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Increase of elastic fibres in muscle spindles of rats following single or repeated denervation with or without reinnervation

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Summary. Muscle spindles in the lower lumbrical muscles of rats were studied by transmission electron microscopy following denervation with or without reinnervation. The number and total area of elastic fibres per muscle spindle increased at 3–12 months following various experimental procedures: (1) denervation and reinnervation after a single crush lesion to the sciatic nerve; (2) reinnervation after four-fold repeated crush injuries; and (3) transection and suture of the nerve. The increased number of oxytalan and elaunin fibres, the precursors of mature elastic fibres, within these muscle spindles provided further evidence for their numerical and dimensional increase. An attachment site of elastic fibres at the spindle pole was identified at the inner cells of the outer spindle capsule. The processes of these cells embraced terminating elastic fibres tightly. Attachment of elastic fibres to intrafusal muscle fibres was less conspicuous since they were not similarly embraced but were rather indistinctly, though closely, associated with the basal lamina along longitudinal surface indentations of intrafusal muscle fibres. It is concluded from this series of experiments that muscle spindles, as dynamic mechanoreceptors, maintain their elastic properties even under pathological conditions. The increase of elastic fibres following denervation and reinnervation represents an obviously meaningful reaction that may compensate for loss of tonic properties of muscle spindles without causing stiffness.

Key words: Muscle spindle – Elastic fibres – Elaunin fibres – Oxytalan fibres – Denervation – Reinnervation

Introduction

Few light or electron microscopic studies on the distribution of elastic fibres (EFs) in normal muscle spindles in man (Cooper and Daniel 1967; Cooper and Gladden 1974; Gladden 1975), cat (Gladden 1972, 1976; Cooper and Gladden 1974; Banks 1984), rat (Cooper and Daniel 1967; Cooper and Gladden 1974), or baboon (Greer 1985) are available. The studies have shown that EFs form a continuous and mainly longitudinally arranged network around intrafusal muscle fibres (IMFs) which continues beyond the end of the spindle capsule. It is connected to a much finer EF network around extrafusal muscle fibres (Barker and Banks 1986). In the polar region of cat muscle spindles, static nuclear bag 2 fibres were noted to be associated with more and thicker EFs than dynamic nuclear bag 1 fibres, although this difference was "not always obvious close to the end of the capsule, but was obvious about 1 mm from the end of the capsule" (Gladden 1976) – the C-region or the extracapsular part of a spindle pole. In contrast to what has been reported for cat muscle spindles, EF distribution in polar regions cannot be used to distinguish nuclear bag 1 from nuclear bag 2 fibres reliably in human (Gladden et al. 1985), baboon (Greer 1985), or newborn kitten muscle spindles (Gladden et al. 1987). In equatorial regions of cat, rat, and human muscle spindles, EFs are dispersed among the inner capsular sheaths (Gladden 1972; Cooper and Gladden 1974). The EFs are said to arise from the spindle pole opposite to their attachment site beyond the primary sensory ending and to cause small sarcolemmal projections for anchoring on bag 1 and bag 2 fibres (Banks 1984). Their precise site of attachment at the spindle pole, and their composition, size, and number have not been determined by electron microscopy.

Changes in the connective tissue elements of muscle spindles appear to be non-specific concomitants of a wide variety of disorders (Boyd and Smith 1984) with hardly any documented change in EFs (Swash 1982). By re-examining muscle spindles from previous studies (Schröder et al. 1979; Dieler and Schröder 1990) we noticed strikingly prominent EFs around IMFs at various time intervals following denervation and reinnervation. This was not found in control muscle spindles indicating an involvement of EFs following alterations of innerva-

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tion that should affect muscle spindle function. This prompted us to reinvestigate the available series of experiments by analysing the intrafusal elastic fibres further at the fine structural level.

Materials and methods

Muscle spindles from the lower lumbrical muscles of Wistar rats were examined following complete denervation or reinnervation after single or repeated crush injuries to the sciatic nerve (Schröder et al. 1979). In addition, 21 muscle spindles from posterior lumbrical muscles of female Sprague Dawley rats weighing 200–250 g at initial surgery were examined following transection and suture of the sciatic nerve (Dieler and Schröder 1990). Thus there were five groups: (1) denervation after 3, 6, and 12 months; (2) reinnervation 3, 6, and 12 months after a single crush lesion; (3) four-fold repeated crush lesions with a time interval of 3 months between crush injuries; (4) reinnervation 3 and 6 months after nerve transection and immediate suture; and (5) control muscle spindles from lumbrical muscles contralateral to the experimental side from all groups.

