REINNERVATION OF MUSCLE FIBER BASAL LAMINA AFTER REMOVAL OF MYOFIBERS

Differentiation of Regenerating Axons at Original Synaptic Sites

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ABSTRACT

Axons regenerate to reinnervate denervated skeletal muscle fibers precisely at original synaptic sites, and they differentiate into nerve terminals where they contact muscle fibers. The aim of this study was to determine the location of factors that influence the growth and differentiation of the regenerating axons. We damaged and denervated frog muscles, causing myofibers and nerve terminals to degenerate, and then irradiated the animals to prevent regeneration of myofibers. The sheath of basal lamina (BL) that surrounds each myofiber survives these treatments, and original synaptic sites on BL can be recognized by several histological criteria after nerve terminals and muscle cells have been completely removed. Axons regenerate into the region of damage within 2 wk. They contact surviving BL almost exclusively at original synaptic sites; thus, factors that guide the axon's growth are present at synaptic sites and stably maintained outside of the myofiber. Portions of axons that contact the BL acquire active zones and accumulations of synaptic vesicles; thus by morphological criteria they differentiate into nerve terminals even though their postsynaptic targets, the myofibers, are absent. Within the terminals, the synaptic organelles line up opposite periodic specializations in the myofiber's BL, demonstrating that components associated with the BL play a role in organizing the differentiation of the nerve terminal.

KEY WORDS active zone · basement membrane · neuromuscular junction · synaptogenesis · x-irradiation

From the orderly and specific way in which synapses are formed, it is apparent that the components of the synapse exchange information as synaptogenesis proceeds. Skeletal muscle is a convenient tissue in which to study this interchange; the neuromuscular junction is a relatively simple and accessible synapse, and adult muscle can be reinnervated following damage to the motor nerve. The influence of nerve on muscle is well documented: many properties of the muscle fiber in general (21) and of the postsynaptic membrane in particular (18, 21, 45) are altered by denerva-

tion and restored to their original state when the muscle is reinnervated. The nerve terminal not only provides but also receives morphogenetically important information as the neuromuscular junction regenerates. Regenerating axons form contacts precisely at original synaptic sites, making few contacts elsewhere on the muscle fiber's surface (2, 15, 19, 27); the axons differentiate into nerve terminals only when they are within 0.1 μm from the muscle fiber;[1] and synaptic vesicles accumulate above the junctional folds that invaginate the postsynaptic membrane.[1]

The experiments reported here were undertaken as part of a study[1] (27, 31, 44) whose aim is to discover the structures that provide information to regenerating motor nerve terminals, and to determine the types of information that they provide. A sheath of basement membrane, composed of basal and reticular laminae, surrounds each muscle fiber, and the basal lamina (BL) extends through the synaptic cleft of the neuromuscular junction. When muscle fibers are damaged, they degenerate and are phagocytized, but their basement membrane sheaths survive; new myofibers then regenerate within the sheaths (9, 24, 50). We have devised a way to damage and denervate frog muscle in which the orientation of the sheaths is preserved; the sites where synapses had been situated can be identified after both myofiber and nerve terminal are removed (31). By X-irradiating the damaged muscle, we inhibited regeneration of myofibers (32, 42, 52) without blocking the regeneration of the damaged axons. We were therefore able to study the reinnervation of "muscle" in the absence of myofibers.

This paper describes several early steps in the reinnervation of the basement membrane sheaths. In the absence of muscle cells, as in their presence, we found that: (*a*) axons can regenerate to the basement membrane sheaths of the muscle fibers; (*b*) regenerating axons contact the BL of the sheaths almost exclusively at original synaptic sites; and (*c*) regenerating axons differentiate into nerve terminals, by morphological criteria, when they reach the original synaptic sites. Further studies revealed that components contained in or connected to the muscle fiber's BL trigger at least some aspects of this differentiation.

[1] Rotshenker, S., and U. J. McMahan. Differentiation of regenerating axon terminals at original synaptic sites on frog muscle fibers. Manuscript in preparation.

MATERIALS AND METHODS

The Cutaneous Pectoris Muscles

Experiments were performed on the paired cutaneous pectoris muscles of 5-cm long male frogs (*Rana pipiens*). These thin, flat muscles lie directly beneath the skin of the thorax, and can be exposed for surgery or dissection by cutting and folding back a flap of skin. Each muscle is composed of ~500 muscle fibers and is innervated by a nerve that contains ~25 motor axons (44). The nerve enters the muscle from the lateral edge and courses across its center, in a direction perpendicular to the long axis of the muscle fibers (Fig. 1). Blood vessels enter and run with the nerve. The nerve branches intramuscularly to provide each muscle fiber with a single neuromuscular junction. Most of the neuromuscular junctions are found along nerve branches in the central portion of the muscle, in a 2-3-mm wide band (27).

Damaging and Denervating Muscle

To induce degeneration and phagocytosis of myofibers, a rectangular slab was cut from the muscle on each side of the main nerve trunk, leaving behind a row of damaged muscle fiber segments, a "bridge" (31), extending between groups of undamaged fibers at the medial and lateral edges of the muscle (Fig. 1, right side). Each bridge was 1-1½ mm wide, 3-4 mm long, and contained 250-400 muscle fiber segments. Generally, each bridge bore 50-150 synaptic sites. The numbers of nerve branches and synaptic sites that were

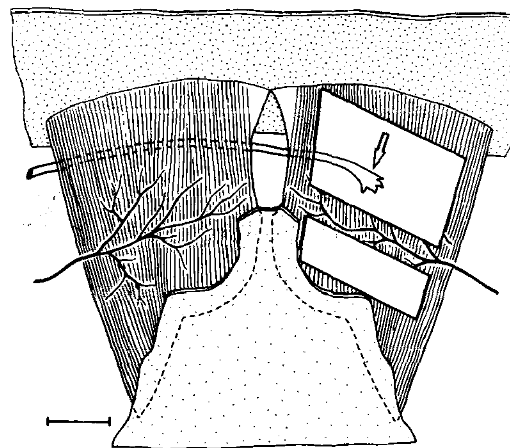

FIGURE 1 Sketch of the paired cutaneous pectoris muscles with attached origin and insertion. The muscle on the left is intact. The nerve enters from the lateral edge. Two rectangular areas have been cut from the muscle on the right, leaving behind a row of muscle fiber segments, a "bridge", between undamaged medial and lateral fibers. In some experiments, a "foreign" nerve (arrow), which normally supplies the forelimb, was implanted near the bridge. Bar, ~3 mm.

preserved could be increased by making the bridge wider, but degeneration of damaged muscle was incomplete if the bridge was much more than 1½ mm wide.

Usually, muscles were denervated by crushing their nerve with fine forceps 1-2 mm from the muscle's lateral edge; reinnervation is prompt following a nerve crush (27). In some experiments, the nerve was cut at the lateral edge of the muscle and a 2-cm long stretch proximal to the cut was evulsed to prevent reinnervation by "native" axons. In these animals, we implanted a "foreign" nerve near the bridge (Fig. 1), and "foreign" axons reinnervated the damaged muscle. The large nerve that supplies the forelimb was cut near the elbow. The central stump was dissected free from its musculature and run under the ipsilateral cutaneous pectoris muscle. The cut end was then laid near the medial edge of the contralateral bridge, where it was held in place with a blood clot.

For all operations, frogs were anesthetized by immersion in 0.1% MS-222 (tricaine methane sulfonate, Ayerst Laboratories, New York) in water. Incisions were closed with 7-0 sutures. Animals were kept at 18°C and were force-fed beef liver twice each week.

X-Irradiation

Frogs were X-irradiated to prevent their damaged muscles from regenerating. Anesthetized frogs were placed ventral side up beneath the source of a Westinghouse Coronado Radiotherapy unit (Westinghouse Electric Corp., Pittsburgh, Pa.). Lead shielding restricted the radiation to a 15-mm wide band (rostral-caudal) centered on the cutaneous pectoris muscles. Radiation was delivered at a rate of 100 rad/min (15 mA, 250 kV, 2 mm Al added filtration, 65 cm from source to center of animal). In a preliminary experiment, we varied the dose of X-rays and the time between muscle damage and irradiation. Doses of 1,600 or 2,000 rads inhibited the regeneration of myofibers almost completely, whereas a 1,200-rad dose was slightly and a 800-rad dose was markedly less effective (Fig. 2*a*). 1,600- or 2,000-rad doses were equally effective when delivered any time between several hours and 4 days after the muscle was damaged. If, however, irradiation was delayed until 5 days after surgery, some myofibers regenerated during the subsequent few weeks (Fig. 2*b*). On the basis of these results, we submitted frogs to a 1,600-rad dose, 3 days after surgery in subsequent experiments.

Microscopy

For light and electron microscopy, muscles were fixed in glutaraldehyde (0.8% in 0.06 M phosphate buffer, pH 7.2) for 30-45 min, rinsed in buffer (0.13 M sodium cacodylate, pH 7.2), refixed in osmium tetroxide (1% in 0.11 M cacodylate buffer, pH 7.2), dehydrated in ethanol, rinsed in propylene oxide, and embedded whole in thin (1 mm or less) wafers of Epon. To stain BL for electron microscopy, ruthenium red (Sigma Chemical Co., St. Louis, Mo.) was added to the osmium tetroxide fixative, at a final concentration of 0.5 mg/ml (28, 31). To demonstrate cholinesterase (ChE) at neuromuscular junctions, Karnovsky's histochemical stain was applied to the muscle after glutaraldehyde and before osmium fixation (25, 27). In a few experiments, glutaraldehyde fixation was omitted and muscles were treated with a zinc iodide and osmium mixture (27, 30) to impregnate motor nerve terminals.

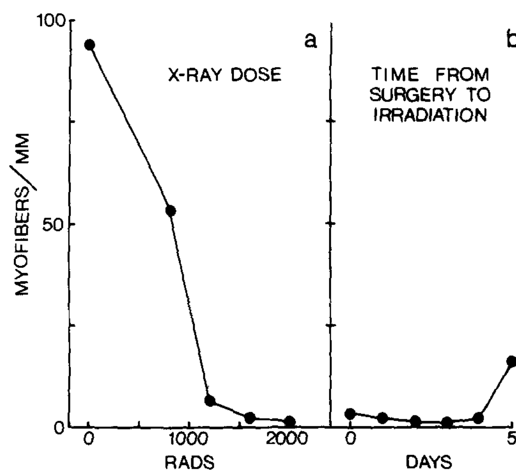

FIGURE 2 X-irradiation inhibits regeneration of myofibers in damaged muscle. Muscles were fixed 16 days after they were damaged and denervated and the number of myofibers per millimeter of bridge length was determined light-microscopically in cross sections. (*a*) Frogs were irradiated at the indicated dose, 2 or 3 days after surgery. (*b*) Frogs received a single dose of 1,600 or 2,000 rads at the indicated times after surgery. Each point is the average of counts from two to five bridges. Normal muscles had 80-100 myofibers/mm.

