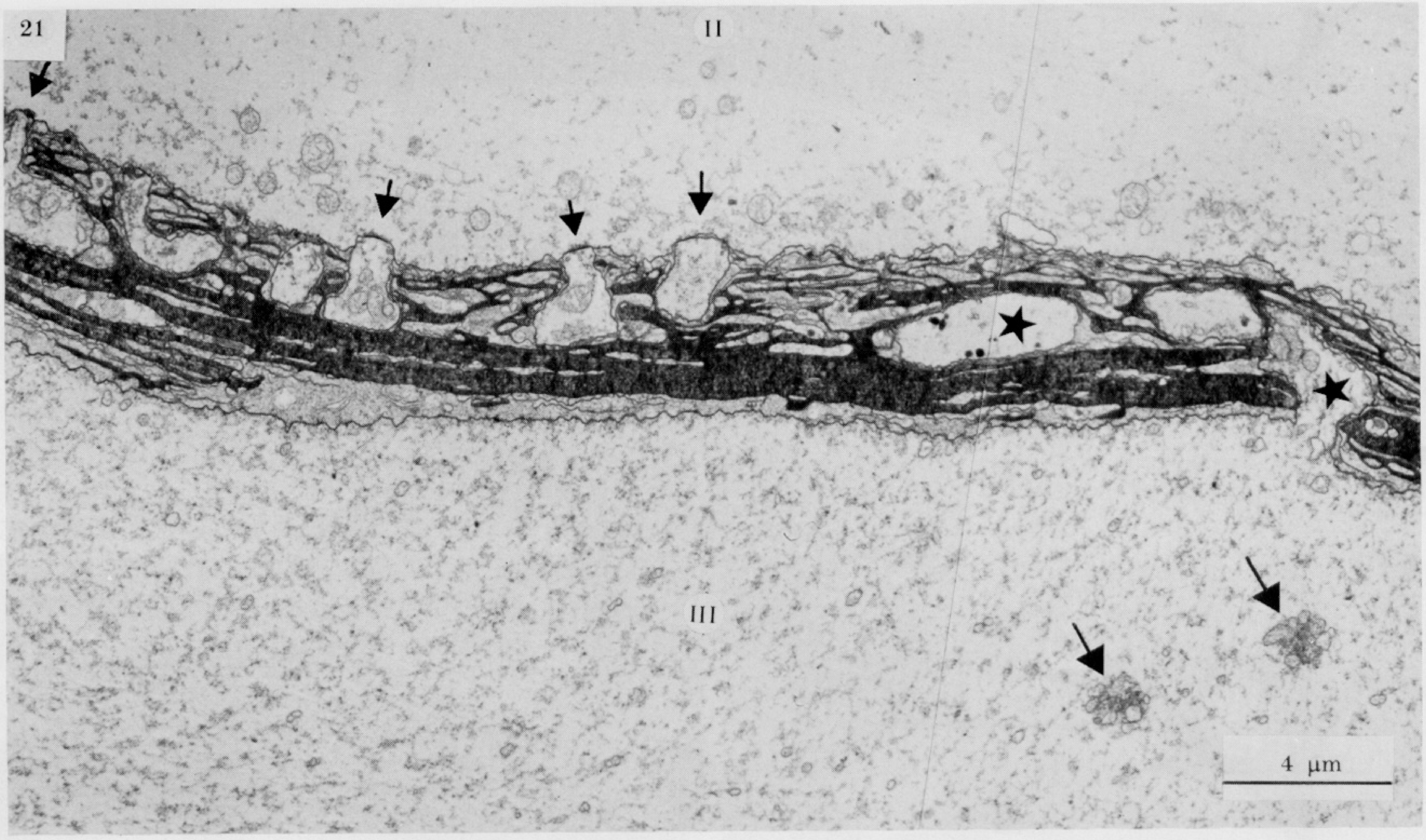

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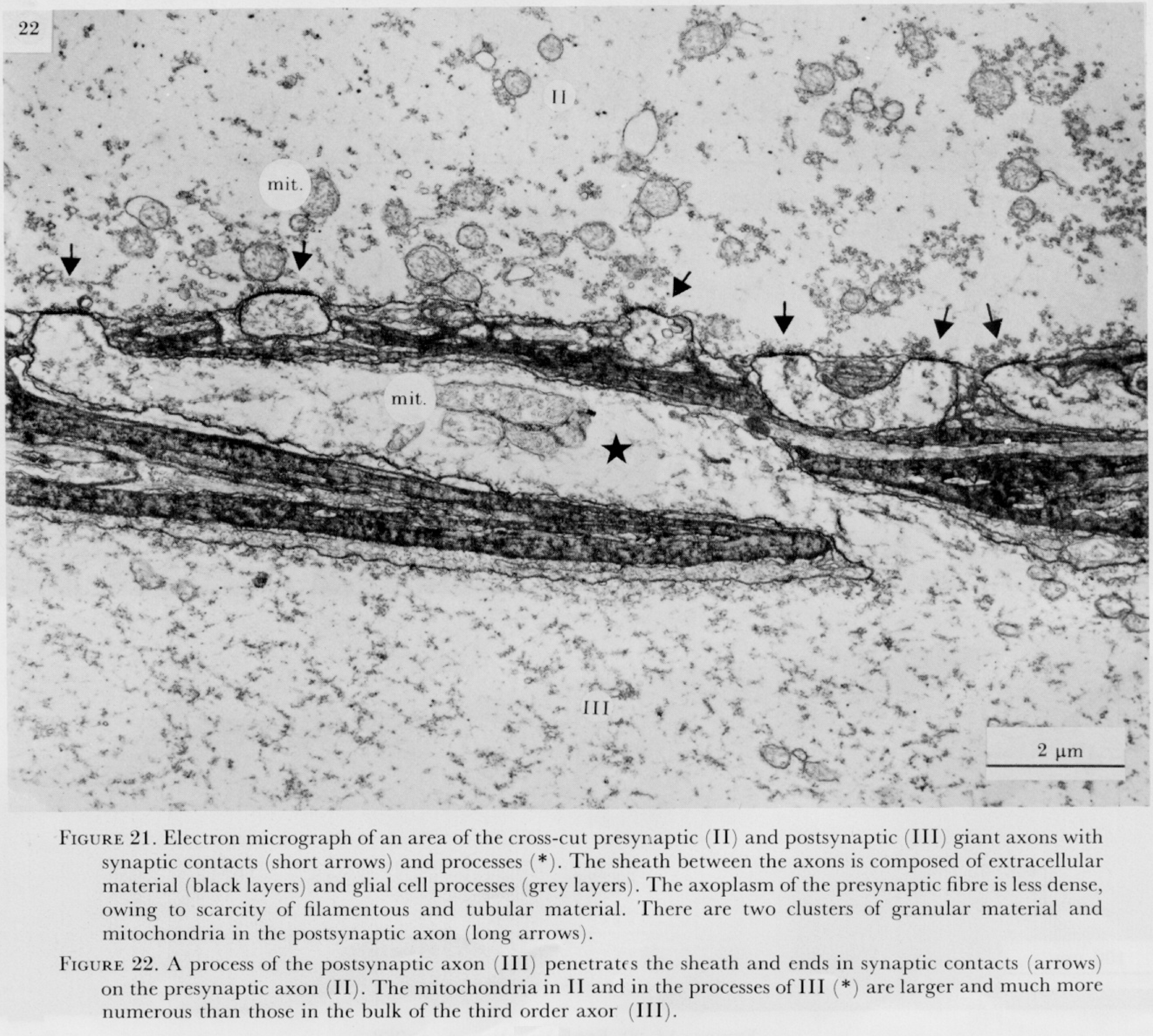

FIGURE 21. Electron micrograph of an area of the cross-cut presynaptic (II) and postsynaptic (III) giant axons with synaptic contacts (short arrows) and processes (*). The sheath between the axons is composed of extracellular material (black layers) and glial cell processes (grey layers). The axoplasm of the presynaptic fibre is less dense, owing to scarcity of filamentous and tubular material. There are two clusters of granular material and mitochondria in the postsynaptic axon (long arrows).

FIGURE 22. A process of the postsynaptic axon (III) penetrates the sheath and ends in synaptic contacts (arrows) on the presynaptic axon (II). The mitochondria in II and in the processes of III (*) are larger and much more numerous than those in the bulk of the third order axon (III).

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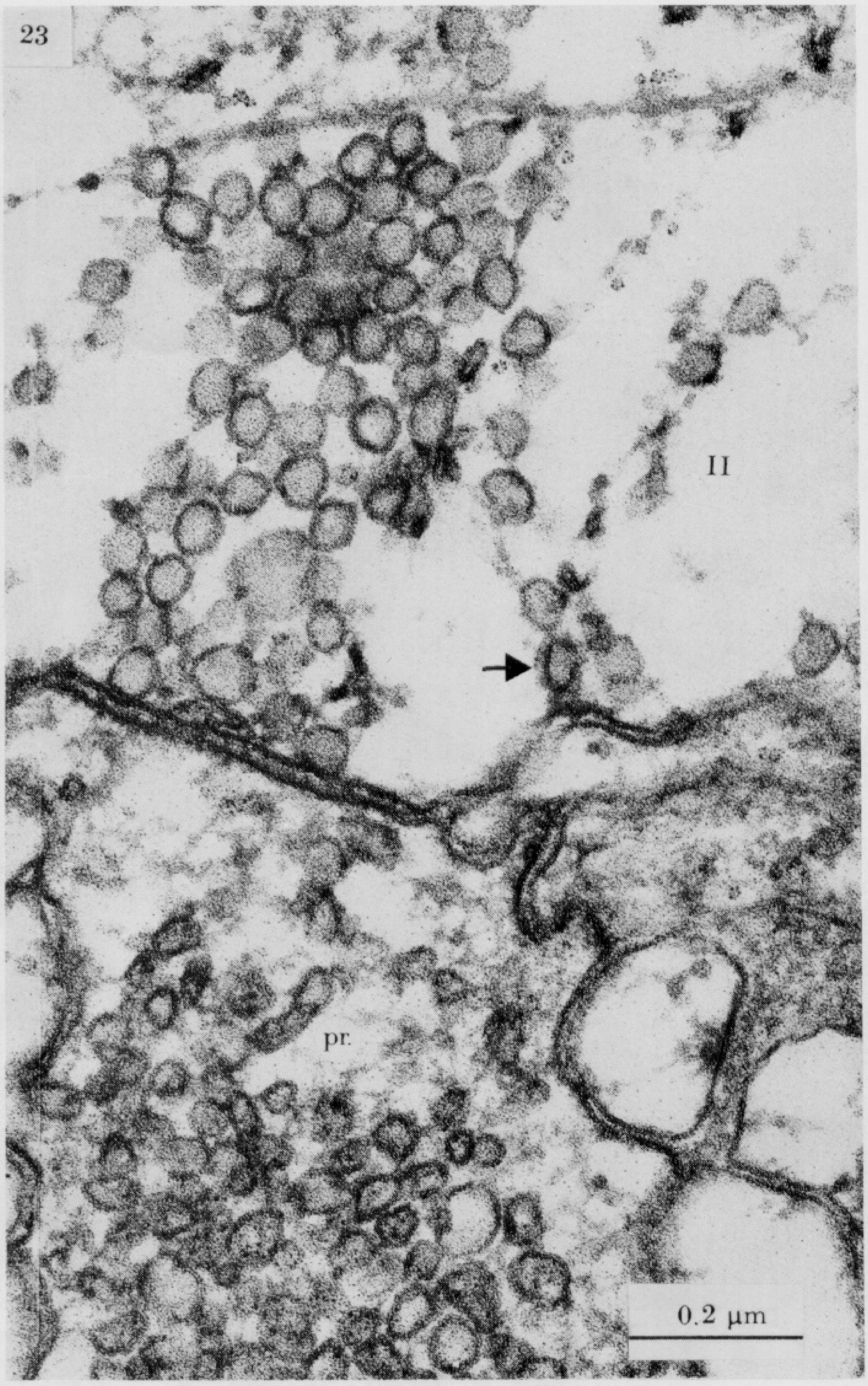

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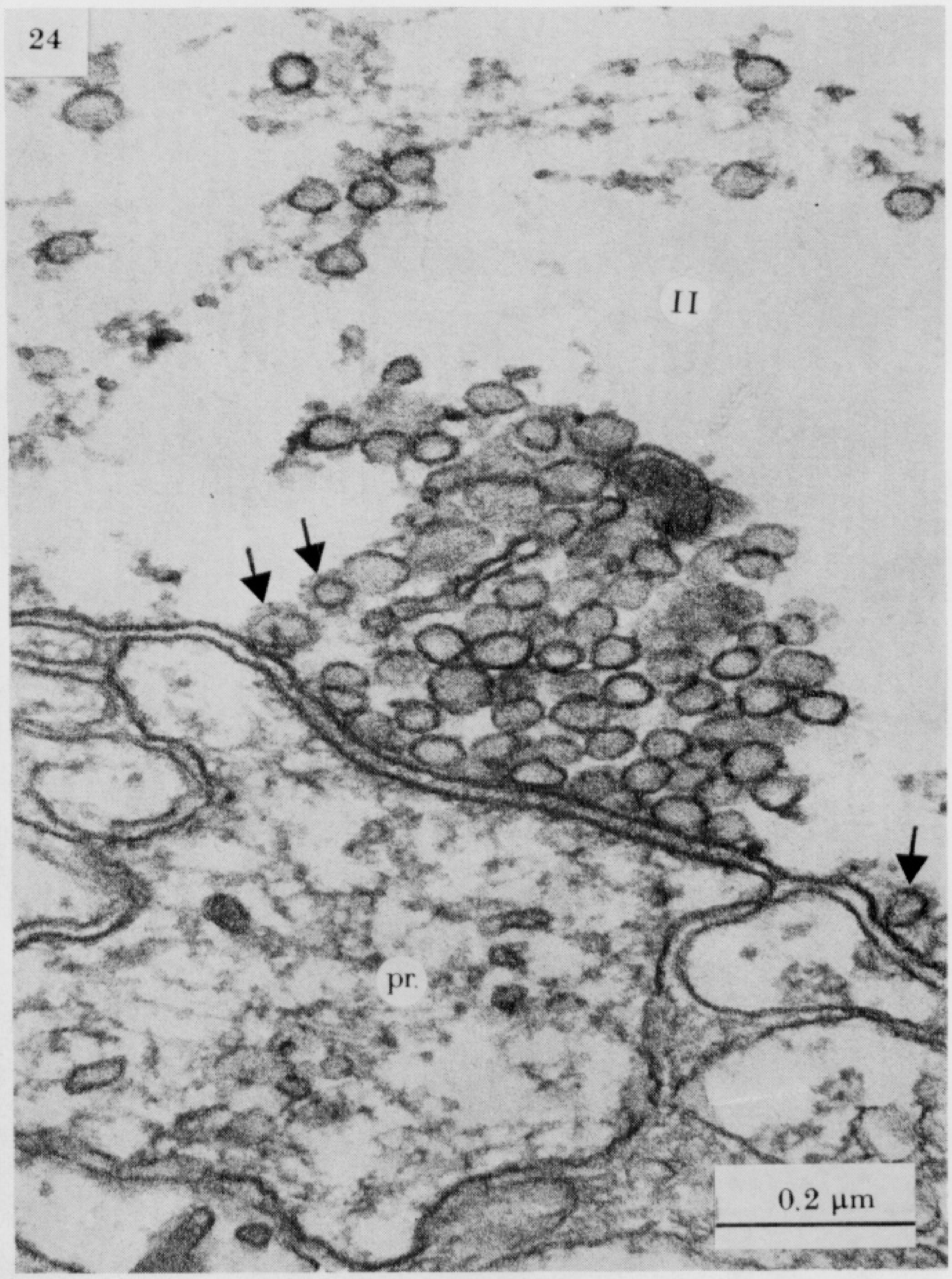

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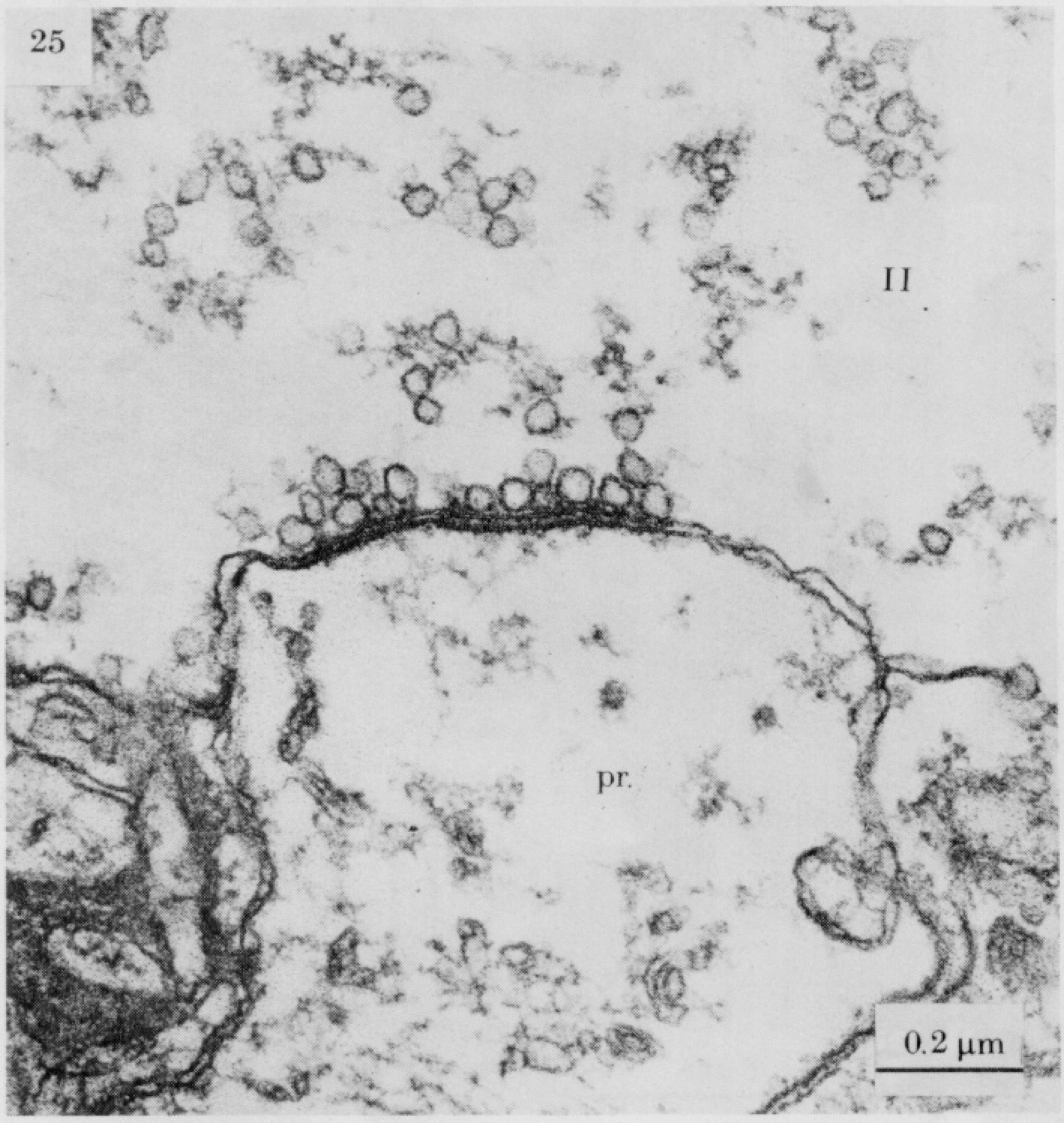