Unilateral crushing, sectioning of sciatic nerves, and preparation of lumbrical muscles were performed as described before (Schröder 1974; Schröder et al. 1979). Unilateral nerve sectioning and suture was accomplished under ether/chloral hydrate anaesthesia by transection of the left sciatic nerve at the mid-thigh level and immediate approximation with two diagonally opposed 9-0 epineurial sutures. The lower extremities were fixed by perfusing the abdominal aorta with a solution containing 3.9% glutaraldehyde in 0.1 M Sorensen's phosphate buffer. Lumbrical muscles were excised and postfixed for 1–3 h in the same fixative, osmicated, dehydrated through graded alcohol solutions, and embedded for single or spaced serial cross-sectioning in epoxy resin. Ultrathin sections were cut with diamond knives, mounted on copper grids, counterstained with uranyl acetate and lead citrate, and examined with a Philips EM 400 T electron microscope at 60 kV.

EFs surrounding IMFs, situated in the periaxial space, or between lamellae of the inner and outer spindle capsule were counted and measured in 20 individual muscle spindles at characteristic regions (the equatorial and intracapsular polar regions). Ultrathin transverse sections of muscle spindles were photographed and enlarged to 9600–21 000:1. Area, perimeter, and maximum and minimum diameter of EFs were measured with a Kontron IBAS 1 interactive image analysing system (Zeiss, Oberkochen, FRG) using the standard software for manual image analysis. The border of the EFs was defined by the most peripherally located filaments and measured by moving the stylus around these structures.