The Surface of the Muscle Fiber: Terminology

Electron microscopy of skeletal muscle (49) has revealed that the surface complex of each muscle fiber, the sarcolemma (7), comprises several concentric layers (Figs. 3, 4*a*, and 5*a*). The innermost layer, a typical lipid-rich, osmophilic plasma membrane, is coated by a thin, carbohydrate-rich glycocalyx, which can be rendered electron dense by any of a number of stains (40), including the ruthenium red-osmium mixture (28) used in this study. Separated from the glycocalyx by a narrow, electron-lucent gap is the BL, which also stains intensely with ruthenium red. This feltlike layer is 10-15 nm thick and, like the BLs of other tissues (47), it is probably collagenous (12). A reticular lamina of indeterminate width lies just beyond the BL. The reticular lamina contains collagen fibrils embedded in an amorphous matrix; the fibrils but not the matrix are visible in our

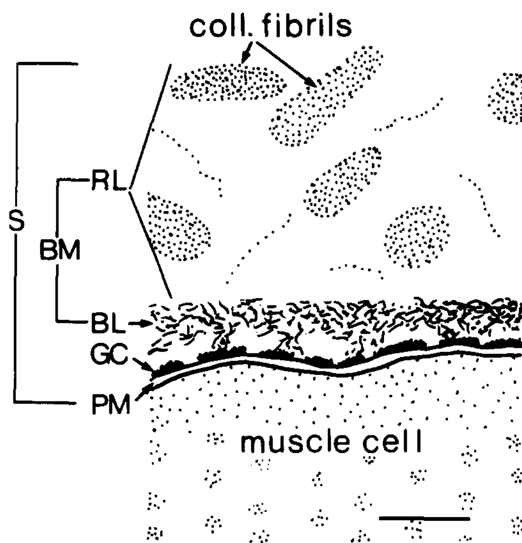

FIGURE 3 The surface or sarcolemma (*S*) of a muscle fiber; drawing from an electron micrograph. Cytoplasm of the muscle cell is surrounded by layers of plasma membrane (*PM*), glycocalyx (*GC*), basal lamina (*BL*), and reticular lamina (*RL*) containing collagen fibrils. The BL and RL together compose basement membrane (*BM*). Bar, ~0.1 μm.

electron micrographs. The BL and reticular lamina together are called the basement membrane. In some cases—but not here—the term basement membrane is used to refer to the BL alone, and the term sarcolemma is applied to the plasma membrane alone.

RESULTS

Degeneration of Damaged Muscle and Nerve

MUSCLE: When muscle fibers are damaged as described in Materials and Methods, the myofibers retract and degenerate, but the sheaths of basement membrane that surrounded them are left behind (Fig. 4*b*). Macrophages invade the sheaths and remove remnants of the disrupted myofibers, including fragments of plasma membrane that adhere to the inner surface of the BL (Fig. 5*b*). The bulk of the sheath is emptied during the first few days after the muscle is damaged.

If the cut muscle is not damaged further, myofibers regenerate within the surviving basement membrane sheaths (Fig. 4*c*), laying down a new layer of BL in areas where they do not completely fill the old sheaths (Fig. 5*c*). By 2 wk after surgery, nearly all of the sheaths contain regenerating myofibers, and the bridge contracts upon direct electrical stimulation. If, however, the muscle is X-irradiated within a few days of surgery, regeneration is markedly inhibited (Fig. 2). The basement membrane sheaths of the original muscle fibers persist in the irradiated bridge (Figs. 4*d* and 5*d*) for at least 5 wk, but myofibers regenerate within only a few of them.

Degeneration and phagocytosis of the damaged muscle continues after X-irradiation, and the sheaths of BL gradually empty (Fig. 4*d*). 7 days after surgery, <2% of the BL's inner surface is apposed by debris or fragments of plasma membrane (Fig. 6). However, the sheaths never empty completely: mononucleated cells, including macrophages and presumptive myoblasts (9, 24, 39, 46) remain inside the sheaths, and send out long, thin processes that can extend for hundreds of micrometers. These processes, broken during fixation or unidentifiable because of their small size, may account for the small number of plasma membrane fragments that adhere to the BL for several weeks after the bulk of the debris has been removed (Fig. 6).

NERVE: The muscle fiber's BL, but not its reticular lamina, extends through the synaptic cleft at the neuromuscular junction (Fig. 7*a*). The nerve terminal is capped by processes of a Schwann cell, and the Schwann cell's basement membrane (BL and reticular lamina) fuses with that of the muscle at the edges of the termina. Within the cleft, projections of BL line the junctional folds that invaginate the muscle cell's surface (Fig. 7*a*). ChE is contained in or connected to the BL of the neuromuscular junction (33); accordingly, in muscles treated with a histochemical stain for ChE, reaction product fills the synaptic cleft (Fig. 7*b*).

Nerve terminals survive for at least a week in muscles that have been damaged and irradiated but not denervated (Fig. 7*c*). If, however, the nerve is crushed at the same time that the muscle is cut, nerve terminals degenerate and are phagocytized by Schwann cells, as occurs in denervated but undamaged muscle (6, 35). After a nerve crush near the muscle's edge, virtually all of the terminals are phagocytized within 4 days, and the Schwann cells come to lie directly on the BL of the synaptic cleft (Fig. 7*d* and *e*). During the subsequent weeks, Schwann cell processes retract from many of the denervated synaptic sites, as they do in undamaged muscle (27, 35, 45), leav-

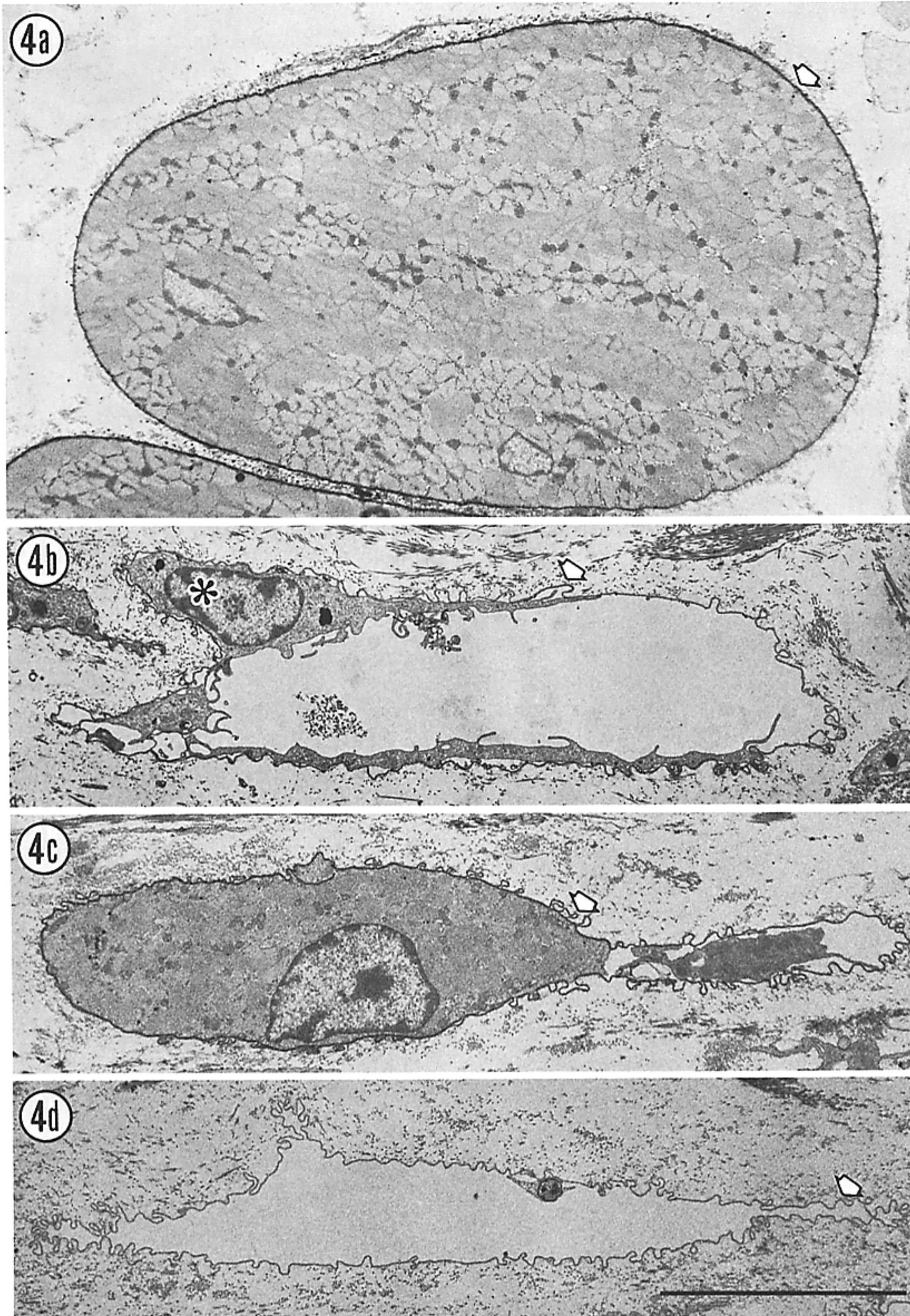

FIGURE 4 Damaged muscle degenerates and then regenerates; x-irradiation prevents its regeneration. Cross sections of muscle fibers and/or their BL sheaths. (*a*) Normal muscle fiber, ensheathed in darkly stained BL. (*b*) 4 days after muscle damage, much of the myofiber has been removed, but its BL survives. A macrophage (*) lies in the lumen of the BL. (*c*) 2 wk after muscle damage, a myofiber has regenerated within the sheath. (*d*) 4 wk after muscle damage and irradiation, the BL sheath remains nearly empty. Arrows indicate regions shown at higher magnification in Fig. 5. Bar, 10 μm.

