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CHANGES IN THE ULTRASTRUCTURE OF SURVIVING DISTAL SEGMENTS OF SEVERED AXONS OF THE ROCK LOBSTER

I. PARNAS[1,*], O. SHAHRABANY-BARANES[1], N. FEINSTEIN[1], P. GRANT[2], H. ADELSBERGER[3] AND J. DUDEL[3]

[1]*The Otto Loewi Minerva Center for Cellular and Molecular Neurobiology, The Hebrew University of Jerusalem, Israel,* [2]*Laboratory of Neurochemistry, NIH, Bethesda, Washington, DC, USA* and [3]*Department of Physiology, The Technical University, Munich, Germany*

*e-mail: HANNA@VMS.HUJI.AC.IL

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Summary

Peripheral axons of lobsters can survive for many months after axotomy. We have investigated the structural and ultrastructural changes seen after axotomy using confocal microscopy and electron microscopy. While the proximal stump had a normal appearance, the distal part of the cut axon became lobulated, and glial cells penetrated the original glial tube (axon tube) in which the axon normally runs. The changes proceeded from the cut end towards the muscle. As time elapsed, the axon tube seemed to be filled with glial cells, but interposed small profiles of the original axon could be identified by injection of a fluorescent dye into the axon. The glial cells send cytoplasmic projections deep into folds of the axolemma, and nuclei were found at the end of these long processes. Proliferation of glial cells was also seen.

Key words: axon, degeneration, glia, axotomy, rock lobster, *Panulirus penicillatus.*

Introduction

In vertebrates, the distal stump of transected axons degenerates within a few days (Waller, 1850). In most cases, the distal stump and its myelin disintegrate, leaving Schwann cells surrounding the basal lamina tube (axon tube) to serve as a conduit for regenerating axons. In invertebrates and some vertebrates, degeneration of the decentralized axon segments is slow, and they survive for long periods and are able to conduct action potentials and release transmitter (invertebrates, Hoy *et al.* 1967; Nordlander and Singer, 1972; Wine, 1973; Bittner and Johnson, 1974; Ballinger and Bittner, 1980; Atwood *et al.* 1989; Blundon *et al.* 1990; Parnas *et al.* 1991; Sheller *et al.* 1991; for reviews, see Bittner, 1988, 1991; vertebrates, Lazar, 1980; Matsumoto and Scalia, 1981; Cancalon, 1982; Lubinska, 1982; Zottoli *et al.* 1987; Blundon *et al.* 1990). What accounts for the very slow process of degeneration and the long-term survival of these distal axon segments?

The possibility that axons possess the machinery for protein synthesis is still a matter of controversy. Some investigators (Gainer *et al.* 1977; Lasek *et al.* 1974, 1977; Sheller, 1989; Sheller and Bittner, 1992) argue that axons lack the machinery for protein synthesis. However, protein synthesis has been demonstrated in different axonal preparations (e.g. Giuditta *et al.* 1968; Crispino *et al.* 1993*a*,*b*), and recently Spira *et al.* (1996) kept isolated *Aplysia californica* and *Aplysia oculifera* axon segments in culture, in the absence of any glia or other neuronal cells, and found that such isolated segments survived for 22 days and were able to synthesize proteins during the entire period tested (7–8 days in culture, longer periods were not tested).

Another mechanism involved in long-term survival of decentralized axons is the transfer of proteins (Bittner, 1977, 1981; Gainer *et al.* 1977; Lasek *et al.* 1974, 1977; Lasek and Tytell, 1981; Lieberman *et al.* 1994; Meyer and Bittner, 1978*a*,*b*; Sheller, 1989; Sheller and Bittner, 1992; Singer and Salpeter, 1966) or other compounds (Tsacopoulos *et al.* 1994) into the axon from surrounding glial cells (for a review, see Lieberman *et al.* 1994). Indeed, after transection, the axon and glia show ultrastructural changes that may facilitate the passage of such materials between glia and axons (Lieberman *et al.* 1994).

A different mechanism for long-term survival was suggested by Atwood *et al.* (1989). These researchers suggested that glial cells merge with the axon stumps, thereby ‘donating’ nuclei and the machinery for protein synthesis. Nuclei were also found in severed sensory axons, but only in rare cases (Govind *et al.* 1992).

In the present study, we reinvestigated the question of long-term survival, focusing in particular on the glial–axon morphological interactions following axotomy. Our observations indicate that the glia undergo profound reorganization; they proliferate and form numerous

cytoplasmic processes, which invade and partition the axon into smaller surviving segments. Our confocal microscopic studies reveal that a glial cell can send a protrusion containing its nucleus deep into foldings of the axolemma. These may be seen (in cross sections) with the electron microscope as floating nuclei in the severed axon.

Materials and methods

Preparation

Most of the experiments reported here were carried out in the Otto Loewi Minerva Center laboratories in the Interuniversity Institute in Eilat, Israel, over a period of 4 years. Rock lobsters *Panulirus penicillatus* from the Gulf of Eilat were kept in pools with running sea water, at temperatures varying from 18 to 21 °C. The lobsters were anaesthetized by placing them on crushed seawater ice, and small apertures (approximately 1 cm^2) were opened in the dorsal shell in abdominal segments II–IV. In each segment, the nerve bundle innervating the deep extensor abdominal muscles (DEAMs) (Parnas and Atwood, 1966) was exposed and cut with fine scissors, distal to the branch of the nerve innervating the superficial muscles. The pieces of shell were replaced and glued with dental cement (carboxylate cement, ESPE Durelon, Germany). In one set of experiments, the nerves from the contralateral side served as a control. In another set of control animals, the shell was removed in three segments and the nerves were exposed, but not cut. Each lobster was placed in a separate container with running sea water and was fed with fish fillets. Between 1 and 500 days after transection of the nerves, the animals were killed and dissected, and the DEAMs and their nerves were isolated as previously described (Dudel and Parnas, 1987). The numbers of animals and nerves taken after different periods are given in Table 1. Although most of the material shown in Table 1 was studied both physiologically and under the electron microscope, the results reported here are limited to the early period, up to 90 days post-axotomy (but see confocal microscopy results, where one animal was studied 1 year after axotomy).

Electrical recordings

Each isolated DEAM preparation with its nerve (proximal to the scar, scar region, distal region and muscle) was placed in the recording chamber with circulating (Gilson pump, 12 °C) lobster saline solution containing (in mmol l^{-1}): 538 NaCl, 12 KCl, 12 $CaCl_2$, 10 $MgCl_2$ and 10 Tris maleate at pH 7.4. Using conventional extracellular stimulation and macropatch recording techniques, we tested whether stimulation at the proximal region produced a recordable action potential at the distal stump. Stimulation of the proximal stump always produced a response in the proximal region, but never in the distal stump. We then stimulated the distal stump, as close as possible to the cut end (approximately 1–2 mm from the scar), and recorded action potentials from the nerve when the external recording electrode was placed along the nerve at different distances from the muscle. This procedure took approximately 30–60 min, after which the nerves were separated from the muscle and fixed for electron microscopy. In three experiments, the nerves were fixed immediately without any electrical recording to test whether the recording procedure had deleterious effects on ultrastructure. When compared with unstimulated nerves from other segments of the same animal, nerves subjected to electrical stimulation displayed no gross changes in the ultrastructure of the axons or glia.

Table 1. *Number of animals tested and nerve preparations examined at different periods after axotomy*

Time (days)	No. of animals	Distal stump	Proximal axon	Contralateral
1–20	19	49	19	8
21–60	14	36	10	3
80–120	8	18	10	3
130–200	7	17	2	1
220–515	4	10	4	2
Total	52	130	45	17

Fura-2 injection

Fura-2, used as a fluorescent indicator, was injected intraxonally as described previously for crayfish axons (Ravin *et al.* 1997). The microelectrode (5–12 MΩ) contained 2 mol l^{-1} KCl and 10 mmol l^{-1} Fura-2. The fluorescent indicator was loaded by passing 0.5 s hyperpolarizing pulses of 0.4 nA, applied at a rate of 1 Hz, for 20 min (or more), until the axon showed good staining. The excitation wavelength was 380 nm, and emission was measured at 510 nm (Ziv and Spira, 1993, 1995).