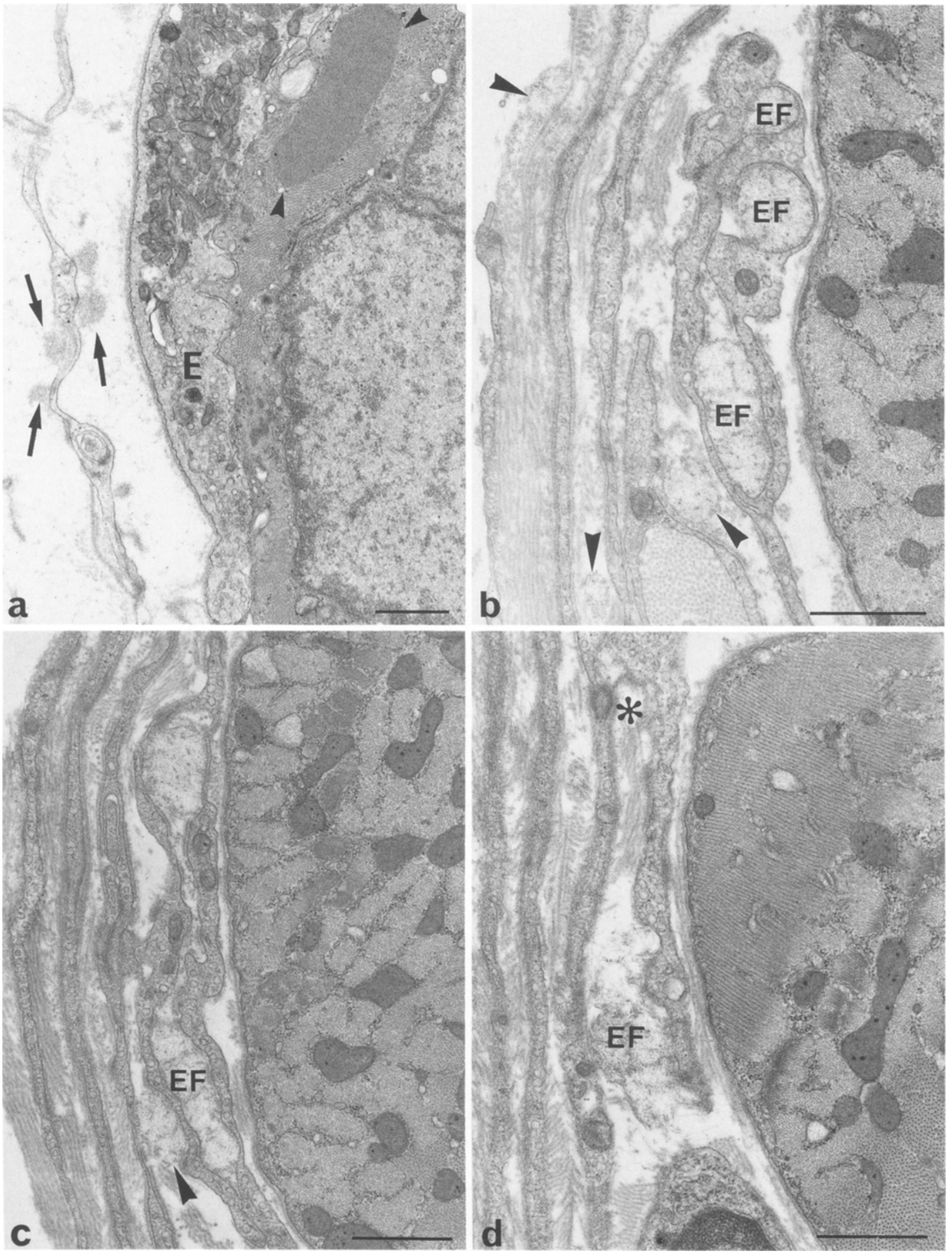
Results

As in previous studies (Boyd and Gladden 1985; Kucera and Walro 1987), the muscle spindles of the rats analysed usually contained 3–4 IMFs, 2 nuclear bag and 2 nuclear chain fibres. In the equatorial region, IMFs were enclosed by an inner spindle capsule sheath composed of a single layer of contiguous, highly branched cell processes devoid of a covering basal lamina. These cells closely resembled endoneurial fibroblasts of peripheral nerves (Boyd and Smith 1984). Usually, 15–18 small EFs composed of numerous fine filaments measuring 10–12 nm in diameter and smaller amounts of centrally located amorphous elastin were situated closely adjacent to fibroblastic processes, more frequently facing the inner axial space than the outer periaxial space (Fig. 1a). Repeatedly, EFs were encountered lying isolated within the periaxial space, or in contact with outer capsule cells or IMFs.

In the juxtaequatorial region, the periaxial space tapered towards the spindle pole. Fibroblastic processes of the inner spindle capsule approached flat cell processes covered by a basal lamina that was continuous with the perineurium of the spindle nerve and formed the outer spindle capsule (Low 1976). The appearance and distribution of EFs was similar to that seen in the equatorial region.

In the intracapsular polar region, fibroblastic processes of the inner spindle capsule were only rarely encountered. Here, they were mainly seen between IMFs, but no longer formed an inner capsular sheath. The outer spindle capsule consisted of three to five layers of concentrically arranged flat perineurial cell processes. They were enclosed on either side by a basal lamina and contained abundant micropinocytotic vesicles, a well-developed rough endoplasmic reticulum, Golgi complexes, and mitochondria. Only a few EFs were seen on the external surface of the outer spindle capsule (Fig. 1b). Numerous collagen fibrils were encountered between cell layers of this capsule. Here, EFs appeared to lie either independently, or closely adjacent to the basal lamina of cell processes of the outer spindle capsule. Some were surrounded by these cell processes without an intervening basal lamina between EF and cell membrane (Fig. 1b–d; see also Fig. 2b, c). In spaced serial ultrathin sections, EFs between cell processes of the outer spindle capsule that were formerly closely surrounded by cell processes tapered and disappeared when followed towards the spindle pole (Fig. 1c, d). EFs in the polar region were usually thicker than in the equatorial region. They consisted of two structurally distinct components: few filaments at the periphery or, less frequently, more in the centre of EFs, and amorphous elastin that was usually electron-lucent (Fig. 1b–d). Inside the spindle cavity, usually 19–21 EFs were situated close to fibroblastic processes, in contact with the basal lamina of an IMF (Fig. 3). They were occasionally seen between two adjacent IMFs, sometimes indenting both to a similar degree.

Fig. 1a–d. Control muscle spindles. **a** Equatorial region. A primary sensory ending (*E*) is closely attached to an intrafusal muscle fibre. It is filled with mitochondria as well as small clear neurogenic vesicles and surrounded by a common basal lamina. Several thin elastic fibres (*EFs*) mainly composed of filaments (*arrows*) are apparent at the inner axial and outer periaxial side of a fibroblastic cell process forming the inner capsular sheath. Note also cytoplasmic body within the intrafusal muscle fibre (*arrowheads*). *Scale bar:* 1 μm. **b** Intracapsular polar region. Several flat cell processes of the outer spindle capsule are covered by a basal lamina and studded with micropinocytotic vesicles. They alternate with layers of collagen fibrils. Three EFs mainly composed of amorphous material of moderate electron density are tightly enclosed by the innermost cell layers (EF). EFs can also be seen between capsular cell layers and at the outer surface of the spindle capsule (*arrowheads*). *Scale bar:* 1 μm. **c, d** Serial sections from the muscle spindle area shown in **b** towards the spindle pole. **c** The upper embraced elastic fiber visible in **b** has disappeared in this plane of section, 13 μm towards the spindle pole. The lower EF appears to be flattened in comparison to **b**. The innermost EF indicated by an *arrowhead*



in **b** has approached the cell membrane of an internal capsular cell process (*arrowhead*). An intervening basal lamina is no longer discernible. *Scale bar:* 1 μm. **d** Another 11 μm towards the spindle pole, the upper EF visible in **c** has disappeared (*asterisk*). The lower EF is slightly enlarged and of uneven contour. There is no clear basal lamina between this obviously terminating EF and the cell membranes of the adjacent capsular cell processes. *Scale bar:* 1 μm

