

ACETYLCHOLINE RECEPTORS IN REGENERATING MUSCLE ACCUMULATE AT ORIGINAL SYNAPTIC SITES IN THE ABSENCE OF THE NERVE

STEVEN J. BURDEN, PETER B. SARGENT, and U. J. McMAHAN

From the Department of Neurobiology, Stanford University School of Medicine, Stanford, California 94305

ABSTRACT

We examined the role of nerve terminals in organizing acetylcholine receptors on regenerating skeletal muscle fibers. When muscle fibers are damaged, they degenerate and are phagocytized, but their basal lamina sheaths survive. New myofibers form within the original basal lamina sheaths, and they become innervated precisely at the original synaptic sites on the sheaths. After denervating and damaging muscle, we allowed myofibers to regenerate but deliberately prevented reinnervation. The distribution of acetylcholine receptors on regenerating myofibers was determined by histological methods, using [¹²⁵I]α-bungarotoxin or horseradish peroxidase-α-bungarotoxin; original synaptic sites on the basal lamina sheaths were marked by cholinesterase stain. By one month after damage to the muscle, the new myofibers have accumulations of acetylcholine receptors that are selectively localized to the original synaptic sites. The density of the receptors at these sites is the same as at normal neuromuscular junctions. Folds in the myofiber surface resembling junctional folds at normal neuromuscular junctions also occur at original synaptic sites in the absence of nerve terminals. Our results demonstrate that the biochemical and structural organization of the subsynaptic membrane in regenerating muscle is directed by structures that remain at synaptic sites after removal of the nerve.

KEY WORDS neuromuscular junctions · basal lamina · Schwann cell · junctional folds · α-bungarotoxin

Acetylcholine receptors (AChRs) on skeletal muscle fibers are selectively concentrated at the neuromuscular junction. The density of receptors in the portion of myofiber plasma membrane beneath the nerve terminal is 1,000–5,000 times greater than in extrajunctional regions (10, 16, 23). During the development of myofibers, this subsynaptic specialization arises as a consequence of interaction between nerve and muscle (2, 3, 19).

In the adult, the accumulation of receptors is stable. After denervation, the density of receptors increases in extrasynaptic areas of myofiber membrane, but the concentration at synaptic sites remains unchanged for weeks (4, 20, 31).

This paper concerns the formation of AChR accumulations at synaptic sites in regenerating adult muscle. After damage to nerve and muscle, both axons and myofibers degenerate and are phagocytized, but the Schwann cells that capped the nerve terminals and the basal lamina sheaths of the myofibers survive (e.g., 9, 11, 21, 27, 35, 38). Elements that remain in the muscle influence the

regeneration of axons and muscle cells. New myofibers develop within the basal lamina sheaths of the original myofibers (7, 11, 21, 41), axons grow to the original synaptic sites on the basal lamina, and functional synapses are formed (27). Even when regeneration of the myofibers is prevented, axons still grow to the original synaptic sites and factors associated with the basal lamina sheath direct differentiation of axon terminals (37).

The experiments presented here were designed to determine whether structures in muscle other than nerve terminals influence the organization of AChRs at synaptic sites on regenerating myofibers. After denervating and damaging muscle, we allowed new myofibers to form within the basal lamina sheaths of the original muscle cells, but we deliberately prevented reinnervation. We found that AChRs in the plasma membrane of regenerating myofibers accumulate preferentially at the original synaptic sites on the basal lamina even though the nerve is absent. The density of receptors at the original synaptic sites is nearly the same as at normal neuromuscular junctions. Furthermore the plasma membrane at sites of receptor accumulation is periodically invaginated; these invaginations resemble junctional folds in the subsynaptic membrane of normal muscle. We conclude that in regenerating muscle the nerve is not necessary for the accumulation of AChRs or for the appearance of folds at the synaptic sites; the formation of these specializations can be directed by elements associated with the synaptic basal lamina and/or by Schwann cells.

MATERIALS AND METHODS

Experiments were performed on the cutaneous pectoris muscle of 5-cm long male frogs (*Rana pipiens*). The frogs were kept at room temperature and were fed crickets. The muscles were dissected in Ringer solution (116 mM NaCl, 1.8 mM CaCl₂, 2.0 mM KCl, 0.17% dextrose, 1.0 mM NaH₂PO₄, pH 7.2).

Operations

Animals were anesthetized in 0.1% tricaine methanesulfonate (Finquel, Ayerst Laboratories, N. Y.) and muscles were damaged as described previously (27). As illustrated diagrammatically in Fig. 1, a slab of muscle on each side of the central region of innervation was removed. A bridge of damaged muscle segments (1–2 mm) remained between intact myofibers at the medial and lateral borders of the muscle. The nerve innervating the cutaneous pectoris muscle was cut at the edge of the muscle and ~5 mm of the central nerve stump was removed. 2 wk later, a 1-cm length of the second spinal

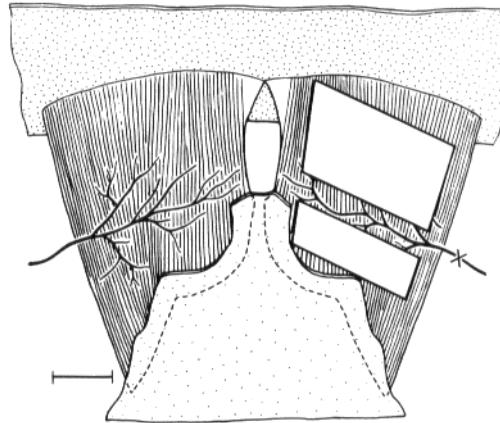


FIGURE 1 Normal and denervated, damaged cutaneous pectoris muscles. The left muscle and its nerve are intact. Slabs have been removed from the right muscle; a bridge of damaged myofiber segments extends between intact myofibers at the muscle's medial and lateral borders. The nerve has been severed (X) near the lateral border. Bar, 3 mm.

nerve was removed near the vertebral column. These procedures resulted in degeneration of the damaged myofiber segments and prevented reinnervation of the muscle for more than a month.

Localization of Acetylcholine Receptors by Horseradish Peroxidase Coupled- α -Bungarotoxin

Acetylcholine receptors (AChRs) were labeled with α -bungarotoxin (α -BGT), a protein that binds strongly and specifically to AChRs of skeletal muscle (8, 12, 32). Horseradish peroxidase (HRP; code HPOFF, Worthington Biochemical Corp., Freehold, N. J.) was coupled to α -bungarotoxin (Boehringer-Mannheim, Indianapolis, Id.) according to the glutaraldehyde procedure of Vogel et al. (40). HRP- α -BGT was separated from free α -BGT on a G-100 column but was not purified further by ion-exchange chromatography as described in the original procedure (40). A few thousand cps of [¹²⁵I] α -BGT were included in the reaction mixture to determine the toxin concentration after coupling. Since the stoichiometry of the conjugate is 1 α -BGT:1 HRP (40), the concentration of the conjugate is equal to the concentration of toxin. AChRs were labeled by incubating muscles with 10⁻⁷ M HRP- α -BGT in Ringer's for 1 h at room temperature. Under these conditions muscles no longer contracted upon electrical stimulation of the nerve, and thus >80% of the AChRs were labeled (1). The muscle was washed in several changes of Ringer's for 10 min, fixed in 1% glutaraldehyde (in 60 mM sodium phosphate, pH 7.0) for 20 min, washed (in 90 mM sodium phosphate, pH 7.3, 24 mM sucrose) for 5 min and then incubated for 2 h at room temperature in 0.05% 3,3' diaminoben-

zidine, 0.02% H₂O₂ in phosphate buffer with sucrose. For light microscopy the muscle was dehydrated, cleared in xylene, mounted whole in Permount (Fisher Scientific Company, Fair Lawn, N. J.) and viewed with bright-field optics. For electron microscopy the muscle was treated with OsO₄ (1% in 90 mM sodium phosphate, pH 7.0), stained en bloc with uranyl acetate (0.5% in 50 mM sodium maleate, pH 5.2) and embedded in Epon. Thin sections were stained with lead citrate (39).