Confocal microscopy

Experiments were carried out in the laboratory of Professor J. Dudel in Munich. Animals were flown from Eilat to Munich, and the nerves were removed the same day. Nerves were stained in lobster saline containing 10 μmol l^{-1} Rhodamine 6G and 10 μmol l^{-1} Acridine Orange (Molecular Probes, Inc.) for 5 min. The preparations were then washed with lobster saline for 15 min. Confocal microscopy was performed with an upright microscope (LSM 10, Zeiss, Oberkochen) equipped with a 40×/0.75 numerical aperture water-immersion lens (working distance 1.9 mm) at an excitation wavelength of 514 nm using an argon laser. Fluorescence was detected using a 575–640 nm bandpass filter.

To reconstruct the images along the *z* axis, we used optical transverse line sections. The thickness of each step was 0.5 μm. The digital line sections were collected on the instrument's computer and presented as a composite picture by aligning them one over the other. The composite picture appears out of focus because the sharpness of the reconstruction is limited by the number of data points per line and by the number of lines generating the picture. The reconstruction was performed in order to obtain an optical transverse section at the region of interest. The *x,y,z* three-dimensional reconstruction had a lower

resolution but confirmed the results observed with single-line optical sectioning.

Electron microscopy

Each nerve was isolated in its entirety to preserve its orientation. The nerve included the proximal region, the scar and the distal stump. For fixation, we used the method of Pearce *et al.* (1986). Before being embedded in epoxy resin, the nerve was cut into 2 mm long consecutive segments. Each segment was flat-embedded in Agar 100/Araldite and polymerized at 60 °C for 2 days. From each of these, 20 semi-thin (2 μm) sections were sampled serially from the region bordering the adjacent segment. These sections were mounted on slides and stained with 1 % Methylene Blue for light microscopy. From each piece of nerve, we also took 50 ultra-thin sections (70–90 nm, LKB III ultra-microtome) from the region adjacent to the semi-thin sections. The ultra-thin sections were collected on 200 mesh thin bar copper grids. They were stained with saturated aqueous uranyl acetate solution and lead citrate. The sections were examined under a Jeol Jem-100CX electron microscope.

Three-dimensional reconstruction

Computer-assisted three-dimensional reconstructions were performed as described by Fried *et al.* (1993). Briefly, the axon profiles were traced, from light microscope prints, onto plastic transparencies. These were aligned in consecutive sections. Outlines were digitized using a summagraphics MM1201 graphics tablet. Serial reconstruction was carried out with Eutectic SSRS software.

Glial cell proliferation

To study glial cell proliferation, we used a modified 5-bromo-2-deoxyuridine (BrdU, Sigma) method for identifying proliferating nuclei (Schubiger and Palka, 1987). Dividing cells incorporate BrdU into their DNA during the S phase of cell division. A stock solution of 10^{-2} mol l^{-1} BrdU was prepared in lobster saline. At different times after transection of the nerves, lobsters were injected on their dorsal side between the thorax and the first abdominal segment with the BrdU solution, to yield a final concentration of approximately 25 mg kg^{-1}. After 24 h, the nerves were removed and immersed in 70 % alcohol for fixation. They were stored in ethanol for more than 1 day before processing for paraffin embedding. Serial sections at 10 μm were taken from the cut end towards the muscle and mounted on subbed slides.

For immunocytochemistry, the slides were passed through xylene to remove the paraffin wax, rehydrated in descending concentrations of ethanol to phosphate-buffered saline (PBS) and washed three times in PBS. They were incubated in 2 mol l^{-1} HCl for 30 min to denature the DNA, washed three times in PBS, then incubated for 1 h in a blocking PBS solution containing 1 % bovine serum albumin (BSA) and 0.2 % Triton X-100. This was followed by a brief immersion in a blocking solution without Triton X-100. Slides were incubated overnight at 4 °C in a drop (100 μl) of monoclonal anti-BrdU antibody (Becton-Dickinson) diluted 1:10 in the blocking solution without Triton X-100. The slides were washed three times in PBS (10 min each) and incubated in a 1:100 goat anti-mouse antibody (in PBS) conjugated with horseradish peroxidase (HRP) (Accurate Chemical, Westbury, NY, USA) for 1 h, washed in PBS and developed with diaminobenzidine. The slides were counterstained with 1 % Methyl Green, dehydrated in ascending ethanol solutions to 100 % ethanol, passed through two changes of xylene and mounted in Entellen (rapid embedding agent), before being examined under a light microscope. The BrdU-positive nuclei were stained brown and the BrdU-negative nuclei were stained green.

Results

Ultrastructure of control axons from non-operated animals

Several studies have dealt with the ultrastructure of normal axons of crustaceans (Atwood *et al.* 1973; Bittner, 1981; Bouton and Bittner, 1981; Kennedy and Bittner, 1974; Nordlander and Singer, 1972). As a baseline, we briefly describe the relevant ultrastructural features of the axons innervating the DEAMs. The nerve bundle innervating the DEAM contains five axons: four excitatory and one inhibitory (Fig. 1A) (Parnas and Atwood, 1966). Each axon is encased in an axon tube (Fig. 1A) composed of glial layers (Fig. 1B). There are approximately ten tightly packed glial layers. Between the glial wrappings are found thin layers of extracellular collagen. At the axon surface, 1–3 layers of adaxonal glial cells are observed. These cells are relatively thick (Fig. 1B) and are penetrated by an elaborate series of canals, which connect the periaxonal space with the extracellular space (Fig. 1D) (Holtzman *et al.* 1970; Nordlander and Singer, 1972). Such a direct connection was demonstrated in lobster nerves by Grossman *et al.* (1979), who showed that lanthanum and HRP applied to the bathing solution penetrate the periaxonal space rapidly *via* these canals. The glial nuclei are oblong and narrow.

Controls from operated animals

For these controls, we exposed the axons by opening and closing the shell, without cutting the axons. Ten days after such exposure, the nerve was isolated and immediately fixed. There were no marked changes in the general appearance of the axon. It still filled the axon tube, although the glial wrappings were looser (Fig. 2). The adaxonal glial layer surrounded the axon and the glial nuclei were still oblong and narrow.

Proximal stump

After cutting the nerve bundle to the DEAM, the proximal and distal parts retracted and separated. As time elapsed after the operation, scar tissue was formed, such that the isolated nerve was composed of the proximal region, the scar region and the distal region. Electrical recordings showed that there was no functional continuity between the original proximal and distal parts. Serial semi-thin sections also showed no structural continuity between the distal and proximal regions, even after