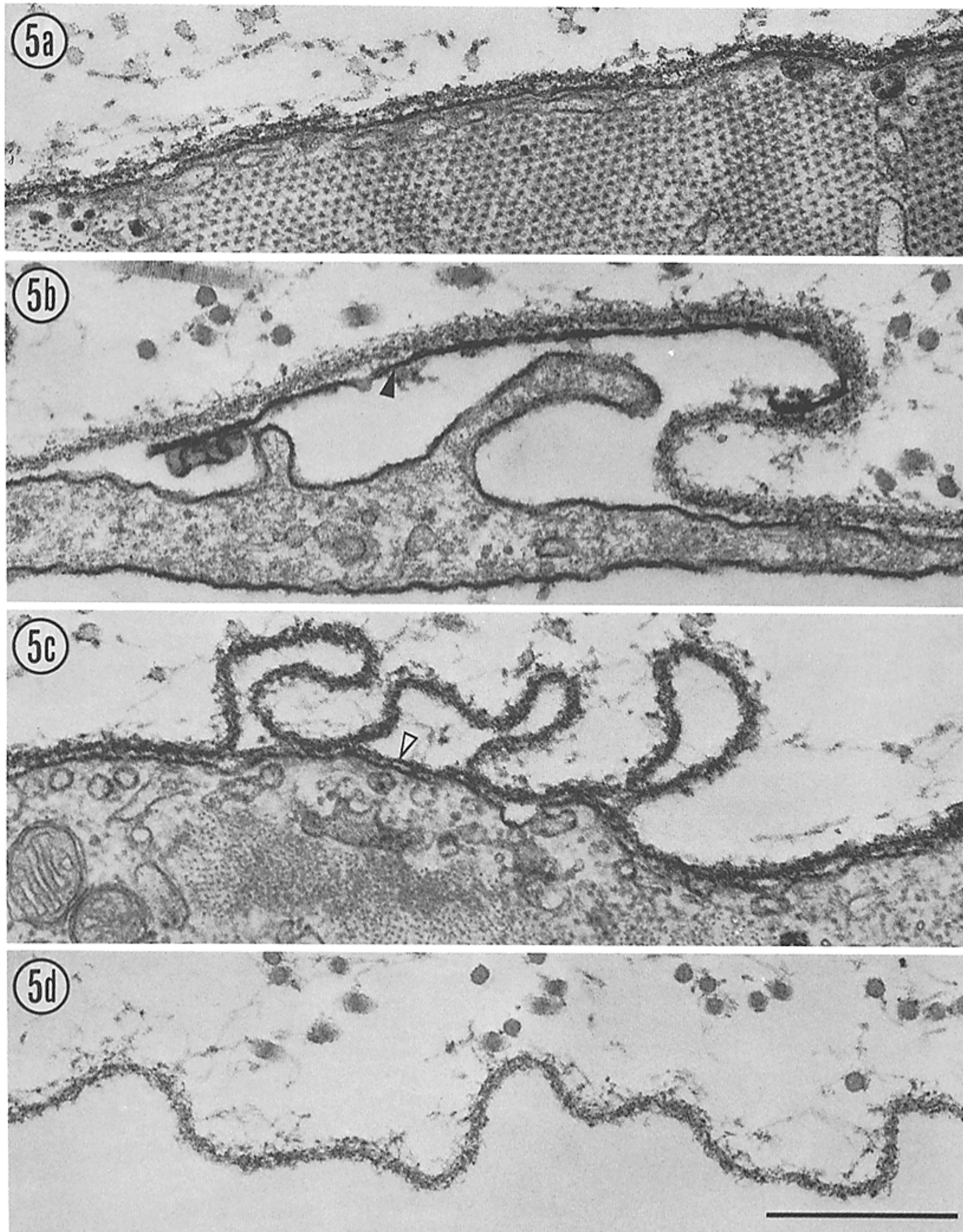

FIGURE 5 BL survives after damaged myofibers degenerate; regions marked by arrows in Fig. 4. (*a*) Undamaged muscle. (*b*) 4 days after muscle damage: a fine process of a macrophage sweeps the inside surface of the BL near a fragment of plasma membrane (arrowhead). (*c*) 2 wk after muscle damage; regenerating myofiber bears new BL (open arrowhead) where it does not fill the surviving BL of the original muscle fiber. (*d*) 4 wk after muscle damage and irradiation; an area of BL free of plasma membrane fragments and debris. Bar, 0.5 μm.

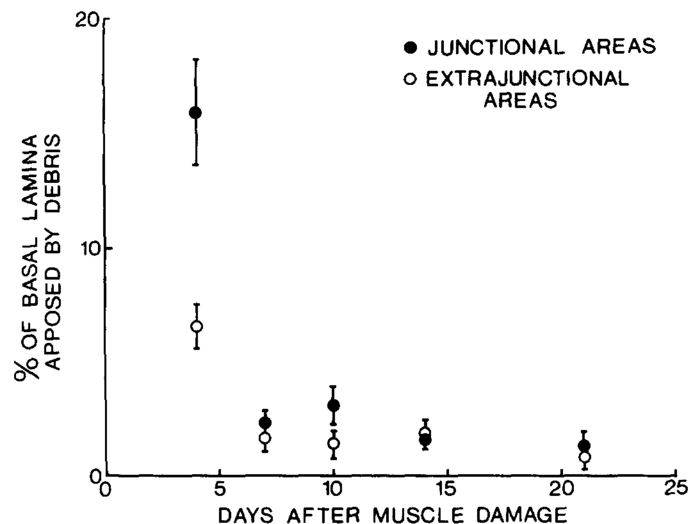

FIGURE 6 Fraction of the inner surface of the myofiber's BL opposed by plasma membrane fragments and amorphous cellular debris, at various times after muscle damage (on day 0) and X-irradiation (on day 3). Electron micrographs of junctional (●) and extrajunctional (○) regions were inspected at × 150,000. Each point shows the mean (±1 SE) of at least 20 samples from two bridges. The average length of BL contained in each sample was 2.7 μm.

ing their BL atop that of the muscle fiber (Fig. 7*f*).

Even after the nerve terminal and myofiber have degenerated, several components of the neuromuscular junction persist. As noted above, processes of the Schwann cells directly oppose the BL of the denervated synaptic cleft (Fig. 7*e*), and the Schwann cells' BL remains behind even when the processes themselves retract (Fig. 7*f*). The projections of BL that extend from the cleft into the junctional folds also survive (Fig. 7*c*, *e*, and *f*). Finally, histochemically demonstrable ChE remains in the synaptic BL of the bridge for many weeks (Fig. 7*d*) (33). These features allow one to recognize synaptic sites on the basement membrane sheaths long after cellular components of the synapse have been removed.

During the first few days after the muscle is denervated and damaged, more debris adheres to the BL of the synaptic cleft than to extrajunctional BL (Fig. 6). The BL of the junctional folds, which could trap membrane fragments or exclude macrophages, might account for this difference. Alternatively, the bonds between BL and plasma membrane may be stronger at the synapse than elsewhere on the muscle fiber. By 1 wk after surgery, however, both junctional and extrajunctional regions of the BL are almost completely clean (Fig. 6). When regenerating axons enter the bridge, as described in the next section, they encounter BL and Schwann cells but few if any remnants of their normal synaptic targets, the myofibers.

Regeneration of Damaged Axons

To determine whether axons regenerate across the bridge, we stimulated the nerve trunk near the lateral edge of the muscle, and looked for contractions in undamaged fibers medial to the bridge (Fig. 1). Stimulation evoked twitches in the medial fibers in 7 of 10 bridges examined 2 wk after nerve crush and muscle damage, and in all of 22 bridges examined a week later (Fig. 8). Thus, the axons were able to grow completely across the region of damage, and to form functional neuromuscular junctions.

The course that the regenerating axons took was studied in preparations stained with zinc iodide and osmium, which impregnates motor nerve terminals and regenerating preterminal axons that have not yet become myelinated (27, 30). Axons generally entered and ran through the bridge in the surviving perineurial tubes of the original axons; the growth of regenerating axons through surviving perineurium is a consistent feature of reinnervation in undamaged muscle following simple nerve crush (19, 27, 48). Within the

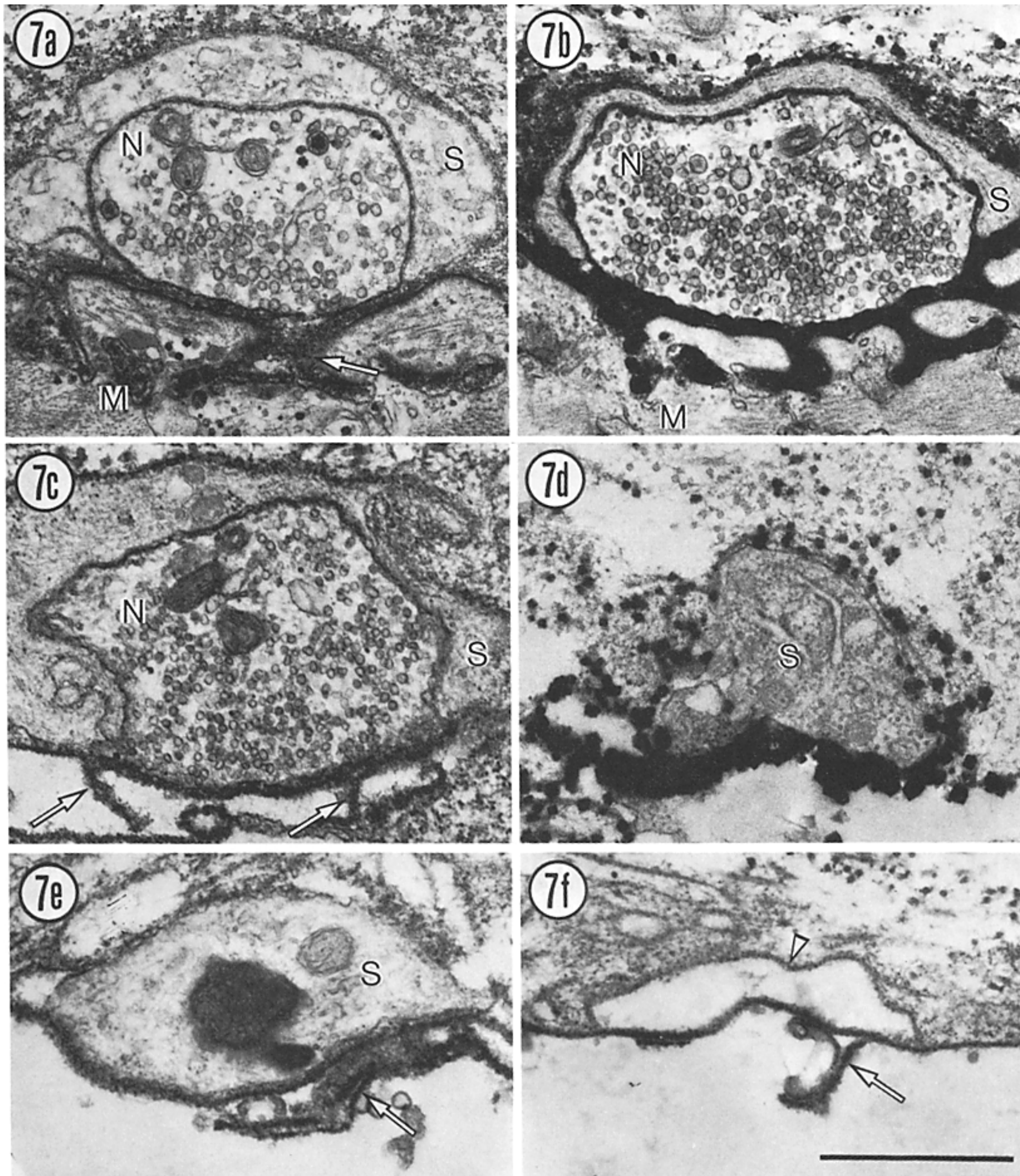

FIGURE 7 Synaptic sites can be recognized in four ways after nerve terminals (*N*) and myofibers (*M*) are removed: (*i*) Projections of BL (arrows) extend into the junctional folds of normal muscle (*a*) and survive in damaged (*c*) and damaged, denervated (*e*, *f*) muscle after myofibers have been removed. (*ii*) The electron-dense reaction product that fills the synaptic cleft in normal muscles stained for ChE (*b*) also stains the BL of the cleft after the muscle is damaged and denervated (*d*). (*iii*) Schwann cell processes (*S*) that cap normal nerve terminals (*a*, *b*) phagocytize the terminals after nerve damage and come to lie on cleft BL (*d*, *e*). (*iv*) The BL that surrounds Schwann cell processes (*a*–*e*) persists when these processes retract from the synaptic site (arrowhead in Fig. 7*f*). Specimens were prepared 4 (*c*, *e*) or 7 days (*d*, *f*) after muscle damage. Bar, 1.0 μm.