Localization of Acetylcholine Receptors by [¹²⁵I]α-BGT

Muscles were incubated in 4×10^{-8} M [¹²⁵I]α-BGT (New England Nuclear, Boston, Mass.; ~100 Ci/mmol) in Ringer's for 1 h at room temperature, washed in Ringer's for 1 h and fixed in 1% glutaraldehyde (in 60 mM sodium phosphate, pH 7.0) for 20 min. They were then stained for cholinesterase (ChE; reference 22) for 15 min to mark synaptic sites, treated with OsO₄ (1% in 90 mM sodium phosphate, pH 7.0), dehydrated and embedded in Epon. Transverse sections (1.0 μm thick) were mounted on microscope slides that had been acid-cleaned and coated with 0.5% gelatin. The slides were then coated with liquid emulsion (Kodak NTB-2 or NTB-3, Rochester, N. Y.) diluted 1:1 with 2% glycerol. The emulsion was cooled to 4°C (5 min), allowed to dry for 1 h at room temperature and exposed for several days at 4°C. The emulsion was developed in Kodak D-19 (undiluted) for 2.5 min at 20°C and fixed in Kodak Rapid Fixer for 1 min. Sections were stained with 1.0% toluidine blue (in 1% sodium borate), and coverslips were mounted with glycerol. Camera lucida drawings were made of the sections; the perimeter of the muscle fibers, the extent of the ChE-stained patches and the position of the autoradiographic grains were indicated. The background grain density was determined over areas of the section that did not contain tissue.

In normal muscle the extrasynaptic grain density was indistinguishable from background, and the grains were concentrated over synaptic sites (marked by ChE stain). We found that 50% of the grains at synaptic sites fell within 3 μm of the ChE-stained patches. The grain density at each synaptic site was determined by first counting the number of grains falling within a window whose border was 3 μm from the stained area and by subsequently dividing the number of grains by the length (in μm) of the ChE patch. In regenerated muscle the extrasynaptic grain density was greater than background, but grains were again concentrated over synaptic sites that were still marked by ChE stain. Extrasynaptic grain density, which is expressed as grains/μm length of membrane, was determined by counting the number of grains falling within 3 μm of the part of the myofiber perimeter that did not stain for ChE. To quantitate the extent to which AChRs were concentrated at synaptic sites on regenerated muscle, we determined the fraction of ChE-stained patches that had clusters of grains. A cluster of

grains at a ChE-stained patch was arbitrarily defined as a group of grains with a density at least ten-fold greater than the average extrasynaptic grain density. To determine whether AChRs were concentrated in extrasynaptic regions of the myofiber surface that did not stain for ChE, we counted the grains falling within a window (10.2 × 6 μm) that circumscribed the mean ChE length (4.2 μm) in cross sections of regenerated muscle. The long axis of the window was centered on the perimeter of the myofiber, and the window was moved around the myofiber surface. For the grain density to be ten or more times the mean extrasynaptic density, it was necessary in our experiments that at least four grains occur within the window.

Cholinesterase Stain Marks the Original Synaptic Sites in Regenerated Muscle

We demonstrated that ChE stain labels only original synaptic sites in regenerated muscle by showing that no new histochemically detectable ChE appears during muscle regeneration. 1 wk after making a bridge, animals were anesthetized and the original ChE was inhibited irreversibly with diisopropyl-fluorophosphate (DFP) by covering the muscles for 30 min with gauze soaked in 10 mM DFP (Aldrich Chemical Corp., Milwaukee, Wisc.) in Ringer's. Original myofibers have been phagocytized by 1 wk after damage, and the incubations were done at this time rather than at the time of the initial operation so that DFP would have better access to ChE in the basal lamina. It has been shown previously that no new detectable ChE appears during the first week after making a bridge (27) and that treatment of muscles with DFP does not inhibit synthesis of ChE (17). One month after making the bridge, the muscles were stained for ChE and were subsequently treated like muscles prepared for autoradiography. No ChE stain was observed in 1-μm sections taken from the vicinity of degenerated nerve bundles. Between 30 and 100 ChE-stained sites were observed in sections taken from comparable areas of bridges not treated with DFP. Therefore, the ChE-stained patches in non-DFP treated muscles represent original ChE and mark original synaptic sites on the basal lamina.

RESULTS

Removal of Acetylcholine Receptors After Degeneration of Damaged Muscle

The removal of myofibers and axons after surgical damage to the cutaneous pectoris muscle and its nerve has been described in detail elsewhere (37). As illustrated in Fig. 2, axons and myofiber segments degenerate completely and are phagocytized by macrophages during the first week after making a bridge of muscle fiber segments and

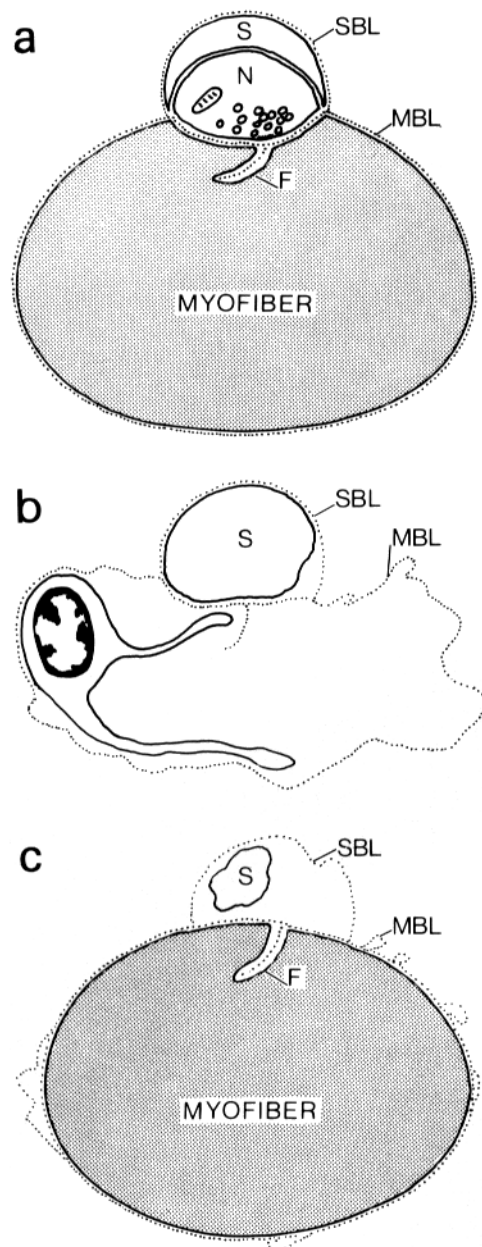


FIGURE 2 Steps in degeneration and regeneration of myofibers in denervated bridges. (a) Cross-section of a normal muscle fiber in its endplate region. N, nerve terminal; F, junctional fold; S, Schwann cell; MBL, myofiber basal lamina; SBL, Schwann cell basal lamina. (b) 7 d after making a bridge and damaging the nerve. The myofiber segment has degenerated and has been phagocytized. The basal lamina sheath remains intact and contains a mononucleated cell. A Schwann cell process occupies the position of the nerve terminal on the presynaptic side of the myofiber basal lamina. (c) 2

severing the nerve (see Materials and Methods). The basal lamina sheaths of the myofibers survive and contain macrophages and myoblasts. Fragments of myofiber membrane are attached to the basal lamina, but they occupy only a small fraction of its surface area. At the synaptic site, basal lamina that projected into the junctional folds persists and is continuous with basal lamina of the synaptic cleft. The axon terminals are phagocytized by Schwann cells, which then occupy the position of the terminal on the synaptic portion of the myofiber basal lamina. Eventually, the Schwann cell processes retract from much of the synaptic basal lamina (26, 33, 34, 37). The basal lamina of the Schwann cell also remains intact and, as in normal muscle, it joins with the basal lamina of the myofiber at the edge of the synaptic site. Thus, by 7 d after damaging nerve and muscle, the principal structures at the synaptic site are the Schwann cell, its basal lamina, and the basal lamina of the myofiber.

AChRs are removed from the synaptic sites along with other components of the postsynaptic membrane. We labeled AChRs with HRP- α -BGT to examine electron microscopically the fate of the receptors on damaged myofibers: HRP produces an electron-dense stain that is readily seen at sites of high receptor density. The HRP- α -BGT stain on normal myofibers is confined to the neuromuscular junction (Fig. 3a). As observed by Lentz et al. (25), stain fills the synaptic cleft (Fig. 3b) and thus is distributed across the synaptic basal lamina. A dense band of stain lines the external surface of the subsynaptic membrane at the top and upper sides of the junctional folds (Fig. 3b), which are the sites of highest receptor concentration (28). 1 wk after damaging and denervating the cutaneous pectoris muscle, synaptic sites no longer displayed the intensity and distribution of HRP- α -BGT staining that is characteristic of normal muscles. At all 35 synaptic sites examined the HRP stain was either absent or limited to the vicinity of small myofiber remnants near the basal lamina (Fig. 3c and d).

