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FACTORS THAT INFLUENCE REGENERATION OF THE NEUROMUSCULAR JUNCTION

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SUMMARY

Regeneration of neuromuscular junctions after trauma occurs in an orderly way and relies on communication between nerve and muscle. This paper summarizes evidence that factors which direct the growth and differentiation of both pre- and postsynaptic components of regenerating neuromuscular junctions are associated with the extracellular matrix of muscles.

INTRODUCTION

Motor axons and skeletal myofibres regenerate after injury and they form neuromuscular junctions similar to normal ones. The way in which regeneration proceeds indicates that the axons and myofibres receive cues from tissue components that persist after degeneration of original pre- and postsynaptic cells. Accordingly, regenerating axons grow through original pathways of Schwann and perineurial cells, regenerating myofibres develop within the basal lamina sheaths of original myofibres, and neuromuscular junctions are formed at the original synaptic sites of the muscle. This paper summarizes findings from a series of experiments performed in this laboratory that were aimed at determining what structures within muscle influence regeneration of the neuromuscular junction. The findings demonstrate that factors which play a role in re-establishing the junction after damage to nerve and muscle are associated with the extracellular components of the muscle and that they are stably maintained in the absence of cells.

The preparation

The experiments we describe were done on the thin, paired cutaneous pectoris muscles of the frog (*Rana pipiens*). Thus, in this section we outline salient features of these muscles and of the frog's neuromuscular junctions. The neuromuscular junction of the frog is the most well understood of all synapses, with regard to structure and function, and nowhere is the arrangement of synaptic components more simple and orderly. The layout of the neuromuscular junctions in the cutaneous pectoris muscles and the methodology available for their study has made these muscles well suited for experiments on synapse regeneration.

The cutaneous pectoris muscles (Fig. 1) lie just beneath the skin of the thorax. A nerve enters each muscle from the lateral edge and courses across it. Each muscle consists of about 500 myofibres and the entering nerve trunk contains about 25

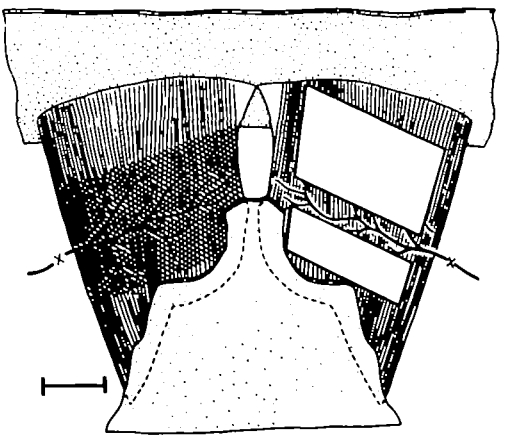

Fig. 1. The cutaneous pectoris muscles. Right muscle: slabs have been cut out leaving a narrow bridge of damaged muscle fibre segments in region of innervation. Left muscle: shaded area is the portion of the muscle that was frozen in order to kill all cells at the neuromuscular junctions. Points of nerve damage are marked by × 's. Bar, 3 mm.

myelinated axons, most of which are motor (Rotshenker & McMahan, 1976). The neuromuscular junctions are situated near the nerve trunk and its large branches, and thus they are confined to a band across the central region of the muscle (Fig. 1).

Our understanding of the structure of the frog's neuromuscular junction is based on several studies including those of Couteaux (1947), Birks, Huxley & Katz (1960*a*), Couteaux & Pécot-Dechavássine (1970), McMahan, Spitzer & Peper (1972), Dreyer *et al.* (1973), and Heuser, Reese & Landis (1974). Examples of terminals as seen in the electron microscope are shown in Figs. 2, 3 and 4. For each muscle fibre, a myelinated motor axon gives rise to several unmyelinated terminal branches (1–3 μm in diameter) that run longitudinally along the fibre surface for up to 300 μm. Each terminal branch lies in its own shallow gutter several micrometres away from its nearest neighbour. The presynaptic membrane is separated from the post-synaptic membrane at points of closest apposition by a 50 nm synaptic cleft. As in other vertebrates, the muscle fibre surface is depressed at intervals along the gutter to form junctional folds. Schwann cell processes cap the nerve terminal; in general their cell bodies are situated alongside the longer terminal branches.

Synaptic vesicles, the most conspicuous of the axon terminal's organelles, are mainly situated in the half of the terminal that faces the muscle fibre (Figs. 2–4). Clusters of vesicles are focused on bands of osmiophilic material, which lie adjacent to the presynaptic membrane, just opposite the mouths of junctional folds in the myofibre surface (e.g. Couteaux & Pécot-Dechavássine, 1970). Accordingly, these bands are distributed at regular intervals along the terminal. Combined anatomical, physiological and biochemical evidence from studies on the neuromuscular junction and other synapses leads to the conclusion that each synaptic vesicle contains acetylcholine and releases its content into the synaptic cleft by exocytosis (See Katz, 1969; Nickel & Potter, 1970; Heuser & Reese, 1973; Heuser *et al.* 1979). When nerves are stimulated, exocytosis occurs at or near the bands of dense material on the

presynaptic membrane (Couteaux & Pécot-Dechavássine, 1970; Heuser *et al.* 1974; Heuser *et al.* 1979) and thus these regions of the terminal are called 'active zones' (Couteaux & Pécot-Dechavássine, 1970).

Each skeletal muscle fibre in frogs, as in mammals, is ensheathed by several concentric layers (Uehara, Campbell & Burnstock, 1976; Sanes, Marshall & McMahan, 1978). The myofibre surface, a typical lipid-rich osmiophilic plasma membrane, is coated by a thin carbohydrate-rich glycocalyx. Separated from the glycocalyx by a narrow gap is the basal lamina (Figs. 2, 3 and 4; also called external lamina). This particulate layer is 10–15 nm thick and like the basal lamina of other tissues (e.g. Carlson *et al.* 1978) probably contains collagen-like proteins. A reticular lamina of indeterminant width and consisting of collagen fibrils lies beyond the basal lamina. The basal lamina and reticular lamina together form the myofibre's basement membrane. The term basement membrane is also used by some investigators to refer to the basal lamina alone.