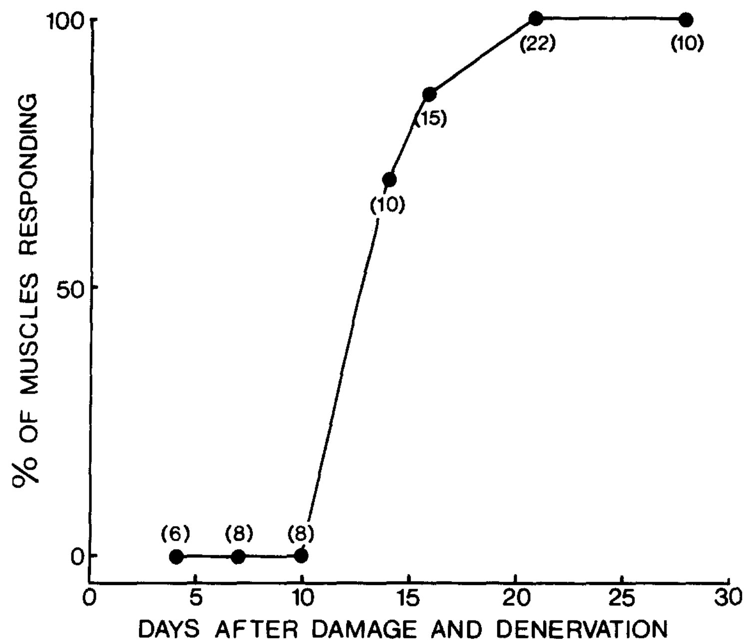

FIGURE 8 Axons regenerate across the bridge after the nerve is crushed. At indicated times after muscle was damaged and denervated (on day 0) and X-irradiated (on day 3), bridges were tested in vitro. The nerve was stimulated electrically near the lateral edge of the muscle and contractions in damaged fibers of the medial edge, across the bridge, were observed through a dissecting microscope (see Fig. 1). Number of bridges tested at each time is shown in parentheses.

bridge, axonal branches left the perineurial tubes to run along the basement membrane sheaths (Fig. 9*b*), a pattern similar to that seen at normal (Fig. 9*a*) and regenerating (27) neuromuscular junctions. Like axons reinnervating undamaged muscle (27) (although unlike normal terminals), axons often left the sheaths and ran for some distance at an angle to the sheath's long axis (Fig. 9*b*) before ending on another sheath or in the connective tissue of the bridge. Thus, in some respects, the bridge was reinnervated in a manner similar to that observed in undamaged muscle.

Nerve terminals in the bridge—i.e., axonal processes closely associated with myofiber BL—were characterized by electron microscopy. Like normal or regenerating nerve terminals in undamaged muscle, terminals in the bridge bear 50-nm diameter agranular synaptic vesicles in a cytoplasmic matrix of relatively low electron density and are coated by a thin glycocalyx that stains intensely with ruthenium red (Figs. 7*a* and *b*, and 10). The terminals varied from ~0.2-1.5 μm in diameter. Some were wrapped in Schwann cell processes which, in turn, lay on the myofiber BL (Fig. 10*a* and *b*). Others were capped by Schwann cell processes but had a portion of their surface directly apposed to BL (Fig. 10*c*). In some cases, two or three terminals lay side-by-side, wrapped or capped by the same Schwann cell process. Finally, terminals frequently lay in the lumen of the BL sheaths (Fig. 10*d*). The wide variation in the size of terminals and in the relationships between terminals and Schwann cells seen in the bridge is also characteristic of early stages of reinnervation in undamaged muscle[1] (17, 27, 29, 45). However, the occurrence of terminals within the sheaths is, to our knowedge, unique to the bridge.

Whatever their size or configuration, terminals in the bridge could be distinguished from Schwann cell processes and other cellular elements because they had (*a*) more 50-nm vesicles, (*b*) a less electron-dense cytoplasm, (*c*) a more prominent glycocalyx, and (*d*) no rough endoplasmic reticulum (Figs. 7 and 10). Our ability to recognize the nerve terminals easily in the electron microscope facilitated quantitative studies of their growth, distribution, and differentiation.

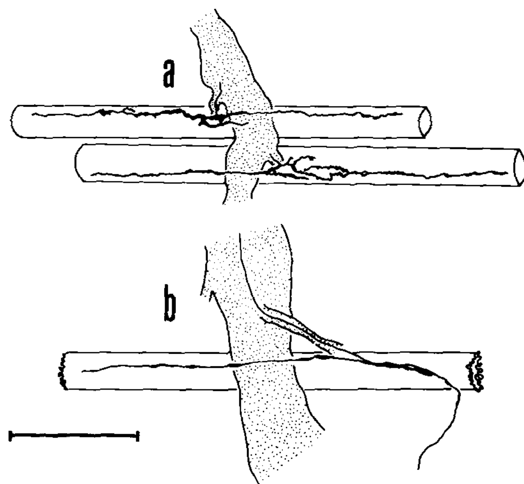

FIGURE 9 Terminal arborizations on myofibers in normal muscle (*a*) and on a basement membrane sheath in a bridge, 4 wk after denervation, muscle damage and X-irradiation (*b*). Camera lucida drawings from whole mounts of zinc iodide/osmium-stained preparations; placement and dimensions of BL in Fig. 9*b* were confirmed by electron microscopy. Axons exit from perineurium (stippled) and run along myofiber (*a*) or its surviving BL (*b*). Like terminals in reinnervated undamaged muscle (21), terminals in the bridge leave BL and continue to grow, while normal terminals end abruptly on the myofiber's surface. Bar, 100 μm.

Precise Reinnervation of Original Synaptic Sites

In vertebrate skeletal muscles (2, 15), including the cutaneous pectoris muscle of the frog (27), reinnervation after a nerve crush is topographically precise: the damaged axons grow back into the muscle and cover a large fraction of the original postsynaptic membrane, while making few contacts elsewhere on the muscle fiber's surface. We have previously shown that this topographic specificity does not require the integrity of the original myofiber for its expression: in unirradiated bridges, regenerating axons contact regenerating myofibers almost exclusively at original synaptic sites on the BL (31). Because irradiation inhibits regeneration of myofibers in the bridge without blocking regeneration of axons, we were able to extend these results.

Our principal finding is that in the absence of muscle, as in its presence, regenerating axons contact the BL almost exclusively at original synaptic sites. This precision is apparent whether one uses the BL of junctional folds (Fig. 10*a* and *c*) or the ChE of the synaptic BL (Fig. 11) as markers of original sites (31). To quantify the precision of reinnervation, we used ChE-stained preparations. Regions rich in ChE-stained synaptic sites were identified in whole mounts, the appropriate blocks of plastic were cut out, and basement membrane sheaths were cross-sectioned for electron microscopy. In a single section from each block, we counted all of the ChE-stained patches of BL–i.e., original synaptic sites–and noted which were apposed by nerve terminals (or by the Schwann cell processes that enwrapped terminals). We then followed the perimeter of each sheath, searching for terminals that lay (or whose Schwann cell wrapping lay) within 0.1 μm of the BL. We also searched many profiles that did not have Che-stained patches but were in the vicinity of regenerating axons. Terminals that lay within the lumen of a BL sheath were excluded from this survey because their relationship to the BL was so clearly aberrant.

Fig. 12 shows the extent to which original synaptic sites were reinnervated after a nerve crush. In normal muscles–i.e., muscles that were neither damaged nor irradiated–nearly all of the original postsynaptic membrane was covered by regenerated nerve terminals within a month after the nerve was crushed. In the absence of myofibers–i.e., in muscles that were damaged and irradiated–reinnervation was slower and less complete. Nevertheless, by 3 wk after the nerve was crushed, ~40% of the ChE-stained patches were apposed by nerve terminals (120 of 298 in seven bridges). Thus, a large fraction of the original synaptic sites were reinnervated in the absence of the myofiber. The subsequent decline (3-5 wk after surgery) will be discussed below.

In control experiments, we studied reinnervation of muscles that were either damaged or irradiated (Fig. 12). Myofibers regenerated within 2 wk in unirradiated bridges (Figs. 2 and 4*c*). Reinnervation began at the same rate in these bridges as in undamaged muscle, but did not proceed to completion. Some sites remained uncovered, perhaps because access to them was blocked by connective tissue that built up after surgery; a similar explanation has been advanced to account for the incomplete reinnervation of atrophied mammalian muscle (19). In uninjured, irradiated muscles, myofibers survived without apparent lesion for at least 5 wk (see also reference 38), but reinnervation was delayed by about a week. Among the factors that might account