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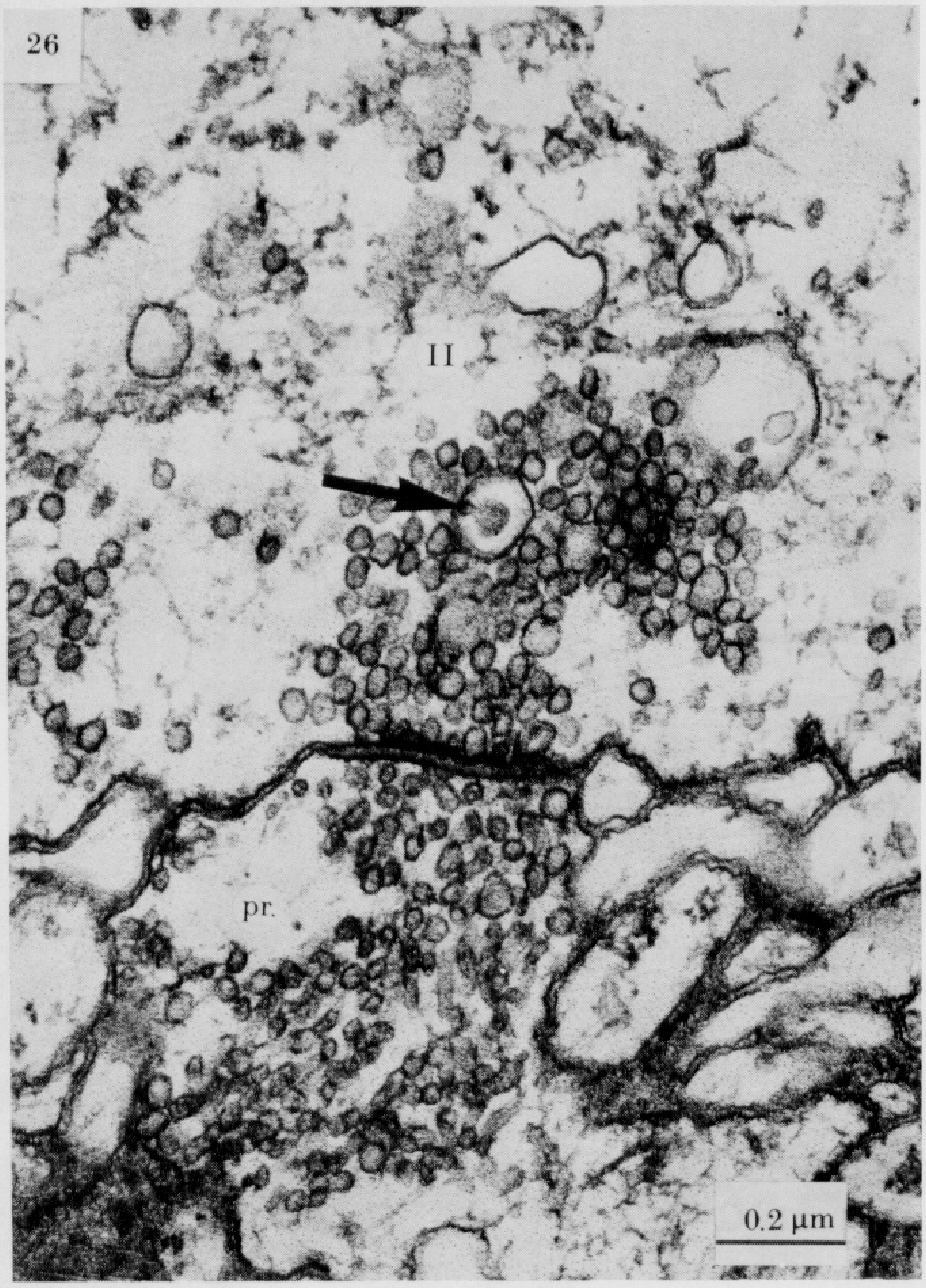

FIGURES 23–26. For description see p. 368

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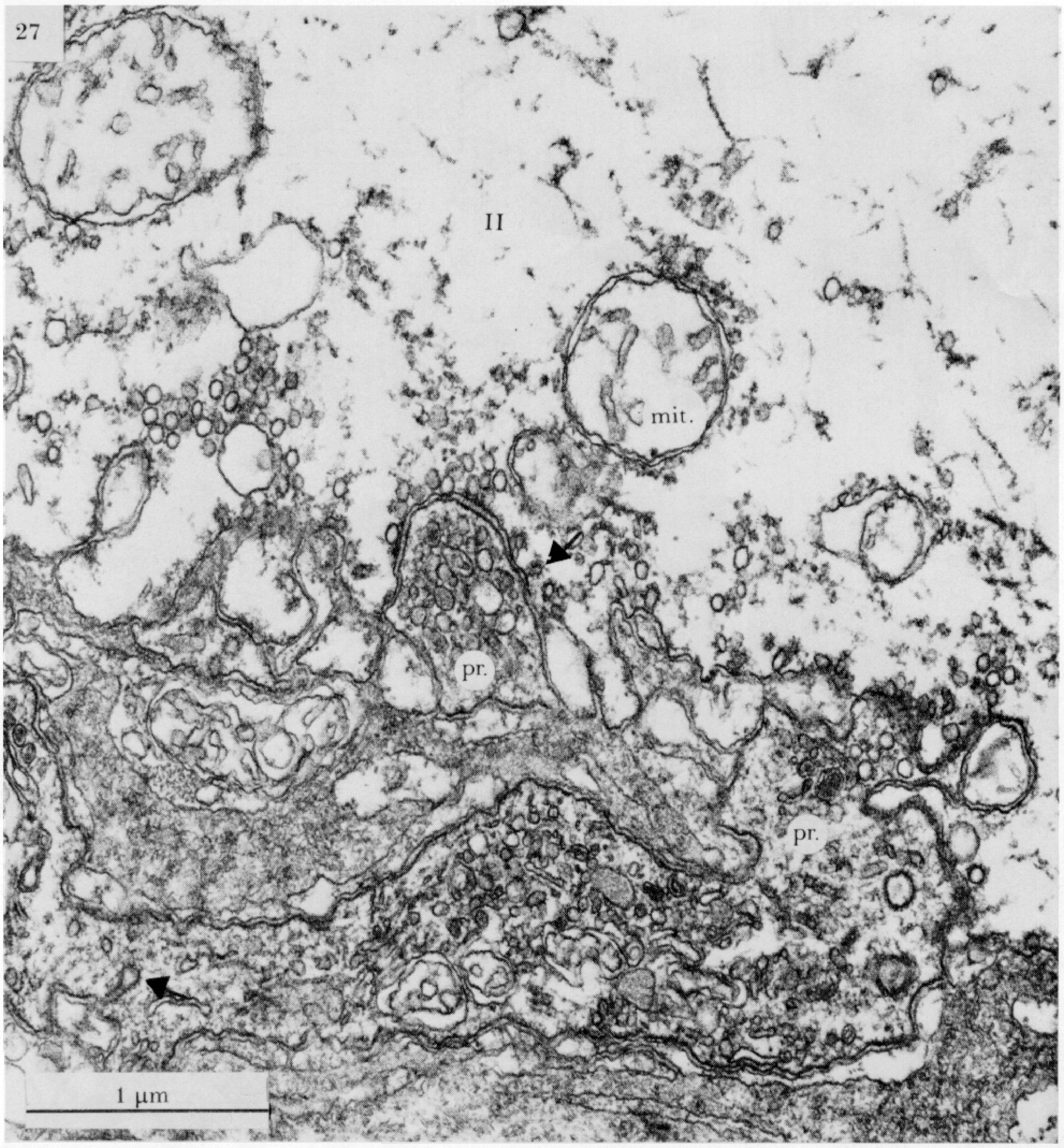

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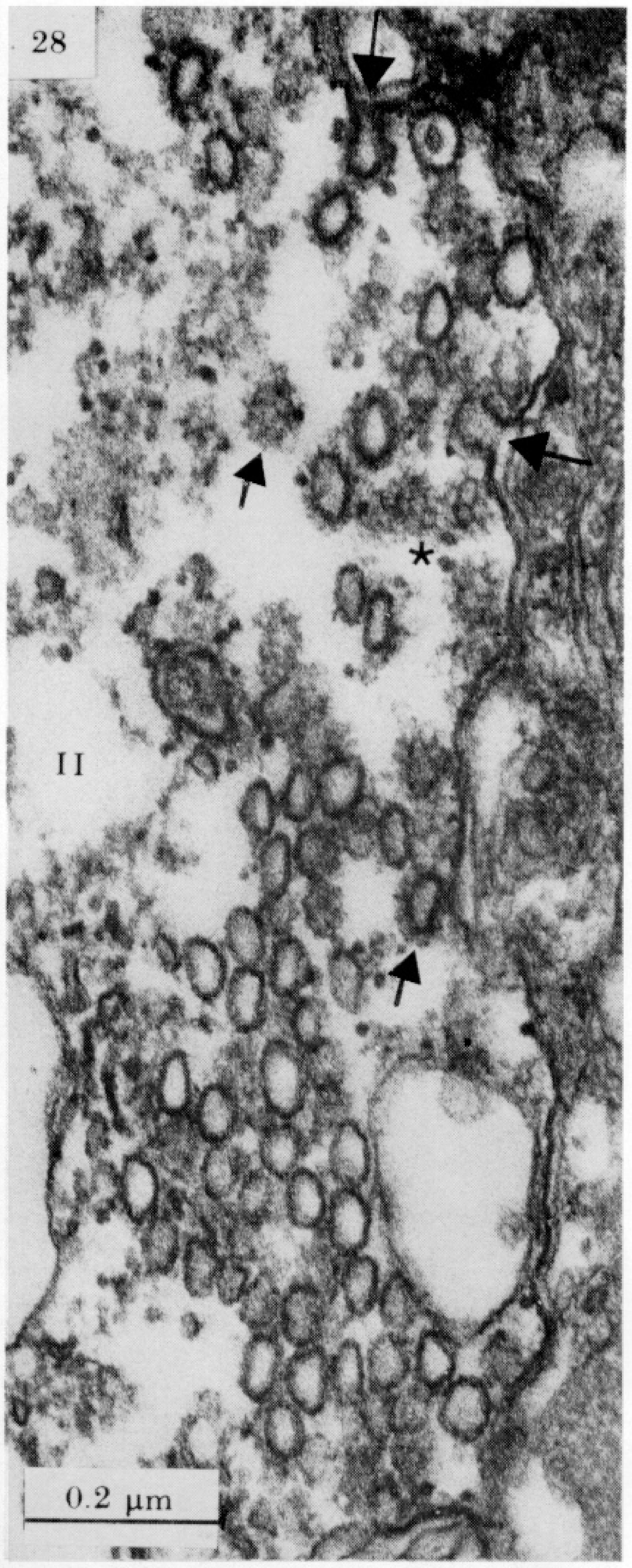

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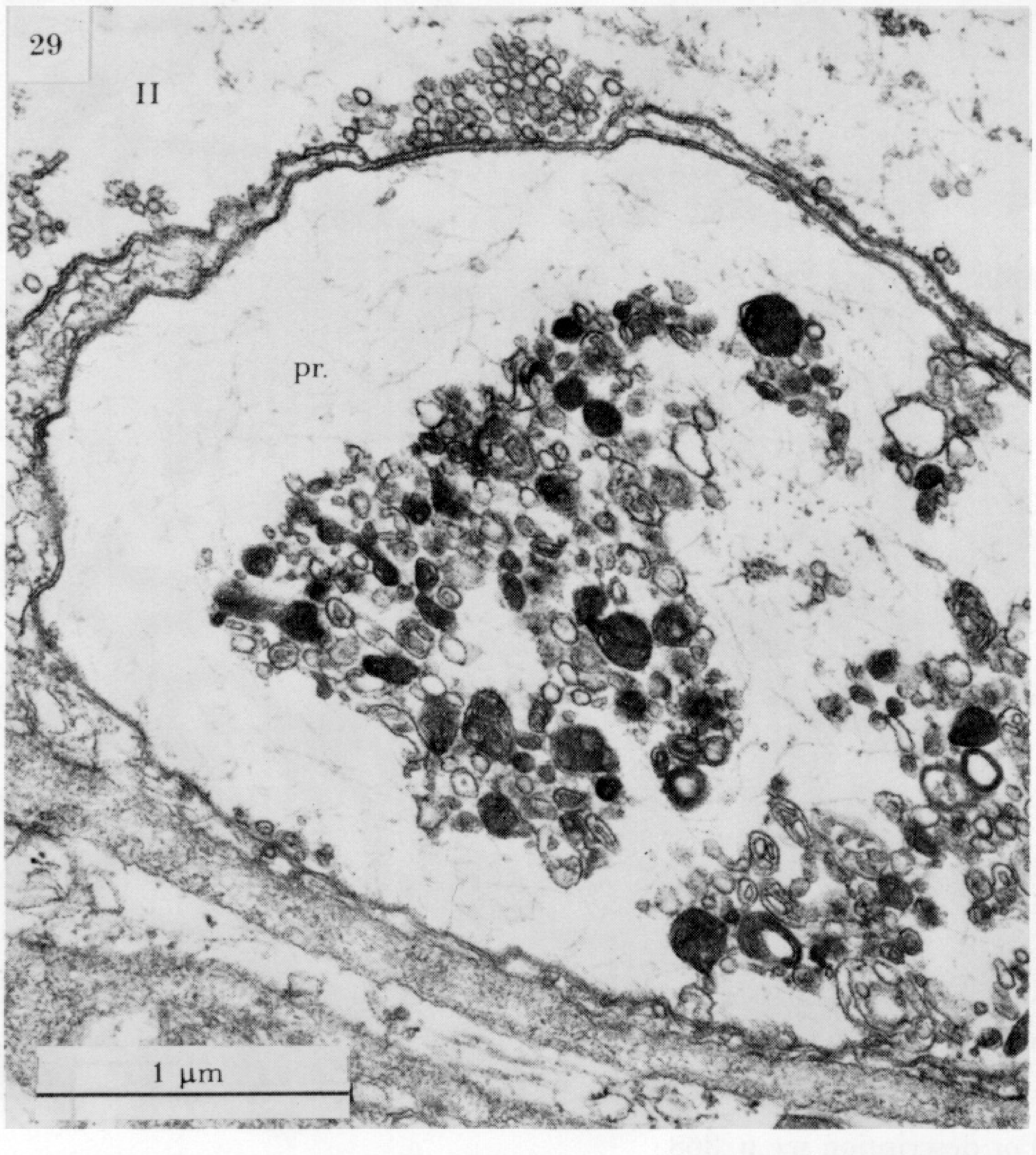

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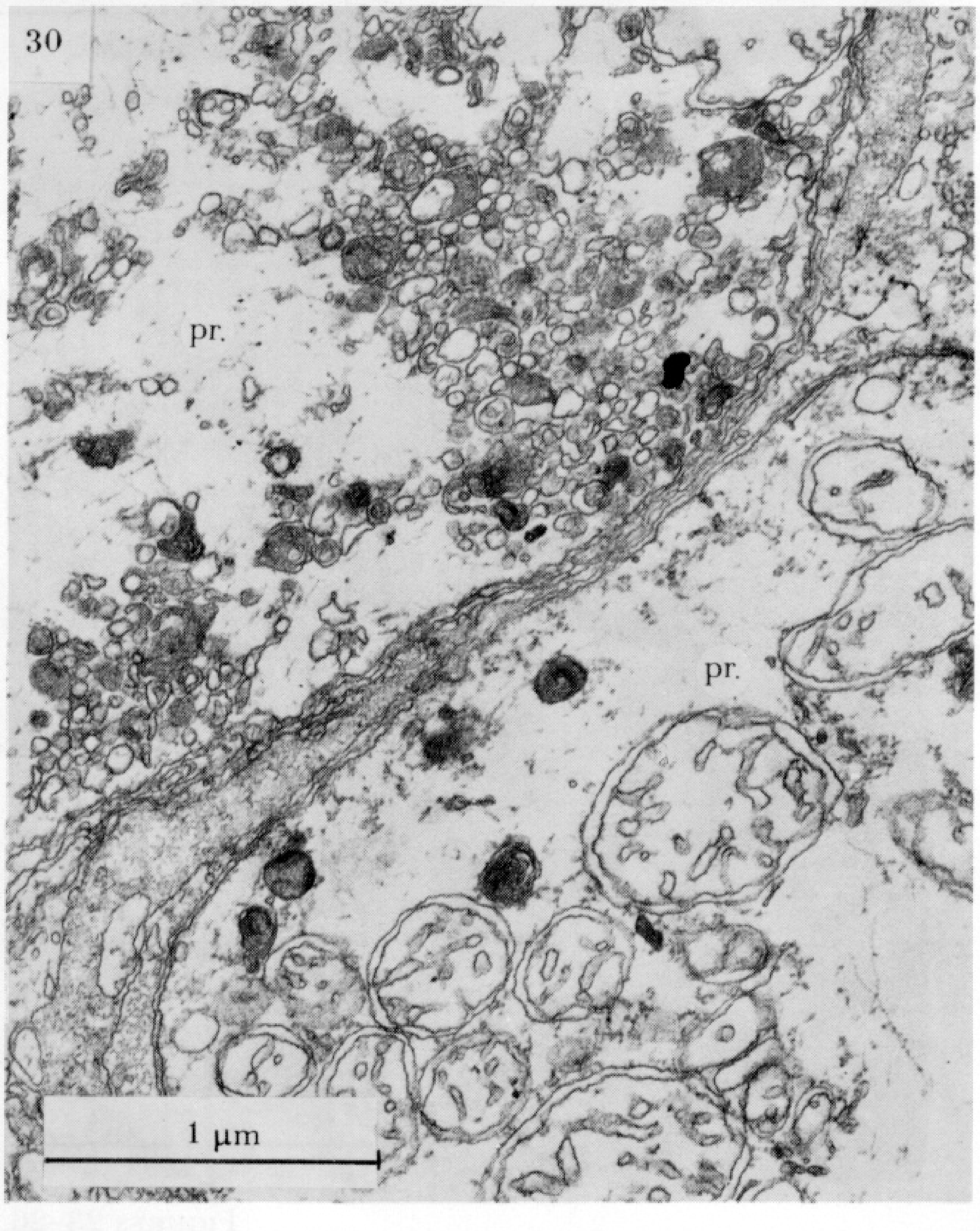