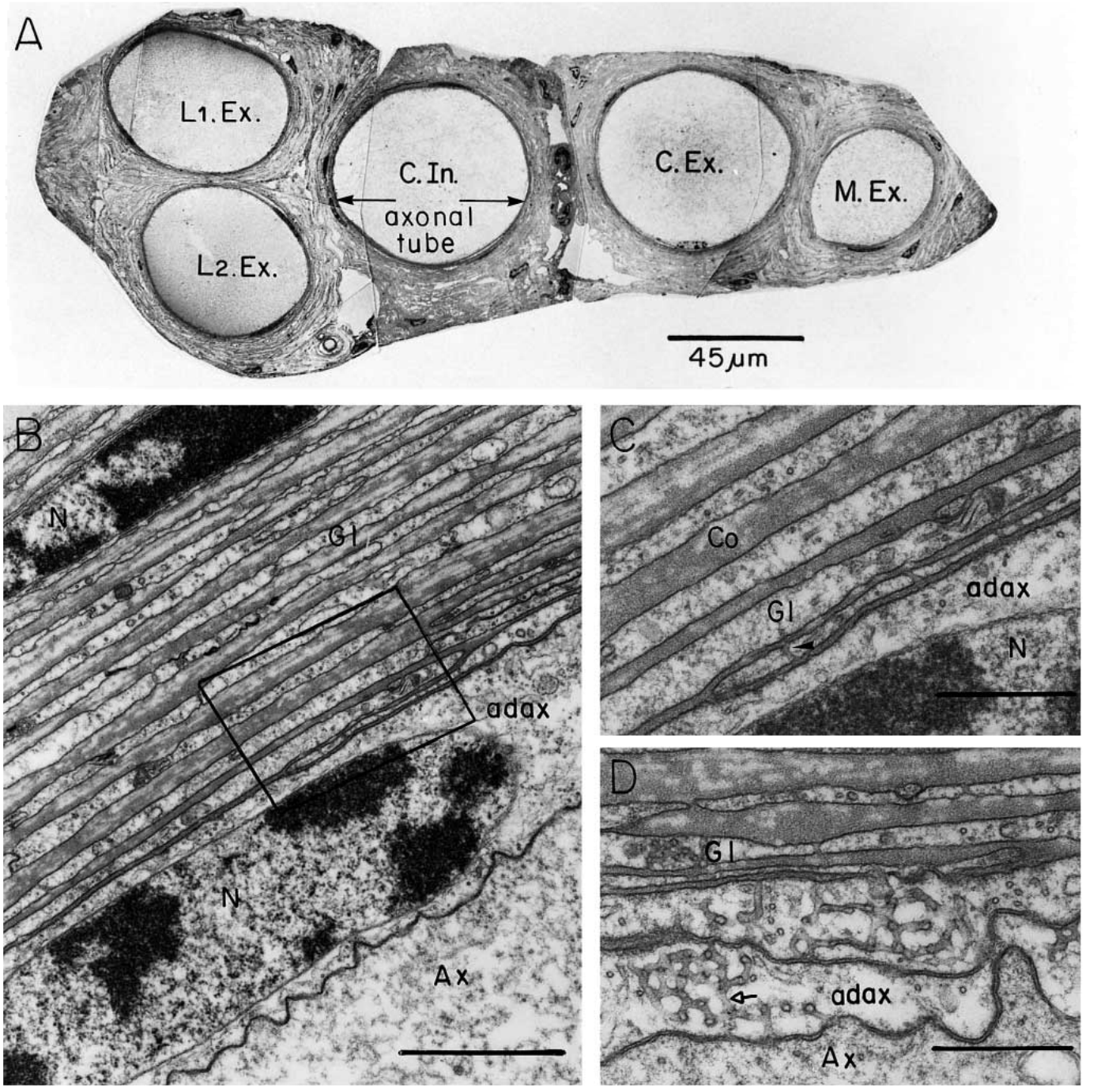

Fig. 1. Control (non-operated animal). Electron micrographs of transverse sections of a nerve (A) and sections of one axon (B,C,D) innervating the deep extensor abdominal muscles (DEAMs). (A) Five axons in the nerve bundle innervating the three muscles (L1, L2, M) of the DEAM: specific excitors L1.Ex., L2.Ex., M.Ex.; the common excitor C.Ex. and the common inhibitor C.In. The boundaries of the axon tube are shown for C.In. Scale bar, 45 µm. (B) The wall of the axon tube consists of tightly packed layers of glial sheath (Gl) and the adaxonal glial layers (adax). Ax, axon; N, nuclei. Scale bar, 1 µm. (C) A magnification of the rectangle in B. The glial sheaths alternate with layers of collagen (Co), which seem to be connected *via* ladder-shaped canals (arrowhead). Scale bar, 0.5 µm. (D) An elaborate canal system (arrow) penetrates the adaxonal glial cells, possibly connecting the external surroundings and the periaxonal space. Scale bar, 0.5 µm.

90 days. Semi-thin sections made from approximately the middle of the scar enabled us to identify the tips of the proximal axons.

The very end of the proximal stump (near the scar tissue) looked damaged (not shown). We did not study the very end of the proximal region. Such changes were described in detail by Spira *et al.* (1993). Approximately 50 µm away from the scar, the proximal axon looked normal (Fig. 3A) and had an axon profile similar to that shown in Fig. 2. The glial wrappings were loose, and the oblong and narrow nuclei were

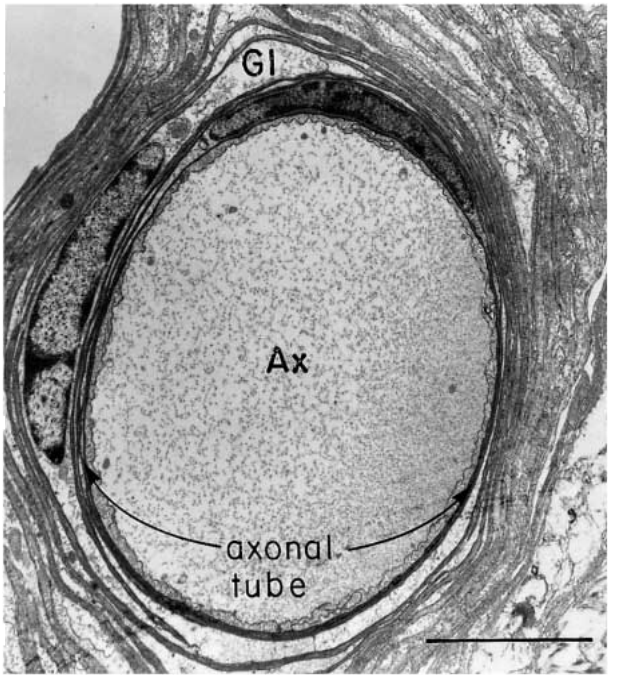

Fig. 2. Control 'non-cut' nerve. Electron micrograph of a transverse section of an axon (Ax). The boundaries of the axon tube, which is fully occupied by the axon, are marked. The morphology is similar to that shown in Fig. 1A, except that the glial sheath (Gl) is rather more loosely wrapped. Scale bar, 5 μm.

arranged around the circumference of the axon tube. We checked 45 proximal cut ends (approximately 50 μm from the scar), ranging from 1 to 520 days after axotomy. In the period from 1 to 90 days, the appearance of all the 20 preparations examined was essentially as in Fig. 3A. Later, sprouting and growth began, but this is not the subject of the present paper.

Ultrastructural changes in the distal stump

As with the proximal stump, we followed the changes in ultrastructure of the distal region, starting with semi-thin sections from the scar tissue and advancing towards the muscle, combining light and electron microscopy. Fig. 3 shows an example 16 days after axotomy. Marked changes were observed in the distal stump. Near the cut end (50 μm), the preparation looked damaged, and we could not identify axon profiles. The axon tubes were distorted and filled with cells and debris (not shown). Approximately 200 μm more distally (Fig. 3B), many axon profiles could be seen within the axon tube (only three profiles are marked in Fig. 3B). These profiles may represent degeneration of this region of the axon. In one of the profiles, one of the nuclei with its surrounding membranes is engulfed by axoplasm. The rest of the axon tube is filled with axon profiles and cells, some containing round nuclei. Two haemocytes were observed between the loose glial wrappings. The presence of haemocytes, which are not normally seen outside blood vessels, suggests that phagocytosis may be occurring.

A more distal section (30 μm distal to the section shown in Fig. 3B) clearly shows one large axon profile (Fig. 3C, other small profiles are not marked). The axon is not round and does not fill the entire axon tube. The tube space not occupied by the axon is filled with cells, some with round nuclei. Note that one of these cells sends a finger-like process containing a large nucleus into an invagination of the axolemma (see confocal microscopy, below). The loose glial wrapping contains oblong nuclei. Approximately 4 mm away from the cut end, the axon appears more normal (Fig. 3D). It is somewhat smaller, and the space between the axon and the original wall of the axon tube is filled with glial cells.

The changes described, including the extreme effects near the cut end and an almost normal appearance near the muscle, are considered typical and were observed in all experiments 2 weeks to 6 months after the cut (subsequently, regeneration was observed, but is not described in this paper). In general, as time elapsed, the changes observed near the cut end soon after axotomy appeared in more distal regions. For example, the findings pictured in Fig. 3B, 200 μm away from the cut end after 16 days, could be found a few millimeters away after 2–3 months. Thus, even though the thin axon processes still conducted action potentials, the lobulation of the axon by the glial cells may represent a slow process of degeneration.