The myofibre basal lamina lies in the synaptic cleft roughly equidistant from the pre- and postsynaptic membrane and sends projections into the junctional folds (Birks *et al.* 1960*a*). It is closely associated with the surface of both the nerve terminal and myofibre by thin strands of material (Figs. 3 and 4; see also Heuser & Salpeter, 1979). The basal lamina of the synaptic cleft is chemically distinctive from that covering extrasynaptic portions of the myofibre. For example, the synaptic basal lamina appears to have different antigenic characteristics (Sanes & Hall, 1979). Other differences will be described below. The Schwann cell also has a thin basal lamina sheath which apparently fuses with that of the myofibre at the edges of the junction (Birks *et al.* 1960*a*). The reticular lamina of the myofibre does not enter the cleft but is continuous with the reticular lamina of the Schwann cell. We found additional extracellular material at the neuromuscular junction when we prolonged aldehyde fixation from 30 min to 2 h and then stained whole muscles with ruthenium red, which binds to glycosaminoglycans and other acidic carbohydrates (Luft, 1966; Luft, 1971; Kanwar & Farquhar, 1979), or stained thin sections of muscles with uranyl acetate in methanol. A network of fine irregular filaments occupies the portion of the synaptic gutter not filled by the terminal and covers the Schwann cell cap (Fig. 2). This filamentous material is continuous with the basal laminae of the myofibre and Schwann cell. It varies in thickness from one portion of a terminal to the next, reaching as much as 1 μm; and some of the collagen fibrils of the reticular lamina are embedded in it. The filamentous material is also present around the short (1–5 μm) unmyelinated preterminal segments of motor axons but it is absent from the myelinated portion of motor axons and elsewhere on the myofibre surface; thus it is a special feature of the neuromuscular junction.

Acetylcholine receptors (AChRs) on skeletal muscle fibres are selectively concentrated at the neuromuscular junction (Peper & McMahan, 1972; Hartzell & Fambrough, 1972; Kuffler & Yoshikami, 1975). The density of AChRs beneath the nerve terminal is at least 500 times greater than in extrajunctional regions (Fambrough & Hartzell, 1972; Kuffler & Yoshikami, 1975; Burden, 1977; Matthews-Bellinger & Salpeter, 1978). Cholinesterase (ChE) of skeletal muscle is also concentrated at the neuromuscular junction (e.g. Couteaux, 1955; Katz & Miledi, 1973). At least some of the ChE is associated with the basal lamina of the synaptic cleft and when all cellular

components (nerve terminal, Schwann cell, and myofibres) have been removed from the synaptic site (Fig. 5*b*) ChE remains attached to the basal lamina for weeks (H[illegible] & Kelly, 1971; Betz & Sakmann, 1973; McMahan, Sanes & Marshall, 1978).

Upon cutting or crushing motor nerves, the nerve endings degenerate and then are phagocytized by adjacent Schwann cells (Birks, Katz & Miledi, 1960*b*). In the frog, both spontaneous and nerve-evoked transmitter release cease within a few days. After a 'silent' period of a few more days, miniature potentials can again be recorded from denervated muscle fibres. It is likely that they arise from ACh released by Schwann cells which, having phagocytized the terminals, are separated from the muscle surface by only the 50 nm cleft. Schwann cells gradually retract from the postsynaptic membrane over the ensuing weeks (Letinsky, Fischbeck & McMahan, 1976) but even after several months, aberrant miniature potentials can be detected (Birks *et al.* 1960*b*). In mammals, Schwann cells retract within a few days after phagocytosis of the terminal and thus Schwann potentials are seldom recorded (Miledi & Slater, 1968).

Many properties of muscle fibres in general and their plasma membrane in particular are altered by denervation and they are restored to their original state when the muscle is re-innervated (e.g. Harris, 1974). For example, the density of AChRs in extrajunctional areas is markedly increased within a few days after denervation. At the site of the junction in mammals, there is little change in the density of AChRs over the first 1–2 weeks following denervation but then it gradually declines (Frank, Gautvik & Sommerschild, 1976; Loring & Salpeter, 1980). The turnover rate of AChRs at the junction, with a half-time of about 10 days normally, decreases after denervation to about 3 days, approaching the turnover rate of extrajunctional receptors, about 1 day (Loring & Salpeter, 1980). Junctional folds become broader and shallower and ChE decreases (Guth, Albers & Brown, 1964; Hall, 1973). In the frog, even though many properties of the muscle cell are altered after denervation, specializations at the synaptic site (a relatively high concentration of AChRs and ChE and the presence of junctional folds) persist for at least two months (Miledi, 1960*a*; Letinsky *et al.* 1976).

Muscle fibres undergo marked degenerative changes when they are damaged in any of a number of ways, including mechanical (cutting or crushing), chemical, thermal, or ischemic injuries (Price, Howes & Blumbert, 1964; Benoit & Belt, 1970; Carlson, 1972; Vracko & Benditt, 1972; Hudgson & Field, 1973; Duchen *et al.* 1974; Snow, 1977). Depending upon the extent of injury, portions of or entire muscle fibres are disrupted. The disrupted cytoplasm and plasma membranes degenerate and are phagocytized by invading macrophages, but the basal lamina sheaths survive (Figs. 5, 6*d* and *e*, 7). Thus injury and its sequelae cleave the surface complex of the myofibre between the plasma membrane and basal lamina; the fate of the glycocalyx is unknown.

The role of extracellular structures in directing growth of axons to original synaptic sites on myofibres

At the site of nerve damage, the portion of the axons that remains connected to their cell bodies sprouts processes which grow into the denervated muscle. Signs of reformation of the neuromuscular junction in the frog can be detected both physiologically and microscopically 7–10 days after cutting or crushing a nerve within 1–2 mm of its muscle, and regeneration is complete by 30 days (Dennis & Miledi, 197[illegible]

Letinsky *et al.* 1976). The re-innervation of original synaptic sites on myofibres is precise; by one month nearly all of the original subsynaptic membrane of the myofibres is covered by nerve terminals and few, if any, contacts occur elsewhere on the myofibre surface (Letinsky *et al.* 1976; Sanes *et al.* 1978). Re-innervation in mammals can also take place within a few days after nerve damage (e.g. Hines, Thomson & Lazere, 1942; Guth, 1956; Dennis & Miledi, 1974) and the axons return to original endplate regions on myofibres (e.g. Tello, 1907; Gutmann & Young, 1944); however, new synaptic sites can form in certain cases (e.g. Saito & Zacks, 1969; Frank *et al.* 1975).

Perineurial cells, Schwann cells and connective tissue elements, that survive in the motor nerve long after damaged axons have degenerated, may provide cues that direct the axons, for regenerating axons often grow through these tubes (Ramón y Cajal, 1928; Holmes & Young, 1942; Gutmann & Young, 1944; Haftek & Thomas, 1968; Letinsky *et al.* 1976). However, regenerating axons can cover long stretches of synaptic surface after they leave the perineurium, lifting off the muscle fibre at the end of the synaptic sites (Letinsky *et al.* 1976). Also axons growing beyond or outside perineurial tubes can 'select' and precisely re-innervate original sites (Tello, 1907; Gutmann & Young, 1944; Letinsky *et al.* 1976). Thus there must be factors at the synaptic site itself that provide cues to regenerating axons.