FIGURES 27–30. For description see p. 368

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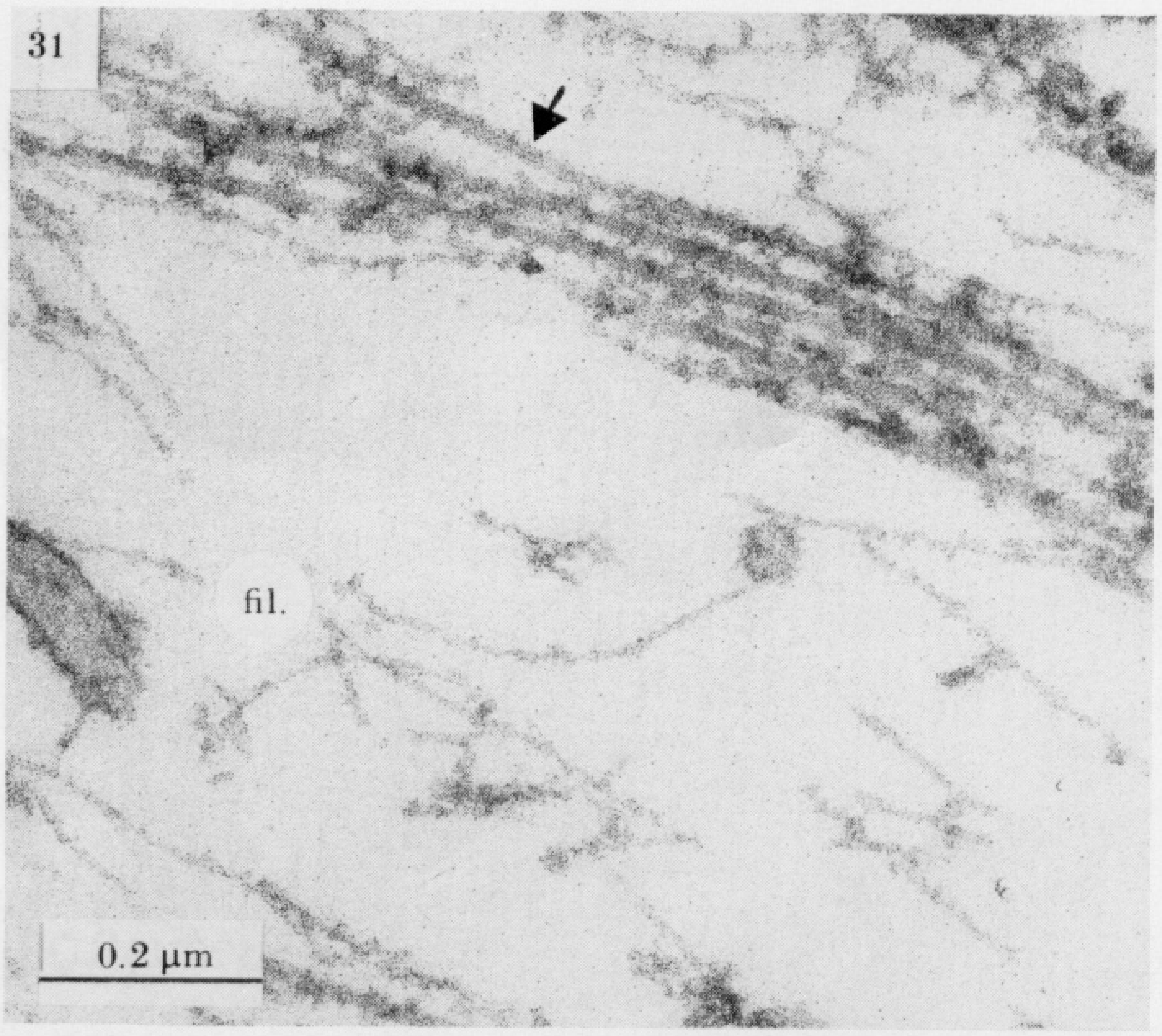

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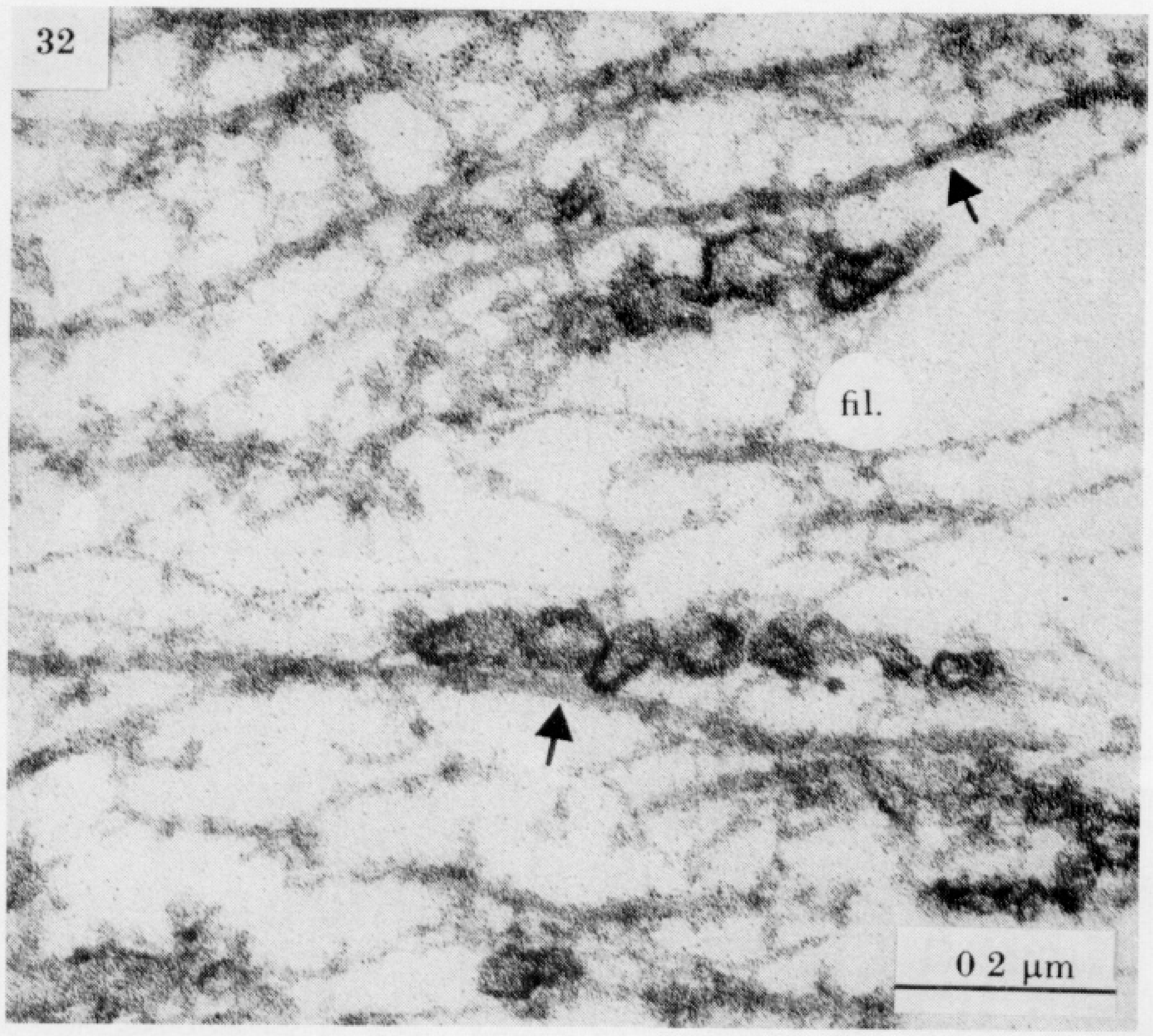

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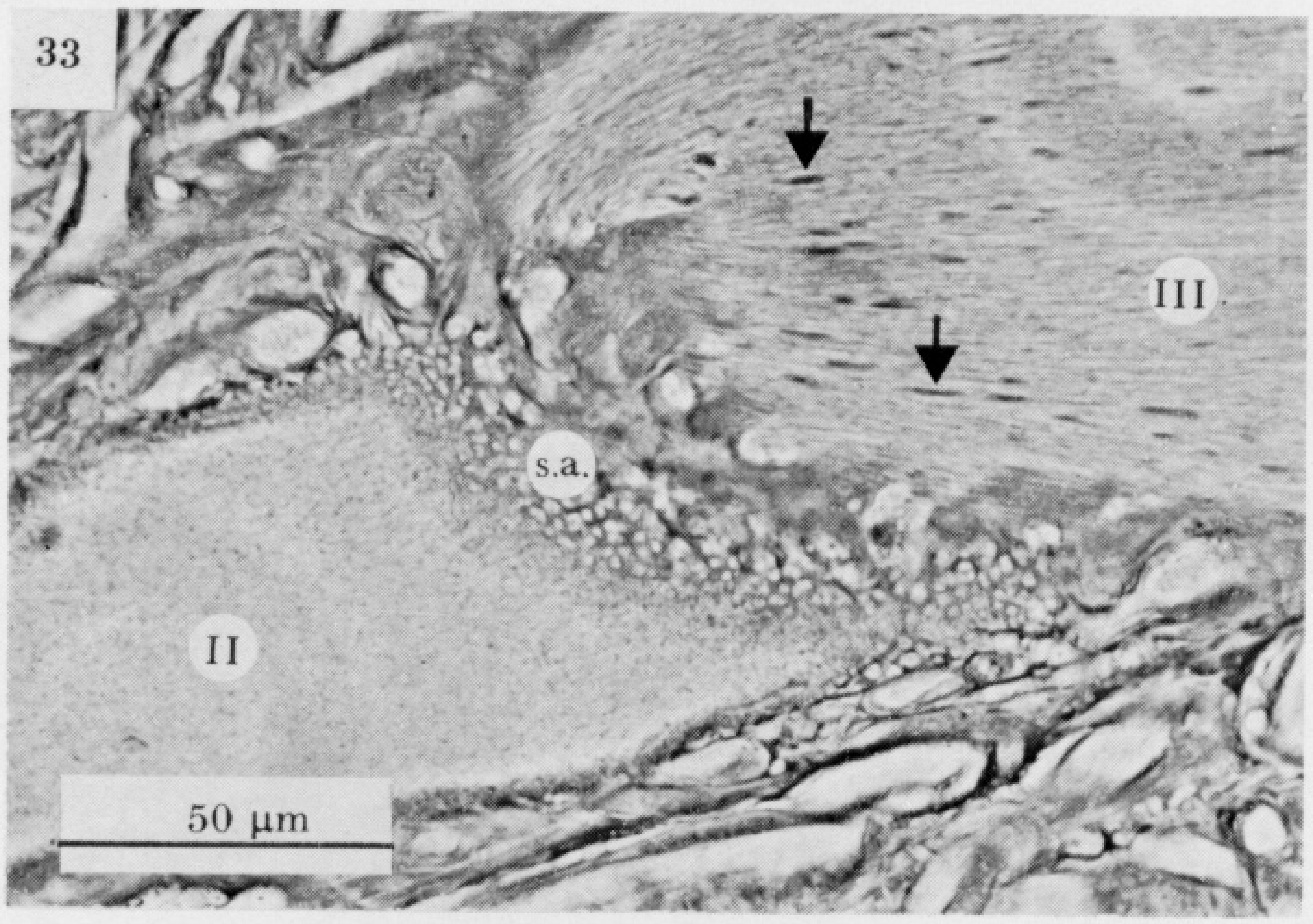

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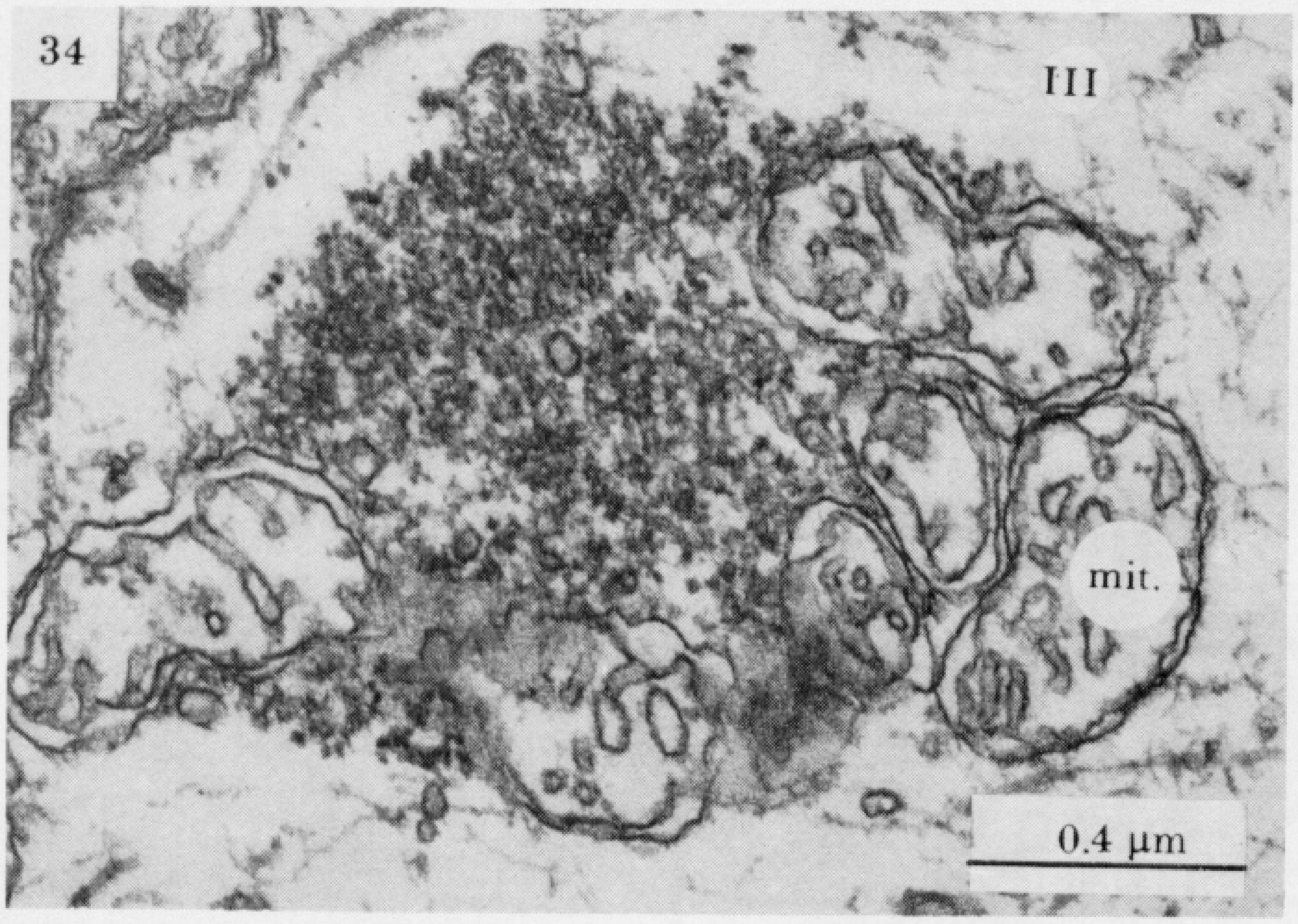

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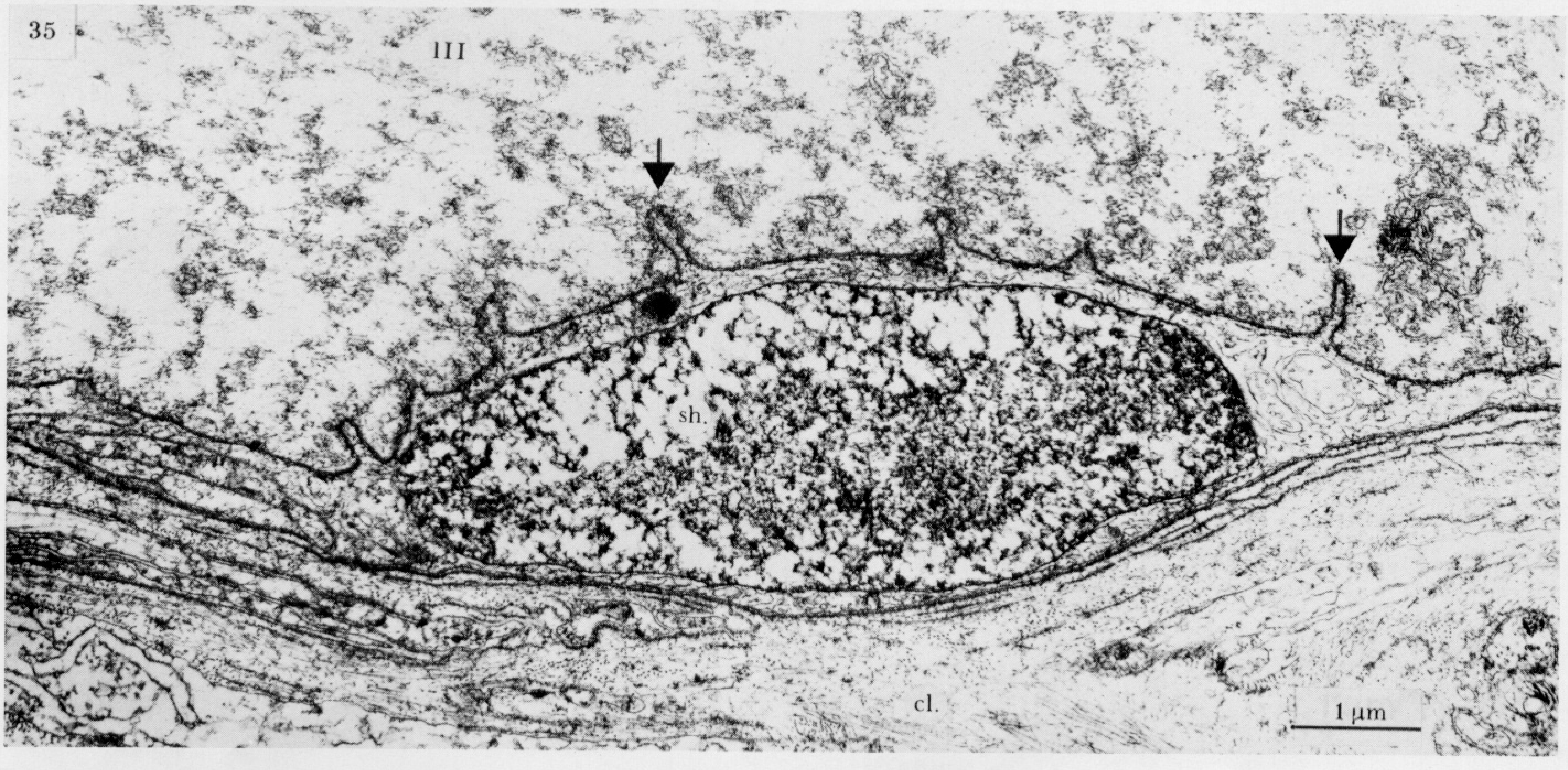

Figures 31–35. For description see p. 368

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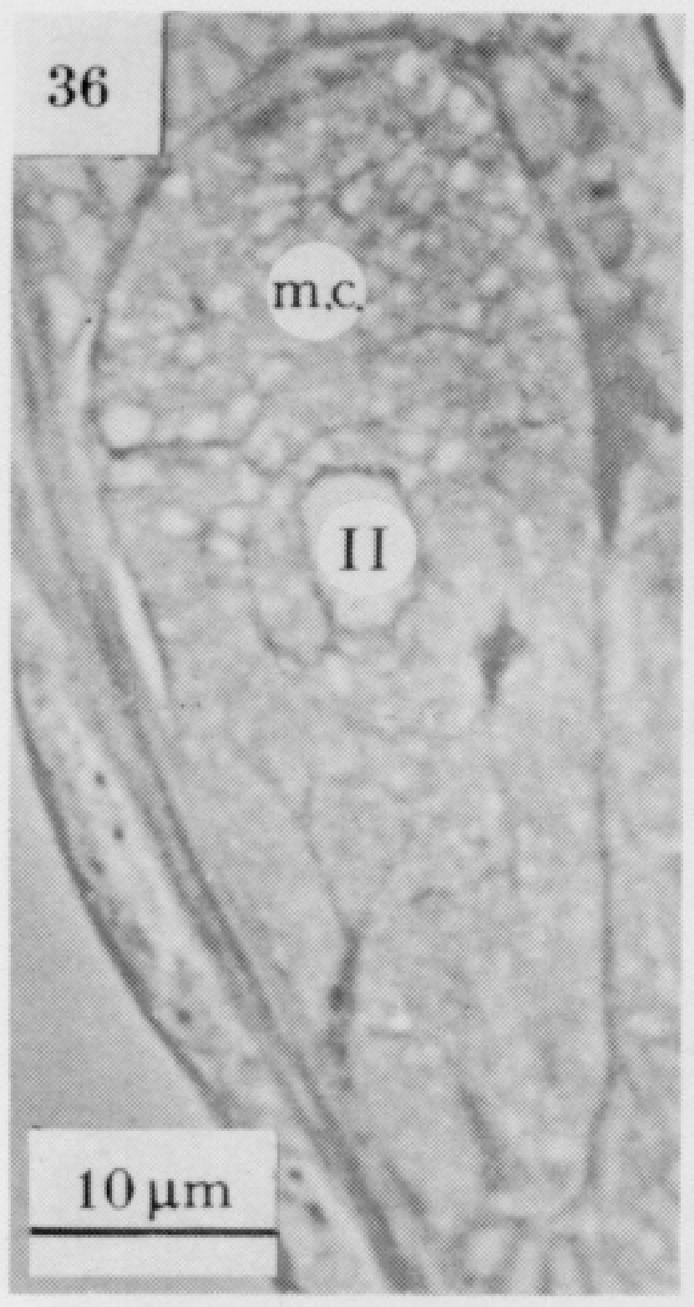