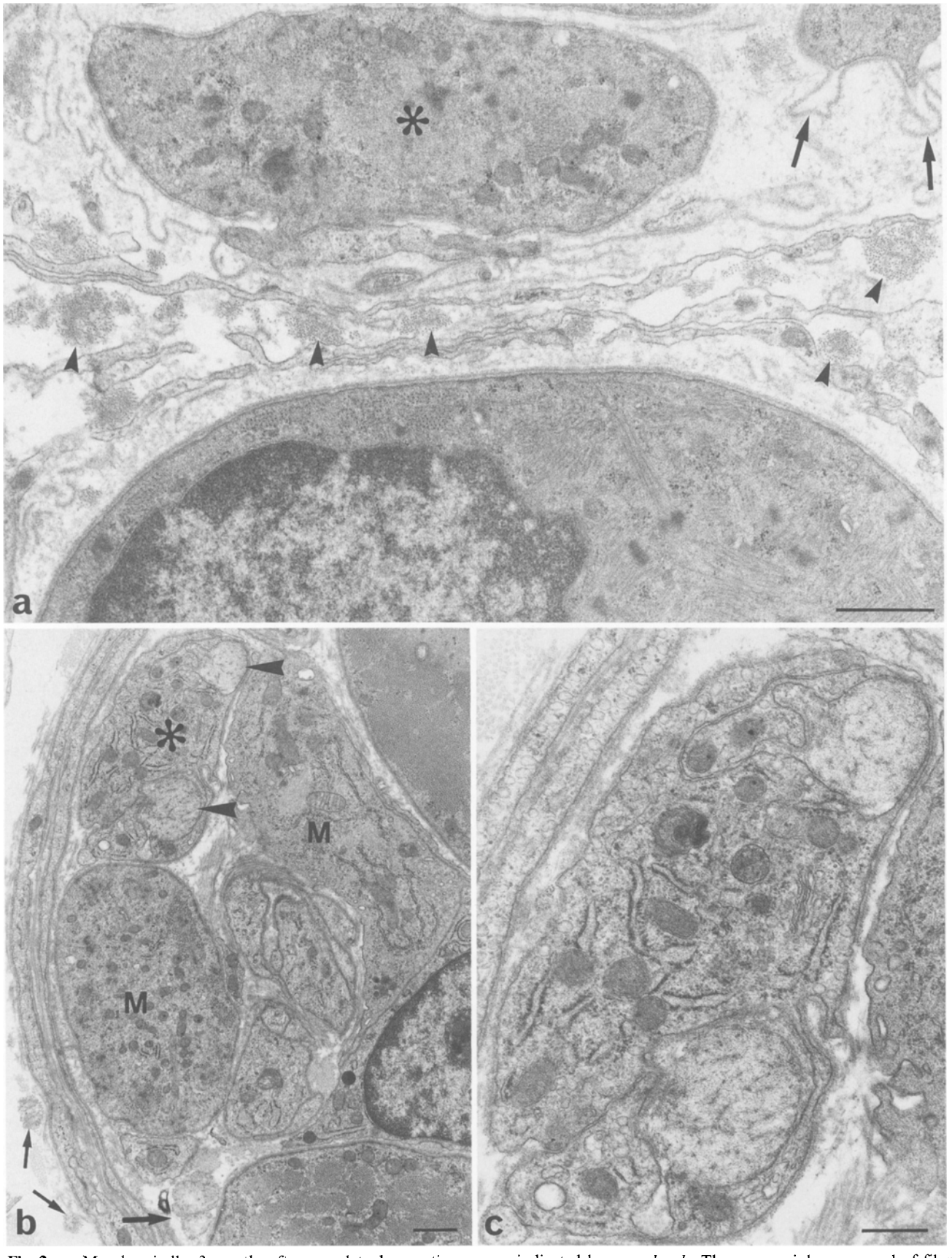


Fig. 2a–c. Muscle spindles 3 months after complete denervation. **a** Equatorial region. A small and presumably newly formed intrafusal muscle fibre (*asterisk*) is separated from the lower one by fibroblastic cell processes forming the inner spindle capsule. On both sides of these cell processes lie abundant EFs some of which are indicated by *arrowheads*. They are mainly composed of filaments with only small amounts of amorphous, centrally located elastin. Note also basal lamina remnants and basal lamina duplications (*arrows*). *Scale bar:* 1 μm.

Following 3 months of complete denervation, the inner axial space at the equatorial region was reduced in width and filled with increased numbers of isolated basal laminae and EFs (Fig. 2a). The total EF area per spindle was increased when compared with controls. The EFs consisted mainly of filaments and minor amounts of centrally located amorphous elastin (Fig. 2a). They lay close to fibroblastic cell processes or between muscle fibres. Their structure and distribution in juxtaequatorial regions was similar to that in equatorial regions, although here they were more often seen close to the basal lamina of intact or newly formed IMFs. At the intracapsular polar level (Fig. 2b, c), EFs were quite prominent, usually situated between IMFs and the inner layer of the outer spindle capsule, often indenting the adjacent IMF. Elastic fibre filaments were more numerous at the surface and within the amorphous elastin component of EFs than in controls.

After 6 and 12 months of complete denervation, EFs were increased in number, thickness, and total cross-sectional area per muscle spindle when compared with controls (Fig. 3).