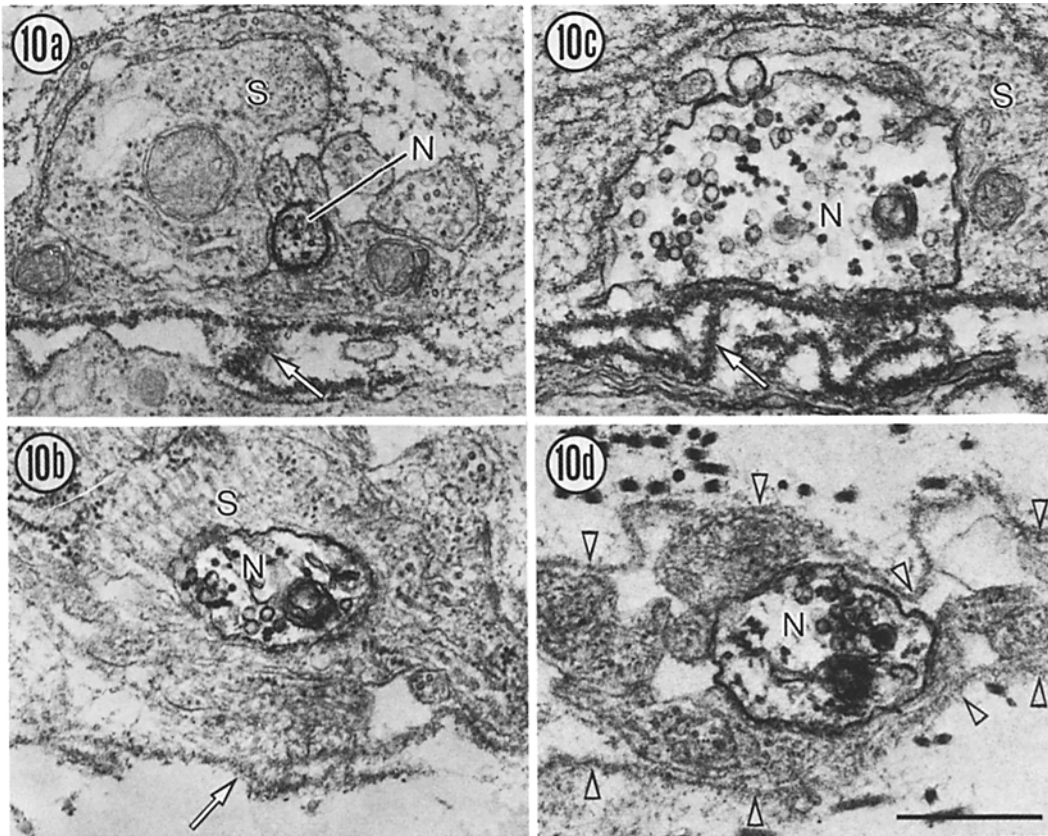

Figure 10 Nerve terminals (*N*) that reinnervate basement membrane sheaths after removal of the myofiber vary in size and in their relationship to the surviving BL. Terminals in Fig. 10*a* and *b* are wrapped in Schwann cell processes (*S*), whereas that in Fig. 10*c* directly opposes the myofiber's BL. Terminal in Fig. 10*d* lies within the lumen of the BL sheath (outlined by arrowheads). Terminals in Fig. 10*a–c* are at original synaptic sites, as shown by BL of junctional folds (arrows). From irradiated bridges fixed 21 days after denervation and muscle damage. Bar, 0.5 μm.

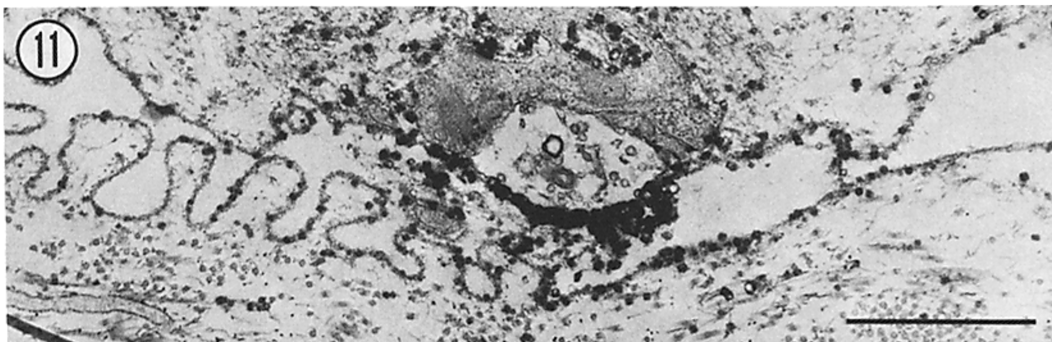

Figure 11 Regenerated nerve terminal in an irradiated bridge, 21 days after denervation and muscle damage. Axon has returned to contact BL of original synaptic cleft, marked here by the histochemically demonstratable ChE that it contains. Bar, 1 μm.

for this delay are poor health of irradiated animals, radiation-induced injury of the motoneurons (14), and inhibition of Schwann cell proliferation (10) that helps to repair perineurial pathways after damage to the nerve (20, 53). Thus, the rate and extent to which sheaths are reinnervated in the absence of myofibers can be largely explained by the separate effects of the two procedures that we use to empty the sheaths: cutting the muscle leads to incomplete reinnervation and irradiation induces a delay.

Fig. 13 shows that nearly all of the terminals that contacted the BL did so at original synaptic sites. This precision was observed in normal muscle, in irradiated but undamaged muscle, in unirradiated bridges where new myofibers regenerated within the sheaths, and in irradiated bridges where only a few percent of the sheaths contained myofibers. Most of the terminals that were not at original sites were probably approaching or leaving them – they were generally near ChE-st ined patches, and collagen fibrils lay between theaterminal (or its Schwann cell sheath) and the BL.

These results suggest that growing axons can reject nonsynaptic BL, a hypothesis that we tested in the following way: a "foreign" nerve was implanted onto the bridge far from the entry of the "native" nerve (Fig. 1) so that regenerating axons would have to grow past long stretches of extrasynaptic BL if they were to reach synaptic sites. 3–4 wk later, the bridges were fixed, stained for ChE to mark original synaptic sites, and cross-sectioned. Electron microscopy revealed that many axonal processes ran through the bridge and some of them approached the basement membrane sheaths. In each of five bridges studied, nearly all of the terminals that were within 0.1 μm of the BL (but were not in the lumen of the sheaths) were situated at ChE-stained sites (Table I). Thus axons contact synaptic BL and reject nonsynaptic Bl when they return to the basement membrane sheaths.

Because the BL is quite clean by the time reinnervation occurs (compare Figs. 6 and 12), it is unlikely that degenerating cellular or membranous remnants of the myofibers account for the precise reinnervation of original synaptic sites. This conclusion gains support from experiments in which we denervated and damaged muscles, delayed reinnervation for up to 2 wk by recrushing the nerve at 4–7-day intervals, and then allowed reinnervation to proceed for 2 or 3 wk. Regenerating axons covered as much original synaptic BL (Fig. 14*a*) and made as few contacts elsewhere on the sheaths (Fig. 14*b*) when reinnervation was delayed as they did when reinnervation was prompt. The cues used by regenerating axons to find or select original sites must therefore be stable for several weeks after the myofiber is removed.

Differentiation of Regenerating Axons into Nerve Terminals

In normal muscle, the portion of the motor axon that contacts the muscle fiber – i.e., the motor nerve terminal – differs from other portions of the axon in many respects. For example, terminals bear active zones (complexes of membrane-associated organelles, thought to be sites of transmitter release; references 11, 22, 37) and numerous synaptic vesicles, whereas preterminal regions of the axon contain no active zones and relatively few vesicles. When undamaged muscle is reinnervated, axons differentiate into terminals where they approach within 0.1 μm of the muscle fiber's surface[1] (29, 45). Electron microscopic analysis showed that terminals in the bridge (axonal processes associated with myofiber BL) also differentiate, becoming morphologically distinguishable from their parent axons.

In the bridge, the density of synaptic vesicles is much higher in terminals than in preterminal axons (Fig. 15). The accumulation of vesicles in terminals is evident (*a*) when terminals are compared with a random sample of axonal profiles (Table II*A*), or (*b*) when they are compared with any of three morphologically distinguishable classes of axons (myelinating axons within perineurial tubes, unmyelinated axons within perineurial tubes, and unmyelinated axons running through the connective tissues of the bridge outside of perineurial tubes; Table II*B*). Also, terminals contain fewer neurofilaments and microtubules than do preterminal axons (Fig. 15).

Another sign of the differentiation of nerve terminals in the bridge is that terminal but not preterminal regions of the axons contain active zones. The active zones are characterized by a cytoplasmic density adherent to the inner leaflet of the plasma membrane, and a focal accumulation of synaptic vesicles (Fig. 16*b*). The densities are elongated and the vesicles that flank them are arranged in rows (Fig. 16*c*). These features are

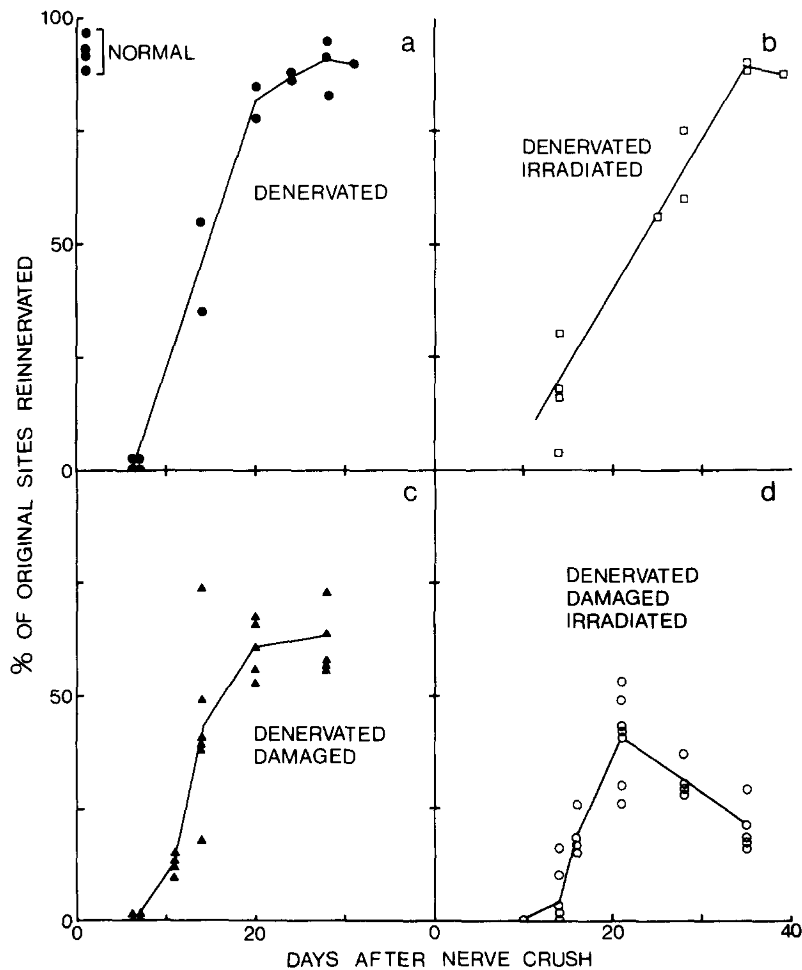

FIGURE 12 Figures 12 and 13 together show that original synaptic sites on BL are reinnervated precisely whether or not myofibers are present. Fig. 12 shows the extent to which original sites, marked by ChE stain, are reinnervated at various times after nerve crush. Each point in Fig. 12*a–d* represents the percentage of ChE-stained patches apposed by nerve terminals in one muscle or bridge; at least 30 patches were counted in a cross section from each preparation. Lines, drawn through mean of all muscles at each time point in Fig. 12*a–d*, are redrawn in Fig. 12*e* for comparison. Four normal muscles were sectioned as controls; >90% of their ChE-stained patches were clearly innervated (*a*). All other muscles were denervated on day 0. Muscles in Fig. 12*c* and *d* were damaged to make bridges at the time of denervation. Muscles in Fig. 12*b* and *d* were X-irradiated on day 3.

similar to those revealed when terminals in normal frog muscle are sectioned (Fig. 16*a*) (11, 22, 37).