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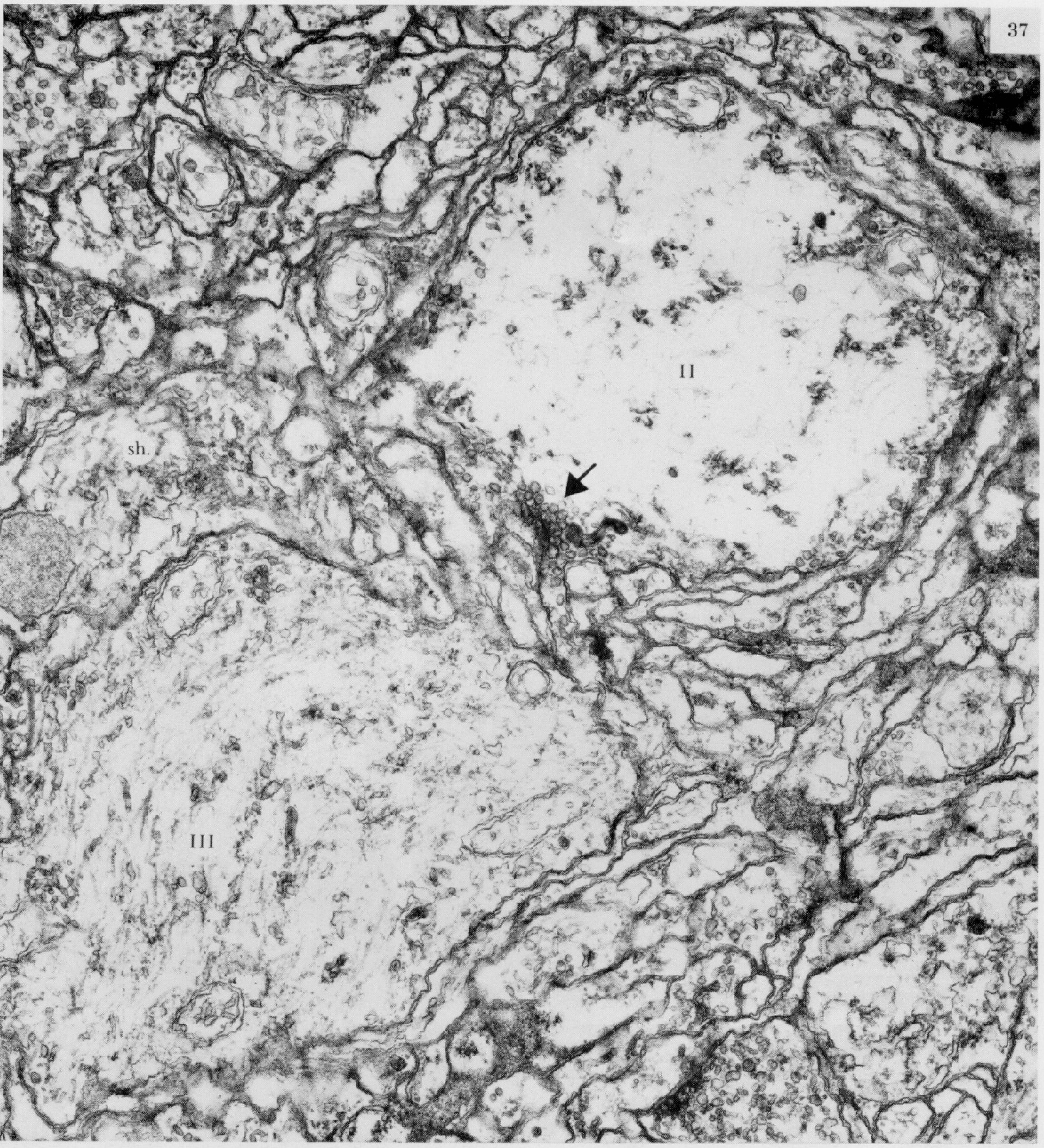

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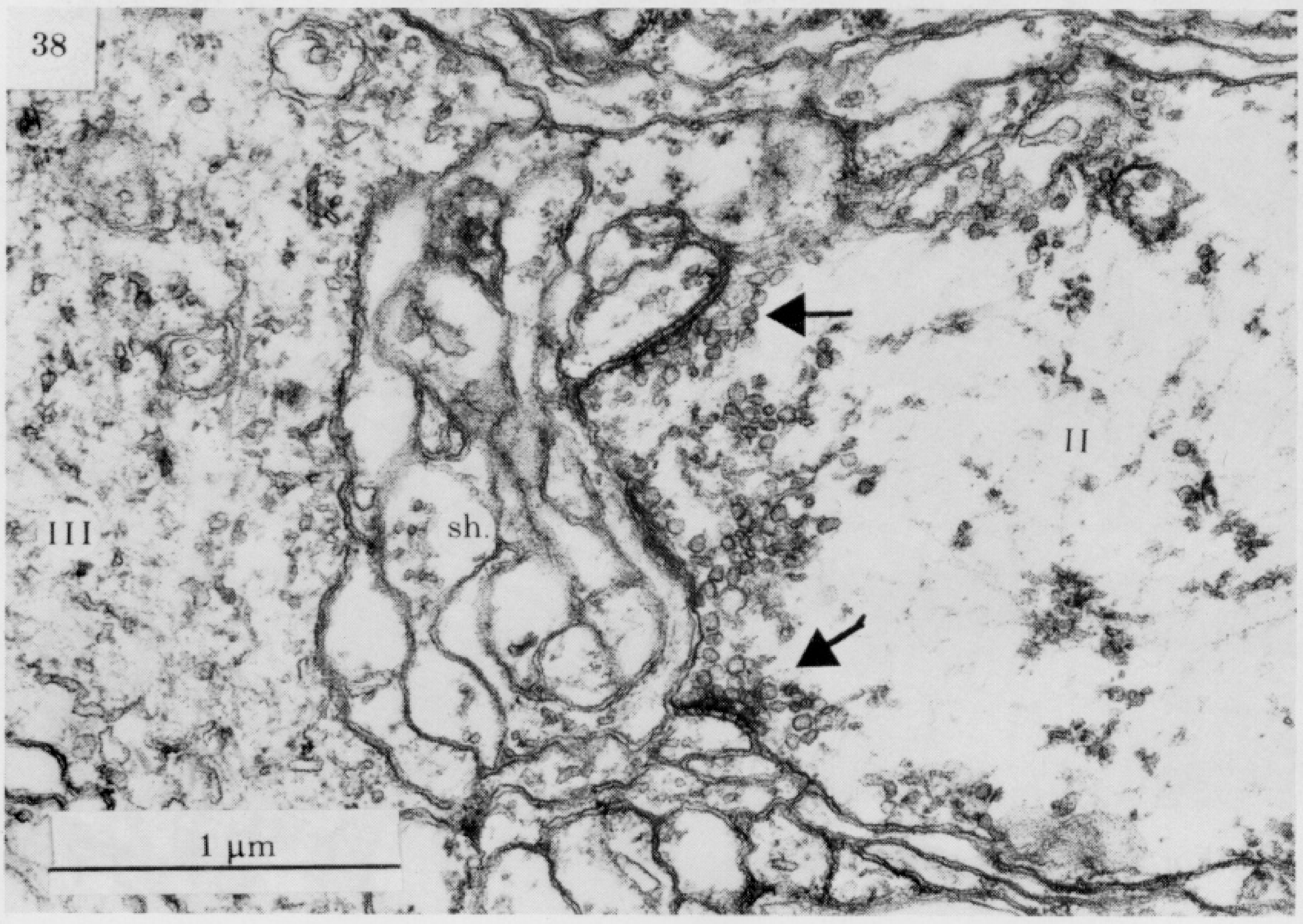

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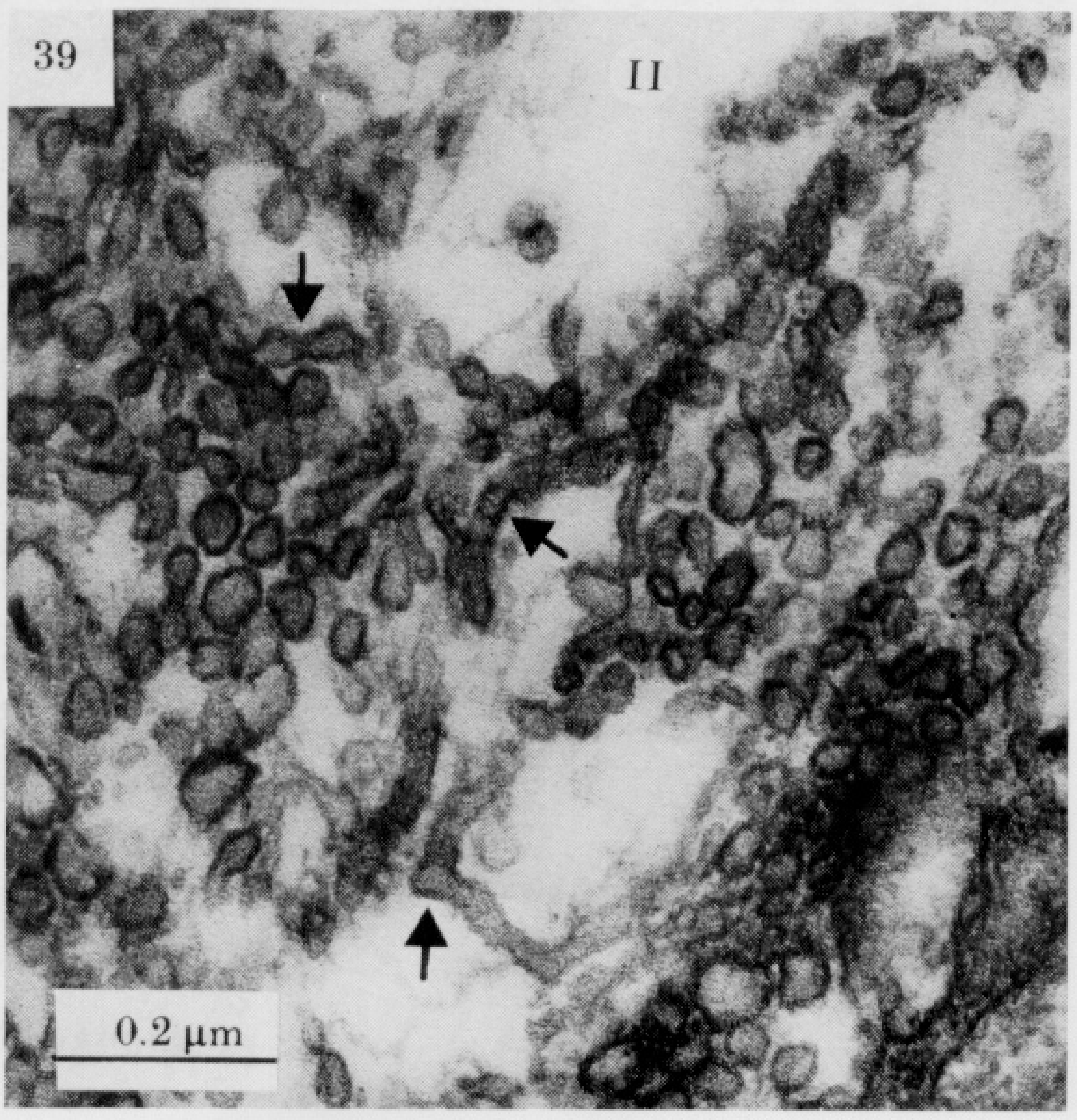

FIGURES 36–39. For description see p. 368

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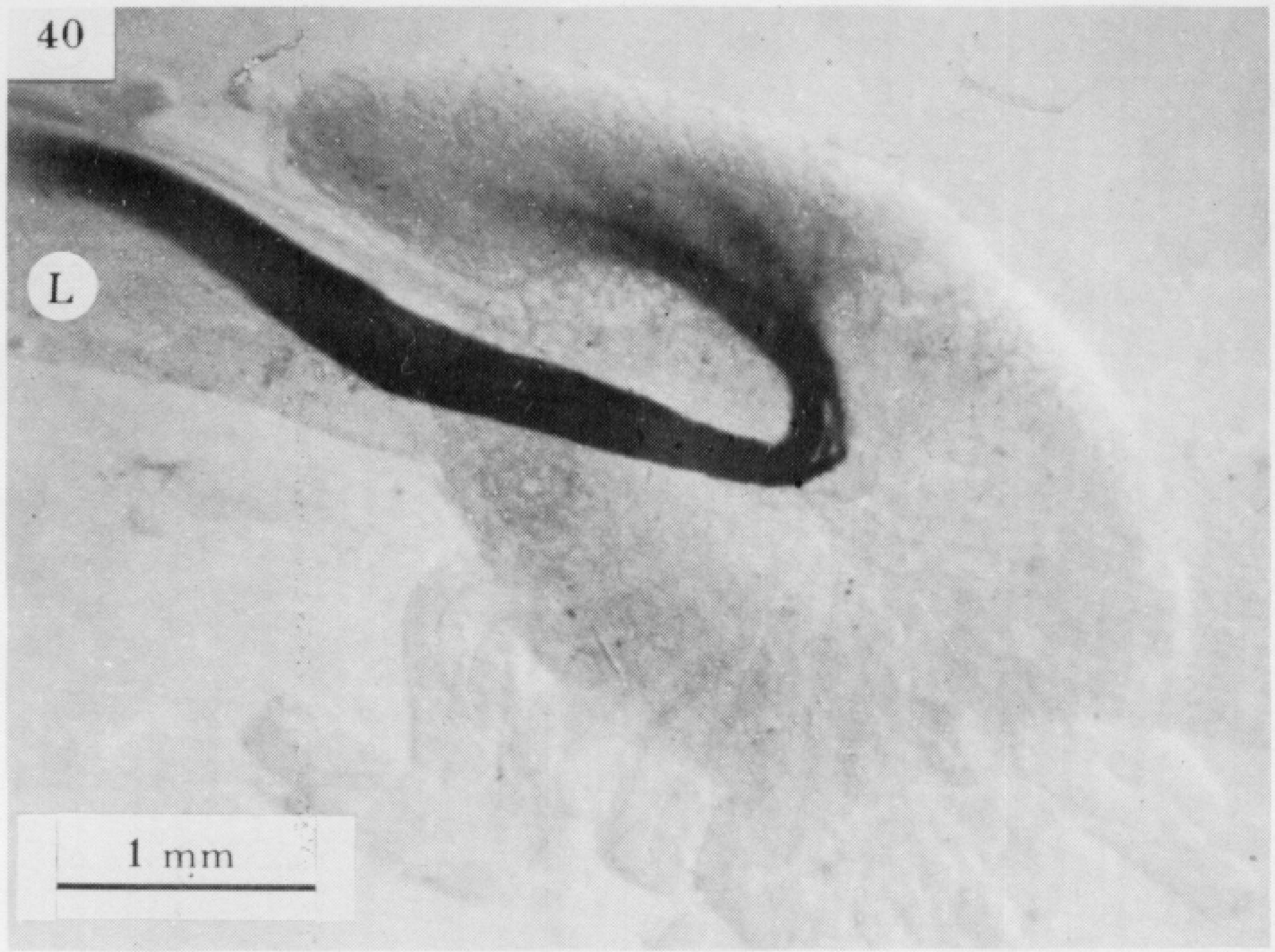

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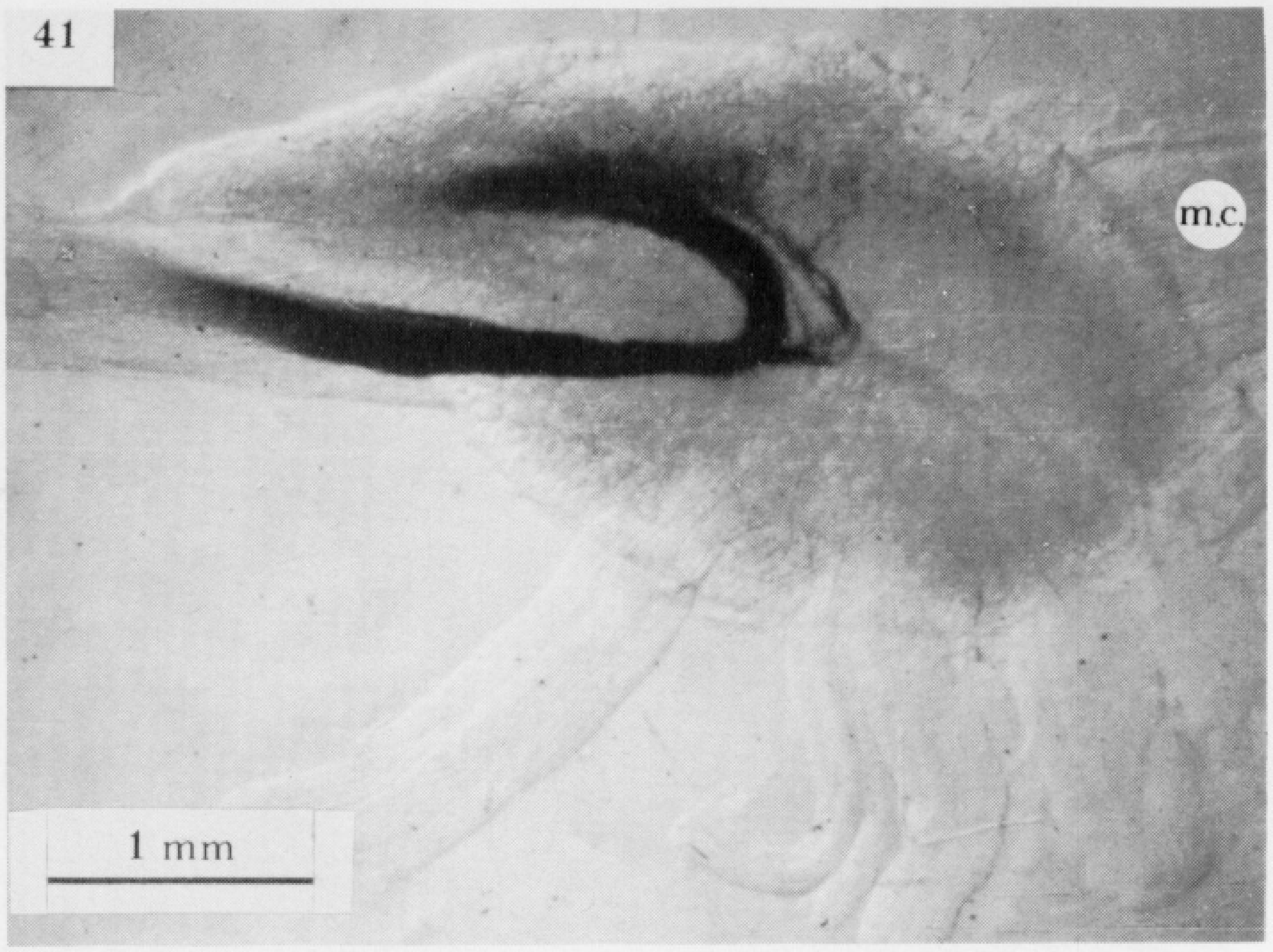