We examined further the removal of AChRs by light microscopic autoradiography after labeling receptors with [125 I] α -BGT and marking the original synaptic sites with ChE stain. In normal mus-

1 wk after making a bridge. A new myofiber has formed within the basal lamina sheath. At the original synaptic site the new myofiber has a fold similar to junctional folds in normal muscle. Not drawn to scale.

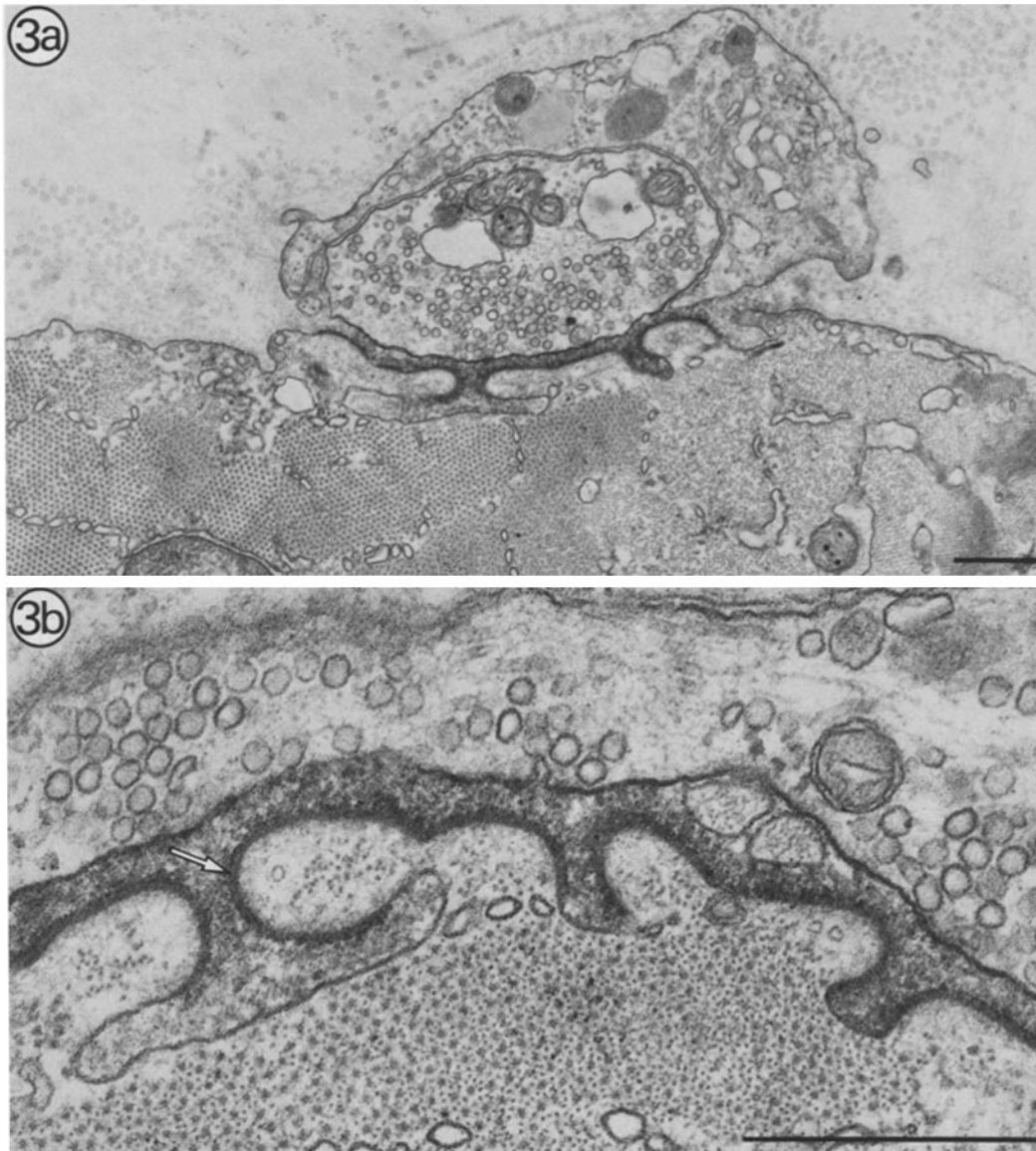
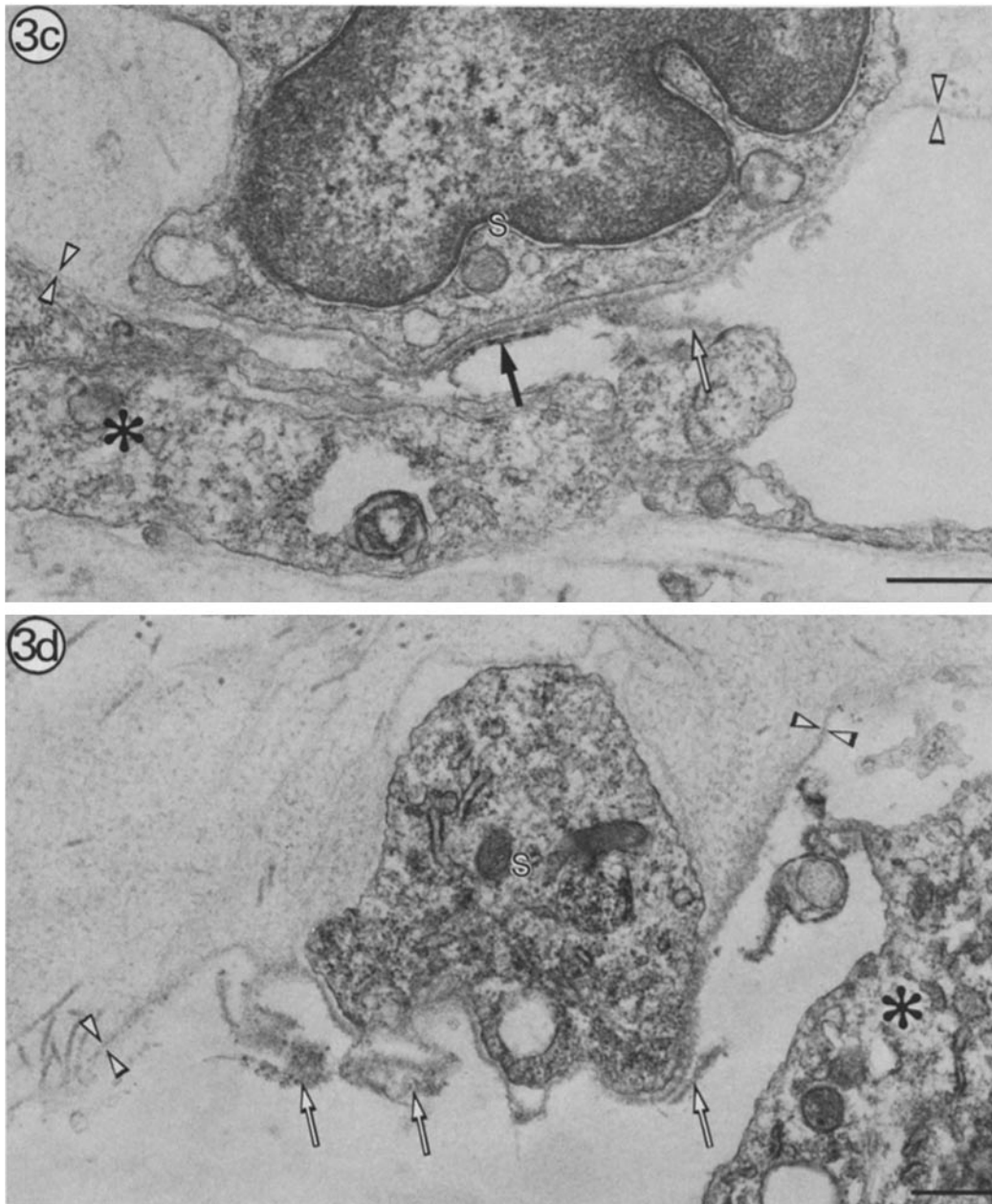


