The Journal of Neuroscience, September 1994, 14(9): 5672–5686

Regenerating Muscle Fibers Induce Directional Sprouting from Nearby Nerve Terminals: Studies in Living Mice

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The principal aim of this work was to better understand how regenerating muscle fibers become innervated in adult animals. To induce muscle regeneration, individual identified muscle fibers in a mouse were damaged with a laser focused through a microscope. The muscle fiber that degenerated and the muscle fiber that was formed in its place were followed by viewing the same site repeatedly over a period of 2 d to 40 weeks. Commonly, the nerve terminal innervating the irradiated muscle fiber partially retracted during muscle fiber degeneration, and then sprouted to innervate the regenerating muscle fiber at the same site it had previously innervated the muscle fiber that was damaged. During the early phase of muscle regeneration we also observed sprouts originating from nerve terminals on adjacent muscle fibers. The new nerve growth was a response to the regenerating muscle fiber rather than to the degenerated fiber it replaced because repeated damage of the same site every 2–3 d over a 10 d period (to prevent regeneration) did not cause any sprouting.

The direction of the sprouts on adjacent muscle fibers showed a bias toward the regenerating muscle fiber, although they avoided the region occupied by the original nerve terminal. Forty percent of the sprouts managed to reach the regenerated fiber. Nonetheless, by 11 d after muscle fiber damage, all sprouts had regressed, leaving the new fiber innervated by the same motor axon that innervated the fiber that was damaged. On the other hand, when the overlying nerve terminal as well as the muscle fiber was damaged, the sprouts from nearby muscle fibers were both more numerous and more stable, and in five cases we observed two or more new synaptic junctions on the regenerating fiber originating from different axons. In one case we witnessed a protracted competition between the original motor axon as it sprouted back and the sprouts from nearby junctions for sole innervation of the regenerate. Ultimately, the surviving sprouts myelinated and became the permanent and exclusive input to the new fiber.

These results indicate that regenerating muscle fibers emit a signal that induces directional sprouting from nearby undamaged nerve terminals. Reinnervation of the regenerating muscle fiber by one axon apparently prevents the maintenance of such neurites. Because the process of muscle regeneration shares many features in common with myogenesis during embryonic development, it is likely that developing muscle fibers present an analogous stimulus to ingrowing motor axons. Furthermore, the well-documented sprouting observed following partial denervation seems similar to the sprouting observed in this study, arguing that denervated muscle fibers use a mechanism to attract axons similar to that used by newly forming muscle fibers.

[Key words: neuromuscular junction, vital staining, muscle regeneration, laser ablation, sprouting, axon outgrowth, competition, synapse elimination]

Sprouting of intact motor axons following denervation of target cells is a well-known phenomenon (Brown et al., 1981; Wernig and Herrera, 1986). It is generally assumed that sprouting is part of the process by which muscle cells, having lost their innervation, initiate their reinnervation, perhaps by reiterating a program similar to the one that occurs in development when target cells are first innervated (for review see Brown, 1984).

The putative signal emanating from denervated muscle that induces motoneurons to sprout is not known, although several growth factors are candidate molecules (Caroni and Grandes, 1990; Gurney et al., 1992; see McManaman and Oppenheim, 1993, for review). For motoneurons, and other sorts of neurons as well, there is some evidence that axons can respond to neurotrophic factors and other chemicals by directional growth (Menesini-Chen et al., 1978; Gundersen and Barret, 1979; Lumsden and Davies, 1986; Kuffler, 1989). Nonetheless, it is not known if sprouts are actually stimulated by denervated muscle fibers to grow toward them.

The possibility of motor axons sprouting in particular directions may be clinically important because of recent interest in implantation of healthy myoblasts as a therapeutic measure for curing diseased muscle (Partridge et al., 1989; Morgan and Partridge, 1992; see also Griggs and Karpati, 1990). Obviously, new muscle fibers generated from implanted myoblasts require innervation. It is not known, however, how (and whether) newly forming muscle generally become innervated in adult muscles. Presumably, undamaged motor axons will need to sprout toward these regenerating muscle fibers and establish synapses. One of our aims in this study was to provide some basic information concerning the way intact axons respond to regenerating muscle fibers in their midst. In the process we have found evidence showing that regenerating muscle fibers have the ability to induce sprouting and cause sprouts to make contact with them.