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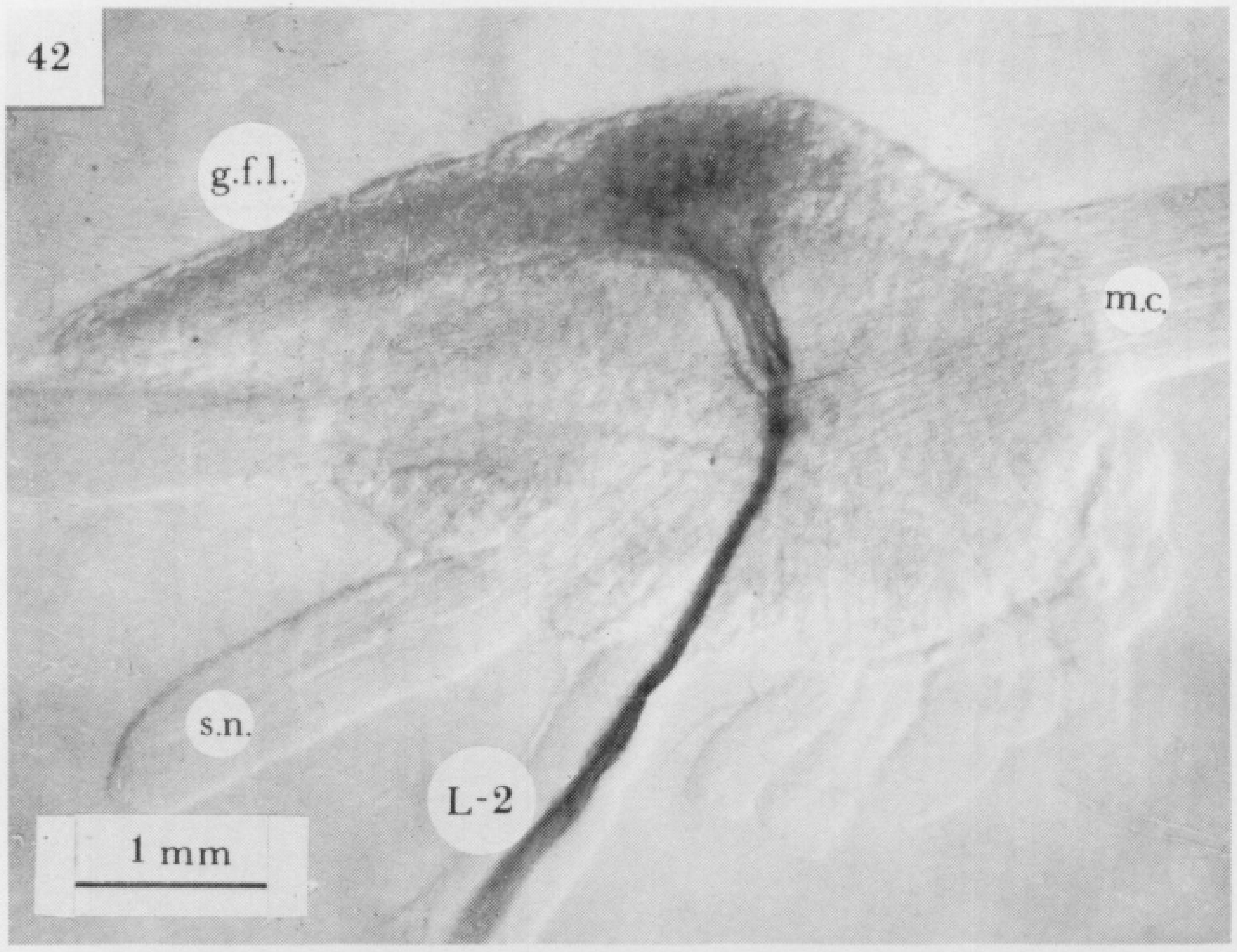

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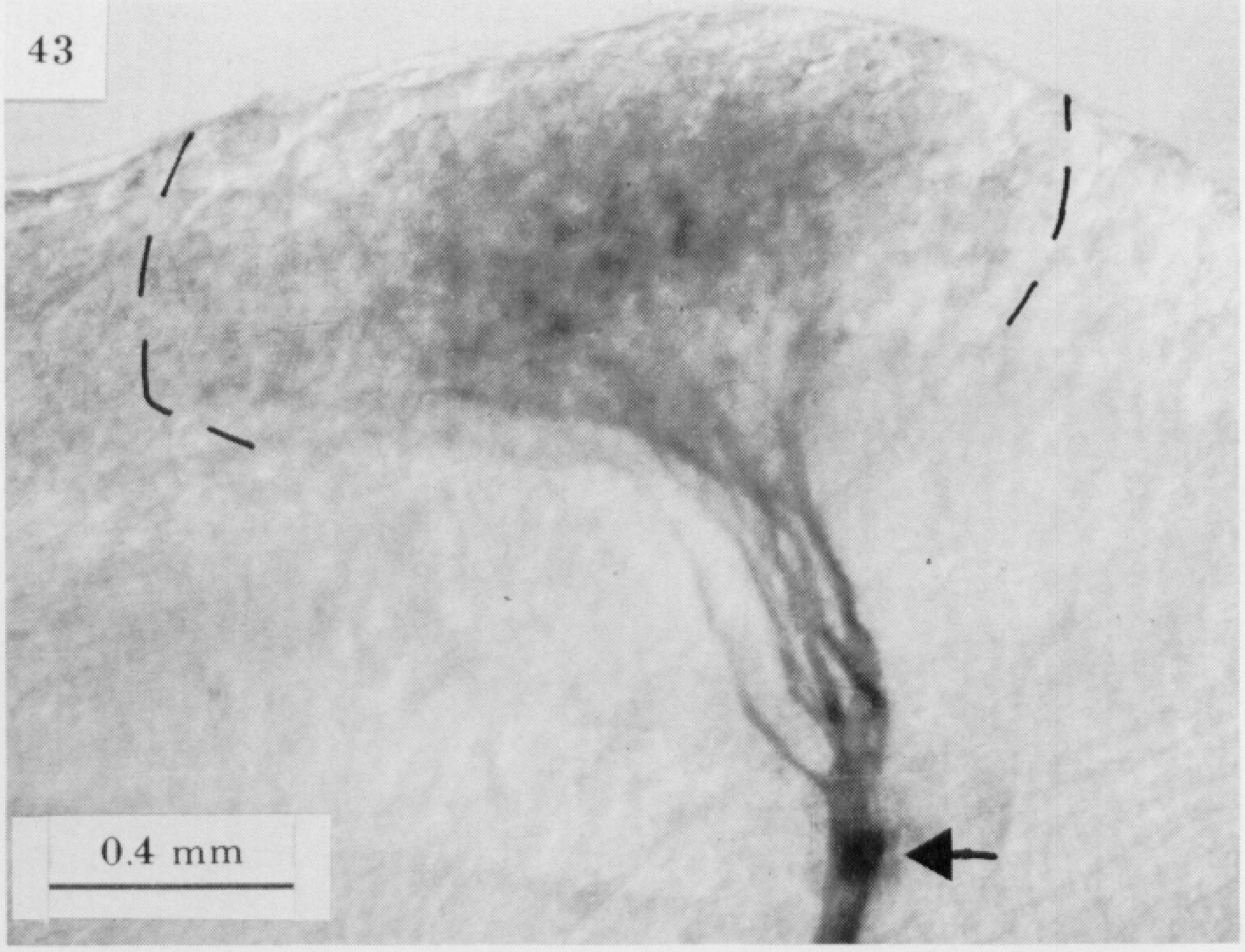

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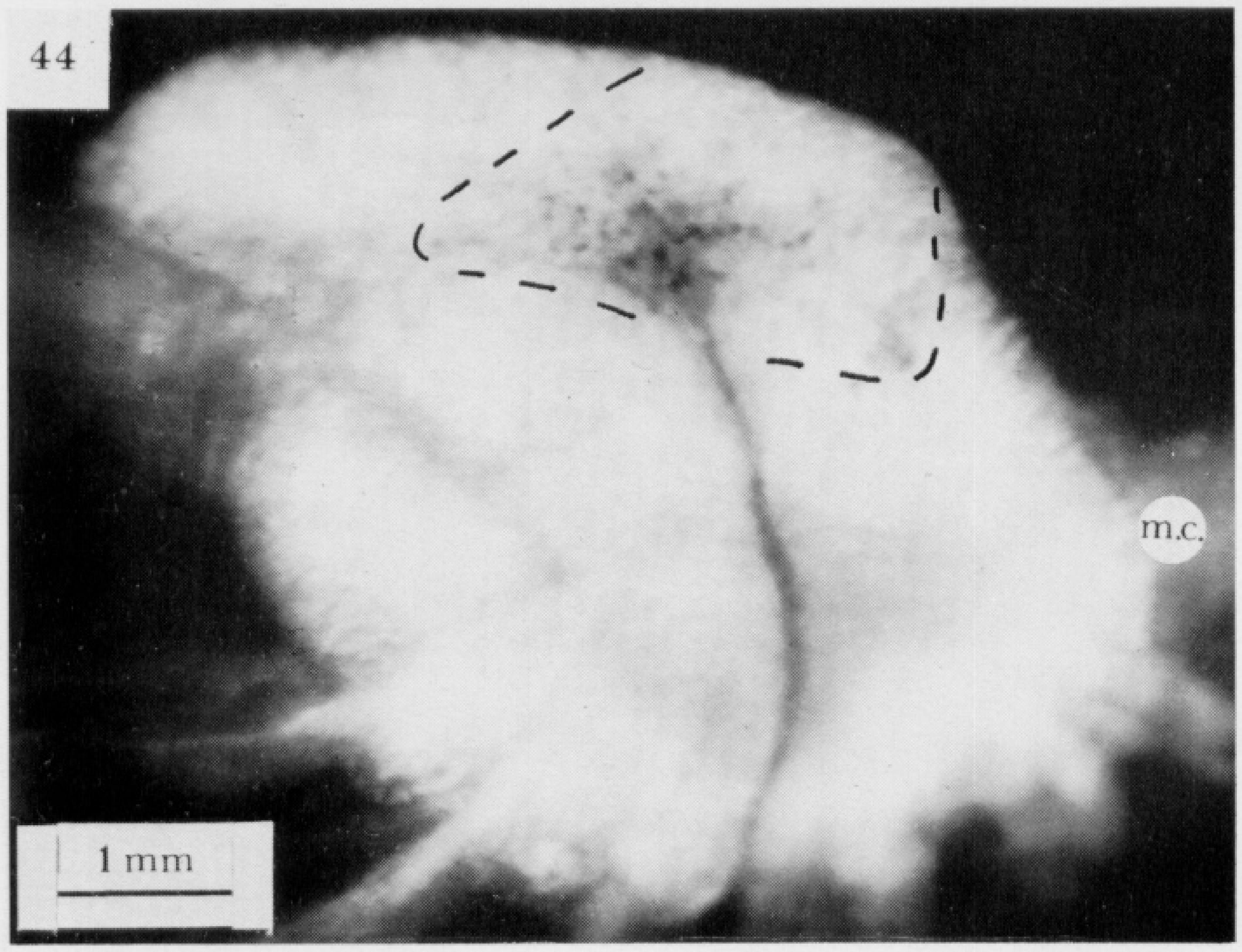

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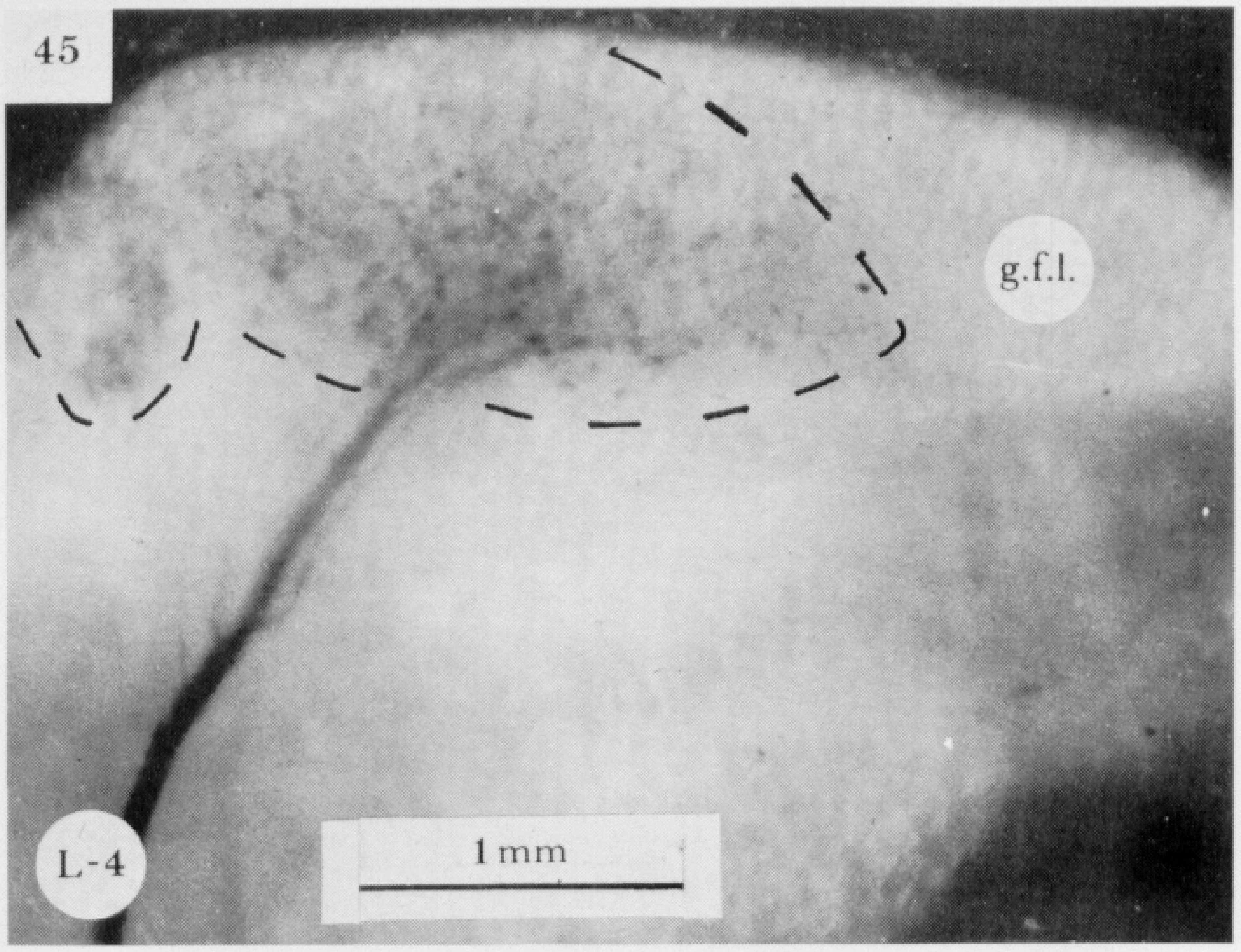

FIGURES 40–45. Cobalt injected third order giant axons. Abbreviations: g.f.l., giant fibre lobe; m.c., mantle connective; s.n., stellar nerve. Figures 40 and 41 show the largest axons (L) in two small squids of 7 cm mantle length, figures 42 and 43 the third largest axon (L-2) in a squid of 8 cm mantle length, figures 44 and 45 the fifth largest axon (L-4) of a squid of 18 cm mantle length, seen from the dorsal (figure 44) and from the ventral side of the ganglion (figure 45).

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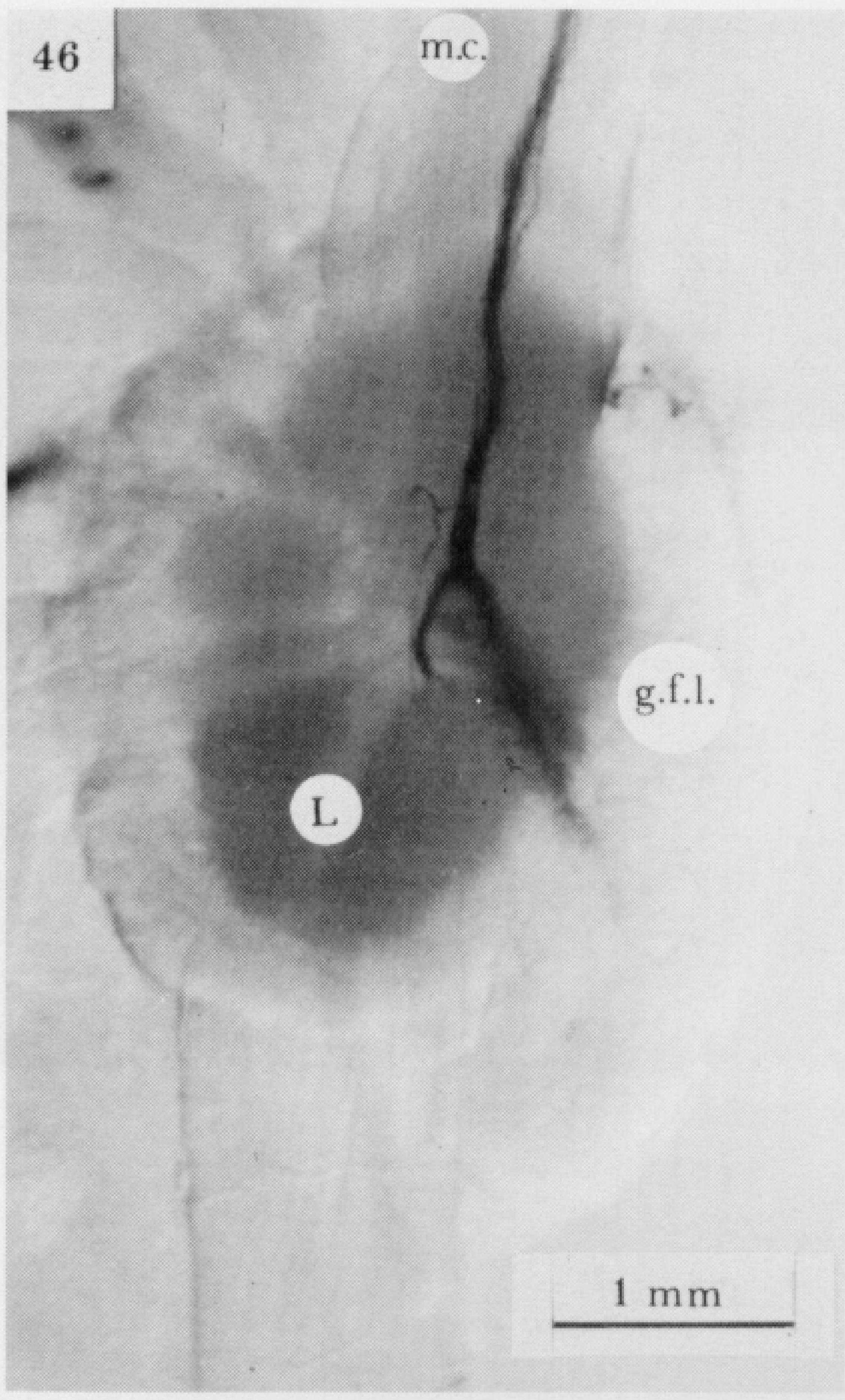