FIGURE 3 HRP- α -BGT staining at synaptic sites is markedly reduced within one week after denervating and damaging the muscle. (*a and b*) Normal neuromuscular junctions. (*c and d*) Synaptic sites 7 d after making a bridge and severing the nerve. (*a*) Stain is confined to the region of the neuromuscular junction and fills the synaptic cleft. (*b*) Dense band of stain (arrow) lines the postsynaptic membrane at the tops and part way down the sides of folds. (*c*) Stain is confined to a small myofiber membrane fragment (solid arrow). (*d*) Synaptic site without stain. Synaptic sites in damaged preparations are identified by Schwann cells (*S*) and basal lamina (open arrows) that projected into junctional folds of original myofiber. Unidentified cells (asterisks; macrophages or myoblasts) are within the myofiber basal lamina sheaths (outlined by arrow heads). Bars, (*a and b*), 0.5 μ m; (*c and d*), 1.0 μ m.

cles, ChE stain is demonstrable at all terminal branches of the neuromuscular junction and it is not seen elsewhere on the muscle fiber surface (13,

30). ChE remains associated with the basal lamina after damaging the muscle, and thus ChE stain enables one to identify the original synaptic sites



on the sheaths even in the absence of myofibers (29). Fig. 4 shows autoradiographic grains produced by $[^{125}\text{I}]\alpha\text{-BGT}$ at ChE-stained patches in cross sections of normal and damaged muscle. Many grains were at ChE-stained patches in normal muscle, but few grains were at ChE-stained

patches in damaged muscle. We measured the density of grains at ChE-stained patches (see Materials and Methods) and found that by 4 d after injury the mean density of receptors at synaptic sites was 10% of normal (Fig. 5a). As shown in the upper histogram of Fig. 5b, the distribution of

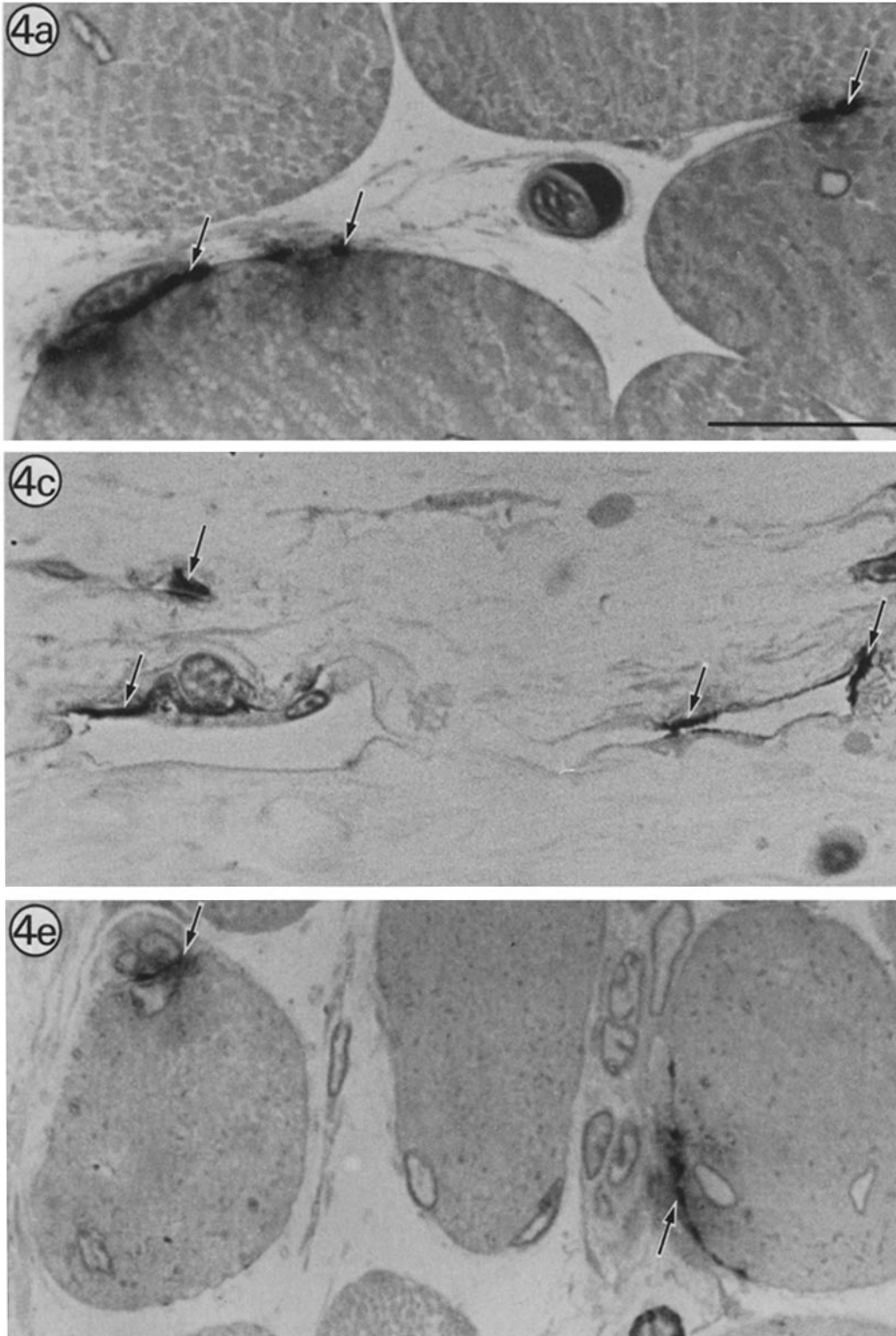
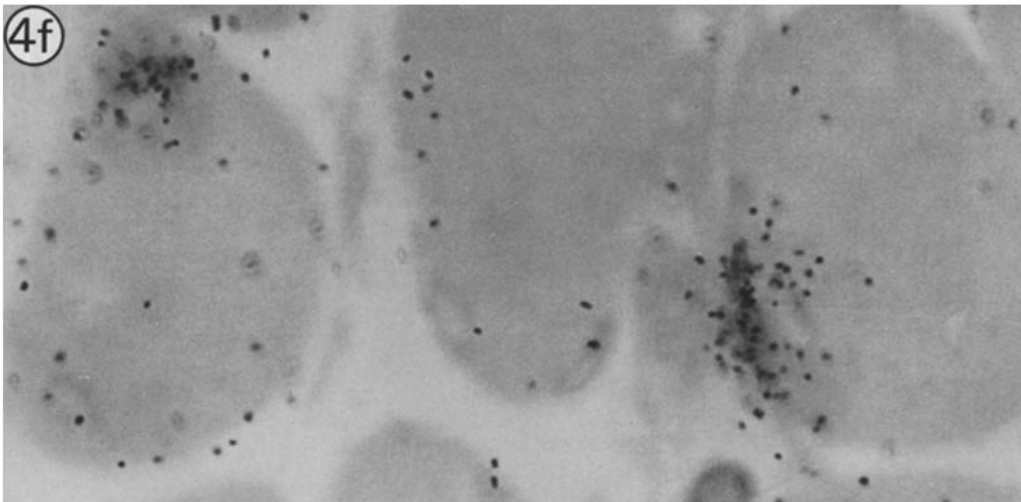
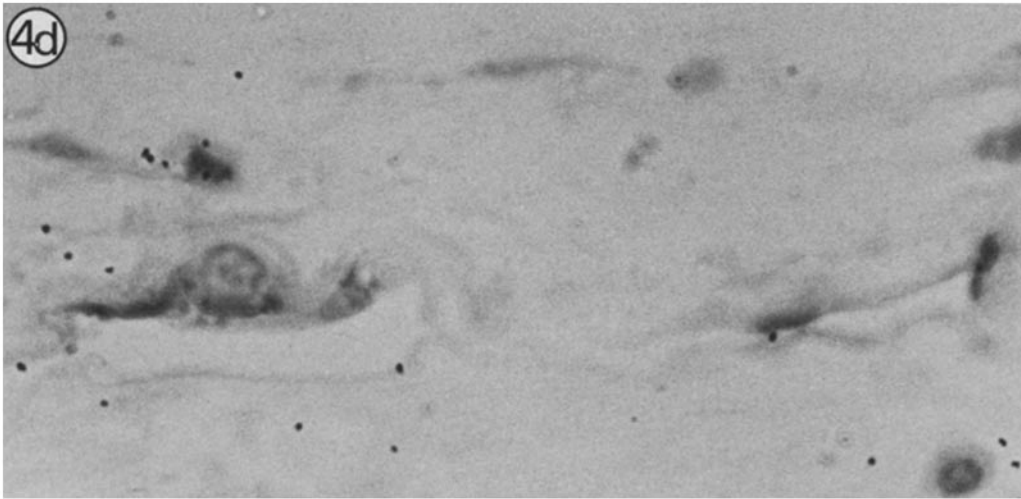
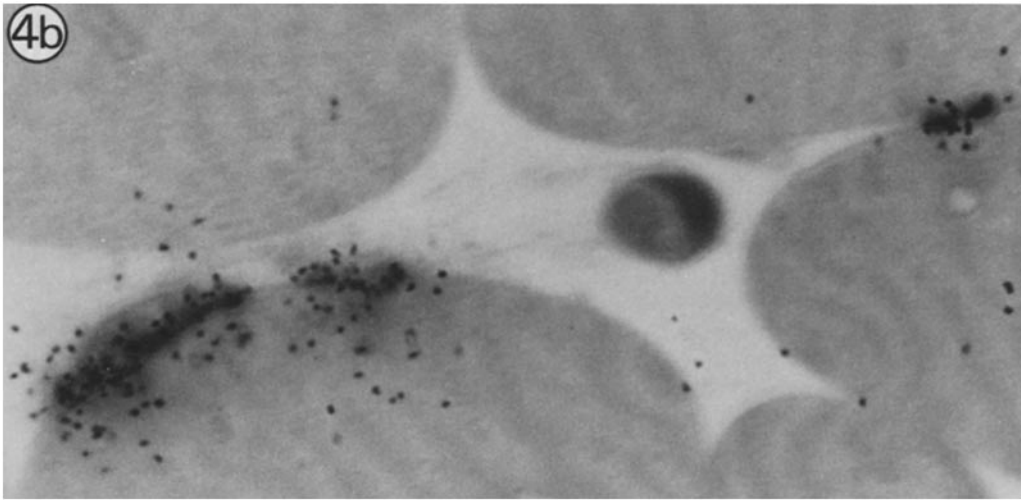