Within this typical cascade of events, there was considerable temporal and spatial variability in the intensity of the response in different preparations. Some showed large changes after only short periods, while others showed almost no response. However, after 3–4 weeks, almost all preparations showed these typical changes. It should be noted that the stages progressed differently over the length of the nerve bundle. For example, in one axon tube, several small axon profiles could be seen near the cut end. More distally, one larger profile was observed, but even more distally several profiles were again seen. These were followed once more by one or two larger profiles and an almost normal appearance near the muscle.

Another obvious feature was that not all five axons in the same nerve responded equally. Fig. 4 shows such an example 90 days after the cut. In the same ultra-thin transverse section, we observed two axon tubes. In one (Fig. 4A), the changes were marked and many axon profiles were seen. In the second (Fig. 4B), one large axon profile occupied most of the axon tube, while some small axon profiles and other cells occupied the remaining space of the axon tube.

Continuity of axon profiles

Small axon profiles have already been described after axotomy and partial degeneration in crayfish (Atwood *et al.* 1973; Bittner, 1981; Bouton and Bittner, 1981; Kennedy and Bittner, 1974; Nordlander and Singer, 1972). It was suggested that these axon profiles are satellite axons or new regenerating axons penetrating the original axon tube (Atwood *et al.* 1973; Bittner, 1981; Bouton and Bittner, 1981; Kennedy and Bittner, 1974; Nordlander and Singer, 1972). However, the semi-thin serial sections and longitudinal sections (Figs 5, 6) show that these profiles belong to the original axon, which was 'divided' into finger-like processes by intruding glial cells. Fig. 5 shows a reconstruction from transverse serial sections. In Fig. 5A, the

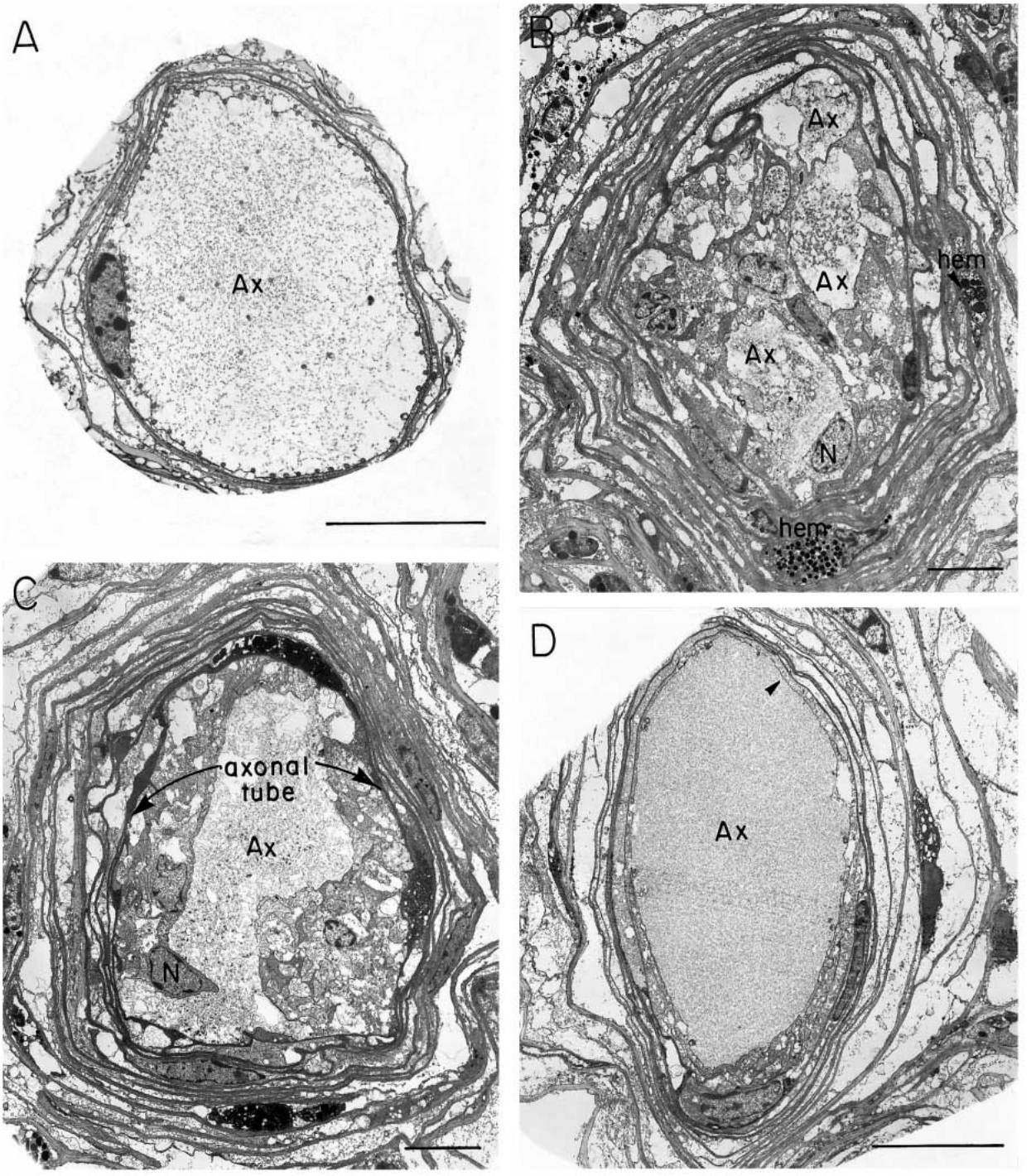

Fig. 3. Ultrastructural changes 16 days after axotomy. (A) The proximal stump of the axon 50 μm proximal to the scar. (B,C,D) Three transverse sections through the distal stump 200 μm, 230 μm and 4500 μm, respectively, from the cut end. (A) An electron micrograph of a transverse section through the proximal stump 50 μm from the scar. The axon (Ax) looks similar to that in a control preparation (Fig. 2). The mitochondria are in the circumference near the axolemma. One or two layers of adaxonal glial cells surround the axon. (B) Electron micrograph 200 μm distal to the cut end. The axon tube contains at least three axon profiles (Ax) surrounded by glial cells. Other, smaller axon profiles are not marked. Some of the glial cells contain nuclei. One nucleus (N) is engulfed by axoplasm. The axoplasm is filled with mitochondria and vacuoles. Haemocytes (hem) are recognizable between the glial wrappings. (C) Electron micrograph of the same axon 230 μm away from the cut end. One larger axon profile (Ax) is seen, but it does not occupy the entire axon tube and it has an irregular shape. Glial cells occupy part of the axon tube. One of the glial cells has a finger-like process protruding into the axon and it contains a large nucleus (N). (D) Electron micrograph of the same distal axon 4500 μm from the cut end. The axon (Ax) occupies almost the entire axon tube and resembles a normal axon. The arrowhead points to the adaxonal glial layers. Mitochondria are less numerous. Scale bars, 10 μm.

view is from the muscle side of the axon towards the cut end. It is clear that one large axon profile emits three finger-like processes, and one of these processes emits a secondary branch. Fig. 5B,C shows the same reconstruction from different angles. Fig. 6 shows a panoramic composite of a longitudinal electron microscope section, through a distal region, 44 days after axotomy. Nearer to the cut end, many finger-like processes (Fig. 6A) are continuous with a smaller number of axonal processes (Fig. 6B), which merge into one large axon nearer to the muscle (Fig. 6C).

Another rapid way to test for continuity is to inject a fluorescent dye into the larger axon near the muscle and to test whether the dye fills the smaller axonal processes.

The results of such an experiment are shown in Fig. 7A. Fura-2 was injected into the axon near the muscle where it filled a single axon profile (a). Further along the nerve, three axon profiles were seen (Fig. 7A, b–d). Profile b is actually composed of two branches that appear as one. In d, these two profiles can be discriminated more clearly. Branch c is out of the plane of focus and, hence, the staining looks less intense so that the branch appears not to be continuous. Normal axons never show such branching along the nerve bundle, and in this region of the nerve the axons always form one continuous cylinder. Branching occurs only at the entry to the muscles and over the muscles. After Fura-2 imaging, the axon was fixed for electron microscopy. Fig. 7B shows three clear axon profiles, corresponding to the two closely adjacent branches (Fig. 7A, b and d) and the single more remote branch (Fig. 7A, c). The distance between the smaller profile of the pair and the single branch in Fig. 7B is 23 μm. In Fig. 7A, the distance between the double profile b and the single profile c is 24 μm. These results support the interpretation that the small axon profiles observed for periods up to 3 months are continuous with the original axon and are not newly growing or satellite axons.