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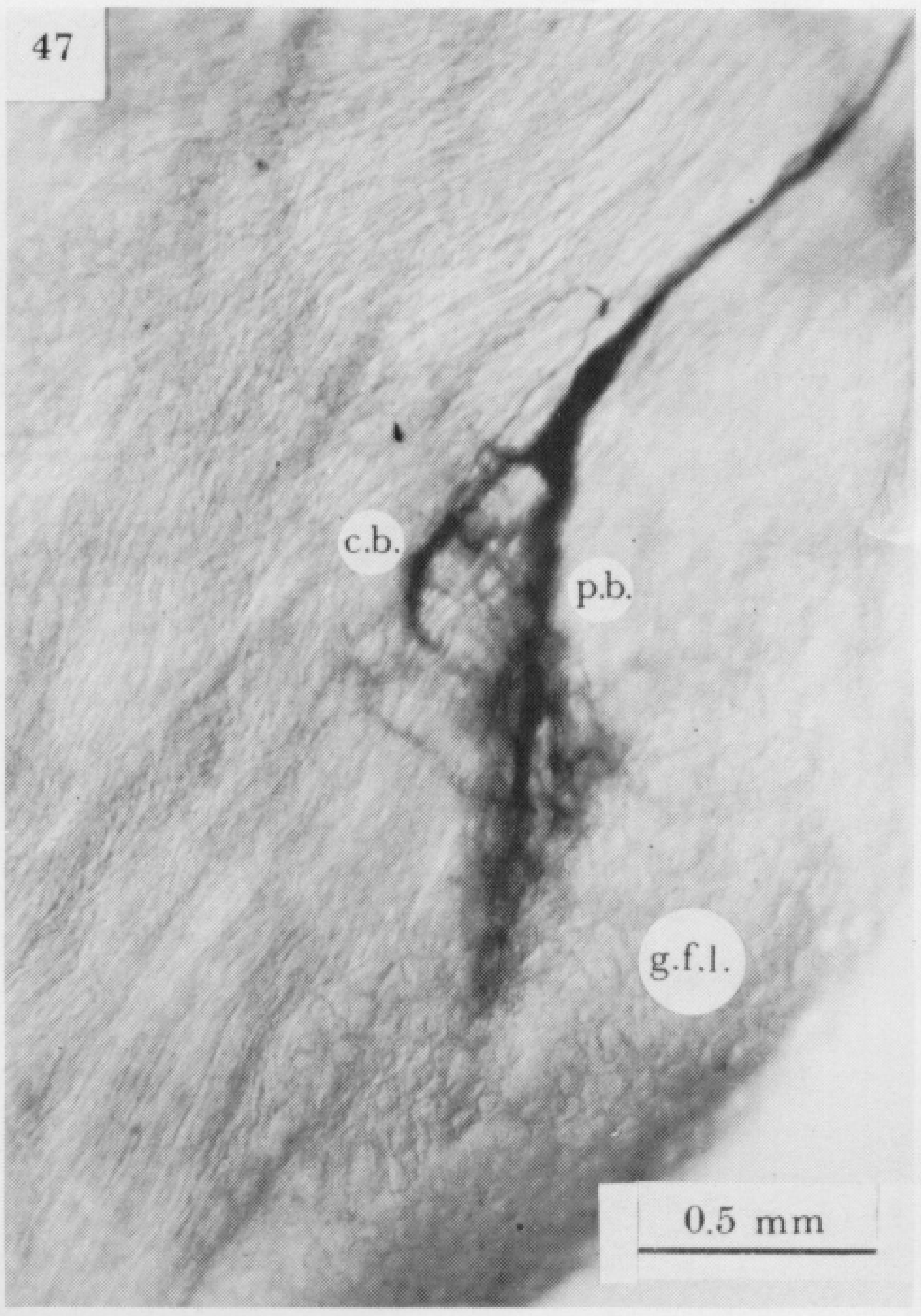

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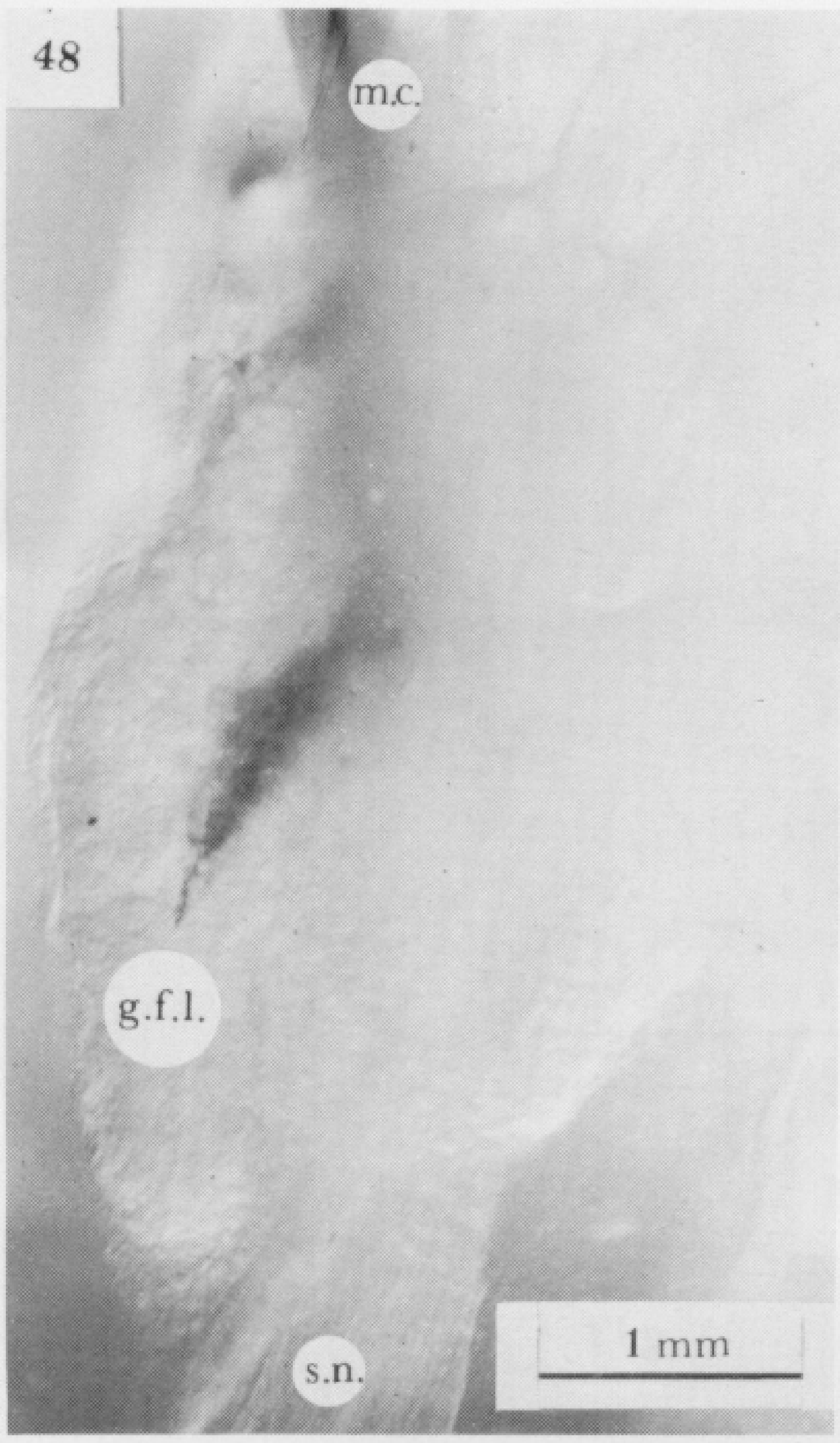

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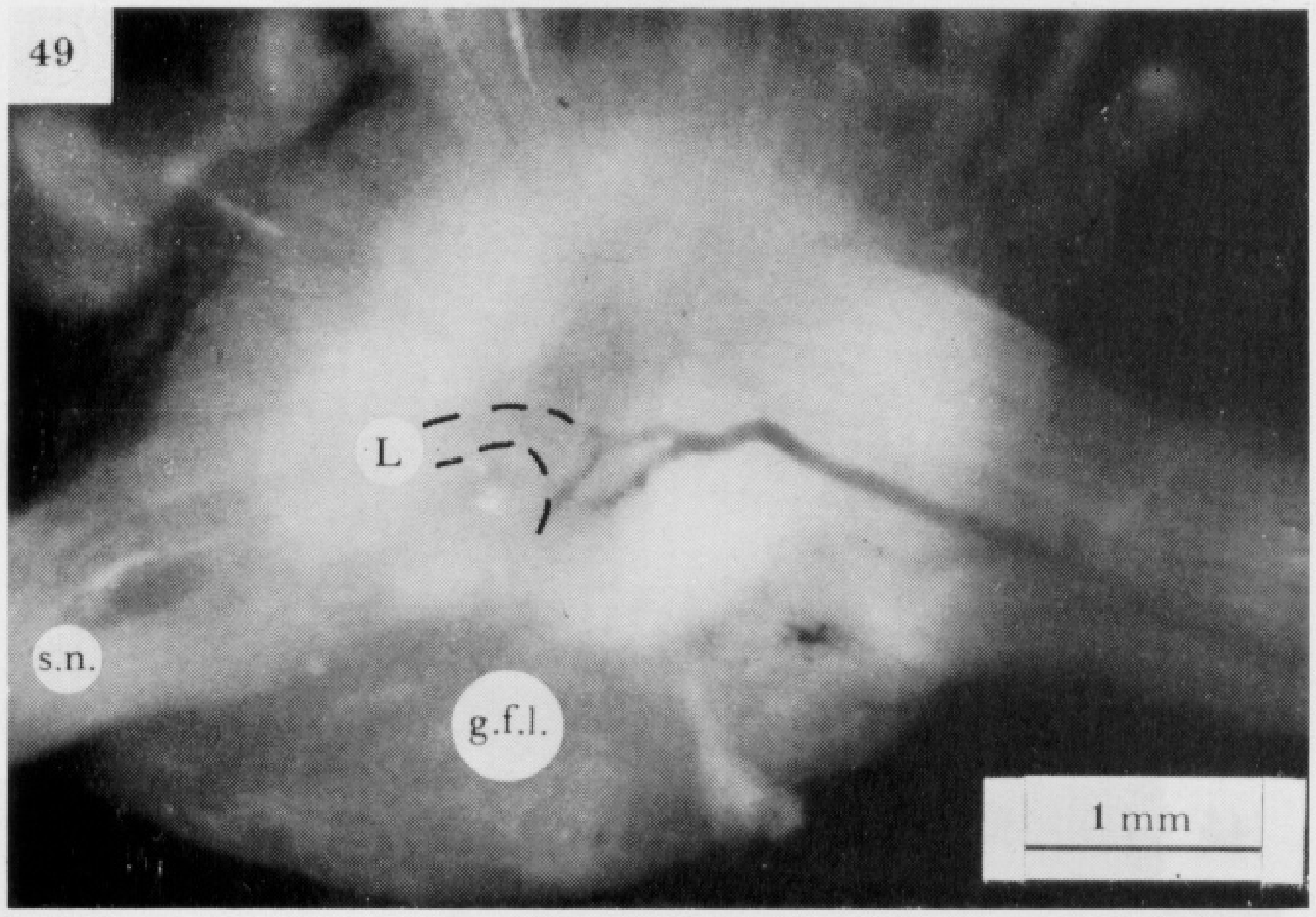

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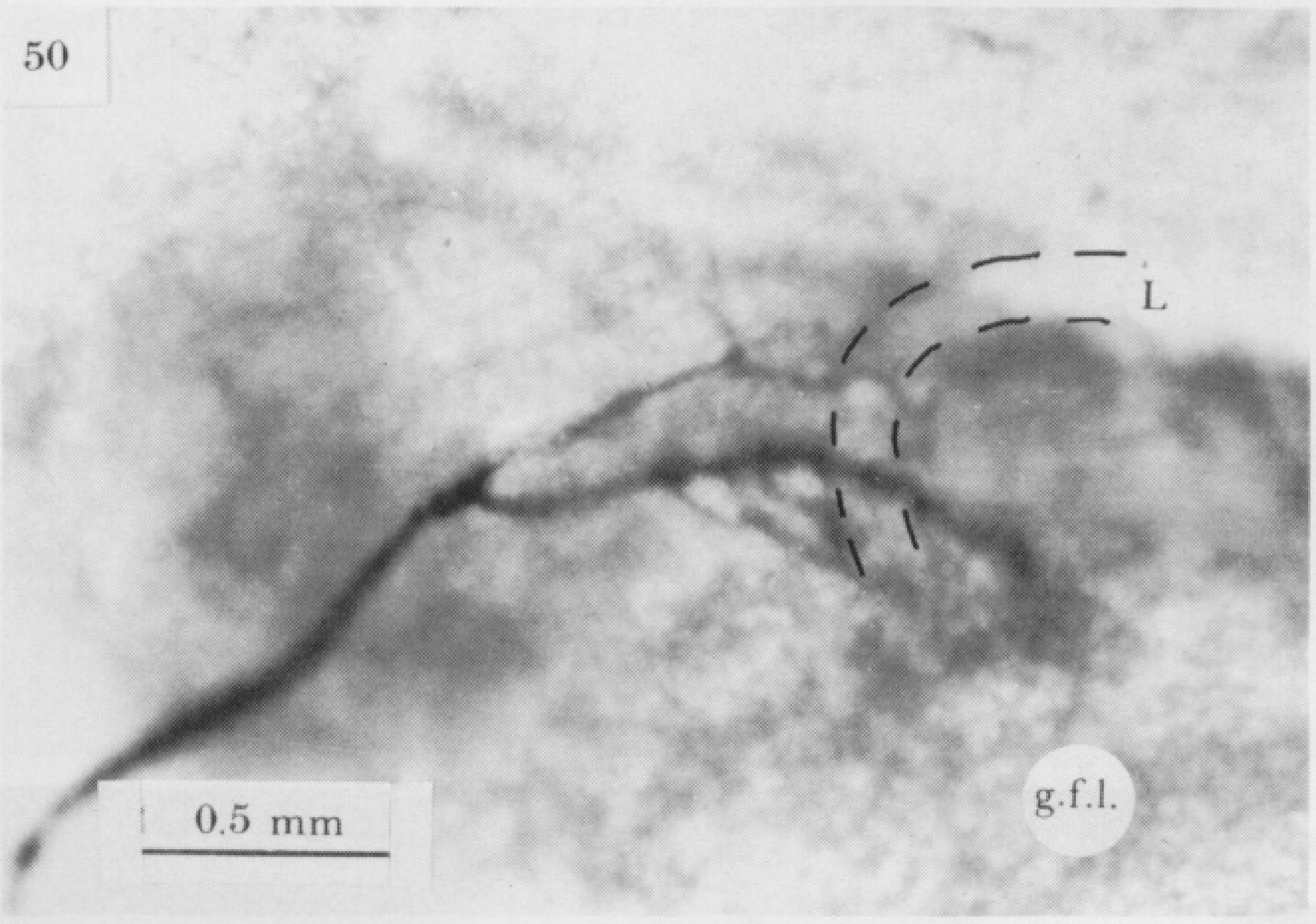

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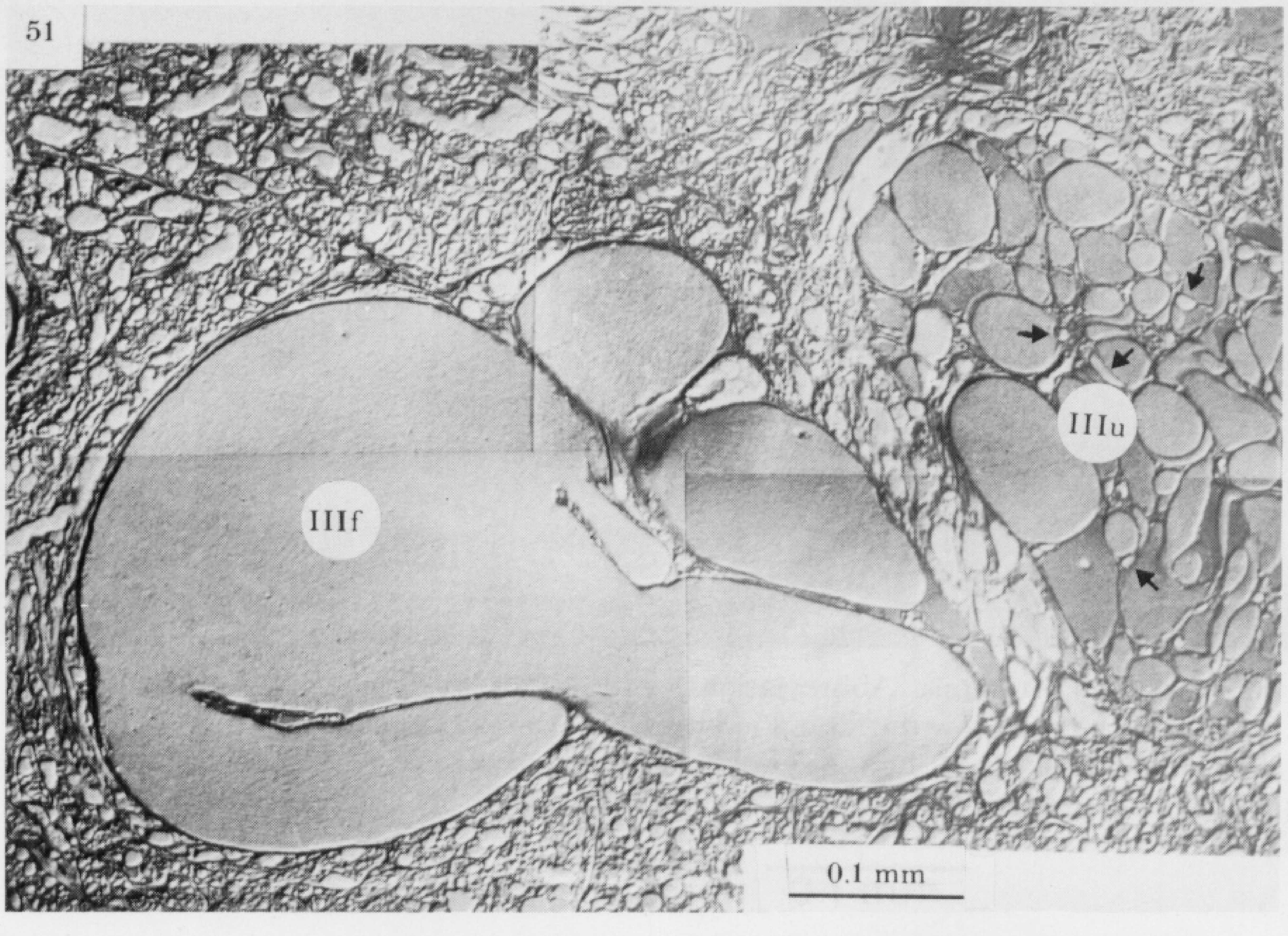