Some of these results have been published before in preliminary form (van Mier and Lichtman, 1989, 1990).

Received Dec. 27, 1993; revised Mar. 17, 1994; accepted Mar. 24, 1994.

We thank V. Creasy, G. Jester, A. Reitman, and S. Turney for technical assistance. This work was supported by the NIH and MDA (J.W.L.). P.V.M was partially supported by a career advancement fellowship of the Netherlands Organization for Scientific Research (NWO).

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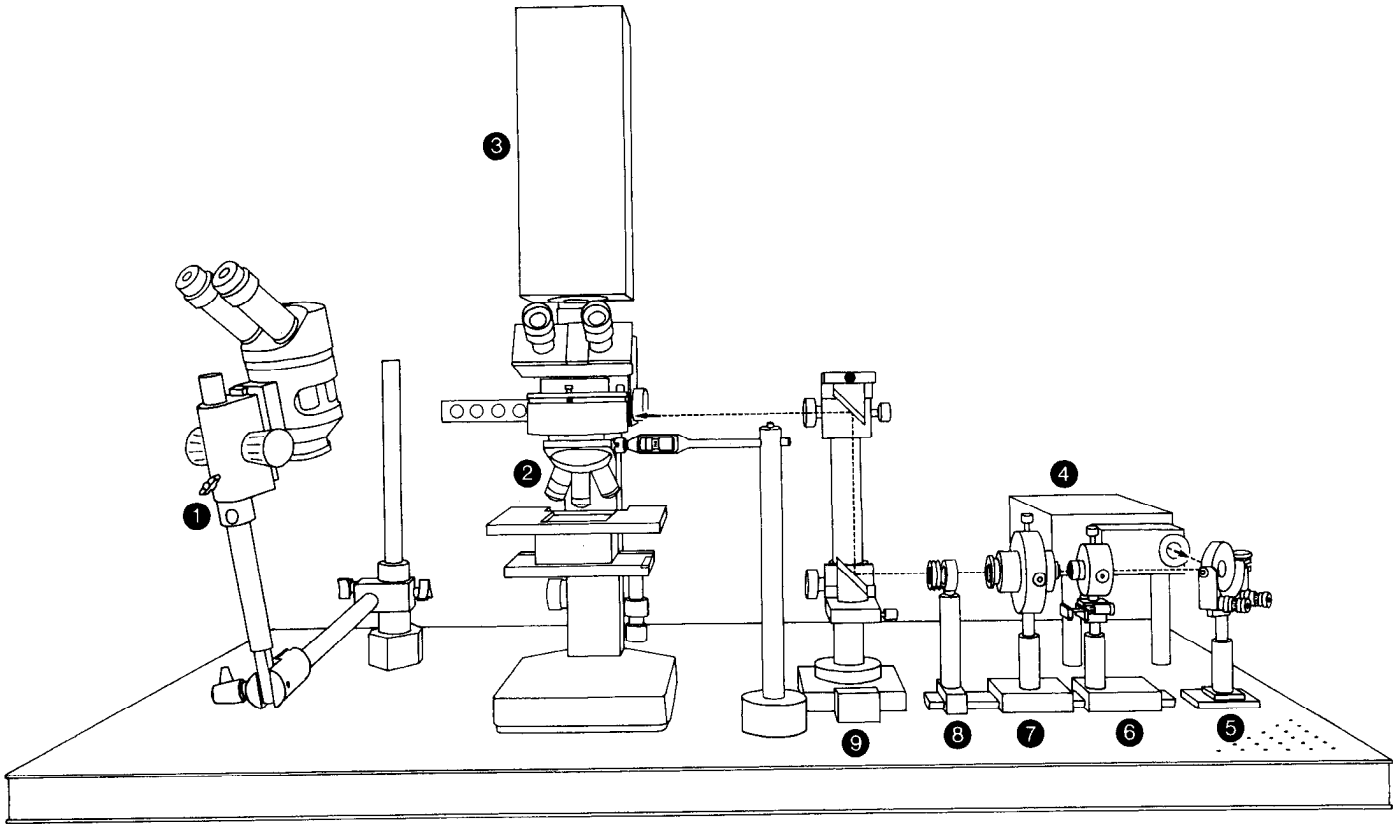