Reinnervated muscle spindles following crush lesions of the sciatic nerve showed increased numbers of thin EFs in the equatorial region 3 months after a single crush lesion (Fig. 4a). Their distribution was similar to that in controls; they lay either close to fibroblastic cell processes of the inner spindle capsule or isolated within the inner axial space. Only rarely were EFs encountered in the outer periaxial space. At the equatorial and juxtaequatorial level, EFs mainly comprised filaments only, whereas in the polar region they were composed of both, filaments and elastin. Compared with controls, the number of filaments in EFs was higher so that the majority of EFs comprised equal proportions of filaments and elastin. Some EFs were entirely formed by filaments (not illustrated). In the equatorial and polar region the total EF area per muscle spindle was nearly doubled when compared to controls.

Following 6 and 12 months of recovery (Fig. 4b, c), EFs were increased in number as well as in total area per muscle spindle when evaluating equatorial and polar regions.

Following four-fold repeated crush injuries of the sciatic nerve at time intervals of 3 months, EFs were considerably increased in number and total cross-sectional area per spindle: at the equatorial region there was an up to five-fold numerical increase and an approximately four-fold increase of the total EF area, whereas at polar levels both values approximately doubled. The amount of filaments in EFs resembled those following 3 months of complete denervation. In the equatorial region, EFs mainly comprised filaments whereas in the polar region elastin predominated. In general, filaments were more abundant and the amorphous elastin component showed a higher electron density than in controls (Fig. 4d).

Reinnervated muscle spindles following transection and suture of the sciatic nerve showed numerous thinly myelinated axons reinnervating extrafusal nerve fascicles and muscle spindles after 3 months of recovery. Certain muscle spindles showed increased numbers of IMFs. EFs in equatorial regions were more prominent than in controls. They were mainly composed of filaments, particularly in polar regions. The number and total area of EFs per muscle spindle was approximately doubled when compared with controls.

Following 6 months of regeneration, the number and total area of EFs were increased in equatorial and polar regions. In equatorial regions EFs comprised filaments and elastin to approximately equal proportions wheras in polar regions elastin of uneven electron density predominated.

Discussion

The present study has shown that EFs within muscle spindles of lower lumbrical muscles of rats increased in number and total cross-sectional area per spindle following complete denervation, after temporary severance of innervation due to single or repeated crush lesions, or after transection with immediate suture of the sciatic nerve.

During normal development of EFs tropoelastin, the soluble precursor of mature elastin, is synthesized in fibroblasts and smooth muscle cells, and linked together in the extracellular space (Pasquali-Ronchetti and Fornieri 1984; Rosenbloom 1984). Ultrastructurally, three types of EFs can be distinguished (Cleary and Gibson 1983; Ghadially 1988): (1) Mature EFs, comprising about 90% elastin, that presents in routine electron microscopic preparations as an amorphous component with an electron density ranging from lucent to moderately electron dense. A few filaments about 11 nm in diameter are also present; (2) elaunin fibres which resemble immature EFs with a prominent filamentous component and a less abundant and compact elastin component (Cotta-Pereira et al. 1976); and (3) oxytalan fibres formed by filaments 10–12 nm in diameter without the amorphous material regularly present in mature EFs (Fullmer and Lillie 1958; Cotta-Pereira et al. 1976). The latter fibres represent early developmental stages of EF formation that occur in the order: oxytalan, elaunin, and mature EFs. In the present study, immature EFs mainly formed by filaments (oxytalan and elaunin fibres)

b Intracapsular polar region. Adjacent to two intrafusal muscle fibres in the upper and lower right corner, there are two newly formed intrafusal muscle fibres (*M*), several Schwann cell processes, and fibroblasts. Two prominent EFs lie adjacent to the lower intrafusal muscle fibres (*large arrow*), two other EFs (*arrowheads*) are enclosed by cytoplasmic processes that are shown at higher magnification in **c**. Note also EFs mainly composed of filaments at the outer surface of the spindle capsule (*small arrows*). *Scale bar:* 1 μm.

c Higher magnification of the area indicated by *asterisk* in **b**. Both EFs are surrounded by one to two cytoplasmic processes that are covered by a common basal lamina. The processes are filled with micropinocytotic vesicles, rough endoplasmic reticulum, Golgi complex, and mitochondria, suggesting that this cell is derived from the outer spindle capsule. *Scale bar:* 0.5 μm