A prominent characteristic of normal (5, 34) and regenerated[1] (45) nerve terminals in undamaged muscle is that organelles are asymmetrically distributed within them: synaptic vesicles are concentrated on the side of the terminal that faces the muscle fibers, mitochondria are concentrated on the opposite side, nearer the Schwann cell cap, and active zones are found only on the presynaptic membrane, directly opposite the muscle fiber's surface. Organelles are also asymmetrically distributed within nerve terminals in the bridge. Portions of terminal profiles enwrapped in Schwann cell processes contained no active zones and had fewer vesicles than portions that contacted the myofiber BL (Fig. 10*a–c*, Table II*C*). In terminals that directly apposed the BL of the synaptic cleft, nearly two-thirds of the synaptic vesicles (65 ± 2%, mean ± SE in 52 terminals)

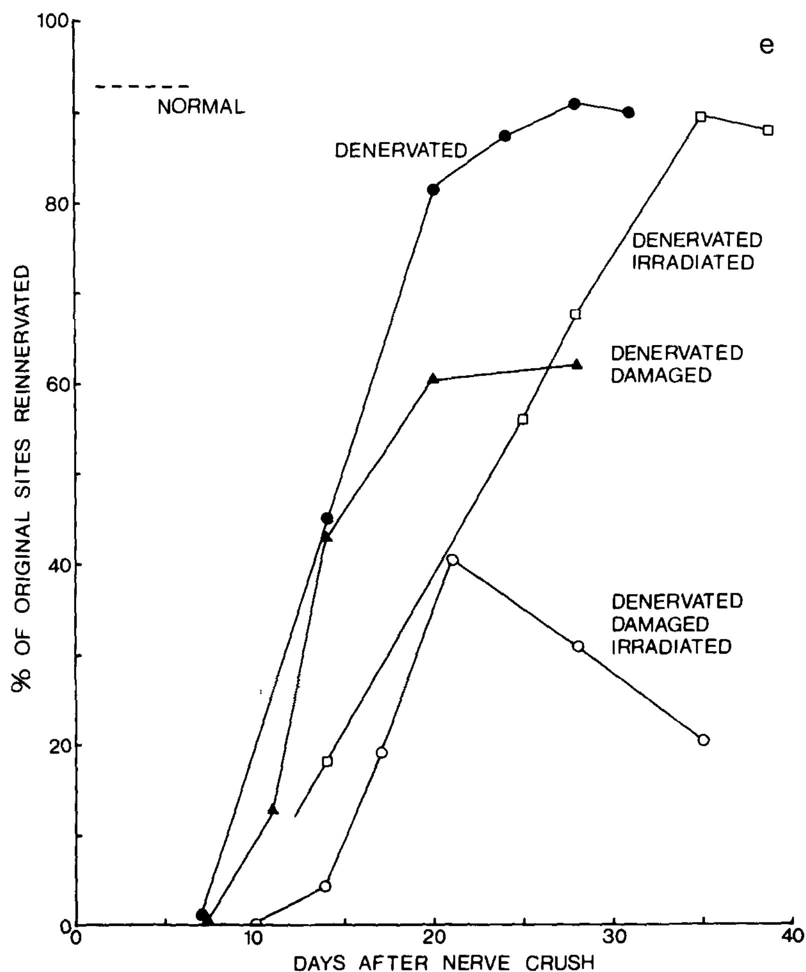

FIGURE 12*e*

lay in the half of the terminal that faced the cleft (Table II*D*). Mitochondria accumulated in the other side of these terminals—61% (36 of 61 in 52 terminals) lay in the half of the terminal that faced the Schwann cell cap. Furthermore, active zones occurred only on the portion of the nerve terminal's surface that abutted the BL of the myofiber sheath.

Thus, the nerve terminals acquire a normal complement of synaptic organelles and become morphologically polarized in a normal way even when there is no myofiber lying beneath the "presynaptic" membrane. The association of synaptic vesicles and active zones with the BL of the synaptic cleft suggests that components in the BL might play a role in triggering the transformation of the regenerating axon into a nerve terminal. The next section describes a test of this proposition.

Differentiation of Nerve Terminals is Organized by Basal Lamina

Synaptic vesicles and active zones are not evenly distributed along the length of normal frog motor nerve terminals. Instead, they are associated with junctional folds: there are more vesicles above folds than in between them (5, 34), and the cytoplasmic densities of the active zones lay precisely opposite the mouths of the folds (5, 11). The folds persist in denervated but undamaged muscle for many months (6, 27, 45). When original synaptic sites are reinnervated, vesicles and active zones are once again found associated with

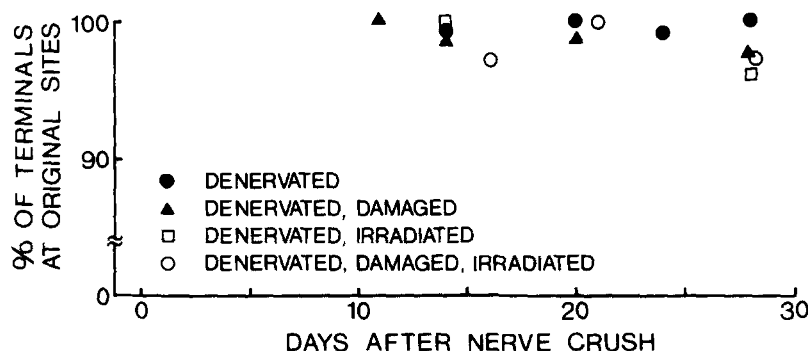

FIGURE 13 Nearly all of the terminals within 0.1 μm of myofiber BL are situated precisely at a ChE-stained patch. Each point represents the summed data from two to five of the preparations presented in Fig. 12; at least 20 cross-sectioned BL sheaths, each bearing one or more ChE-stained patches, were examined in each preparation. In most preparations, nearby sheaths without ChE-stained patches were also surveyed. Symbols as in Fig. 12.

TABLE I

Precise Reinnervation of Original Synaptic Sites in the Bridge by Axons Regenerating from a "Foreign" Nerve

	Axonal processes		
Bridge	Total	Within 0.1 μm of myofiber BL	At original synaptic sites on myofiber BL
1	226	11	11
2	79	12	10
3	136	10	9
4	129	12	10
5	118	8	7
Total	688	53	49

Axonal processes were counted in a single cross section from each of five irradiated bridges fixed 3–4 wk after surgery. 92% (49/53) of the processes that were within 0.1 μm of surviving BL sheaths were situated at the ChE-stained original synaptic sites.

the original synaptic folds.[1] Thus, there must be factors near the folds that organize the differentiation of nerve terminals.

When muscles are cut to make a bridge, the plasma membrane and cytoplasm of the folds are lost. However, as shown above, the projections of BL that extend into the folds survive after the myofibers degenerate (Fig. 7*c*, *e*, and *f*), marking the sites where folds had been: regenerating axons form new terminals directly opposite these sites (Figs. 10 and 15). If factors that organize the differentiation of nerve terminals were located in the BL, one might expect that vesicles and active zones would be preferentially localized above intersections of cleft and fold BL.

To seek such relationships, we studied cross sections of five bridges that were fixed 3 wk after they were denervated, damaged, and X-irradiated. We collected micrographs that showed nerve terminals directly apposed to the BL of the muscle fiber's sheath and projections of BL whose connection to the sheath lay beneath the terminal. Micrographs that met these criteria (e.g., Figs. 10*c* and 15*b*) were analyzed as shown in Fig. 17*a*. To calculate the density of synaptic vesicles near to and far from intersections of fold and cleft BL, the distance along which the nerve terminal's plasma membrane contacted the myofiber's BL was measured, and all synaptic vesicles situated (entirely or in part) within 100 nm of this membrane were counted. Vesicles within 200 × 100-nm rectangles centered on the intersections (Fig. 17*a*) were considered to be near intersections whereas those elsewhere in the juxtamembranous strip were classified as far from intersections. To quantify the association of active zones to fold sites, we measured the distance from the midpoint of each active zone's cytoplasmic density to the nearest intersection of fold and cleft BL.

Synaptic vesicles accumulated above the intersections of fold and cleft BL: the density of vesicles was over twice as high within 100 nm of the intersections as elsewhere along the region of contact between nerve and BL (Fig. 17*b*). Comparison of regions within single cross sections showed that the ratio of vesicle densities near to and far from intersections varied from terminal to terminal (Fig. 17*c*). However, the mean ratio, 2.9 ± 0.4 to 1 (mean ± SE, n = 84), was signifi-

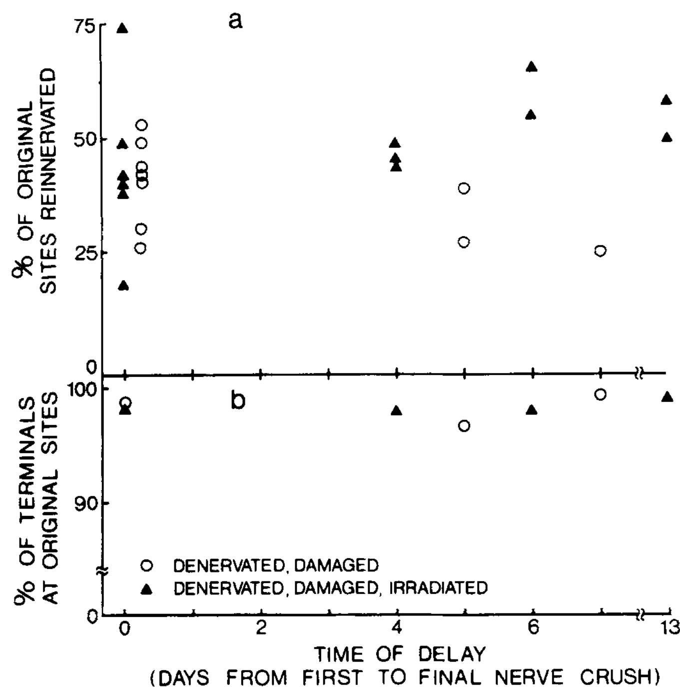

FIGURE 14 Original synaptic sites in the bridge are reinnervated precisely whether or not reinnervation is delayed by repeated nerve crush. Nerve was recrushed at 4–7 day intervals. Bridges were examined 14 (unirradiated bridges, ▲) or 21 days (irradiated bridges, ○) after the final nerve crush. Data was gathered by methods detailed in legends to Figs. 12 and 13.

cantly ($P < 10^{-5}$; Student's t test) greater than the ratio of 1 that would be expected if vesicles were not specifically associated with intersections. The high median ratio (=2; Fig. 17*c*) provides assurance that the associations we saw did not result from the disproportionate contribution of a small population of terminals with particularly high ratios. Furthermore, even in sections that did not display active zones (see below), the mean ratio was 2.2, significantly >1.