FIGURE 4 $[^{125}\text{I}]\alpha\text{-BGT}$ binding at synaptic sites decreases upon denervating and damaging the muscle and increases after myofiber regeneration. (*a and b*) Normal muscle. (*c and d*) Vacated basal lamina sheaths 4 d after damage. (*e and f*) Denervated, regenerated myofibers 30 d after damage. Synaptic sites are marked by ChE stain (arrows in *a, c, and e*; myofibers in focus), and $[^{125}\text{I}]\alpha\text{-BGT}$ binding sites on the same cross sections are marked by autoradiographic grains (*b, d, and f*; emulsion in focus). The specific activity of $[^{125}\text{I}]\alpha\text{-BGT}$ was ~ 100 Ci/mmol and the emulsion was exposed for 4 d. Bar, 10 μm .



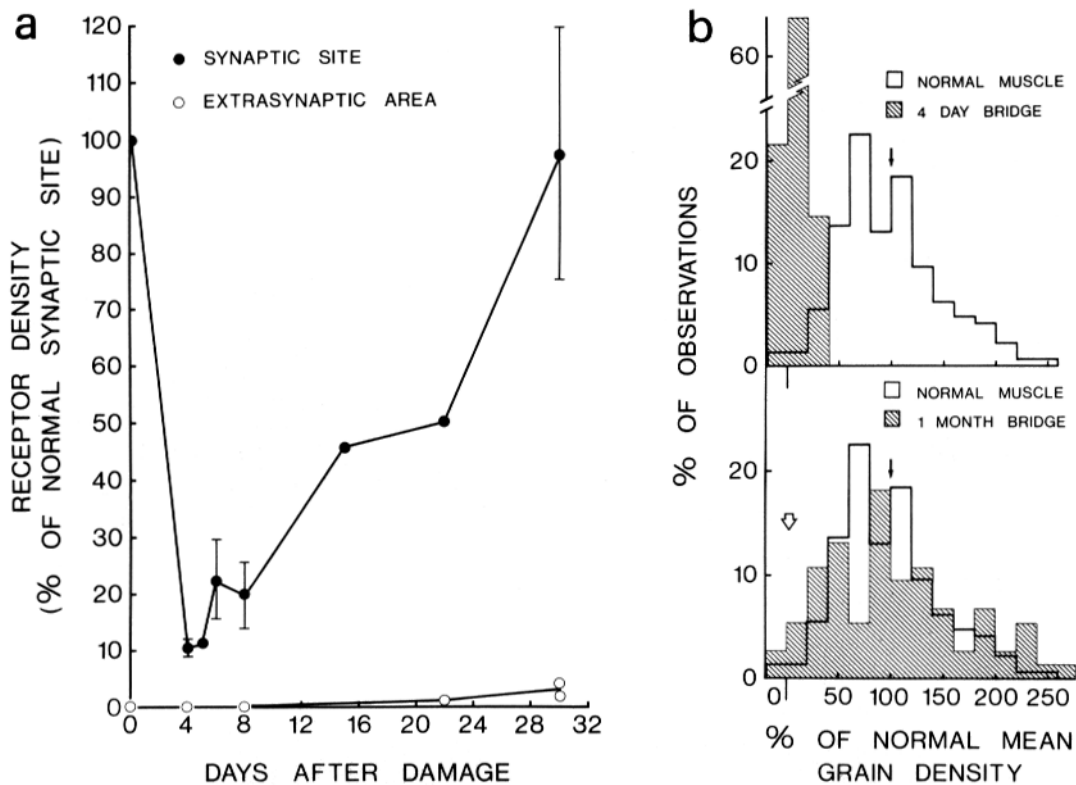


FIGURE 5 The density of AChRs at original synaptic sites during degeneration and regeneration of myofibers. AChR density at ChE-stained sites was determined by autoradiography after incubation of muscles with [125 I] α -BGT (details in Materials and Methods). Normal muscles were treated in parallel with experimental ones, and all values are expressed as a fraction of the mean at normal neuromuscular junctions. (a) Mean AChR density at synaptic (●) and extrasynaptic (○) areas as a function of time after muscle damage. For extrasynaptic sites each point represents the mean for a single muscle. For synaptic sites the 15-d and 22-d points represent the mean from a single muscle, the 5-d point from two muscles and the remaining points represent the mean and the standard error from three to five muscles. The autoradiographic grain density at an average of 60 synaptic sites was determined for each experimental muscle, and the mean grain density was expressed as a fraction of the mean density measured at normal synaptic sites. The mean density of AChRs at original synaptic sites falls to 10% of normal within 4 d of damage but subsequently increases as regeneration proceeds; by 30 d after damage the mean AChR density is $98 \pm 22\%$ of normal. Extrajunctional AChRs were undetectable in normal muscle and during the first two weeks after damage. By 30 d after damage the density of extrajunctional AChRs was $\sim 3\%$ of the density at normal synaptic sites. (b) A comparison of the distribution of autoradiographic grain densities at individual synaptic sites in normal muscles and in muscles examined 4 d (top) and 30 d (bottom) after damage. The histograms were generated from the analysis of 195 synaptic sites in normal muscle, 215 synaptic sites in 4-d damaged muscle and 73 synaptic sites in 30-d regenerated muscle. Since data were pooled from several different experiments to generate the histograms, the density at each site was expressed relative to the mean density at synaptic sites in the paired normal muscle. The solid arrows indicate the mean of all observations in normal muscles (100%), and the open arrowhead in the lower histogram indicates the mean extrasynaptic grain density in denervated, regenerated muscle. The distribution of AChR densities at synaptic sites in 4-d damaged muscle is markedly shifted to the left relative to normal. The distribution of AChR densities at original synaptic sites in 30-d regenerated muscle is similar to the distribution found in normal muscle.