Confocal microscopy

Fig. 8 is an overview of three axons within the same nerve bundle, 6 weeks after axotomy. Axon 1 is filled with small organelles, axon 2 is invaded by cellular (probably glial) fingers and contains exposed nuclei, whereas axon 3 appears normal.

The transverse optical line sections showed different stages of change after axotomy. The changes were similar to those seen in the electron microscope sections. A control axon in Fig. 9A is surrounded by glial layers. An axon 10 weeks after axotomy (Fig. 9B) is invaded by glial cells (section taken approximately mid-way to the muscle), which separate it into compartments. In Fig. 9C,D, 10 weeks after axotomy, strongly fluorescing nuclei float in the axoplasm (see also axon 2 in Fig. 8). Optical serial line sections revealed that such nuclei were connected *via* a short stalk (Fig. 9D, arrowhead) to glial cells. In another preparation 6 weeks after axotomy, an optical longitudinal section (Fig. 9E) shows an axonal region invaded by glial cells and a nucleus apparently isolated in the free axonal space, but a shifted transverse optical section of the same region confirms that this nucleus was still connected to a glial cell with a small stalk (Fig. 9F, arrowhead). In other cases, we observed free-floating nuclei (not connected to glial cell).

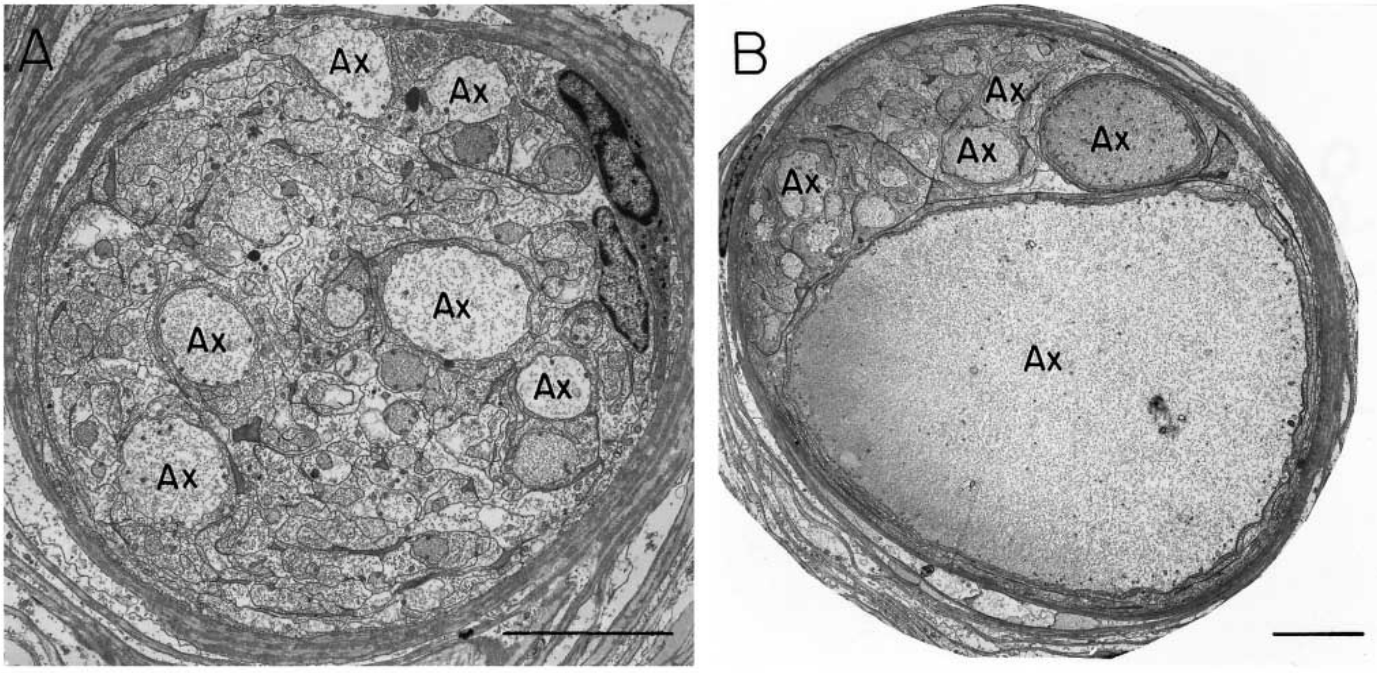

Fig. 4. Electron micrographs of two adjacent axons from the same transverse section, 1500 μm distal to the cut end, 90 days after axotomy. (A) The axon tube is filled with cells and axon profiles. Some of the axon profiles are marked (Ax). (B) A large axon (Ax) profile is prominent in the second tube. Small axon profiles (Ax) and cells occupy the space between the larger axon profile and the wall of the axon tube. Scale bars, 10 μm.

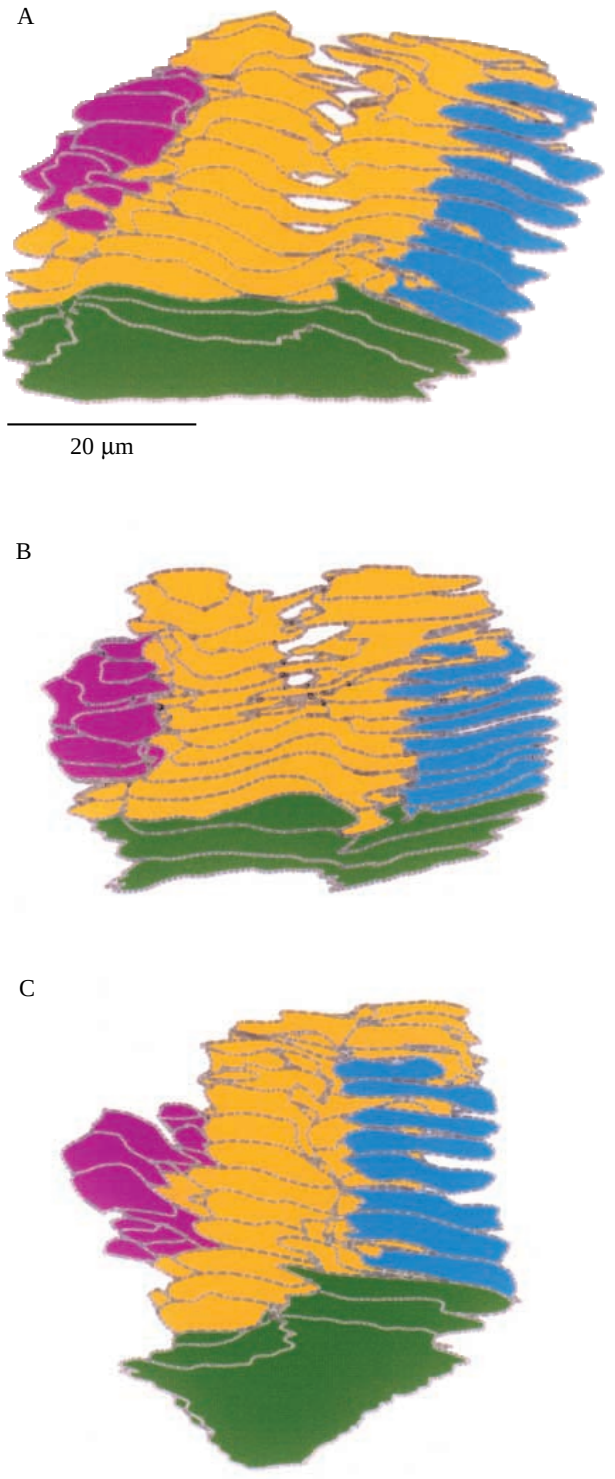

Fig. 5. Three-dimensional computer reconstruction from 15 serial sections of the distal segment 16 days after axotomy, same axon as in Fig. 3 (region between B and C). The thickness of each section was 2 μm. Only the larger axon profiles were traced. (A) The view is from the muscle end towards the cut end. Note that one axon profile (green) emits three fingers (two yellow, one blue) and that from one of the fingers a secondary branch is observed (purple). (B) Same but with upward tilting of the front end. (C) Tilting to the left, shows that one yellow branch emits another finger-like process (purple). Scale bar, 20 μm.