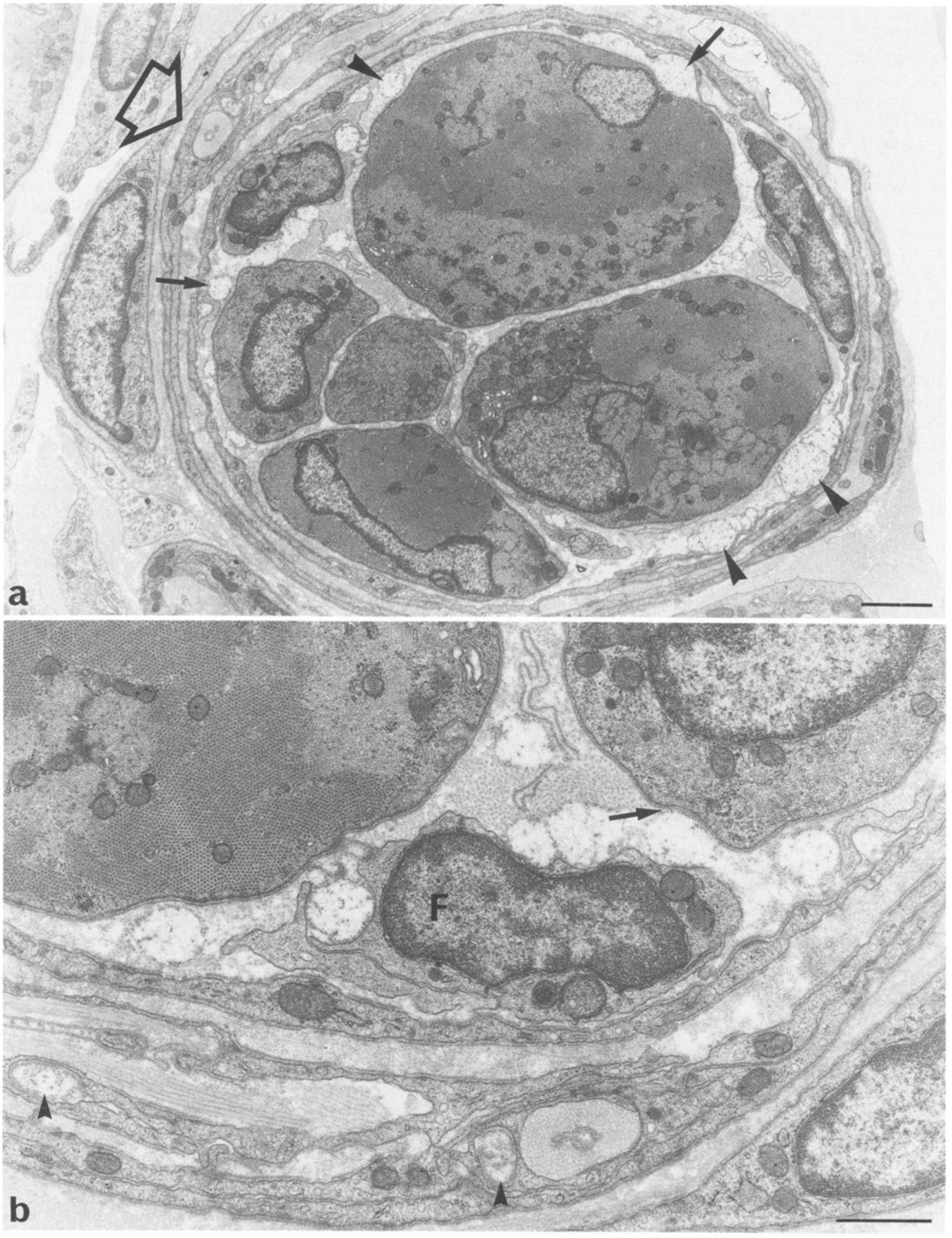


Fig. 3. a Polar region of a muscle spindle 6 months after complete denervation. Note prominent EFs (*arrowheads*) mainly at the outer circumference of five intrafusal muscle fibres. Some of the EFs are seen indenting the muscle fiber surface (*arrows*). *Scale bar:* 2 μm. **b** Higher magnification of area indicated by *open arrow* in **a.** Prominent EFs can be seen attached to a fibroblast (*F*) or to the basal lamina of an intrafusal muscle fibre (*arrow*). They are surrounded by collagen fibrils. Some EFs are enclosed by cytoplasmic processes of the outer spindle capsule (*arrowheads*). *Scale bar:* 1 μm

were encountered following 3 months of denervation, and at the various stages of reinnervation. Thus, neoformation of EFs had apparently occurred, as was shown by an increase in the number and total area of EFs per muscle spindle; by an increased variability of the cross-sectional area; by an increase of oxytalan and elaunin versus mature EFs; and by a higher electron density of the amorphous elastin component. Newly laid down elastin is known to show a variable but greater intensity of staining with uranyl acetate and lead citrate than mature or aged EFs (Ghadially 1988).

The stimulus causing EF proliferation appeared to be denervation rather than reinnervation of muscle spindles, since numerical and dimensional EF increase was more prominent following early stages of denervation than at later stages of reinnervation. In addition, following repeated crush lesions to the sciatic nerve, the increase in number and total area of EFs per spindle was more pronounced than in the other experimental groups, even in polar regions (Fig. 4d) where EFs were usually less prominent than in the other groups studied. Since four consecutive crush lesions were performed, each followed by 3 months of reinnervation, four temporary periods of denervation occurred and these caused more extensive EF neoformation than 12 months of reinnervation after a single crush lesion.