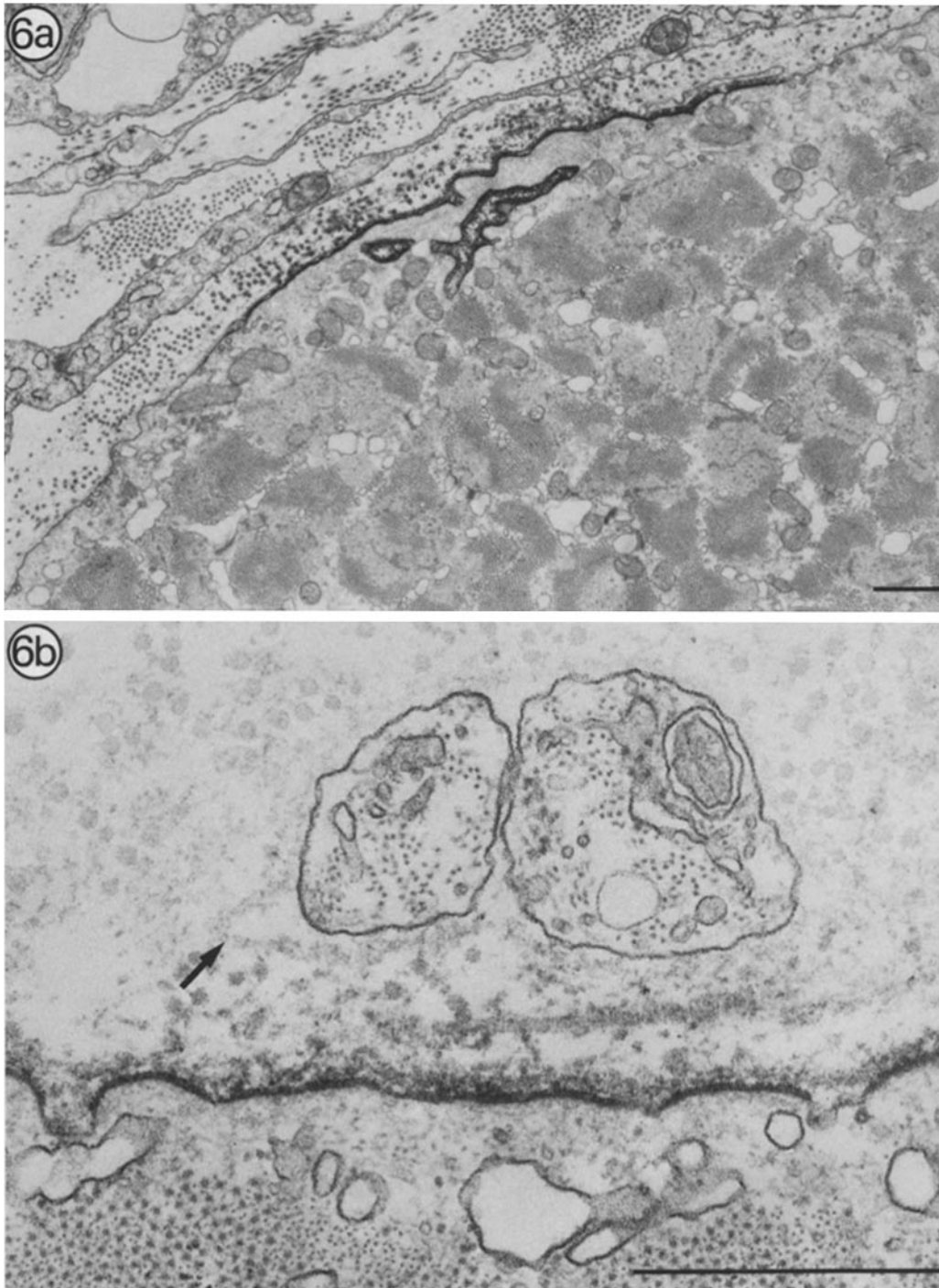


FIGURE 6 Patches of HRP- α -BGT staining on denervated, regenerated myofibers 30 d after damage to the muscle. (a) HRP- α -BGT stain is limited to a discrete area on the myofiber surface. Stain is also present in folds whose continuity with the cell surface is not seen in this section. Nearby collagen fibers are also stained owing to diffusion of the reaction product. (b) Dense band of stain lines the external surface of the myofiber membrane as at normal junctions (compare with Fig. 3 b). Schwann cell processes that capped nerve terminals are nearby. A basal lamina (arrow) distinguishes Schwann cells from connective tissue cells, which have no basal lamina. Bars, 1.0 μ m.

grain densities at 4 d was markedly shifted to the left relative to normal, and >80% of the receptors had been removed from 86% of the synaptic sites.

AChRs on Denervated, Regenerating Myofibers Accumulate at Original Synaptic Sites on the Basal Lamina Sheaths

During the second week after damage, new myofibers form within the basal lamina sheaths of the original muscle cells (reference 37; illustrated schematically in Fig. 2c). By 1 mo after the operation, the plasma membrane of myofibers has discrete areas of high receptor density. We observed by electron microscopy well defined patches of stain (Fig. 6a) after treatment of the bridges with HRP- α -BGT. As at normal neuromuscular junctions, the entire thickness of the basal lamina was stained and a dense band of stain lined the external surface of the myofiber plasma membrane (Fig. 6b). At many of the receptor patches there were periodically distributed folds in the myofiber surface (Fig. 7). These folds resemble junctional folds at the normal neuromuscular junction (compare Figs. 3b and 7).

Two observations indicated that patches of receptors were situated at original synaptic sites on the basal lamina sheaths. Some HRP- α -BGT-stained areas of plasma membrane were near Schwann cell processes that had originally capped nerve terminals (Fig. 6b). Moreover, in whole-mount preparations examined with the light microscope, we observed patterns of HRP- α -BGT stain corresponding in size, shape and arrangement to stained endplate arborizations in normal

muscle (Fig. 8). The stained areas in the whole-mounts of both regenerated and normal fibers had a periodic substructure corresponding to the folds in the plasma membrane.

We confirmed that receptors accumulate preferentially at original synaptic sites by staining preparations for ChE, labeling receptors with [125 I] α -BGT and examining cross sections by light microscope autoradiography. In this way, we were able to measure AChR density in synaptic and extrasynaptic areas of the basal lamina sheaths. Since no new ChE appears during the course of muscle regeneration (see Materials and Methods), the stained areas on regenerated myofibers mark the location of original synaptic sites. We found that as in normal myofibers, denervated regenerated muscle fibers had dense clusters of grains associated with ChE-stained spots (Fig. 4e and f). The average grain density at extrasynaptic areas of basal lamina was considerably lower than at ChE-stained spots, but, as shown in Figs. 4 and 5, it was higher than the extrasynaptic grain density in normal muscle. By defining a grain cluster as having more than ten times the average extrasynaptic density, we found that 91% of ChE-sites (41 of 45 sites from three muscles) had grain clusters. The mean grain density at ChE-sites was about 30-fold greater than the average grain density in extrasynaptic areas (Fig. 5a). We then examined how frequently extrasynaptic areas had grain clusters as large or larger than the mean size of clusters at original synaptic sites (details in Materials and Methods). In cross sections through portions of two bridges, 7% of the surface of myofibers that had synaptic sites stained for ChE, yet 88% of the

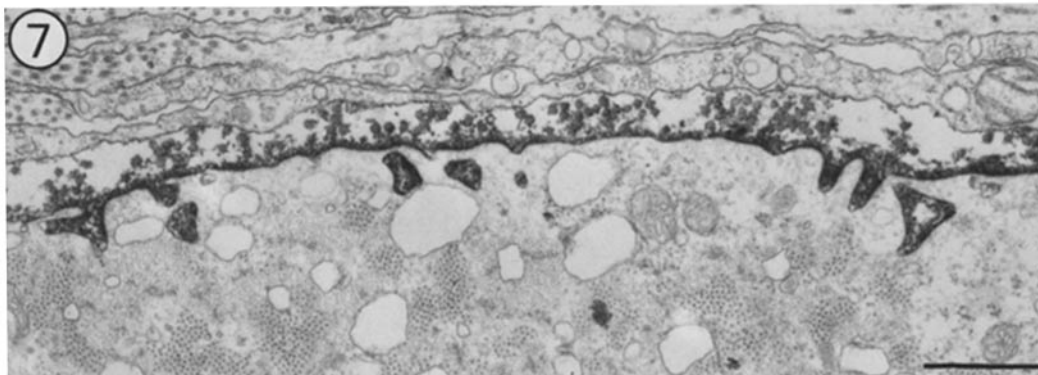


FIGURE 7 The plasma membrane at patches of HRP- α -BGT stain in denervated, regenerated muscle has periodic folds that resemble junctional folds at normal neuromuscular junctions (compare with Fig. 3a). 30 d after damage. Bar, 1.0 μ m.