One possibility is that the target cells – the myofibres – guide re-innervation of original synaptic sites (Tello, 1907; Ramón y Cajal, 1928; Fischbach, 1974). We (Marshall, Sanes & McMahan, 1977) examined the role of the myofibre in nerve regeneration by studying the precision of re-innervation of original synaptic sites in damaged cutaneous pectoris muscles.

A rectangular slab was cut from the muscles on each side of the nerve trunk leaving behind a bridge (3–4 mm long; 1–1·5 mm wide) of muscle fibre segments extending between strips of undamaged fibres at the muscle's medial and lateral edges (Fig. 1). The nerve was then crushed with fine forceps 2–3 mm from the muscle's lateral border. By 4 days after the operation, phagocytosis of myofibres was extensive and profiles of the basal lamina sheaths enclosed numerous small cells that included macrophages and myoblasts. The myoblasts are probably derived from mononucleated satellite cells which are situated at intervals along the surface of myofibres in normal muscles and remain intact in the bridge. By 2 weeks after the operation, new myofibres had formed within the basal lamina sheaths by fusion of myoblasts, and nerves had grown back to the muscle. Both electron microscopy and electrophysiology demonstrated that the myofibres became innervated. The most convenient way to identify original synaptic sites on the sheaths was to stain for ChE (Figs. 5, 6*a*; Karnovsky, 1964); over 95% of the stained patches in these preparations marked the old sites (see Marshall, Sanes & McMahan, 1977, for experimental details). When we scanned the perimeter of cross-sectioned sheaths, we found that 99% of the terminals lay within 1 μm of a ChE patch and 40% of the patches were occupied by nerve terminals (Fig. 6*b*). These results demonstrate that the presence of the original myofibre is not necessary for the expression of topographic specificity in denervated skeletal muscle; instead, factors that guide re-innervation of synaptic sites are maintained external to the muscle cell.

We have extended these studies by examining re-innervation of damaged muscle after removal of Schwann cells as well as the original muscle cells, leaving only

extracellular components of synaptic sites intact (Edgington, Kuffler & McMahan, full manuscript in preparation). Instead of cutting muscle fibres as described above we froze the region of innervation in the cutaneous pectoris muscles (Fig. 1; e.g. Vracko & Benditt, 1972; McMahan *et al.* 1980). A brass bar cooled in liquid nitrogen was repeatedly applied to the muscle for 10 min. By 10 days the portions of muscle fibres in the frozen region along with nerve terminals and their Schwann cells (as well as all cellular components of intramuscular nerve bundles) degenerated and were phagocytized but the basal lamina sheaths remained intact. Two weeks after freezing, myofibres had regenerated within the basal lamina sheaths (Fig. 5*c*) and were contacted by regenerating axon terminals that evoked muscle twitches when the nerves were stimulated.

We examined the perimeter of cross-sectioned myofibres that had been stained for ChE and found that by 3 weeks about 50% of the ChE spots were apposed by nerve terminals (Fig. 6*c*). Moreover, more than 98% of the terminals situated within 1 μm of the basal lamina were at ChE spots. To determine whether all of the ChE spots represented original synaptic sites or whether any new spots had been formed, we treated a set of muscles *in situ* with an irreversible inhibitor of ChE at the time we damaged them (Marshall, Sanes & McMahan, 1977) which eliminated all detectable ChE activity at the old sites. Three weeks later we stained the muscles for ChE, reasoning that any reaction product would represent new ChE produced subsequent to the operation. The inhibitor, diisopropyl fluorophosphate (DFP), dissolved in Ringer to a concentration of 10 mM was applied topically by injecting it beneath the frozen muscle and by placing pieces of gauze soaked in it directly on the muscle for 2 h. DFP rapidly hydrolyzes in aqueous solutions; therefore we made changes in DFP soaked gauze and injections of freshly dissolved DFP every 30 min. Electron microscopy showed that both nerve and muscle cells degenerated in the DFP treated muscles as they did in unpoisoned preparations and by 3 weeks regenerated terminals contacted myofibre basal lamina. At all sites there was some staining indicating that new ChE had been formed at the regenerated synapses. However, measurements made on electron micrographs showed that the amount of stain in all of the synaptic clefts in DFP treated preparations was less than that in the clefts of preparations run in parallel but not treated with DFP. Thus the cholinesterase spots marked original synaptic sites and the axons returned precisely to these sites in the absence of the original Schwann cells and myofibres.

In muscles damaged by freezing, nearly all of the original synaptic sites became re-innervated by 42 days. On the other hand, in muscles damaged by cutting myofibres, only about 50% of the sites were re-innervated. Perhaps sites remained uninnervated in the bridged preparations because access to them was blocked by connective tissue that built up after cutting the muscle; a similar explanation has been advanced to account for the incomplete re-innervation of atrophied mammalian muscle (Gutmann & Young, 1944).

The growth of axons and their guidance to the original synaptic sites on denervated fibres are complex processes and we do not know how many factors are involved. Our observations showing that none of the original cells (myofibres and Schwann cells) of the synaptic sites need be present for precise re-innervation of sites means that at least one factor important for guidance is located extracellularly. This factor

may have been produced by muscle cells and/or Schwann cells, or its production may have required their presence, or its expression may require the presence of one or the other cell type, but clearly it is stably maintained for a week or more in the absence of cells. Extracellular factors that might play a role in directing topographically precise re-innervation include (*a*) the reticular lamina which coats extrasynaptic portions of the myofibre basal lamina and therefore may act as a mechanical barrier to regenerating axons, (*b*) the basement membrane of the Schwann cell which is not impermeable to axons (Gutmann & Young, 1944; Letinsky *et al.* 1976) but may provide mechanical guidance and (*c*) the synaptic basal lamina of the myofibre or the extracellular material of the Schwann cell which may contain molecules that growing axons specifically recognize.

The role of the basal lamina in differentiation of motor nerve terminals

Regenerated nerve terminals contain a high density of synaptic vesicles (Saito & Zacks, 1969) and their active zones are situated opposite junctional folds of myofibres (e.g. Rotshenker & McMahan, 1976). Preterminal portions of axons and portions of terminals that grow beyond the end of the synaptic gutter (to become separated from the myofibre plasma membrane by more than 100 nm) have relatively few vesicles and no active zones (Letinsky *et al.* 1976; Rotshenker & McMahan, unpublished observations). Thus, regenerating axons, like normal axons, have synaptic specializations only at neuromuscular junctions.