In all, 15 axons (length 1–1.5 cm) from eight animals were surveyed for nuclei (times after axotomy were: 3 weeks, two animals; 6 weeks, three animals; 10 weeks, two animals; and 1 year, one animal). The number of nuclei found per axon was very small. In most axons, only one or two nuclei were found. For example, in the 1 year post-axotomy animal, two axons were surveyed. One axon contained one free nucleus and one connected nucleus, and the second axon contained only one nucleus connected to a glial cell. Ten weeks after axotomy, we found three free nuclei in one axon (the largest number of nuclei per axon); in the other three axons, one contained two connected nuclei and each of the other two axons contained one connected nucleus. Six weeks after axotomy, in one animal, two axons each contained a free nucleus; in the other two animals, one axon had two connected nuclei and the other three axons had one connected nucleus each. Three weeks post-axotomy, two axons contained one connected nucleus each, while one axon contained two connected nuclei.

Thus, 15 axons contained a total of 21 nuclei, of which 15 were connected with a stalk to glial cells and the remaining six appeared to be floating free in the axon. This finding by itself does not mean that the nuclei had no connection to glial cells. Possibly the connections were too small to be detected or they were not in the path of the optical line section. It should be noted that, along a stretch of an axon as long as 1–1.5 cm, only 1–3 nuclei were observed with the confocal microscope, which explains why it is so difficult to observe floating (Atwood *et al.* 1989; Govind *et al.* 1992) or dangling nuclei in the electron microscope sections.

Glia cell proliferation

Since the axon tube was invaded by glial cells and the number of the cells seemed to increase, we investigated whether glial cells in the axon sheaths or within the axon tube undergo cell division. To test this, we used the BrdU technique, which identifies nuclei that have replicated DNA during the BrdU exposure (see Materials and methods). Replicating nuclei show brown staining and non-replicating nuclei are stained green.

Fig. 10 shows profiles of cross sections (10 μm thick) of single axon tubes 3 (Fig. 10A) and 7 (Fig. 10B) days after axotomy. We counted the total number of nuclei in sections of the whole nerve bundle containing the five axons and the surrounding connective tissue in control intact contralateral nerve bundles and in distal stumps 1, 3, 7 and 16 days after axotomy. Table 2 summarizes our results. Even though only one animal was used for each period, the trend is clear. In the controls, only 1.4 % of nuclei stained positive. In damaged nerves, the number of BrdU-positive nuclei increased to 5.6 % 1 day after axotomy and rose to 23–25 % 3–7 days after nerve damage. Thus, it appears that there is a peak of nuclear division 3–7 days after axotomy, which then declines back towards the control level after 16 days. These findings show that, after axotomy, there is glial cell proliferation at an accelerated rate in the distal region, but they do not exclude the possibility that, in addition to glial cell division at the distal region, other glial

Table 2. *Numbers of nuclei in distal axon stumps over time after axotomy*

	Time (days)	No. of sections	Total no. of nuclei	No. of nuclei per section	No. of BrdU-stained nuclei	No. of BrdU-stained nuclei per section	% BrdU-stained nuclei
Contralateral control	3–7	38	823	21.6	12	0.3	1.4
Distal stump	1	56	671	11.9	38	0.68	5.6
	3	24	428	17.8	108	4.5	25.2
	7	26	1049	40.3	241	9.27	23
	16	36	1296	36	64	1.77	5

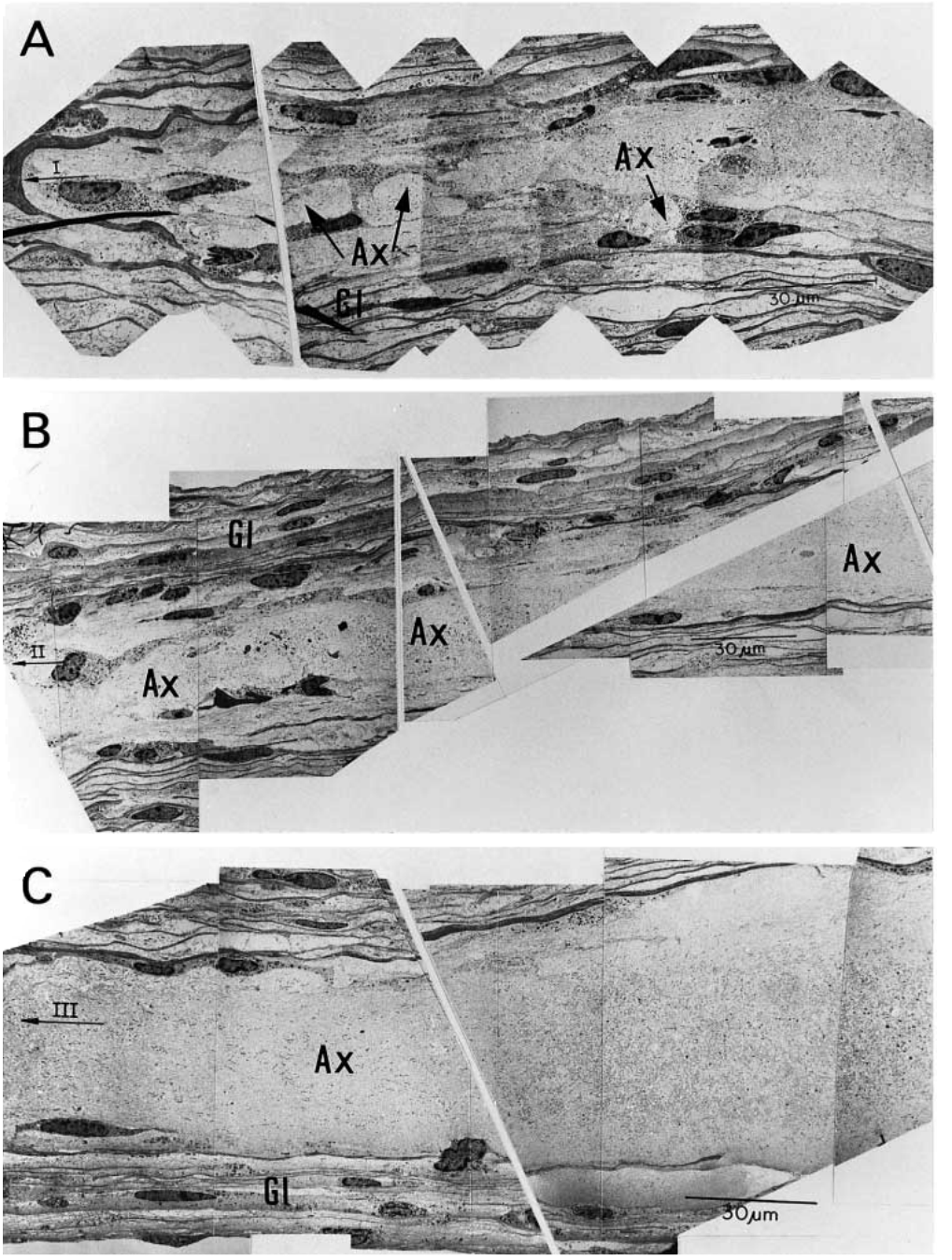

Fig. 6. A panoramic composite of a longitudinal electron microscope section of a distal axon segment of approximately 0.6 mm in length 44 days after axotomy. Because of its size, the figure is shown in three parts with the most proximal part in A and the part nearer to the muscle in C. (A) Arrow I, on the left in A, marks an edge approximately 1 mm from the scar. Arrows II (B) and III (C) indicate the continuity of the longitudinal section. Only some of the axon profiles are marked by Ax. Note that the axon nearer to the scar is partitioned by glial cells, while one larger branch is prominent on the right. (B) The axonal 'fingers' on the left are continuous with the larger axon on the right (the straight white partitions were produced by the supporting grid). (C) A clear continuation of one larger axon. Ax, axon; Gl, glia. Scale bars, 30 µm.