Inside denervated or reinnervated intrafusal nerve fascicles, mature EFs were not seen. The outer spindle capsule is continuous with the perineurium of the spindle nerve (Low 1976; Ovalle and Dow 1983) and the inner is formed by endoneurial fibroblasts (Boyd and Smith 1984). The endoneurial connective tissue space in the spindle nerve is thus continuous with the axial and periaxial space of the spindle capsule (Barker and Banks 1986). Hence, connective tissue changes in the endoneurium of peripheral nerves following nerve injuries (Röyttä et al. 1987; Salonen et al. 1987; Röyttä and Salonen 1988) are of interest with regard to those occurring in muscle spindles. Long-term endoneurial changes in distal stumps of transected and non-reinnervated rat sciatic nerves included an increase of collagen fibrils and the occurrence of 10–12 nm microfibrils (Röyttä and Salonen 1988). Amorphous material resembling elastin associated with these microfibrils has not been reported in nerve stumps even 50 weeks after surgery (Röyttä and Salonen 1988). Formation of EF elements in peripheral nerves therefore appears to be restricted to the immature forms of EFs, that is to say, fibrils formed of EF filaments. Since fibroblasts are involved in EF production and EFs in the present study were found to be intimately associated with cytoplasmic processes of the inner capsule, the increase of EFs can most likely be attributed to a denervation-induced stimulation of intrafusal fibroblasts. However, influences from outer capsule cells, IMFs, and Schwann cells cannot be excluded. The importance of intrafusal fibroblasts is supported by a previous ultrastructural study of normal vertebrate muscle spindles where an intimate association of EF filaments to cytoplasmic processes of the inner spindle capsule was discussed as determining EF polymerization and their pattern of organization (Ovalle and Dow 1983).

The minute surface projections of nuclear bag fibres possibly functioning as attachment sites of EFs reported in cats by Banks (1984) were not encountered in the present cross sectional study. At intracapsular muscle spindle poles, however, EFs were repeatedly seen to be indenting the IMF surface to some extent. Here, they were often tightly associated with the basal lamina of IFMs. This association was therefore regarded as a possible attachment site of EFs at IMFs. Such an attachment is also suggested from longitudinal indentations of IMFs by presumptive EFs, dichotomous branching of which has been discussed in a scanning electron microscopic study of teased IMFs (Schröder et al. 1989). However, EFs in polar regions were regularly seen intimately associated with or sometimes tightly embraced by outer capsular sheath cells. In these instances, there was no basal lamina between EFs and the capsular cell membrane (Fig. 1b). In addition, for the first time EFs could be shown by ultrathin serial sections to terminate at this site (Fig. 1c, d). These areas are therefore considered to represent the attachment site of EFs at the outer spindle capsule.

Since complete denervation eliminates both afferent and efferent impulses from or to IMFs (Boyd 1985), muscle spindles follow passive movements due to stretch and contraction of their muscle during locomotion. The numerical and dimensional increase of EFs and the occurrence of immature EFs within muscle spindles, as seen in the present study and in human muscle spindles of patients suffering from neurogenic muscular atrophy (unpublished observations), can thus be regarded as a more meaningful reaction following loss of contractile power before reinnervation occurs than simple fibrosis due to an increase of collagen fibrils. Oxytalan fibres are known to be formed in other areas where connective tissue is subjected to mechanical stress (Jonas and Riede 1980; Cleary and Gibson 1983; Ghadially 1988). Since the present study revealed an approximately five-fold numerical increase of EFs at the equatorial region following repeated crush lesions there would be a change in elasticity of IMFs especially at the portion that is normally covered by primary and, to a lesser extend, secondary sensory endings. The predominant increase of EFs at the equatorial region might compensate to a certain degree for the disturbance of tonic motor innervation following denervation.

It is concluded from the present series of experiments that EFs form an integral part of the muscle spindle, and that muscle spindles as dynamic mechanoreceptors maintain elastic properties even under pathological conditions. The increase of the number and the total cross-sectional area of EFs following a rather non-specific stimulus such as denervation with or without reinnervation appears to represent an obviously meaningful biological reaction, since their increase may compensate for the loss of tonic properties of denervated IMFs without causing rigidity or non-extensibility. Hampered movement would follow simple fibrosis by an increase of collagen fibrils that occurs in peripheral nerves (Salonen et al. 1987; Röyttä and Salonen 1988). But this was not seen in the muscle spindles investigated.