We examined the factors that influence differentiation of the nerve terminal by asking whether axon terminals accumulate synaptic vesicles and form active zones when they re-innervate synaptic basal lamina in the absence of myofibres (Sanes, Marshall & McMahan, 1978). For these experiments muscles were damaged by cutting muscle fibres as described above. To inhibit mitosis of myoblasts and thus their subsequent fusion to form myofibres, the muscles were x-irradiated after damage and denervation. As in non-irradiated muscles, myofibres and axons degenerated and were phagocytized within 1 week but basal lamina sheaths remained devoid of myofibres for more than a month. Axons grew to the 'empty' basal lamina sheaths and by 3 weeks occupied more than 40% of spots marked by ChE stain (Fig. 6*d*). Moreover, more than 95% of the terminals within 0·1 μm of myofibre basal lamina were situated at stained patches.

A set of muscles were treated with DFP when they were damaged and denervated to determine whether all of the ChE spots represented original synaptic sites or whether some had been formed by regenerating axons that might have contacted non-synaptic areas of basal lamina. At the 25 terminals examined in DFP treated preparations at 3 weeks, there was little ChE staining, while in preparations not treated with DFP, staining was intense (Figs. 6*d* and *e*). Thus the ChE stain was a reliable marker for original synaptic sites in the myofibre-free preparations.

The portions of axons contacting the 'empty' sheaths differentiated, acquiring concentrations of synaptic vesicles and active zones (Fig. 7*a*, *b*). Moreover, the active zones were preferentially situated at or near intersections of synaptic cleft basal lamina and basal lamina that projected into the folds of the original myofibre as at normal neuromuscular junctions; of 32 active zones examined, 90% were within 200 nm of an intersection, which is a much higher percentage than predicted if

the active zones were randomly distributed along the 'presynaptic' membrane. There may be a number of factors in these preparations that play a role in the differentiation of the nerve terminals, including factors provided by the myoblasts and/or Schwann cells; neurones growing *in vitro* can be stimulated to differentiate by glial and muscle cells (Patterson & Chun, 1974; Giller *et al.* 1977). Nonetheless, these results indicate that morphological differentiation of the nerve terminal is directed by molecules attached to or a part of the synaptic portion of the myofibre basal lamina sheath and that these molecules are situated at intervals along this region of the sheath.

The role of the basal lamina in organizing the postsynaptic membrane of regenerating myofibres

Restoration of neuromuscular transmission after damage to both nerve and muscle implies that there is a high concentration of AChRs in the postsynaptic membrane of regenerating myofibres, and therefore the postsynaptic membrane becomes functionally differentiated. Since factors associated with the synaptic basal lamina influence the differentiation of the nerve terminal, it is only natural to wonder whether or not the basal lamina can also organize the AChRs on regenerating myofibres. We examined this problem in cut muscle preparations (Burden, Sargent & McMahan, 1979). Bridges were made as previously described and re-innervation was prevented by cutting the nerve and evulsing the central stump. By one month, regenerating myofibres occupied nearly all of the basal lamina sheaths of the original myofibres. To determine whether or not the regenerating myofibres accumulate AChRs at original synaptic sites, we labelled the AChRs with ^{125}I-α-bungarotoxin (^{125}I-α-BGT), which binds specifically to AChRs, and examined cross sections of ChE stained muscles by light microscopic autoradiography. Control experiments showed that no new histochemically detectable ChE is formed on the regenerated fibres, so the stain marks only original synaptic sites. Fig. 8 shows autoradiographic grains produced by ^{125}I-α-BGT at ChE stained patches in denervated regenerating muscle 30 days after damage; there is a dense accumulation of grains at original synaptic sites. A comparison of grain density between synaptic sites in normal muscle and original synaptic sites in damaged muscle revealed that by 1 week after damage the density had fallen to 10% of normal but that by 30 days the density had returned to nearly normal levels. Our analysis showed that the average grain density in extrasynaptic areas of basal lamina was considerably lower (30X) than at ChE spots, but it was higher than the extrasynaptic grain density in normal muscle. The high extrasynaptic grain density is not surprising since denervated but undamaged myofibres also have an increased density of extrasynaptic receptors. By defining a grain cluster as having more than ten times the average extrasynaptic grain density, we found that more than 90% of the ChE stained sites had grain clusters and almost 90% of the grain clusters were at ChE stained sites. Thus nearly all of the original synaptic sites (as marked by ChE stain) had dense accumulations of AChRs, few accumulations of AChRs of comparable size and density occurred elsewhere on myofibres, and the density of AChR accumulations at original synaptic sites was nearly the same as at normal neuromuscular junctions.

Neuromuscular junctions that regenerate in damaged muscle are also characterized by junctional folds (Fig. 6*b* and *c*; e.g. Jirmanová & Thesleff, 1976), another aspect of

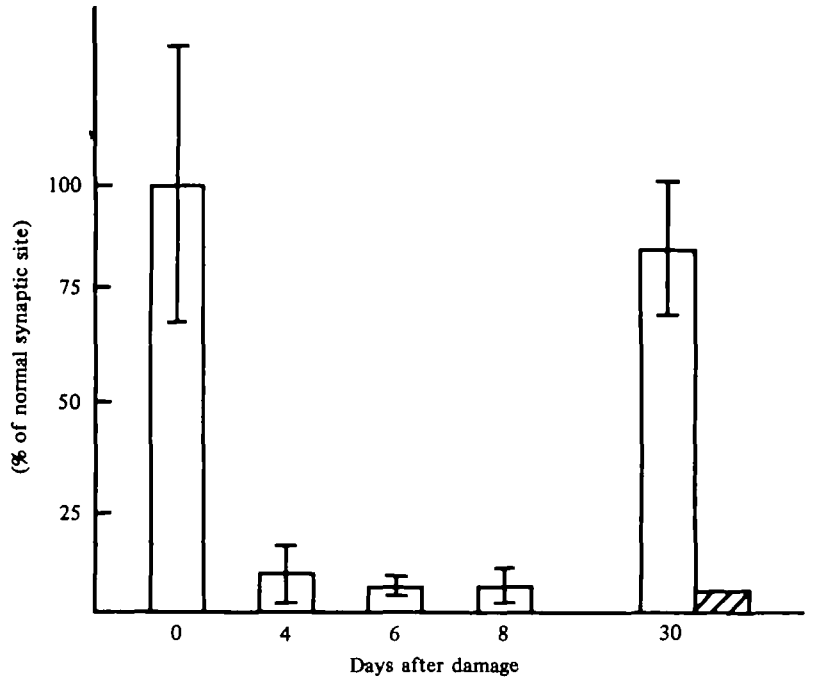

Fig. 9. The density of the AChRs at original synaptic sites during degeneration and regeneration of myofibres in the absence of Schwann cells and nerve terminals. AChR density was determined by autoradiography after incubation of muscles with ^{125}I-α-BGT. The values for experimental muscles (4, 6, 8, 30 days after damage) are expressed as a fraction of the AChR density at synaptic sites in paired, normal muscles (0 days). AChR density was measured at approximately 50 synaptic sites per muscle; error bars represent the standard error of the mean for 3–8 muscles. The lined bar at the extreme right indicates the mean extrasynaptic AChR density of regenerated myofibres at 30 days.

postsynaptic differentiation. To determine whether or not factors that remain at synaptic sites in the absence of nerve terminals direct the formation of the folds as well as the accumulation of AChRs, we treated non-innervated regenerating myofibres with horseradish peroxidase-α-bungarotoxin and stained for horseradish peroxidase (Burden, Sargent & McMahan, 1979). Electron microscopic examination revealed that there were numerous folds at the stained receptor patches and none elsewhere on the surface of the myofibres. Thus the presence of nerve terminals is not required for the formation of junctional folds at original synaptic sites in regenerating muscles.