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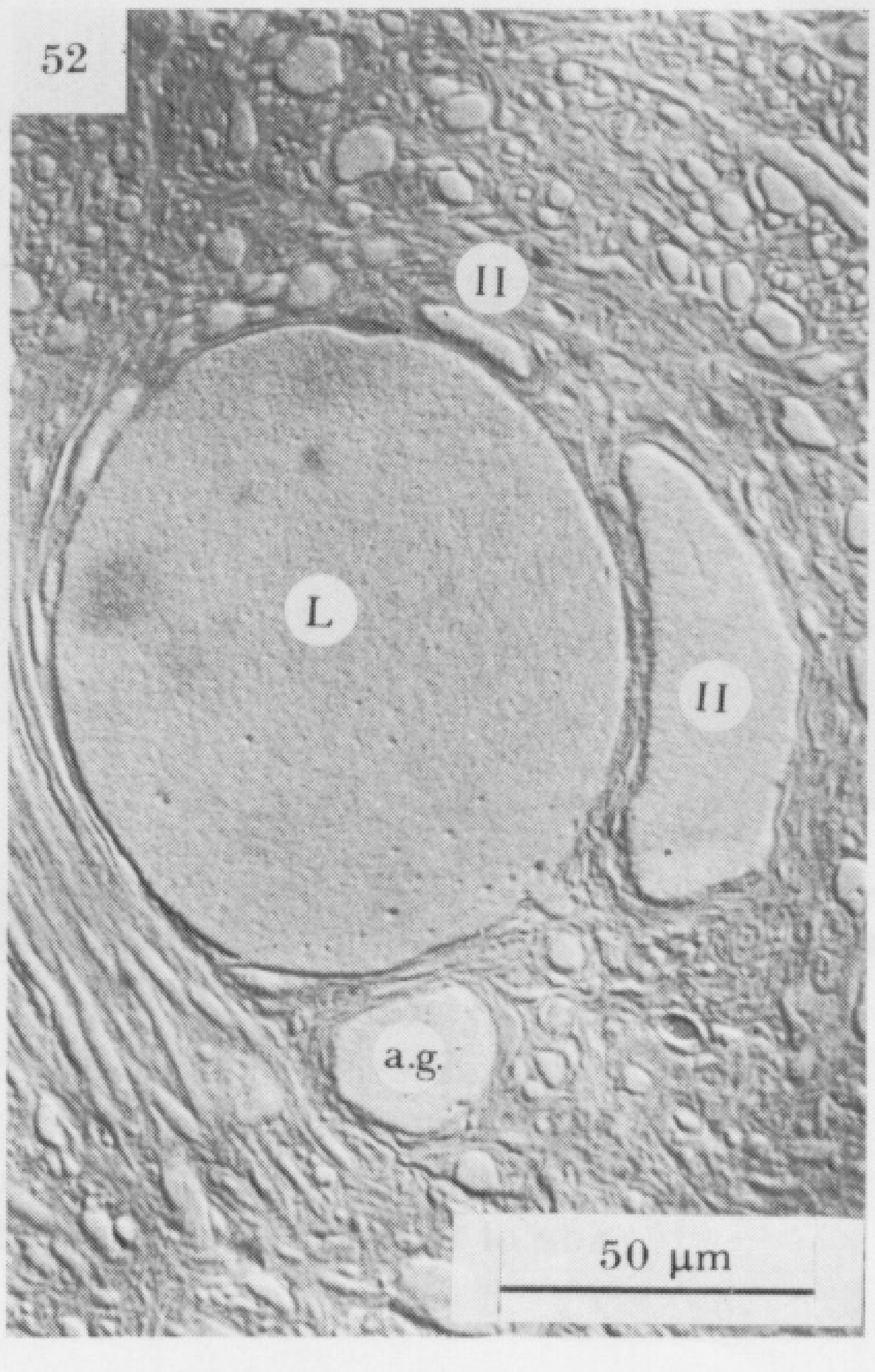
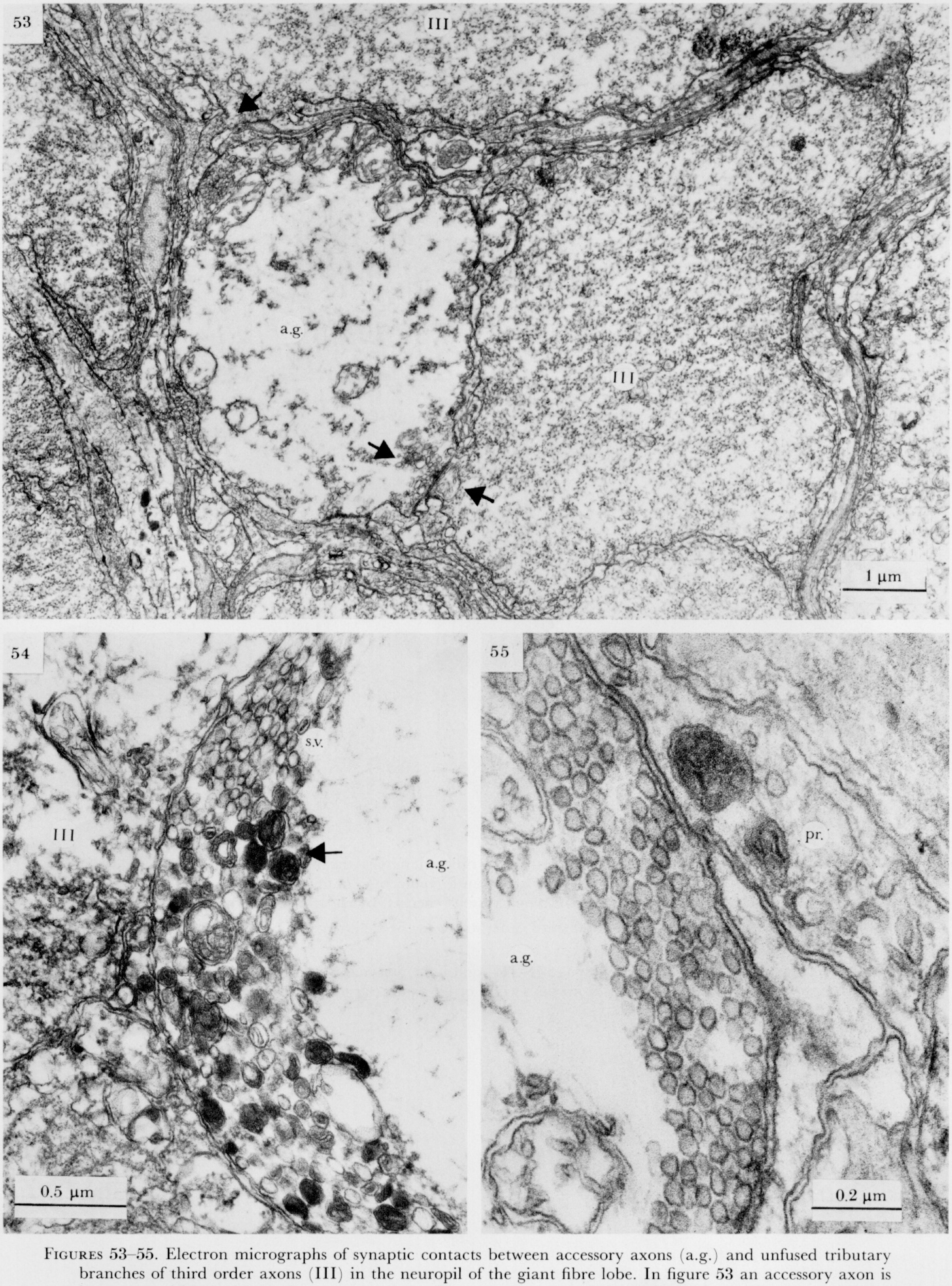
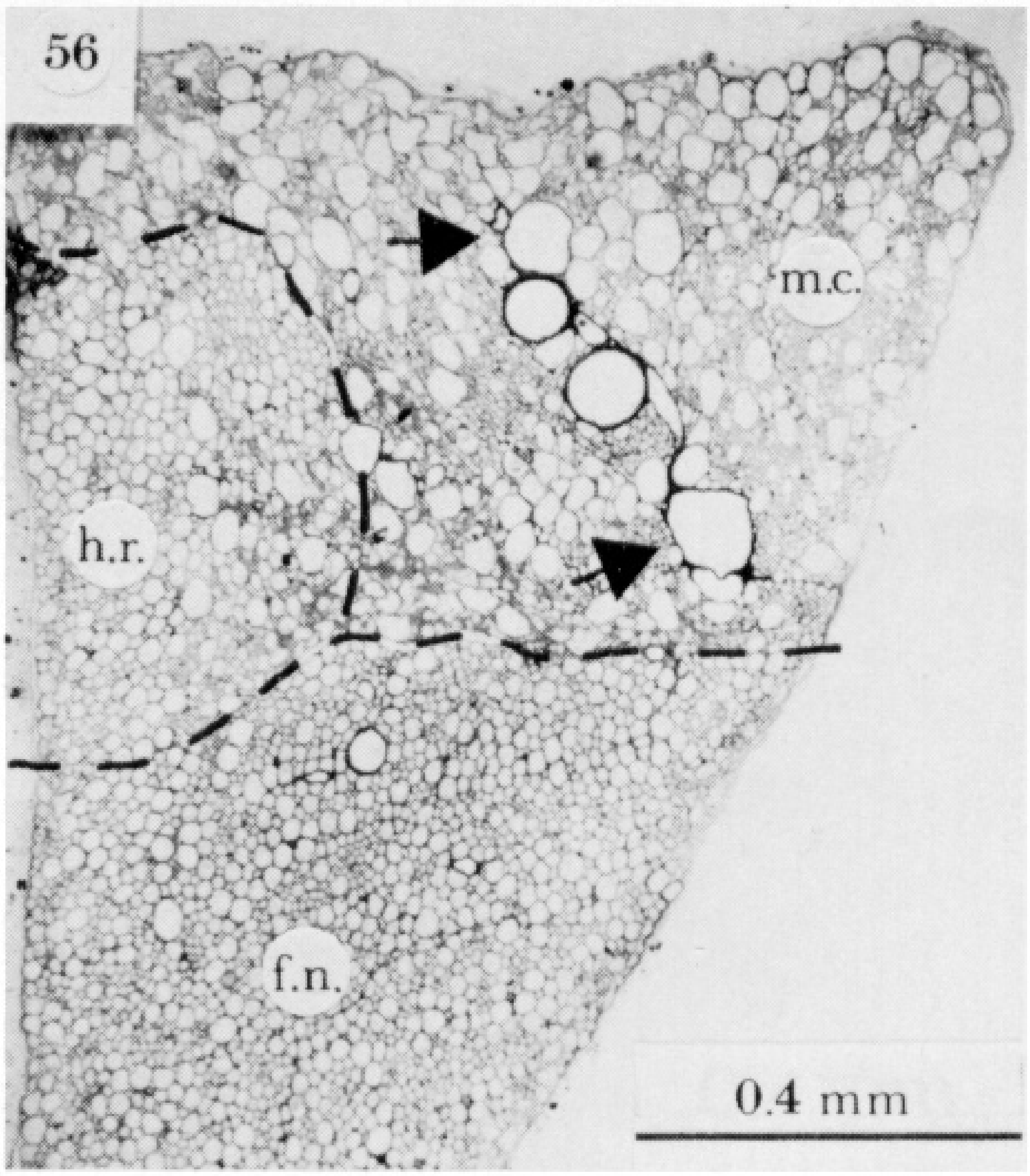
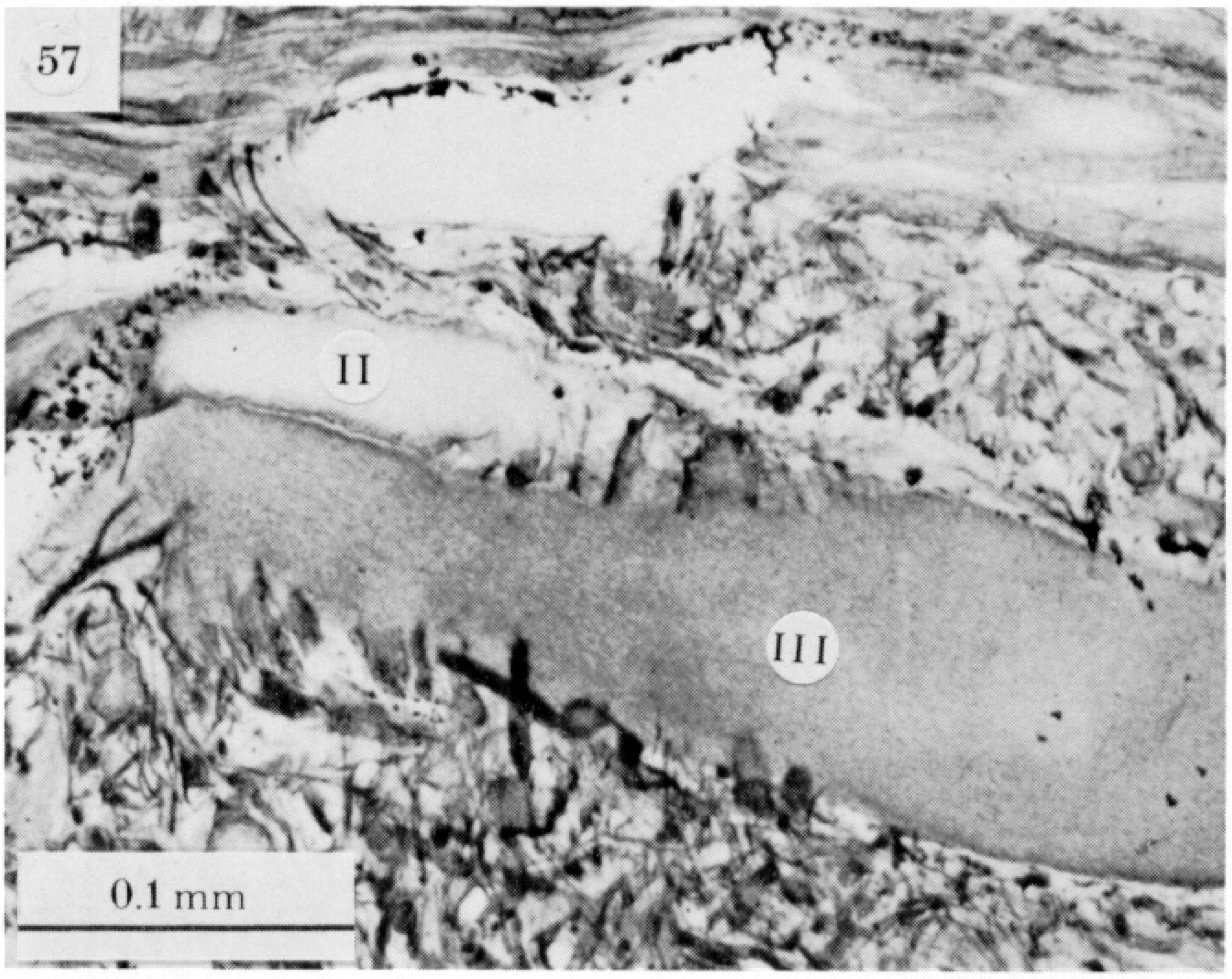


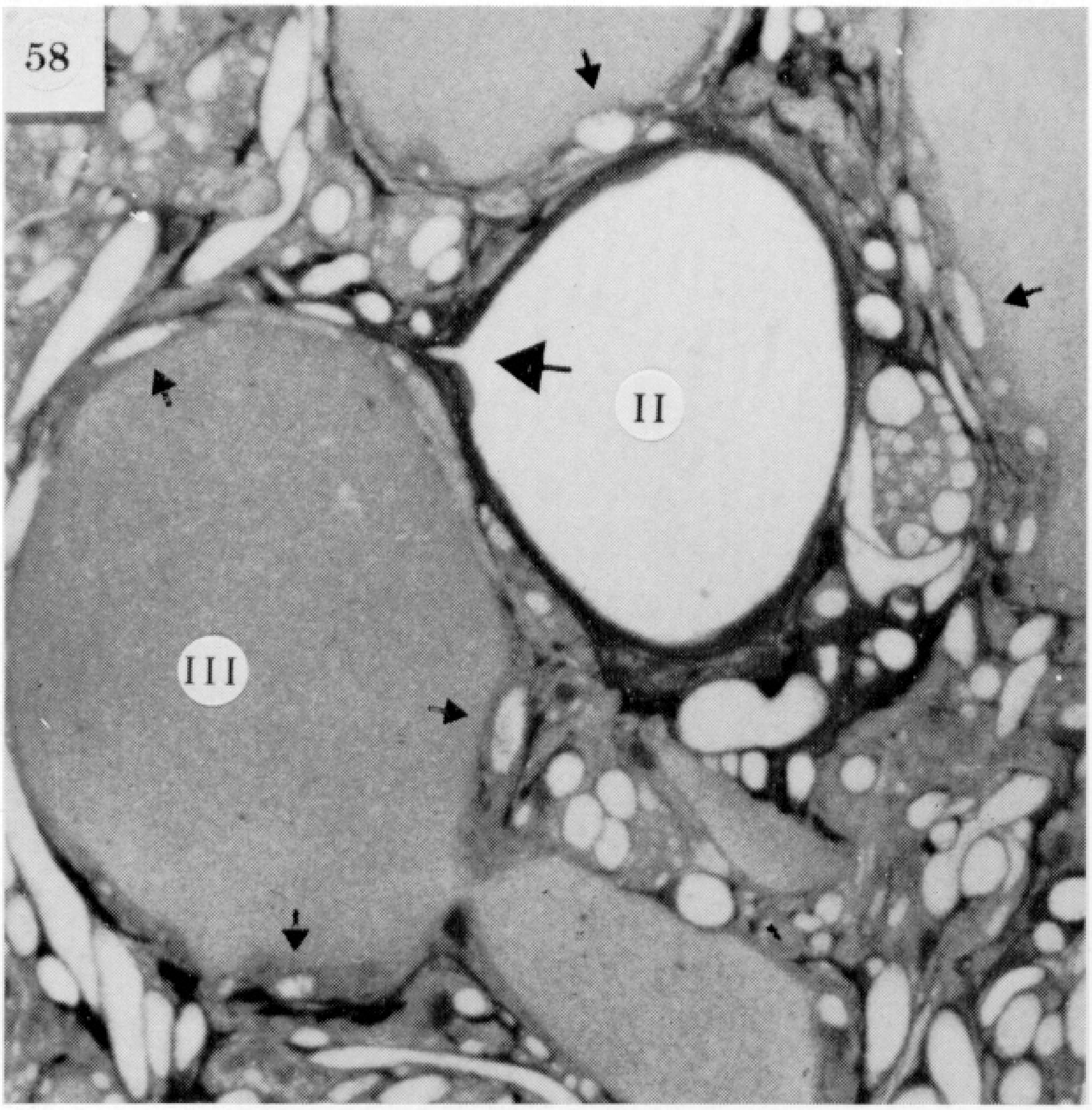
FIGURES 46–52. For description see p. 369.



FIGURES 53–55. Electron micrographs of synaptic contacts between accessory axons (a.g.) and unfused tributary branches of third order axons (III) in the neuropil of the giant fibre lobe. In figure 53 an accessory axon is in contact with two third order axons (arrows). The accessory axon in figure 54 was injected with cobalt. The dark precipitate is included in membrane-bound organelles (mitochondria?) in a group of synaptic vesicles (s.v.). Figure 55 shows an enlargement of a synaptic contact between an accessory axon and a postsynaptic process (pr.).