We found a total of 32 active zones in our set of micrographs. The region of contact between BL and terminals with active zones averaged 1.2 μm in length. Intersections of fold and cleft BLs were broadly distributed along this region of contact. If active zones were randomly distributed over this distance, one would expect (from probability theory) a mean distance of nearly 0.4 μm between the center of an active zone's cytoplasmic density and the nearest intersection. Instead, the mean separation was only 81 nm, and 88% (28/32) of the cytoplasmic densities were within 100 nm of an intersection (Fig. 16*d*). If anything, our measurements overestimate the distance from active zones to intersections, since some projections of BL may have been damaged or unidentifiable. Furthermore, active zones may have been closer to intersections in adjacent, unexamined sections than to the intersections that we saw.

Thus, the clustering of synaptic organelles that occurs above the mouths of junctional folds when undamaged muscle is reinnervated[1] occurs above intersections of fold and cleft BL when axons reinnervate the bridge. It is therefore difficult to escape the conclusion that components contained in or tightly connected to the BL of the synaptic cleft play a role in organizing the differentiation of the regenerating axon into a nerve terminal.

Fate of Regenerated Nerve Terminals

The experiments described so far demonstrate that axons regenerate to basement membrane sheaths in the bridge and that, once there, they differentiate into nerve terminals. We have not studied bridges after long periods of reinnervation, and we therefore do not know the eventual fate of the nerve terminals. However, the amount of original synaptic surface that regenerating ter-

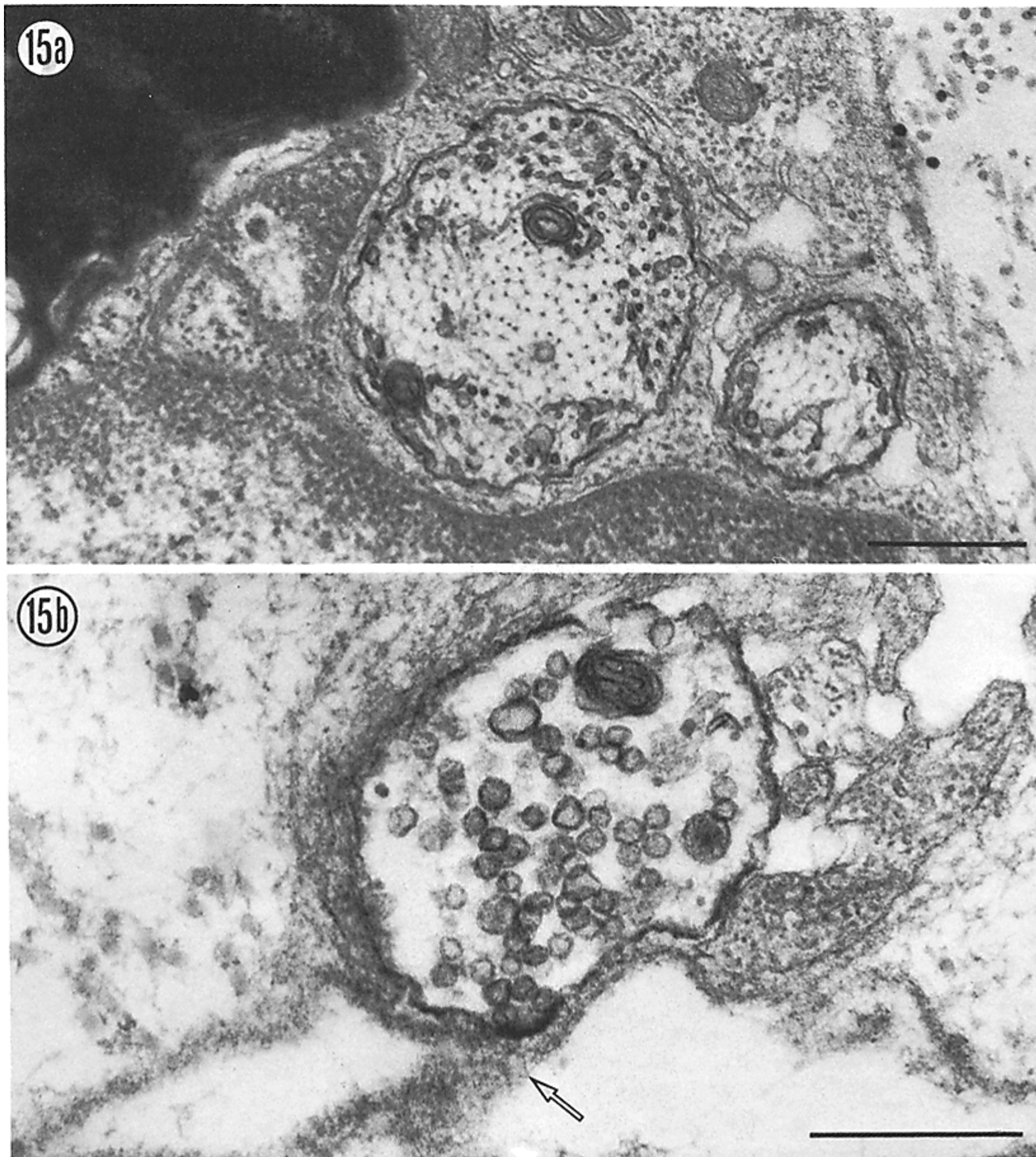

FIGURE 15 Preterminal and terminal portions of axons reinnervating the irradiated bridge 21 days after muscle was damaged and denervated; by morphological criteria, terminals have differentiated. (*a*) Axons in the nerve trunk, wrapped by a Schwann cell, contain many neurofilaments and microtubules but few synaptic vesicles. (*b*) Nerve terminal, apposed to BL of the myofiber sheath, 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.

minals cover decreases 3-5 wk after nerve crush, suggesting that some terminals may retract and/or degenerate after they form. Electron microscopy of ChE-stained preparations showed that 41 ± 4% (mean ± SE of counts from seven bridges) of the original synaptic BL is reinnervated at 3 wk, 31 ± 2% (n = 4) at 4 wk, and 20 ± 3% (n = 5) at 5 wk; the difference between 3 and 5 wk is highly significant ($P < .002$; Student's t test). Although the procedures that we use are traumatic, the decline is not simply a consequence of surgery or irradiation, since it was not observed in muscles

TABLE II

Distribution of Synaptic Vesicles in Axons Reinnervating Irradiated Bridges

Region	n	Vesicles/profile	Vesicles/μm^2*
A. All axonal profiles			
Preterminal axons	95	6.1 ± 0.8	6.7 ± 1.3
Terminals	95	17.6 ± 2.2	50.0 ± 5.3
B. Preterminal axons			
Myelinating	11	18.3 ± 3.3	2.2 ± 0.4
Unmyelinated, in perineurium	38	5.6 ± 1.0	4.7 ± 0.8
Unmyelinated, outside of perineurium	46	3.7 ± 0.8	9.5 ± 2.5
C. Terminals			
Wrapped in Schwann cell process	43	9.5 ± 2.7	26.9 ± 4.1
Abutting myofiber BL	52	24.2 ± 3.1	69.1 ± 8.2
D. Terminals abutting myofiber BL			
Half-profiles closest to myofiber BL	52	15.4 ± 1.3	81.8 ± 8.6
Half-profiles farthest from myofiber BL	52	8.2 ± 1.3	52.8 ± 7.6

Profiles were selected at random from cross sections through three irradiated bridges fixed 3 wk after denervation and muscle damage. Data is given as mean ± 1 SE for indicated number of profiles. Numbers joined by brackets differ significantly ($P < 0.01$; Student's t test).

* Means of values calculated separately for each terminal.

that were only damaged or irradiated (Fig. 12). This observation suggests that myofibers may play a role in the maintenance and/or maturation of nerve terminals, a hypothesis that we are currently testing.

DISCUSSION

The Bridge of Basement Membrane Sheaths

When we cut the cutaneous pectoris muscle to make a bridge (Fig. 1), the muscle fiber's cytoplasm clumps and retracts, leaving behind the sarcolemmal sheaths of the damaged segments. (In this respect, our observations repeat those of Bowman, who discovered the sarcolemmal sheath in 1840 while studying mechanically injured muscle fibers [7].) As in injured mammalian muscle (9, 24, 39, 46), the myofibers' disrupted cytoplasm and plasma membrane degenerate and are phagocytized in the bridge, but the BL of the sheaths survives. Thus, injury and its sequelae cleave the surface complex of the myofiber between the plasma membrane and the BL (see Fig. 3); we do not know the fate of the glycocalyx.

Muscles can be damaged in many ways, but the bridge is particularly well suited for studies that concentrate on nerve-muscle interactions. Cutting the muscle into short segments results in more uniform and complete degeneration of myofibers than occurs after chemical, thermal, or ischemic injury (4, 9, 13, 24, 39, 46, 50). In contrast to procedures in which the muscle is excised, minced, and then reimplanted in its bed to insure complete degeneration (9), the orientation of the damaged segments and the integrity of the intramuscular connective tissue are relatively unaffected when the bridge is made. The bridge also preserves the main nerve trunk, which expedites reinnervation after nerve crush (20, 23, 53), and at least some of the vascular supply, which speeds phagocytosis of disrupted myofibers by blood-borne macrophages (9, 39). In addition, because the bridge spans the center of the muscle's innervation band, many synaptic sites are preserved. Synaptic specializations of the basement membrane sheath—ChE (33), BL of junctional folds (13, 31), and BL of Schwann cells—permit identification of these sites even after the myofiber and terminal degenerate and Schwann cell processes retract.

X-irradiation of the bridge inhibits the regeneration of myofibers (32, 42, 52) that would otherwise occur (9, 24, 31) but does not block phagocytosis of damaged nerve and muscle. At the dose we used, irradiation does not prevent regeneration of motor axons, even though the motoneurons that innervate the cutaneous pectoris lie in the thoracic spinal cord and are irradiated along with the bridge.