Since Schwann cells remain at the synaptic sites in the bridge preparation and Schwann cells are thought to release acetylcholine after they phagocytize nerve terminals, it seemed plausible that the organization of the postsynaptic membrane in the absence of nerve terminals could be directed by diffusible factors released from the Schwann cells. We tested this possibility by examining the organization of acetylcholine receptors and the formation of folds in preparations where the muscles were damaged by freezing; thus, myofibres regenerated in the absence of both nerve terminals and Schwann cells (McMahan *et al.* 1980; Burden, Sargent & McMahan, in preparation). A comparison of grain densities at ChE stained synaptic sites in normal and damaged muscles showed that in the absence of Schwann cells, as in their presence, the concentration of AChRs at original synaptic sites fell to 10% within

10 days after freezing and returned to near normal levels (80%) by one month when myofibres had regenerated (Fig. 9). Junctional folds were also selectively localized to the ChE stained sites. Since the only structure that remained at the synaptic site in this experiment was the basal lamina of the myofibre, factors that direct the organization of the subsynaptic apparatus must be associated with the basal lamina.

Concluding remarks

The experiments described in this review demonstrate that factors which influence the direction of axonal growth and the differentiation of synaptic specializations in regenerating nerve and muscle are associated with the extracellular matrix of the muscle. At least some of these factors are a part of or connected to the synaptic portion of the myofibre's basal lamina sheath. Knowledge of where the factors that influence regeneration of neuromuscular junctions are situated is a step towards characterizing them and determining how they are regulated.

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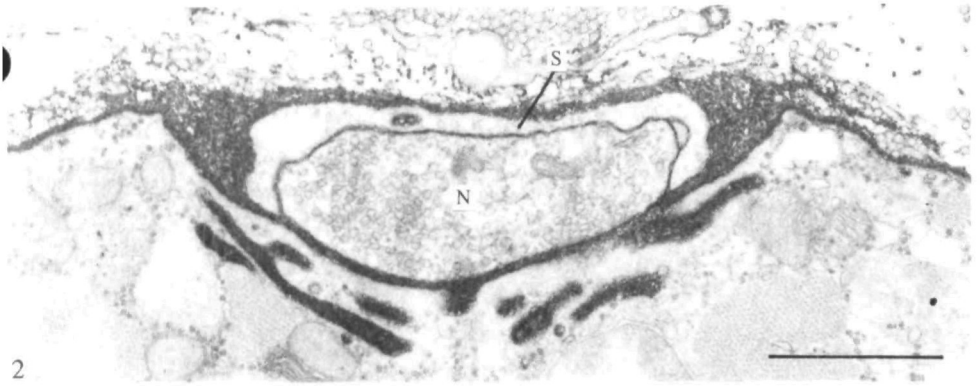

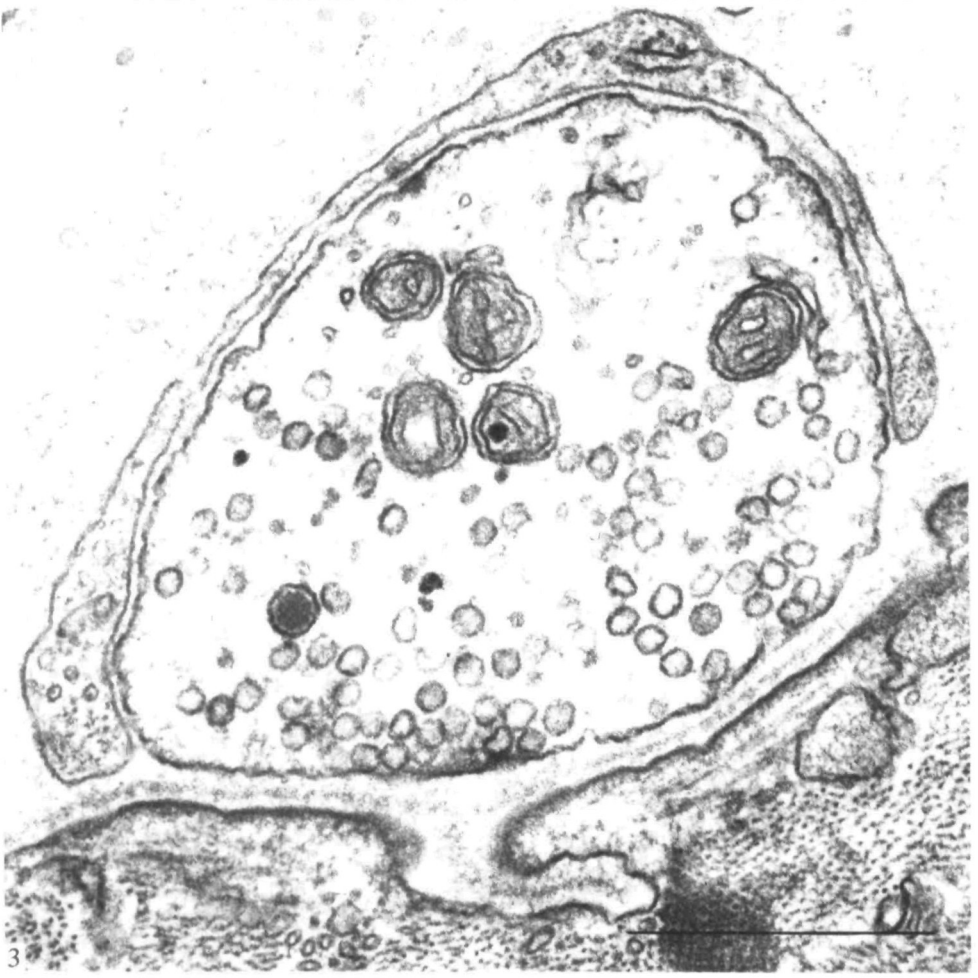

Fig. 2. Each motor nerve terminal (N) in skeletal muscles of the frog is capped by Schwann cell processes (S) and is situated in a gutter in the myofibre surface. Filamentous material fills the portion of the synaptic gutter not occupied by the nerve terminal. The preparation was stained with ruthenium red in osmium tetroxide. Bar, 1 μm.