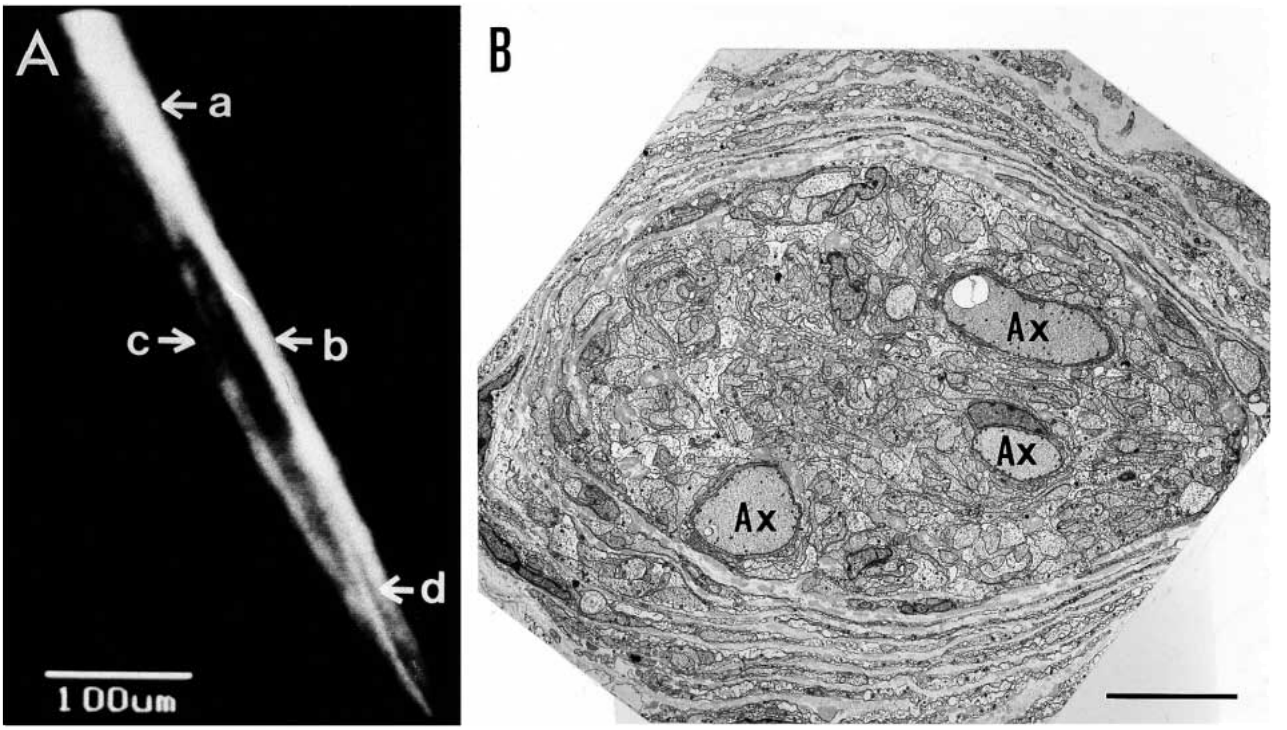

Fig. 7. (A) Fura-2 fluorescence of a distal part of a severed axon 90 days after axotomy. Fura-2 was injected into the axon nearer to the muscle. 8 mm distal to the cut, the axon appears as one cylinder (a) but subsequently divides into three branches. Two adjacent axon profiles, one above the other, appear as a single profile (b). A smaller axon branch, because it is partly out of focus, looks intermittent (c); at point d, the two profiles composing b are resolved. Scale bar, 100 μm. (B) An electron micrograph of a transverse section of the same axon as in A (Fura-2 is not electron-dense). The axon tube is filled with glial cells. Some of the cells contain a nucleus. Note the three clear axon profiles (Ax) which correspond to a transverse section at approximately the level b–c. Scale bar, 15 μm. See text for further detail.

cells migrate to the distal region of the severed axon after replication elsewhere.

Discussion

There have been several reports describing the ultrastructural changes in severed axons in crustaceans (Nordlander and Singer, 1972; Atwood *et al.* 1973; Kennedy and Bittner, 1974; Ballinger and Bittner, 1980; Govind *et al.* 1992), but our study describes in greater detail some of the changes that occur over time along severed axons.

Long-term survival

The degenerative process of cut axons in crustaceans is very slow. We found that the distal segment of the cut axon survived for many months. Survival of an anucleated axonal structure demands synthesis of proteins or at least the maintenance of proteins essential for survival and transmitter release. The occurrence of protein synthesis in axons remains controversial (for a review, see Van Minnen, 1994). Although dendrites contain the machinery required for protein synthesis (Torre and Steward, 1992; Steward and Banker, 1992), the demonstration that axons contain functional ribosomes (Giuditta *et al.* 1991; Crispino *et al.* 1993*a*,*b*) has met with criticism (Gainer *et al.* 1977, 1994). Recently, however, Spira *et al.* (1996) demonstrated that, even 7–8 days after axotomy, protein synthesis occurred in axons isolated from *Aplysia californica* and *Aplysia oculifera* maintained in culture in the absence of glial cells. Protein synthesis for longer periods was not tested. The possibility that proteins are synthesized in severed axons is, therefore, quite reasonable.

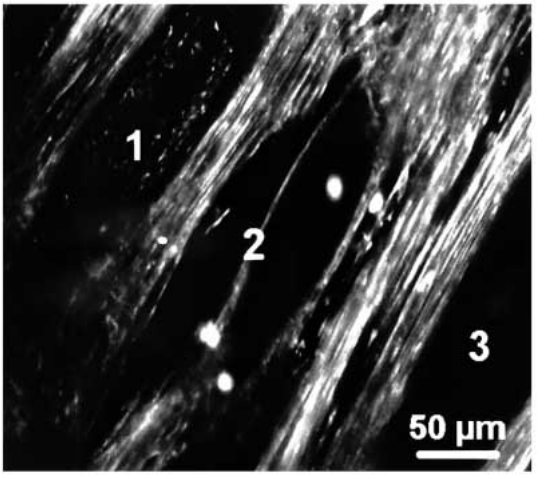

Fig. 8. Confocal image of a longitudinal optical section. Overview of three axons, 6 weeks after axotomy. Different stages at the same time within one nerve. Axon 1 is filled with small organelles, axon 2 is invaded by glial fingers and contains ‘floating’ nuclei, whereas axon 3 appears empty. Scale bar, 50 μm.

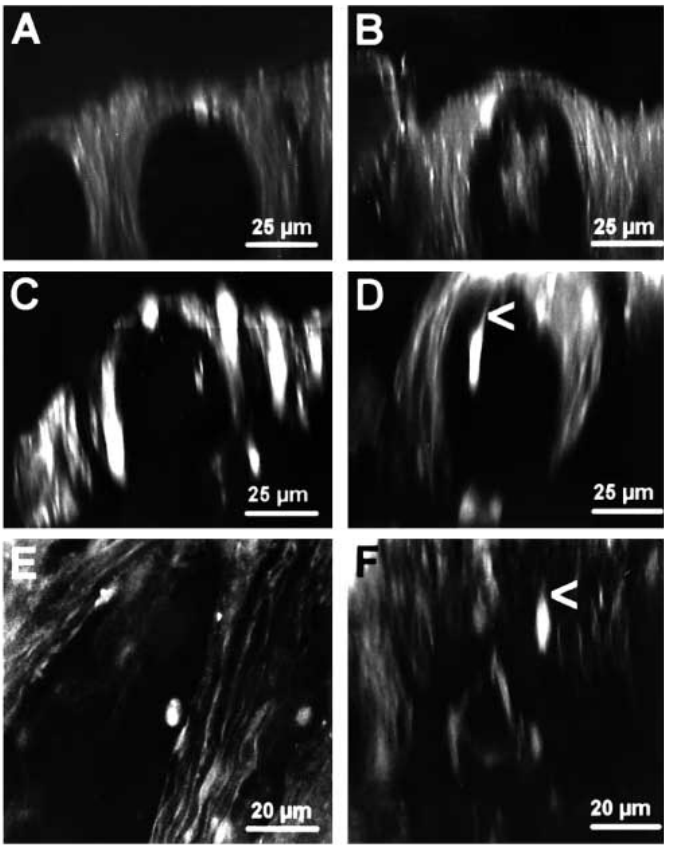

Fig. 9. Confocal images of optical transverse sections (A–D) showing different degrees of change after axotomy. (A) A control axon. Note the fluorescent nucleus at the upper end. (B) An axon 10 weeks after axotomy, invaded by glia. (C,D) The initial (C) and complete (D) invasion of the axon by brightly stained nuclei. A small connection between the nucleus and the glia is retained (arrowhead in D). Both axons 10 weeks after axotomy. (E,F) Another preparation. (E) Optical longitudinal section presenting a region with invading glia and a 'floating' nucleus in the axon, 6 weeks after axotomy. (F) Optical transverse section of the same region as in E showing that the nucleus is connected by a small bridge (arrowhead) to a glia cell. Scale bars, A–D, 25 µm; E,F, 20 µm.