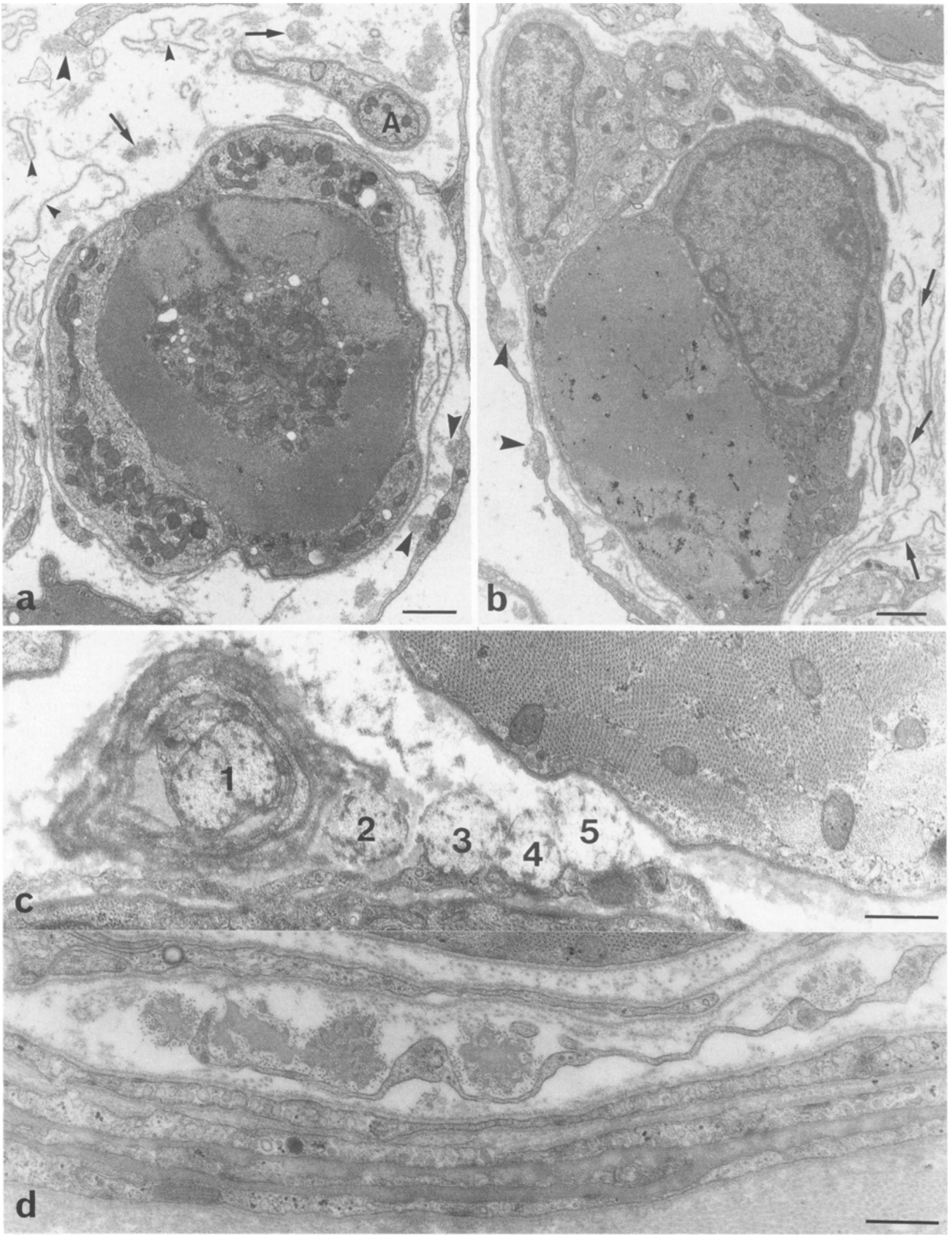


Fig. 4. a Equatorial region. An intrafusal muscle fibre 3 months after crushing the sciatic nerve is almost completely covered by a regenerated primary sensory ending. A preterminal axon (*A*) is surrounded by Schwann cell processes. EFs mainly composed of electron-dense filaments are situated either isolated within the inner axial space (*arrows*) or in close apposition to fibroblastic cell processes (*large arrowheads*). Note also remnants of basal laminae within the inner axial space (*small arrowheads*). *Scale bar:* 1 μm.

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b Juxtaequatorial region of a muscle spindle, 6 months after crushing the sciatic nerve. Close to the intrafusal muscle fibre, Schwann cell processes are associated with eight regenerated non-myelinated axons. EFs mainly composed of filaments are seen on either side of a fibroblastic cell process (*arrowheads*). Note also abundant basal lamina remnants (*arrows*). Within the intrafusal muscle fibre, there are some prominent intermyofibrillar glycogen granules. *Scale bar:* 1 μm. **c** Polar region of a muscle spindle, 12 months after a single crush lesion of the sciatic nerve. Five prominent EFs (1–5) are seen near to (2–5) or surrounded by (1) cell processes of the outer spindle capsule. EFs are mainly composed of amorphous material of uneven electron density. *Scale bar:* 0.5 μm. **d** Polar region following repeated crush lesions of the sciatic nerve at time intervals of 4 × 3 months. Here, EFs are mainly composed of filaments at the periphery or in between amorphous elastin of higher electron density than that shown in **c**. The EFs lie closely adjacent to a fibroblastic cell process that is situated between cell layers from the outer spindle capsule. *Scale bar:* 0.5 μm