Figure 1. Microscope for *in situ* imaging and laser ablation. The dissecting microscope (*1*), used to expose the muscle surgically, is mounted on the left side of the compound microscope (*2*); the top half, including the objectives, can swivel, allowing the dissecting microscope access to the microscope stage. In these experiments, an anesthetized mouse is placed on the stage, and its sternomastoid muscle exposed and, following vital staining, viewed with standard epifluorescence optics. Video images are taken with an SIT camera (*3*) on top of the microscope; images are digitized and stored with a computer (not shown). Ablations of muscle fibers and nerve terminals are performed with the aid of a pulsed dye-pumped laser (*4*) placed on the right side of a stiff, vibration-isolated table top. After deflection by a mirror (*5*) the laser beam is passed through a set of two objectives (*6* and *7*), a filter holder (*8*) and then by a beam steerer (*9*) deflected toward the side of the light port in the back of the microscope. *Dashed line* indicates laser light path.

Materials and Methods

Experimental procedures

Six- to 10-week-old female mice (CF1/B strain, Harlan Sprague-Dawley) were anesthetized with a single intraperitoneal injection of chloral hydrate (0.5–0.6 mg/kg body weight), or a mixture (5.0 ml/kg body weight) containing 0.17 mg of ketamine (Ketaset, Aveco) and 1.7 mg of xylazine (Anased, Lloyd Laboratories) per milliliter of 0.9% sodium chloride solution. The anesthetized mouse was placed on its back on the stage of the microscope (Fig. 1), modified so that the upper part swings away. The mouse was then intubated and mechanically ventilated for the duration of the experiment. A midline incision was made from the sternum to the apex of the mandible, and the left sternomastoid muscle was exposed by lateral deflection of the skin and salivary glands. The muscle was gently lifted on a small platform (Lichtman et al., 1987). Nerve terminals and muscle fibers were stained for 3 min with a 4–10 μM solution of 4-Di-2-Asp (Molecular Probes, Eugene, OR), a fluorescent vital mitochondrial marker (Lichtman et al., 1987; Magrassi et al., 1987). After staining, the wound was washed with lactated Ringer and a coverslip was lowered on the surface of the muscle. With the microscope swung back in its normal position, the neuromuscular junctions were visualized with conventional epifluorescence and video microscopy. Using a low-light-level SIT camera (Dage/MTI) and a Trapix digital image processor (Recognition Concepts) connected to a microVAX (Lichtman et al., 1987), video images were digitized, averaged (32–64 frames), and stored on optical disk. Image processing was done with IMAGR, a computer language developed by J. Voyvodic, and further modified by W. Sunderland, L. Heydayati, and S. Turney.

At the end of each experiment the wound was sutured and the mouse allowed to recover under a heating lamp. Once recovered, the mouse was returned to our animal facility.

Laser ablation procedure

For the selective damage of single muscle fibers and/or nerve terminals, a dye pumped Nitrogen laser (model LS 337, Laser Science, Cambridge; Fig. 1) was used in conjunction with a modified conventional epifluorescence microscope (Laborolux, Leitz). All ablations were done with blue light using the dye Coumarin 500 (Laser Science, Cambridge, MA), which dissolved in *p*-dioxane produces light with a wavelength of about 470 nm when excited at 337 nm. Typically, the duration of the light flashes delivered by this system were 3 nsec long, the pulse power was about 120 μJ.

Setup. Laser and microscope were mounted on a high-stiffness honeycomb vibration isolation table (Micro-G, Peabody, MA). The microscope was modified so that a special movable holder containing a dielectric coated mirror (99% reflectivity at 450–700 nm; Oriel, Stratford) was inserted in the light port of the microscope, deflecting the laser beam toward the front side of the microscope. Here, the light was deflected by a fluorescein filter/beamsplitter cube (Leitz I2 cube) toward the back focal plane of a 100× water-immersion objective (NPL Fluotar, Leitz; 1.2 NA).

To manipulate its focus, the laser beam was passed through a set of two microscope objectives (Fig. 1), which collimates, expands, and focuses the beam. First, the laser beam was focused through a 25× objective (NPL Fluotar, Leitz; 0.55 NA) onto an inverted 5× infinity corrected objective (NPL Fluotar, Leitz; 0.09 NA), which then converted the focused laser light into a slowly converging beam (4–10° over 1 m). The laser beam was focused through the 100× objective onto the muscle to a spot adjusted to be about 1 μm (the spot size could be increased or decreased by adjusting the distance between the two focusing objectives; labeled 6 and 7, Fig. 1).