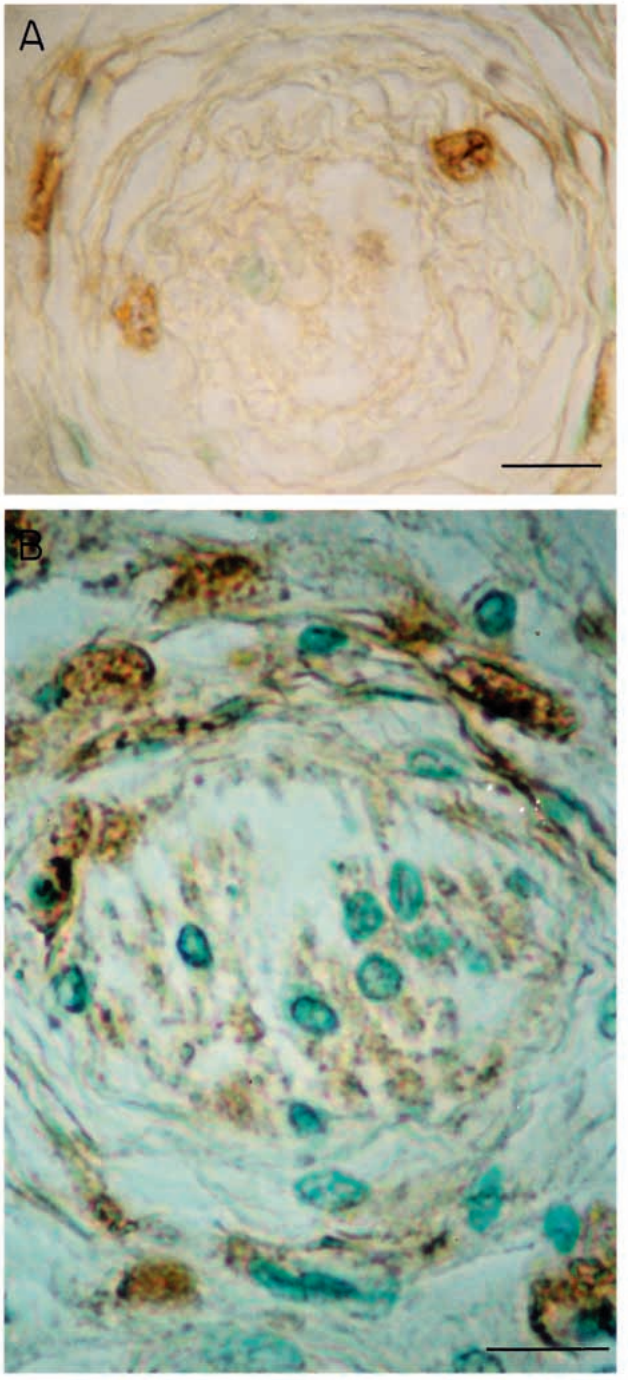

Fig. 10. Glia cell proliferation in cut distal stumps. Sections (10 µm) of axon profiles immunostained with anti-BrdU 3 days after axotomy (A) and 7 days after axotomy (B). (A) Three BrdU-stained nuclei are prominent, two inside the axon profile and one along the glial sheath. Also note the faintly stained green nuclei (negative BrdU staining). (B) Inside the axon tube, many profiles of non-dividing nuclei are seen (green). Many dividing nuclei (brown) are seen in the glial sheath. Scale bars, 10 µm.

It has been suggested that glial cells surrounding the axon produce the necessary proteins to maintain axonal function and that these proteins are transferred to the anucleated axons (Bittner, 1988; Lieberman *et al.* 1994). The cascade of changes in the glial cells that surround the axons and penetrate the axon tube supports such an explanation. Lobulation of the axon into finger-like processes by the invading glial cells increases the surface area between the axon membrane and the glial elements. However, these changes might be processes that lead finally to the degeneration or collapse of the severed axon. Nevertheless, such finger-like axonal processes survived and continued to respond to electrical stimulation and to conduct action potentials for many months.

Small axonal profiles within the axon tube of the severed crustacean nerve have been seen in previous studies (Nordlander and Singer, 1972). Since no serial sections were made in that study, these profiles were considered to belong to satellite axons growing into the original axon tube (Atwood *et al.* 1973). Our semi-thin serial sections (Figs 3B,C, 5, 6) and

the fluorescent dye injection (Fig. 7A) actually show that these profiles belong to the original axon.

A previous article from our laboratory (Atwood *et al.* 1989) suggested that glial cells 'donate' their nuclei to the severed axon and that these 'kamikaze' nuclei (Sheller *et al.* 1991) are responsible for protein synthesis in the severed axon. Such 'donation' of nuclei was also described in sensory axons of crustaceans (Govind *et al.* 1992) and in the giant axons of crayfish (Sheller *et al.* 1991). However, it was emphasized that such donations were observed only in rare cases and that this mechanism may not operate under normal conditions (Govind *et al.* 1992).

The use of confocal microscopy allows us to offer a different explanation for the appearance of nuclei floating in the axon cytoplasm. We found that the glial cells send into the axon a thin grape-like process containing the nucleus. This was found for 15 out of 21 nuclei detected by the confocal microscope. An ultra-thin section through such a dangling nucleus will appear as a floating nucleus in the axoplasm (especially if the glial and axonal membranes around the nucleus were not well preserved). Such dangling nuclei have never been observed before and they may explain why such nuclei were found only in rare cases in electron microscope sections (Atwood *et al.* 1989; Govind *et al.* 1992; Sheller *et al.* 1991).

Proximal–distal axon response

The changes observed after axotomy progressed over time and were limited to the distal stumps. The proximal stumps, except for the very tip (20–50 μm), looked almost normal. The surrounding glial cells did not show marked proliferation or invasion of the axon tube. This suggests that a signal from the cell body prevents these changes from occurring in the proximal stump. The mechanism producing the changes in the distal stump cannot be simply the absence of a signal arriving from the cell body since the changes did not occur simultaneously all along the length of the distal axon, but occurred first near the cut end and then progressed towards the muscle over time. The axon profiles over the muscle looked normal for many months. It is possible that the lack of a central signal together with exposure of the internal milieu of the axon to external factors entering through the cut end or through the depolarized membrane near the cut (e.g. Ca^{2+}, Ziv and Spira, 1993, 1995) are responsible for the gradual effect. However, even this cannot be the complete explanation since it is known that cut axons form a new membrane and regain their original membrane potential within a few hours and that excess Ca^{2+} is rapidly sequestered (Ziv and Spira, 1993, 1995); however, distal stump changes occurred over a period of several weeks or months. There was also considerable variation in the rate of response of the five axons residing in the same bundle and cut at the same time (Fig. 4A,B). We have no explanation for this variation. The complicated cascade of events observed in the distal stump, together with the changes observed in the glial cells, indicates that several signals must be involved in slowing degeneration and in maintaining the long-term survival of the cut axons. Whether these changes have a physiological role in the survival of the muscles until reinnervation occurs is not clear.

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