Precise Reinnervation of Original Synaptic Sites

Axons regenerating to denervated muscle form new neuromuscular junctions precisely at the sites

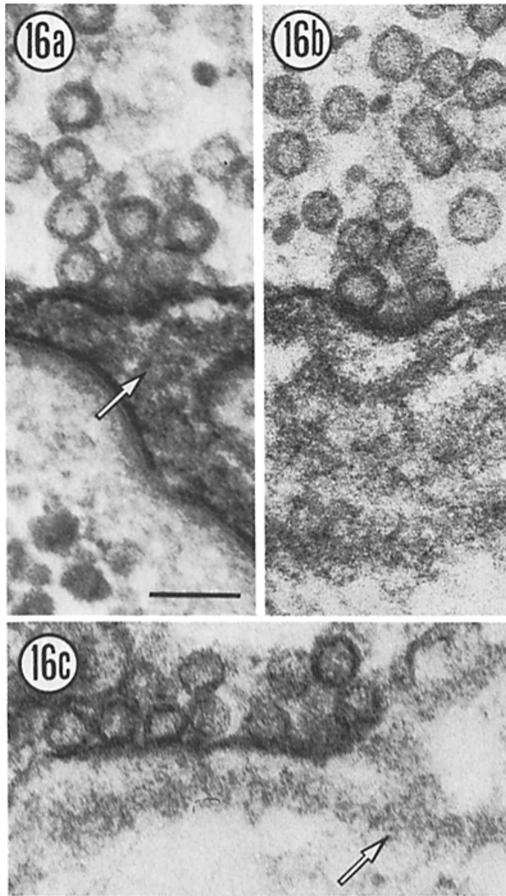

FIGURE 16 Active zones in nerve terminals of the bridge resemble those in normal neuromuscular junctions. (*a*) Normal muscle. (*b, c*) Irradiated bridge, 21 days after denervation and muscle damage. Cross-sectioned active zones in Fig. 16*a* and *b* show cytoplasmic densities on the plasma membrane flanked by synaptic vesicles that approach the membrane closely. Longitudinally sectioned active zone in Fig. 16*c* shows vesicles arranged in a row. Cytoplasmic densities of active zones are near intersections (arrows) of synaptic cleft and junctional fold BL, both in normal muscle (*a*) and in the bridge (*c*). Bar, 0.1 μm.

of the original synapses where new nerve terminals cover the old junctional folds. Although synapses sometimes form at completely new sites (2, 15), preferential reinnervation of original sites has been documented in mammalian, avian, and amphibian muscles reinnervated by their own or by foreign nerves (1-3, 15, 17, 19, 26, 29, 41, 48). This topographic specificity is nowhere more striking than during reinnervation of frog muscle after a nerve crush as occurred in this study: regenerating axons cover nearly all of the postsynaptic membrane while making few if any contacts elsewhere on the muscle fiber's surface (Figs. 12 and 13 and reference 27).

One aid to precise reinnervation is provided by tubes of perineurial and Schwann cells that survive in the motor nerve long after damaged axons have degenerated. Regenerating axons often grow through these tubes (19, 20, 23, 27, 41) and (like regenerating sensory axons; see, for example, reference 51) are thus guided to original synaptic sites. However, regenerating axons can unerringly cover long stretches of synaptic surface after they leave the perineurium, lifting off the muscle fiber at the end of the synaptic site (27). Also, axons growing beyond or outside perineurial tubes can "select" and precisely reinnervate original sites (1, 19, 27). Thus, there must be factors at the synaptic site itself that provide cues to regenerating axons.

The nature and precise location of these cues is not known. One attractive possibility has been that the postsynaptic cell – the myofiber – contains the factors that guide reinnervation of original synaptic sites (1, 15, 41). Our results show, however, that the myofiber need not be present for precise innervation of original sites to occur. Myofibers may originally produce (or be required for the production of) factors that make axons prefer original sites, but these factors are present and can be maintained for several weeks outside of the myofibers.

What and where might these factors be? Among the possibilities that remain to be tested are the following: (*a*) the reticular lamina, which coats extrasynaptic but not synaptic portions of the BL, might act as a mechanical barrier to regenerating axons, shielding them from contact with extrasynaptic areas of the muscle fiber; (*b*) the basement membrane of the Schwann cell, which forms a tunnel over the synaptic site (Fig. 7*f*) is certainly not impermeable to axons (19, 27), but it may, like Schwann tubes in the nerve trunk (20), provide mechanical guidance to restrain and/or orient axonal growth; (*c*) complementary molecules on the surfaces of Schwann cells and axons might provide a basis for recognition by intercellular adhesion, as has been proposed for a variety of neural and nonneural tissues (8, 43); (*d*) axons may recognize extracellular molecules that are differentially distributed between synaptic and ex-

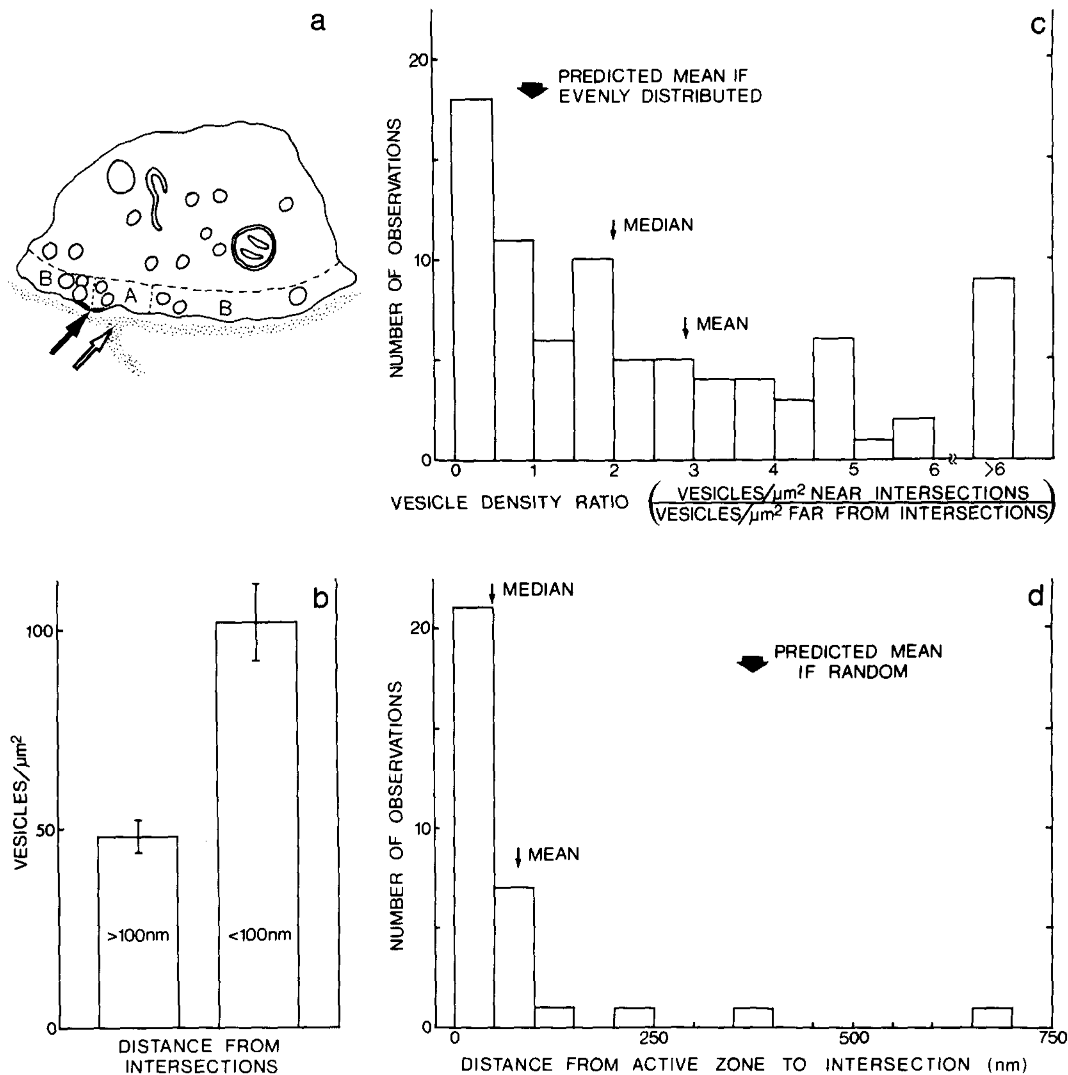

FIGURE 17 Synaptic vesicles and the cytoplasmic densities of active zones are associated with intersections of synaptic cleft and junctional fold BL in the absence of myofibers. (*a*) Tracing of nerve terminal from 1 of 84 electron micrographs from which data shown in Fig. 17*b–d* was collected, to show method of analysis. Micrographs were obtained from five irradiated bridges fixed 3 wk after denervation and muscle damage. (*b*) Vesicles situated in the 100-nm high strip of nerve terminal cytoplasm nearest the myofiber's BL were counted. Vesicle density (vesicle/μm^2) was calculated separately for areas within 100 nm of interactions between synaptic cleft and junctional fold BL (see *A* in Fig. 17*a*), and for areas farther than 100 nm from intersections (see *B* in Fig. 17*a*). Graph shows mean vesicle density (±SE) in each of these two areas. (*c*) Vesicle density ratios (density within 100 nm of intersection/density further than 100 nm from intersection but within the juxtamembranous strip) was calculated separately for each of the 84 terminals. Mean and median ratios are both clearly higher than the mean ratio of 1 that a random distribution would produce. (*d*) For each of 32 active zones, the distance from the center of the cytoplasmic density (see closed arrow in Fig. 17*a*) to the nearest intersection of fold and cleft BL (see open arrow in Fig. 17*a*) was measured. Average separation would be nearly 400 nm if active zones were randomly distributed alone zone of nerve terminal-BL contact (see text); in fact, most cytoplasmic densities are <100 nm from an intersection.

trasynaptic regions of the basal lamina. Regenerating axons might be repelled by molecules that are concentrated in extrasynaptic BL, or be immobilized by (i.e., adhere to) molecules (such as ChE [33]) that are concentrated in the synaptic BL.

Differentiation of Nerve Terminals: A Morphogenetic Role for Basal Lamina

Synaptic vesicles accumulate and active zones form in portions of regenerating axons that approach basement membrane sheaths in the bridge. At least by these morphological criteria, axons can differentiate locally into "presynaptic" nerve terminals in the absence of a "postsynaptic" cell. The spatial relationships of synaptic vesicles and active zones in these terminals to extra-axonal structures (e.g., Schwann cells and BL) leave little doubt that extrinsic factors trigger and organize the transformation of axons into nerve terminals.

The differentiation of the nerve terminal is a complex process, and we do not know how many factors are required for its occurrence. Among the cells that might play a role in this differentiation are the Schwann cells that remain at denervated synaptic sites and the unidentified cells – probably including presumptive myoblasts (9, 39, 46) – that lay within the basement membrane sheaths in the bridge. Neurons growing in vitro can be stimulated to differentiate by glial (36) and muscle (16) cells. It is possible that similar trophic interactions occur in the bridge; our results neither demonstrate nor rule out their existence. We have, however, obtained evidence that at least one morphogenetically important factor is located and stably maintained extracellularly, in the BL of the original synaptic cleft.

The involvement of the BL in axonal differentiation is suggested by the observation that synaptic vesicles accumulate preferentially and active zones appear only in portions of terminals that abut the BL of the myofiber sheath. More direct evidence comes from analyzing the distribution of these synaptic organelles along regions of contact between terminals and BL at original synaptic sites. In normal (5, 11, 22, 34, 37) and reinnervated[1] muscles, vesicles and active zones are concentrated directly opposite the mouths of the junctional folds that periodically indent the postsynaptic membrane. After the cytoplasm and plasma membrane of the myofiber have been removed, these sites can be recognized as points where the projections of BL that once lined the folds join the BL of the synaptic cleft. The vesicles and active zones of terminals in the bridge are closely associated with these points (Fig. 17), demonstrating that molecules periodically arranged along the BL of the original synaptic cleft play some role in triggering and/or organizing the differentiation of regenerating nerve terminals. The extracellular location and apparent stability of these molecules is likely to facilitate attempts to isolate them and to elucidate their mechanism of action.

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