Fig. 3. Most synaptic vesicles are in the half of the nerve terminal that faces the myofibre. They are focused on a narrow band of electron dense material (seen here in cross-section) that lines the presynaptic membrane and defines one of the terminal's active zones. The active zone lies directly opposite a junctional fold in the myofibre surface. The basal lamina of the myofibre bisects the synaptic cleft and sends a projection into the fold. The Schwann cell has a thinner and less distinct basal lamina than the myofibre. Bar, 0·5 μm.

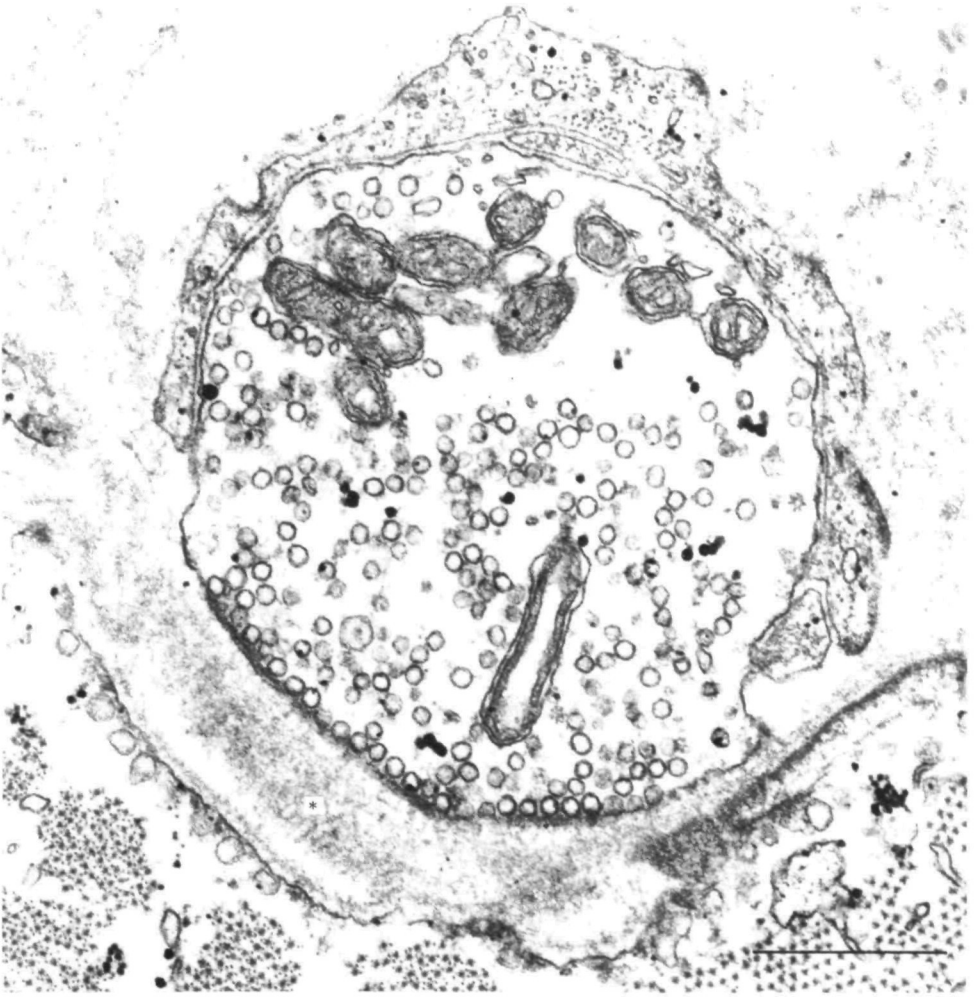

Fig. 4. The active zone in this nerve terminal profile is sectioned longitudinally. The section also passes longitudinally through a junctional fold and the sheet of basal lamina that projects into it. Bar, 0·5 μm.