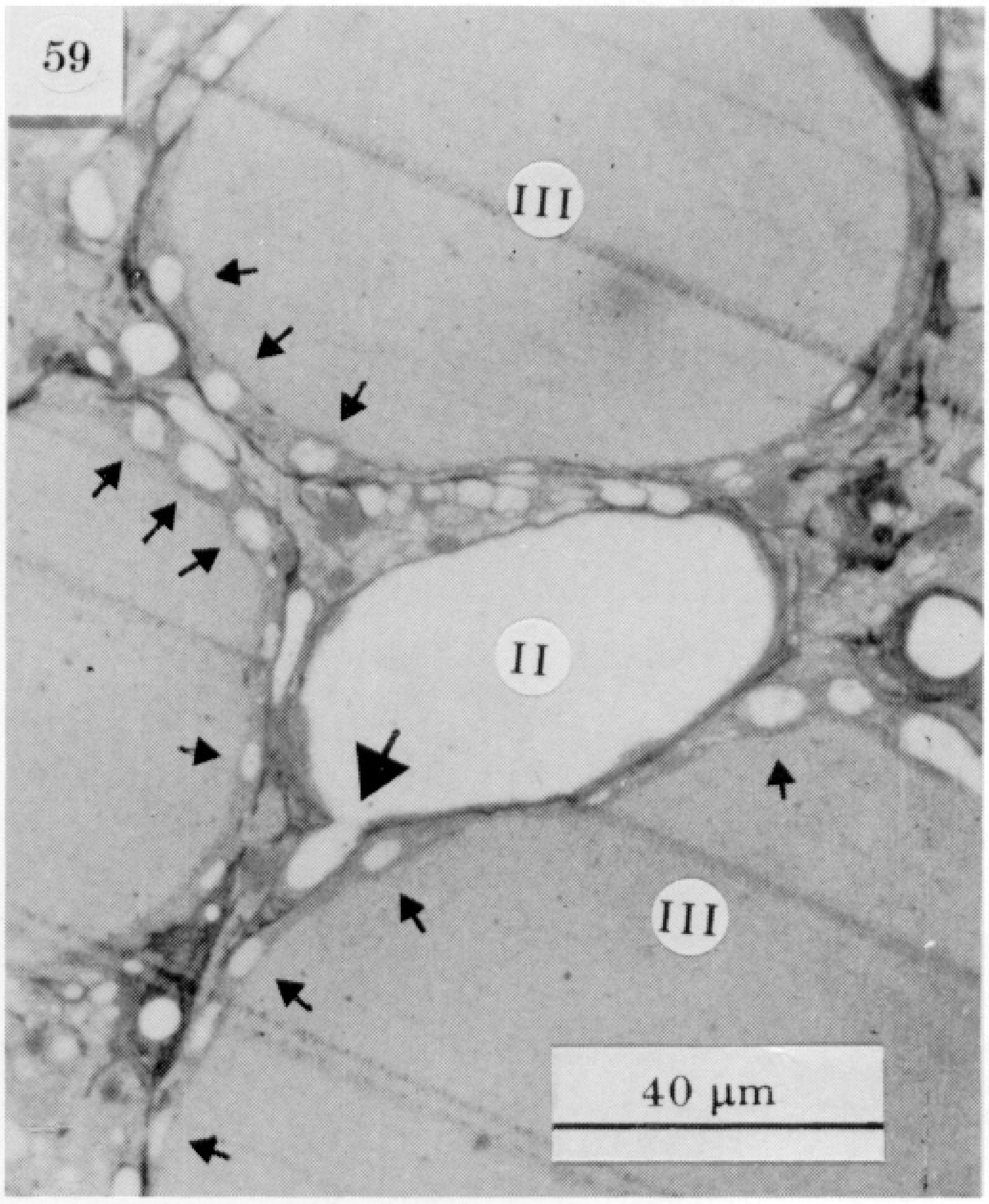
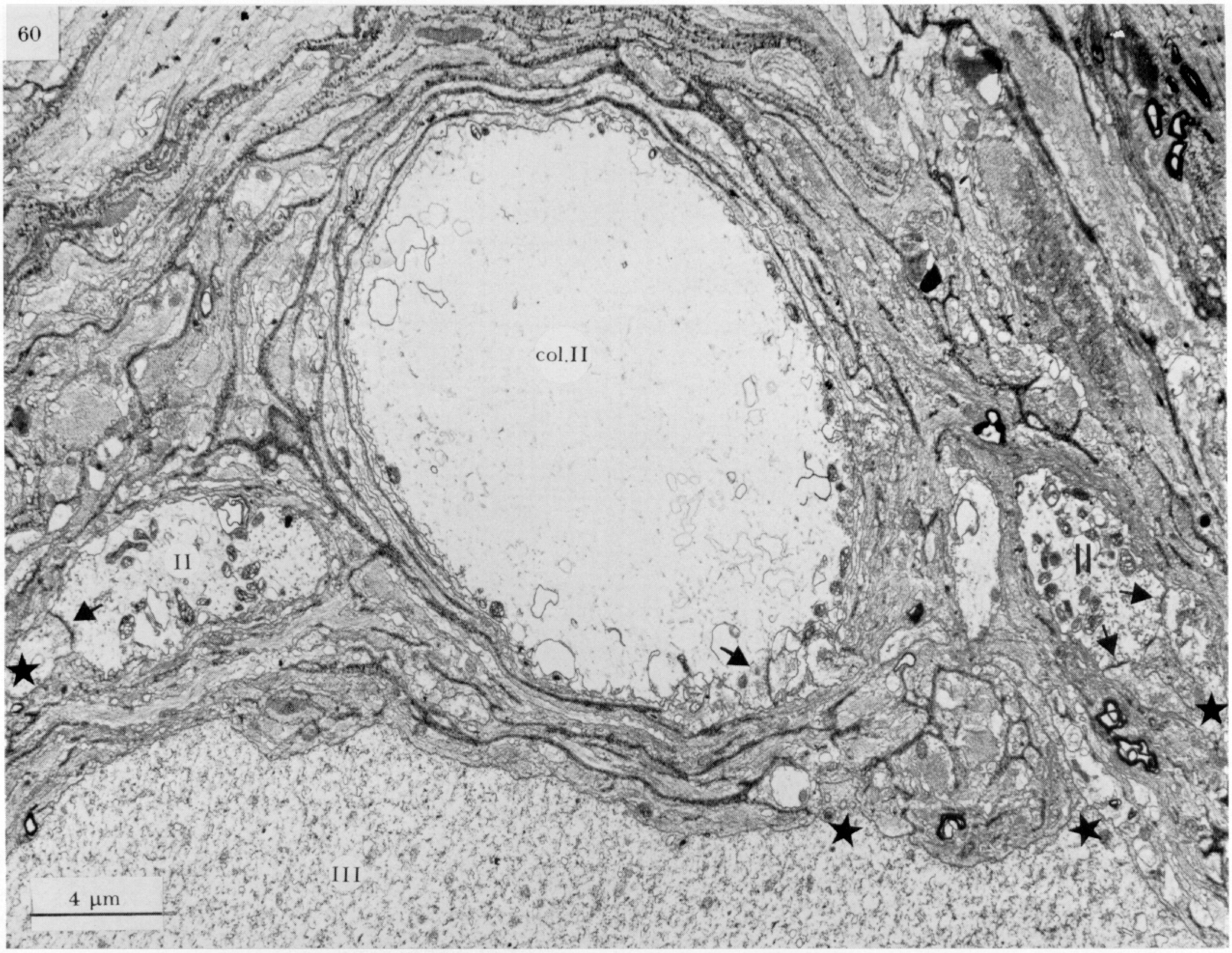
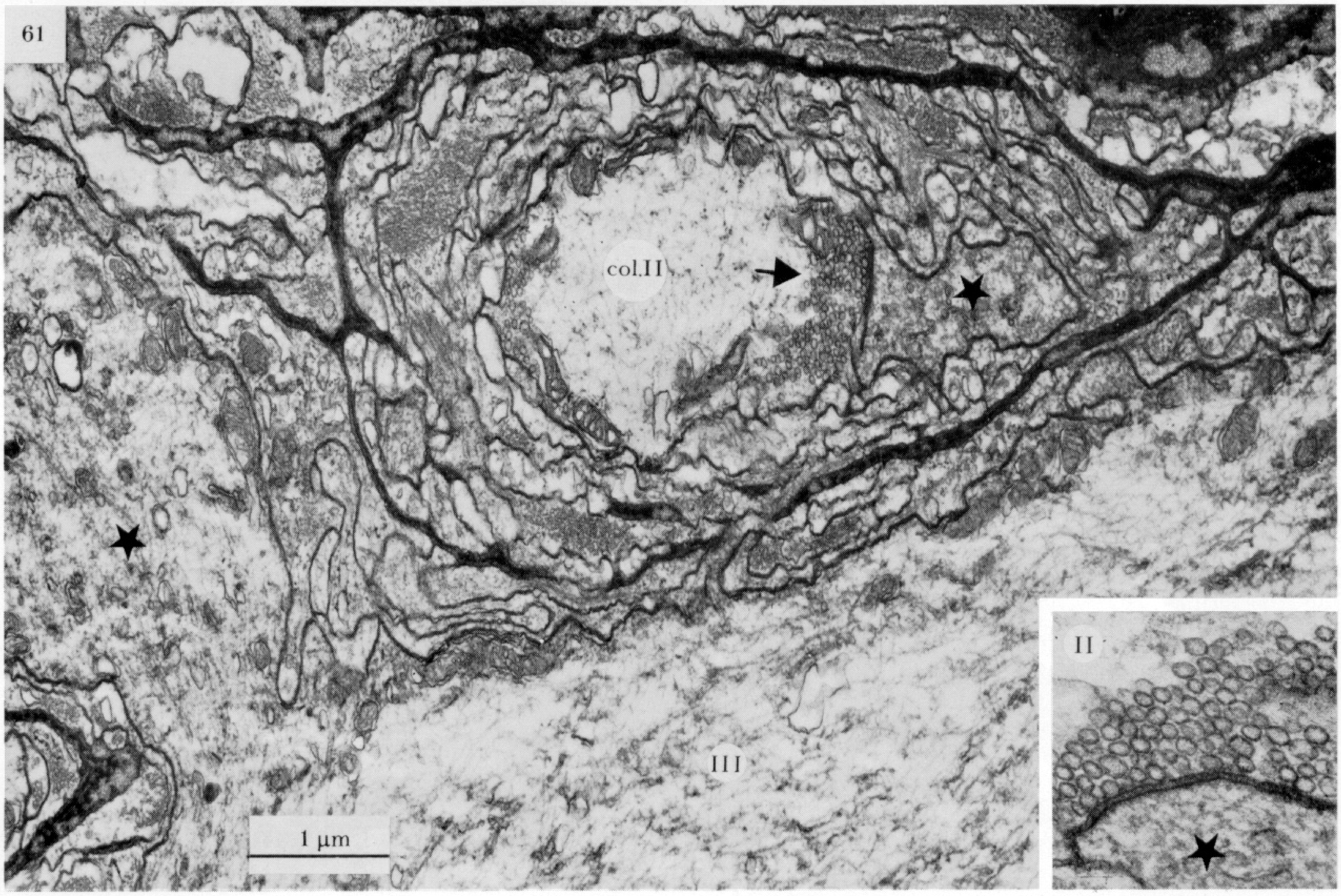


FIGURE 56. Cross-cut pallial nerve of *Todarodes* with three different nerves: the mantle connective (m.c.), the posterior head retractor (h.r.), and the fin nerve (f.n.). The mantle connective contains two giant axons (arrows), between these two profiles there are two cross-cut blood vessels (checked by electron microscopy). Toluidine blue.

FIGURE 57. Longitudinally cut second and third order axons in the stellate ganglion of *Todarodes*. Bodian-stained paraffin section.

FIGURES 58 AND 59. Groups of dark postsynaptic (III) and light presynaptic (II) axons in the stellate ganglion of *Todarodes*. Small branches bud off from the second order axons (large arrows) and run into the sheath of the third order axons (small arrows).





FIGURES 60 AND 61. Electron micrographs of the giant synapse of *Todarodes*. In the sheath of the third order axon (III) there are several collaterals of the second order axon (col.II) that are in synaptic contact (arrows) with processes (*) from the third order axon. Inset: a synaptic contact.

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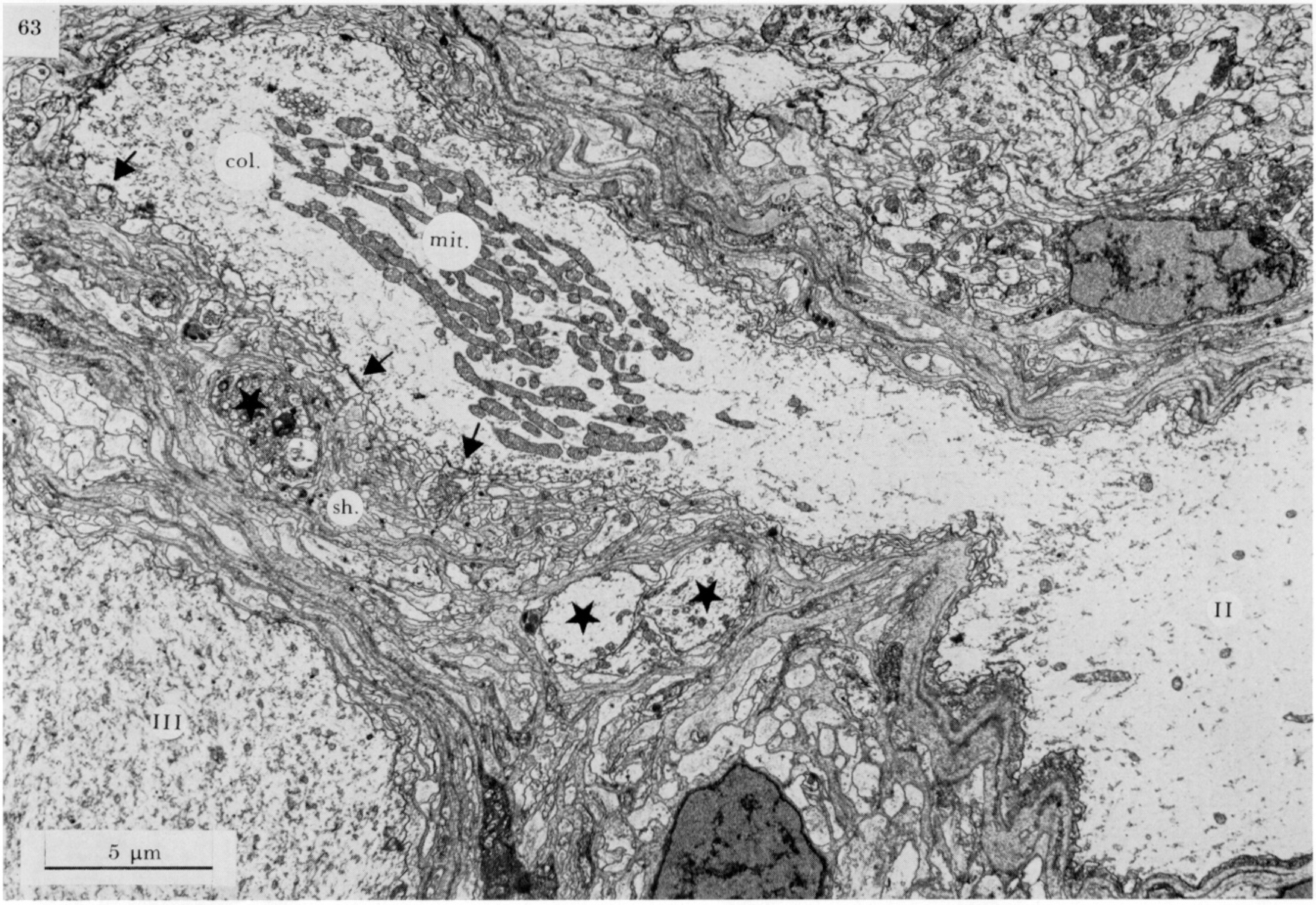

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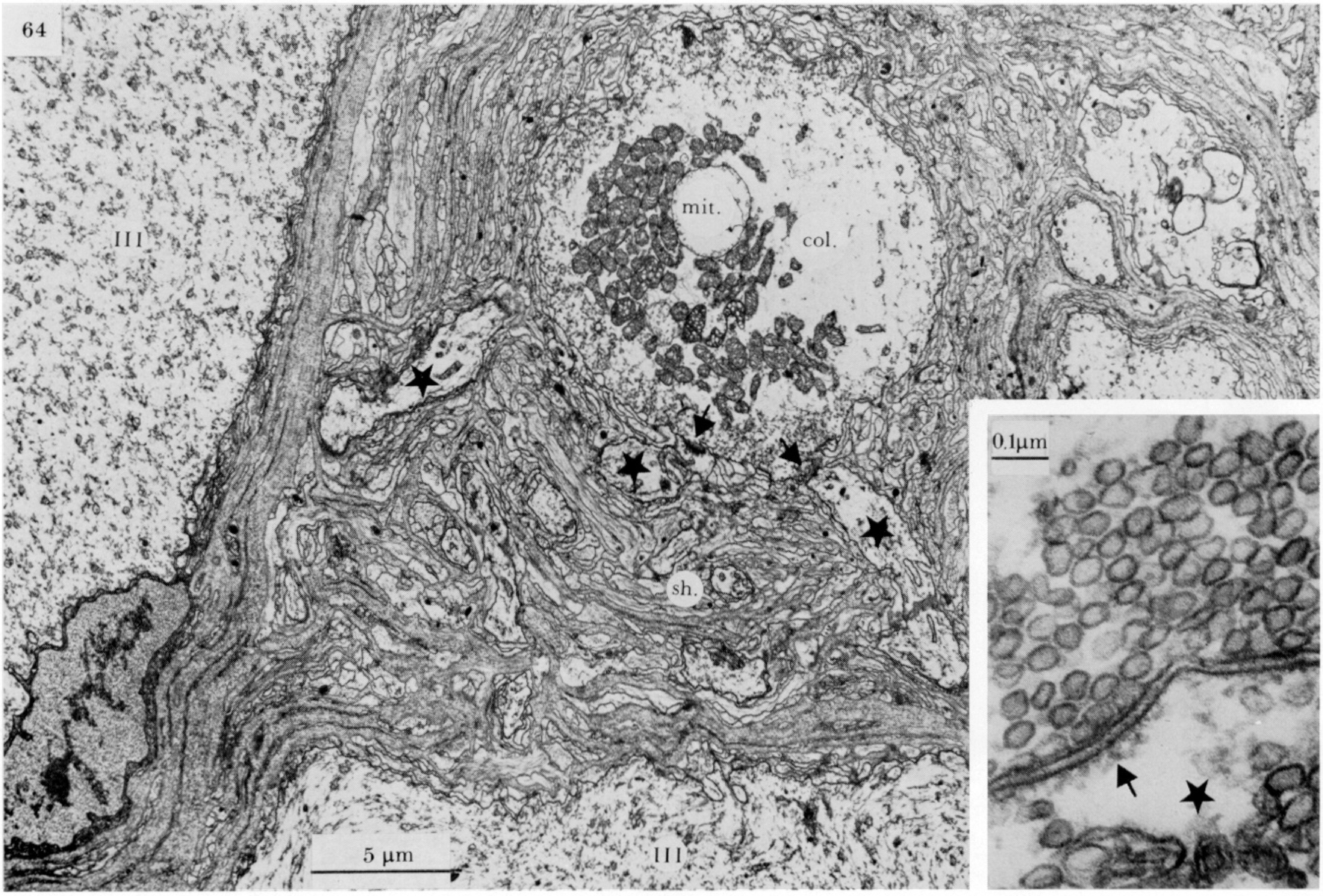

FIGURES 63 AND 64. Electron micrographs of the giant synapse of *Sepia*. Collaterals (col.) of the second order axon (II) with many elongated mitochondria (mit.) run into the sheath (sh.) of the third order axon where they meet processes (*) of the third order axon in numerous synaptic contacts (arrows, see also inset).