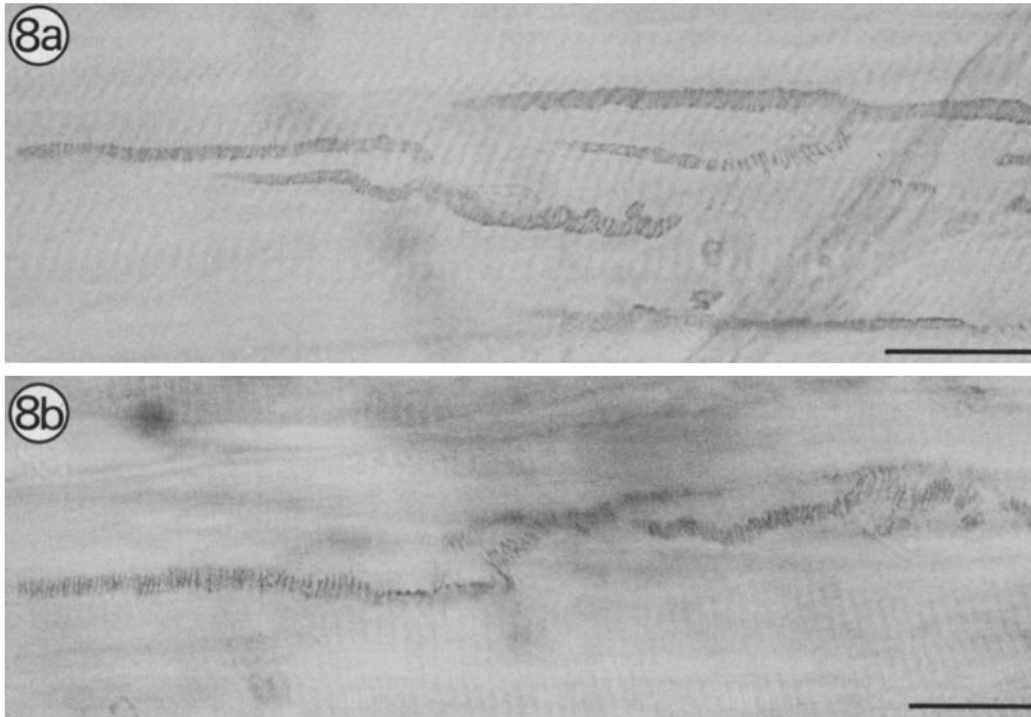


FIGURE 8 Pattern of HRP- α -BGT staining in whole-mounts of denervated, regenerated myofibers is similar to normal. (a) Normal myofiber. (b) Regenerated myofiber 30 d after denervating and damaging the muscle. Stain is arranged in elongate areas. Often the stain on regenerated myofibers is more patchy than that illustrated in (b) but the general pattern of stain, with its characteristic arborizations, is always evident. In normal muscle each area of stain underlies a nerve terminal (see Fig. 3 a). The stained areas on both normal and regenerated fibers are striated owing to folds in the myofiber membrane. Bars, 20 μ m.

grain clusters were associated with the stain. Thus, nearly all of the original synaptic sites had accumulations of receptors, and receptor accumulations that were comparable in size and density were rarely situated elsewhere.

We compared the autoradiographic grain density at ChE-stained sites in regenerated muscle to the grain density at normal neuromuscular junctions. The grain density rose gradually during muscle regeneration from a minimum of 10% of normal at 4 d after muscle damage (Fig. 5 a). By 30 d after damage, both the mean density (Fig. 5 a) and distribution of densities about the mean (Fig. 5 b, lower histogram) were the same in regenerated and normal muscle. The size of the ChE-stained sites was similar in cross sections of regenerating and normal muscle ($4.2 \pm 1.8 \mu$ m for regenerated sites, $4.3 \pm 1.8 \mu$ m for normal sites; mean \pm standard deviation) and consequently the number of receptors per synaptic site is similar in regenerated and normal muscle. Thus, not only do

AChRs accumulate preferentially at original synaptic sites, but also their density and number is as high as that at normal neuromuscular junctions.

Folds in the Myofiber Membrane

The membranes of regenerated myofibers have periodically arranged folds at regions of high receptor density (see Figs. 7 and 8). If elements at the original synaptic sites in the muscle can direct the formation of folds as well as the accumulation of AChRs, one might expect to find folds restricted to spots of high receptor density. We searched for such a correlation with the electron microscope by comparing the density of folds in stained and unstained areas of myofiber membrane after incubating muscles in HRP- α -BGT. Folds were defined as having a breadth <200 nm where they joined with the surface of the myofiber and having a depth >100 nm. These dimensions were chosen to exclude vesicles fusing with the plasma membrane and gradual undulations of the cell surface.

On 13 myofibers examined, we found 26 folds in 100 μm of HRP-stained membrane but no folds in 850 μm of unstained membrane. Thus, folds occur selectively, if not exclusively, at original synaptic sites.

DISCUSSION

Our results demonstrate that postsynaptic differentiation in regenerated muscle is directed by factors maintained external to the nerve. This finding extends the work of Sanes et al. (37), who showed that axons regenerate to and differentiate at original synaptic sites in the absence of myofibers. Thus, after removal of both nerve terminals and myofiber elements remain at the synaptic sites that can direct the formation of pre- and postsynaptic specializations in regenerating nerve and muscle.

After mechanical damage myofibers degenerate and are phagocytized within their basal lamina sheaths. The mean density of AChRs at original synaptic sites on the basal lamina falls to 10% of normal by 4 d after damage. The AChRs that are present at this time are likely to be the original receptors since HRP- α -BGT staining at 7 d is associated only with membrane fragments. We do not know whether these original AChRs are subsequently removed; muscle regeneration begins after this time, and the original receptors were not distinguished from new receptors that appear on regenerated myofibers by our histological methods. Nevertheless, at the time that the muscle regeneration begins, >90% of the original receptors have been removed from synaptic sites.

Muscle cells that regenerate within their basal lamina sheaths in the absence of the nerve have patches of receptors at the original synaptic sites. That regenerating myofibers actively accumulate receptors at these sites is demonstrated by the tenfold increase in receptor density that occurs between 4 and 30 d after muscle damage. Original synaptic sites are remarkably effective in directing the accumulation of receptors on regenerating muscle fibers: Receptor accumulations were at virtually all original synaptic sites, they were only rarely in extrasynaptic areas and the density and number of receptors at synaptic sites in regenerated muscle were similar to those at normal neuromuscular junctions.

The distribution of AChRs on the surface of regenerated, denervated myofibers differs from that on normal myofibers in that regenerated myofibers have a detectable density of AChRs on the

extrasynaptic surface. Developing muscle cells and denervated or inactive myofibers also have AChRs in extrasynaptic areas (4, 15, 31). Thus, it is not surprising that muscle fibers that regenerate in the absence of the nerve have extrasynaptic AChRs.

The accumulation of AChRs is not the only manifestation of myofiber differentiation that occurs at original synaptic sites: regenerated myofibers also have periodically distributed folds in the plasma membrane that resemble junctional folds at normal neuromuscular junctions. These folds may form by a mechanical interaction between the plasma membrane and basal lamina that formerly projected into the junctional folds of the original myofibers.

Surviving elements at the original synaptic site that could direct the organization of AChRs on regenerating myofibers include the synaptic portion of the basal lamina and the adjacent Schwann cells. (a) *Basal lamina*. Sanes et al. (37) showed that factors associated with the synaptic portion of the basal lamina influence the morphological differentiation of motor nerve terminals that reinnervate original synaptic sites in the absence of muscle. Within the nerve terminal, active zones and accumulations of synaptic vesicles line up opposite periodic specializations in the myofiber basal lamina (37). This result raises the possibility that the accumulation of AChRs in regenerating muscle is also influenced by elements contained in or connected to the synaptic portion of the basal lamina. (b) *Schwann cell*. Schwann cells respond to denervation by engulfing motor nerve terminals, after which they occupy for a time the position of the nerve terminal on the presynaptic side of the basal lamina sheath (9). There is good evidence that Schwann cells at denervated synaptic sites release acetylcholine spontaneously (9). Some substance released by Schwann cells may act to organize AChRs on the myofiber surface.

Many of the steps in the formation of neuromuscular junctions are similar during development in vivo (5, 6, 24) and in vitro (18, 36) and during regeneration in adults (14). During the formation of neuromuscular junctions in cell culture, the developing nerve causes AChRs to accumulate on the muscle cell surface at the site of the newly formed synapse (2, 3, 19). Knowledge of where the factors that direct postsynaptic differentiation are situated and how they act during regeneration may provide information not only about how synaptic specializations arise during regeneration, but also how they form during embryonic development.

We are grateful to Robert Marshall and Gerald Barry for excellent technical assistance.

This work was supported by United States Public Health Service grant No. NS 14506, and was carried out during the tenure of Postdoctoral Fellowships from Muscular Dystrophy Association to S. J. Burden (Paul Cohen Fellowship) and P. B. Sargent.

Received for publication 10 January 1979, and in revised form 19 March 1979.