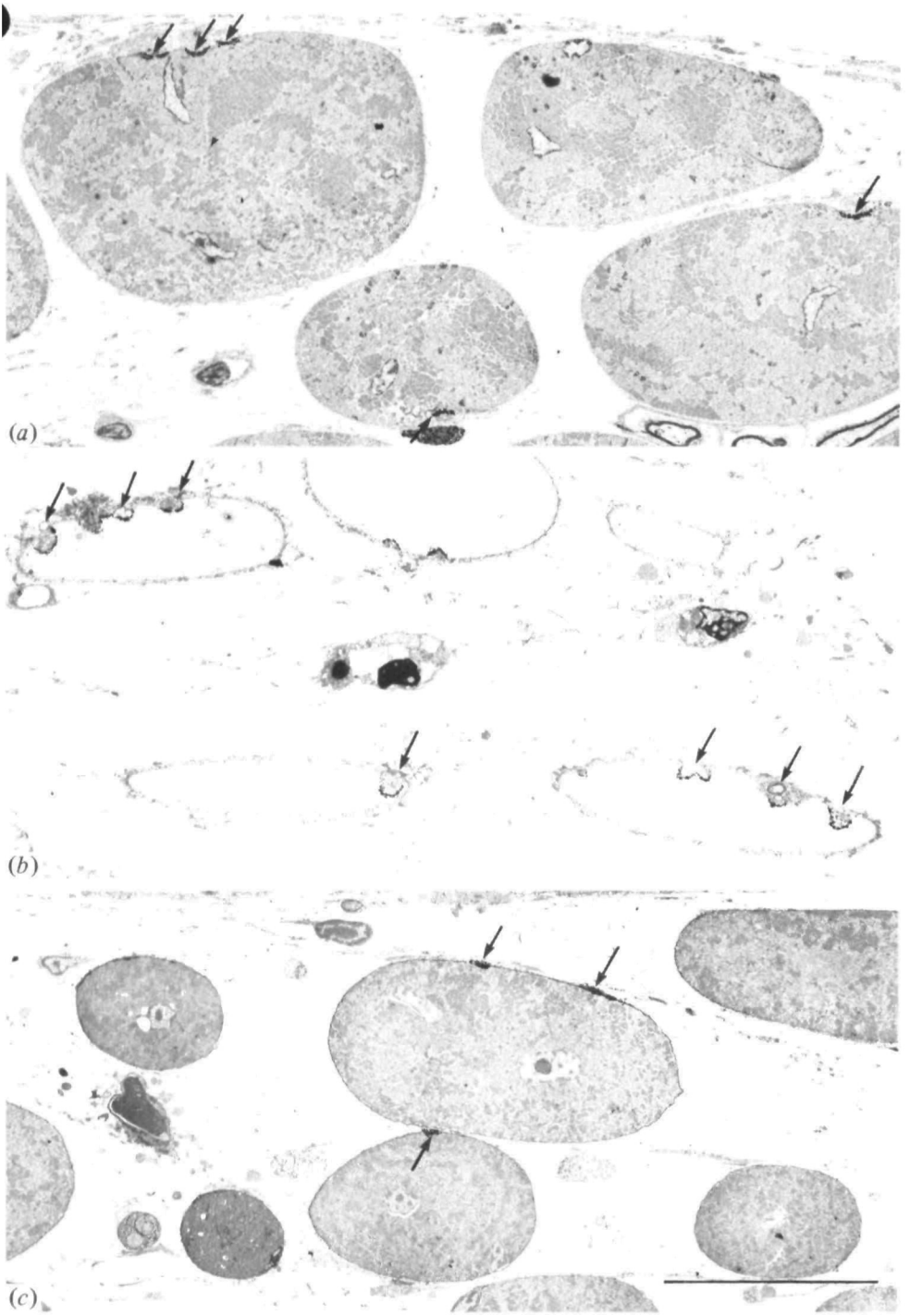

Fig. 5. When myofibres (*a*) are damaged they degenerate and are phagocytized but extracellular sheaths remain intact (*b*). Regenerating myofibres (*c*) form within the basal lamina sheaths of the original myofibres. Muscles shown in *b* and *c* had been damaged by freezing. ChE stain (arrows) marks synaptic sites on the myofibre basal lamina sheaths. Bar, 30 μm.

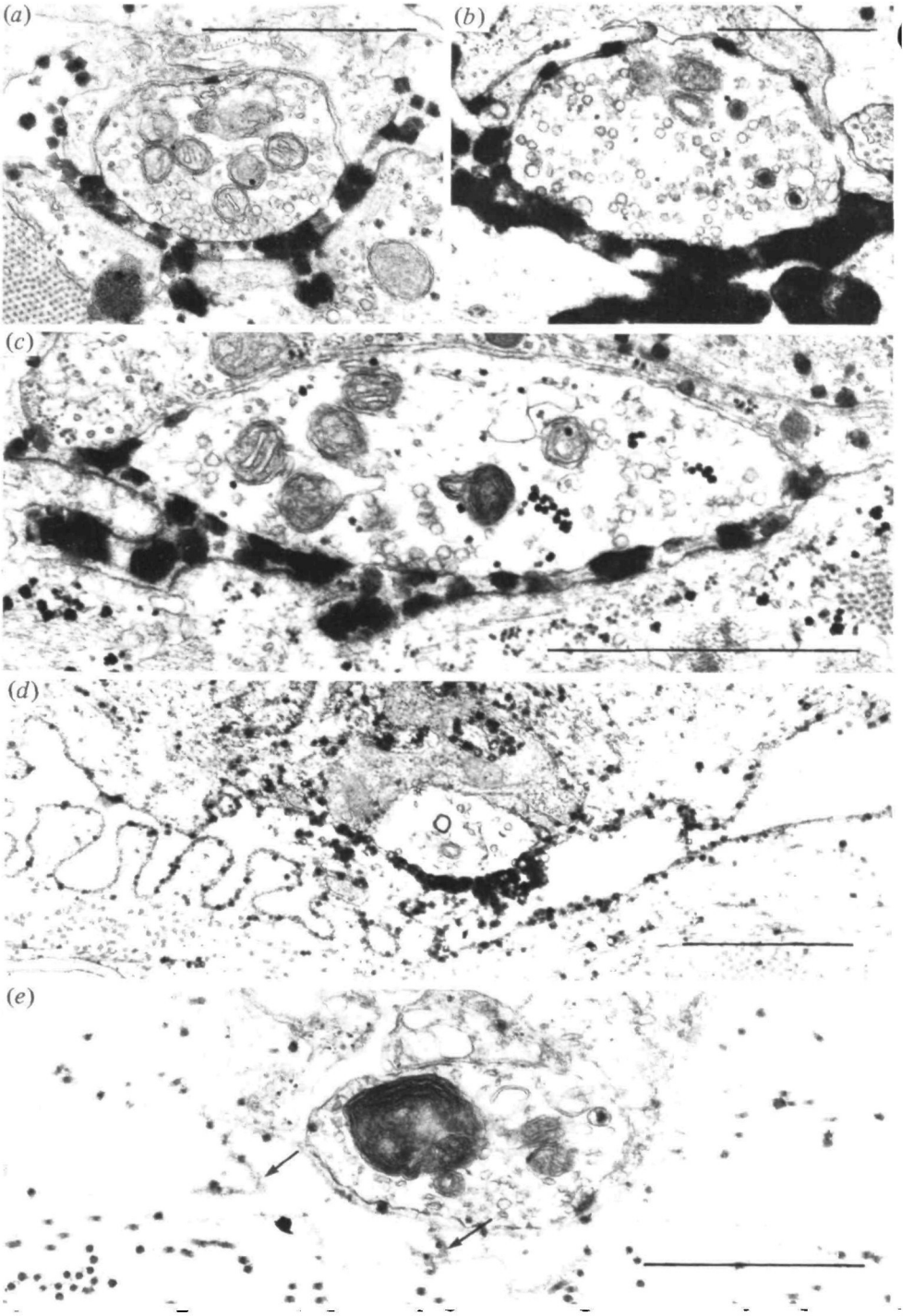

Fig. 6. As in normal muscles (*a*), nerve terminals that re-innervate damaged muscles (*b–d*) are situated at sites that stain for ChE. Myofibres in *b* (from a bridge preparation) and *c* (from a frozen preparation) have regenerated. The myofibres of *d* and *e* were damaged by making a bridge and regeneration was prevented by x-irradiation; thus the terminals have innervated collapsed basal lamina sheaths of the myofibres. In *e*, the muscle had been treated with DFP at the time of damage and shows little ChE staining, demonstrating that ChE marks the original synaptic site in *d*. The basal lamina that projected into the original junctional folds (arrows) is apparent in *e*. Bars, 1 μm.

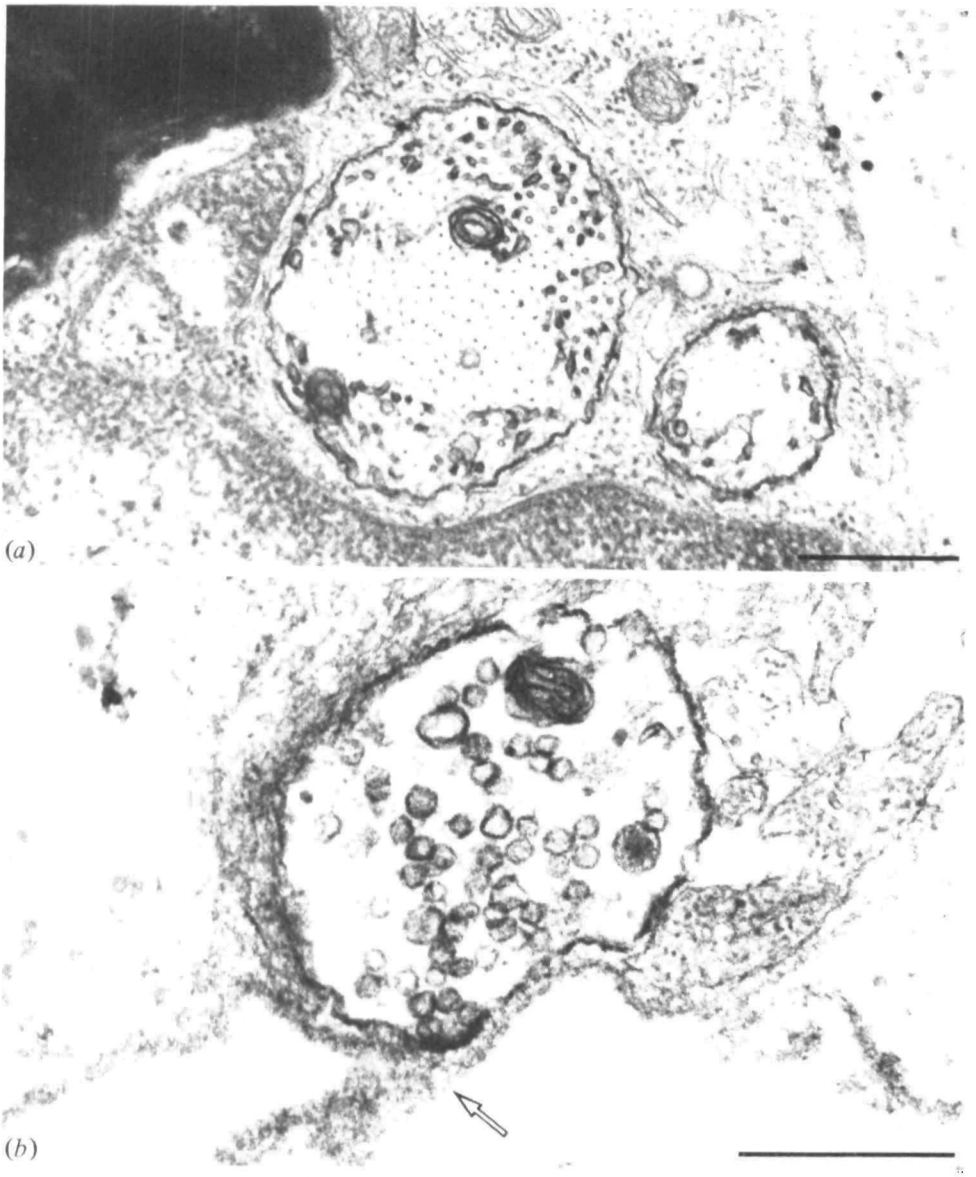

Fig. 7. Preterminal and terminal portions of axons re-innervating an irradiated bridge 21 days after muscle was damaged and denervated; by morphological criteria, terminals have differentiated. (*a*) Axons in the nerve trunk, wrapped by a Schwann cell, contain many neurofilaments and microtubules but few synaptic vesicles. (*b*) Nerve terminal, apposed to an empty BL sheath of a myofibre, contains numerous vesicles, some of which are focused on an active zone that lies opposite an intersection (arrow) of synaptic cleft and junctional fold BL. Bar, 0·5 μm.

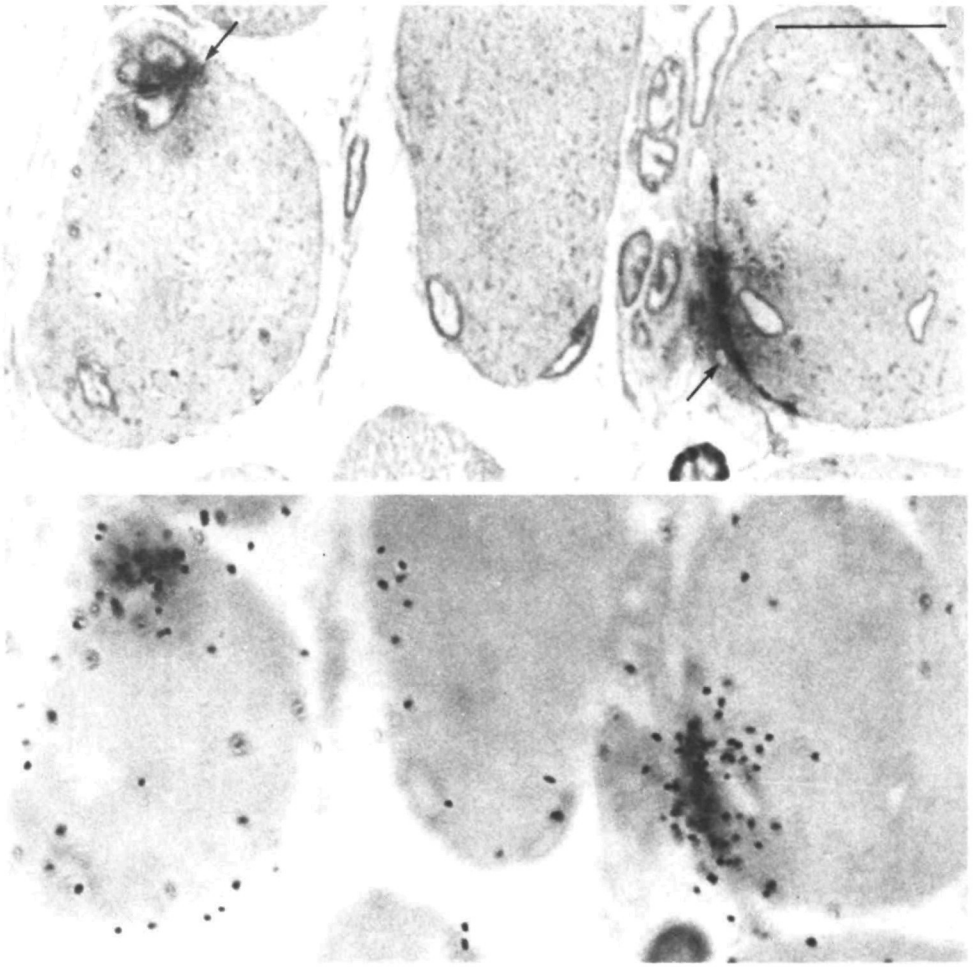

Fig. 8. Regenerating myofibres accumulate AChRs at original synaptic sites in the absence of nerve. Top: original synaptic sites marked by ChE stain (arrows). Bottom: autoradiographic grains at same sites produced by ^{125}I-α-BGT bound to AChRs. 30 days after muscle damage. Bar, 10 μm.