REFERENCES

1. ALBUQUERQUE, E. X., E. A. BARNARD, S. E. JANSSON, and J. WIEC-KOWSKI. 1973. Occupancy of the cholinergic receptors in relation to changes in the endplate potential. *Life Sci.* 12:545-552.
2. ANDERSON, M. J., and M. W. COHEN. 1977. Nerve-induced and spontaneous redistribution of acetylcholine receptors on cultured muscle cells. *J. Physiol. (Lond.)* 268:757-773.
3. ANDERSON, M. J., M. W. COHEN, and E. ZORYCHTA. 1977. Effects of denervation on the distribution of acetylcholine receptors on cultured muscle cells. *J. Physiol. (Lond.)* 268:731-756.
4. AXELSSON, J., and S. THESLEFF. 1959. A study of supersensitivity in denervated mammalian skeletal muscle. *J. Physiol. (Lond.)* 149:178-193.
5. BENNETT, M. R., and A. G. PETTIGREW. 1974. The formation of synapses in striated muscle during development. *J. Physiol. (Lond.)* 241:515-545.
6. BENNETT, M. R., and A. G. PETTIGREW. 1975. The formation of synapses in amphibian striated muscle during development. *J. Physiol. (Lond.)* 252:203-239.
7. BENOIT, P. W., and P. BELT. 1970. Destruction and regeneration of skeletal muscle after treatment with a local anesthetic, bupivacaine (Marcaine). *J. Anat.* 107:547-556.
8. BERG, D. K., R. B. KELLY, P. B. SARGENT, P. WILLIAMSON, and Z. W. HALL. 1972. Binding of α -bungarotoxin to acetylcholine receptors in mammalian muscle. *Proc. Natl. Acad. Sci. U. S. A.* 69:147-151.
9. BIRKS, R., B. KATZ, and R. MILEDI. 1960. Physiological and structural changes at the amphibian myoneural junction, in the course of nerve degeneration. *J. Physiol. (Lond.)* 150:145-168.
10. BURDEN, S. 1977. Development of the neuromuscular junction in the chick embryo: the number, distribution, and stability of acetylcholine receptors. *Dev. Biol.* 57:317-329.
11. CARLSON, B. M. 1972. The regeneration of skeletal muscle—a review. *Am. J. Anat.* 137:119-149.
12. CHANG, C. C., and C. Y. LEE. 1963. Isolation of neurotoxins from the venom of *Bungarus multicinctus* and their modes of neuromuscular blocking action. *Arch. Int. Pharmacodyn. Ther.* 144:241-257.
13. COUTEAUX, R. 1955. Localization of cholinesterases at neuromuscular junctions. *Int. Rev. Cytol.* 4:335-375.
14. DENNIS, M. J., and R. MILEDI. 1974. Characteristics of transmitter release at regenerating frog neuromuscular junctions. *J. Physiol. (Lond.)* 239:571-594.
15. DIAMOND, J. and R. MILEDI. 1962. A study of foetal and new-born rat muscle fibers. *J. Physiol. (Lond.)* 162:393-408.
16. FAMBROUGH, D. M., and H. C. HARTZELL. 1972. Acetylcholine receptors: number and distribution at neuromuscular junctions in rat diaphragm. *Science (Wash. D. C.)* 176:189-191.
17. FILOGAMO, G., and G. GABELLA. 1966. Cholinesterase behaviour in the denervated and reinnervated muscles. *Acta Anat.* 63:199-214.
18. FISCHBACH, G. D. 1972. Synapse formation between dissociated nerve and muscle cells in low density cell cultures. *Dev. Biol.* 28:407-429.
19. FRANK, E., and G. D. FISCHBACH. 1977. ACh receptors accumulate at newly formed nerve-muscle synapses *in vitro*. In *Cell and Tissue Interactions*. J. W. Lash and M. M. Burger, editors. Raven Press, N. Y. 285-291.
20. FRANK, E., K. GAUTVIK, and H. SOMMERSCHILD. 1975. Persistence of junctional acetylcholine receptors following denervation. *Cold Spring Harbor Symp. Quant. Biol.* 40:275-281.
21. JIRMANOVÁ, I., and S. THESLEFF. 1976. Motor endplates in regenerating rat skeletal muscle exposed to botulinum toxin. *Neuroscience* 1:345-347.
22. KARNOVSKY, M. J. 1964. The localization of cholinesterase activity in rat cardiac muscle by electron microscopy. *J. Cell Biol.* 23:217-232.
23. KUFFLER, S. W., and D. YOSHIKAMI. 1975. The distribution of acetylcholine sensitivity at the post-synaptic membrane of vertebrate skeletal twitch muscles: iontophoretic mapping in the micron range. *J. Physiol. (Lond.)* 244:703-730.
24. KULLBERG, R. W., T. L. LENTZ, and M. W. COHEN. 1977. Development of the myotomal neuromuscular junction in *Xenopus laevis*: An electrophysiological and fine-structural study. *Dev. Biol.* 60:101-129.
25. LENTZ, T. L., J. E. MAZURKIEWICZ, and J. ROSENTHAL. 1977. Cytochemical localization of acetylcholine receptors at the neuromuscular junction by means of horseradish peroxidase labeled α -bungarotoxin. *Brain Res.* 132:423-442.
26. LETINSKY, M. K., K. H. FISCHBECK, and U. J. MCMAHAN. 1976. Precision of reinnervation of original postsynaptic sites in muscle after a nerve crush. *J. Neurocytol.* 5:691-718.
27. MARSHALL, L. M., J. R. SANES, and U. J. MCMAHAN. 1977. Reinnervation of original synaptic sites on muscle fiber basement membrane after disruption of the muscle cells. *Proc. Natl. Acad. Sci. U. S. A.* 74:3073-3077.
28. MATTHEWS-BELLINGER, J., and M. M. SALPETER. 1978. Distribution of acetylcholine receptors at frog neuromuscular junctions with a discussion of some physiological implications. *J. Physiol. (Lond.)* 279:197-213.
29. MCMAHAN, U. J., J. R. SANES, and L. M. MARSHALL. 1978. Cholinesterase is associated with the basal lamina at the neuromuscular junction. *Nature (Lond.)* 271:172-174.
30. MCMAHAN, U. J., N. C. SPITZER, and K. PEPPER. 1972. Visual identification of nerve terminals in living isolated skeletal muscle. *Proc. R. Soc. Lond. Biol. Sci.* 181:421-430.
31. MILEDI, R. 1960. The acetylcholine sensitivity of frog muscle fibers after complete or partial denervation. *J. Physiol. (Lond.)* 151:1-23.
32. MILEDI, R., and L. T. POTTER. 1971. Acetylcholine receptors in muscle fibers. *Nature (Lond.)* 233:599-603.
33. MILEDI, R., and C. R. SLATER. 1968. Electrophysiology and electron-microscopy of rat neuromuscular junctions after nerve degeneration. *Proc. R. Soc. Lond. B. Biol. Sci.* 169:289-306.
34. NICKEL, E., and P. G. WASER. 1968. Elektronenmikroskopische untersuchungen am diaphragma der maus nach einseitiger phrenikotomie. I. Die degenerierende motorische endplatte. *Zellforsch. Mikrosk. Anat.* 88:278-296.
35. RAMON Y CAJAL, S. 1928. Degeneration and regeneration of the nervous system. (Translated and edited by R. M. May, 1959.) Hafner Press, N. Y.
36. REES, R. P., M. B. BUNGE, and R. P. BUNGE. 1976. Morphological changes in the neuritic growth cone and target neuron during synaptic development in culture. *J. Cell Biol.* 68:240-263.
37. SANES, J. R., L. M. MARSHALL, and U. J. MCMAHAN. 1978. Reinnervation of muscle fiber basal lamina after removal of muscle fibers. *J. Cell Biol.* 78:176-198.
38. TELLO, F. 1907. Degeneration et regeneration des plaques motrices après la section des nerfs. *Trav. Lab. Rech. Biol. Univ. Madrid.* 5:117-149.
39. VENABLE, J. H., and R. COGGESHALL. 1965. A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.* 24:407-408.
40. VOGEL, Z., G. J. MALONEY, A. LING, and M. P. DANIELS. 1977. Identification of synaptic acetylcholine receptor sites in retina with peroxidase-labeled α -bungarotoxin. *Proc. Natl. Acad. Sci. U. S. A.* 74:3268-3272.
41. VRACKO, R., and E. P. BENDITT. 1972. Basal lamina: the scaffold for orderly cell replacement. *J. Cell Biol.* 55:406-419.