Each experiment was started by making a low-magnification map of

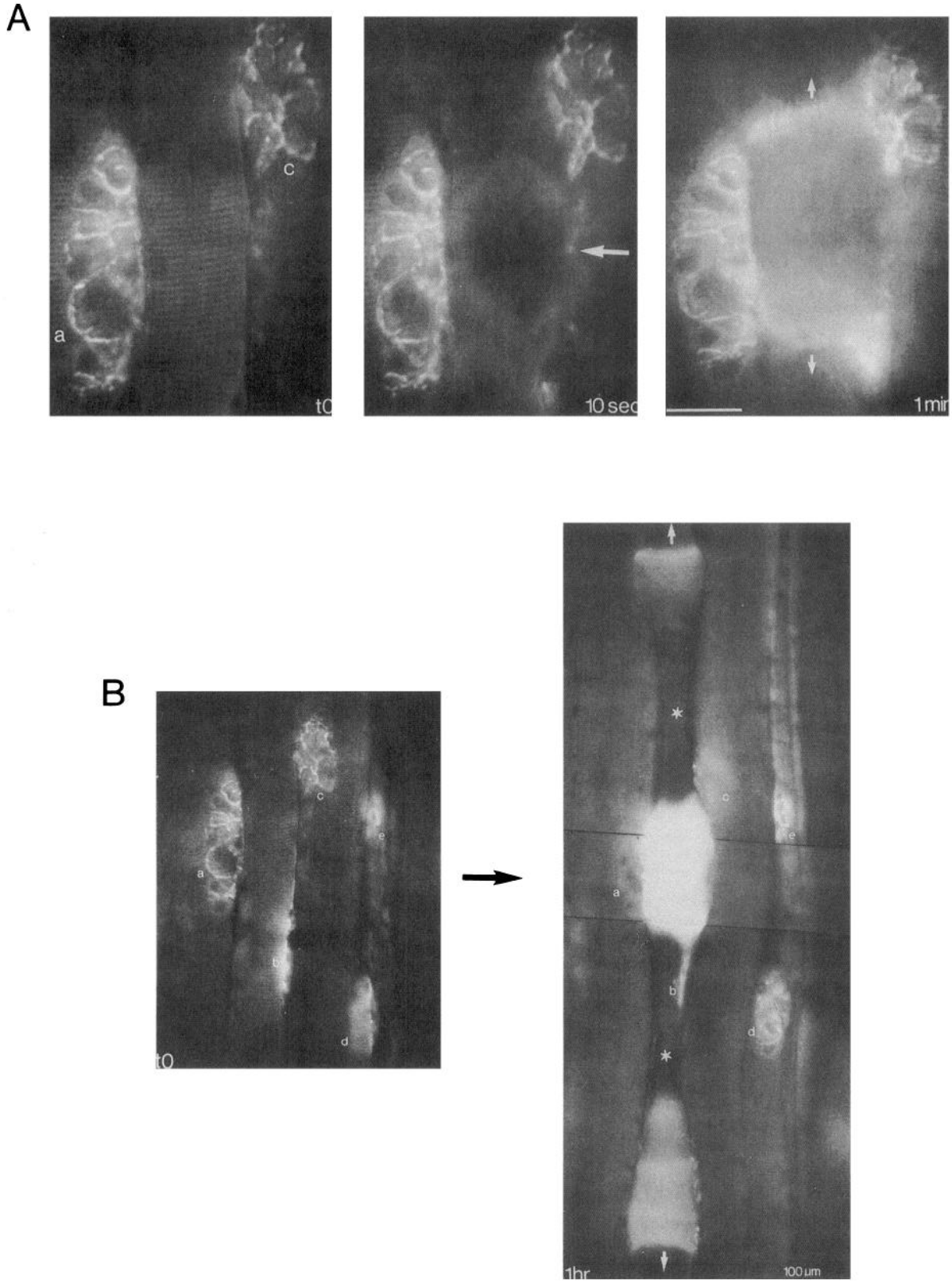

Figure 2. Focused laser pulses cause rapid damage to individual muscle fibers in the sternomastoid muscle of living mice. Shown are 4-Di-2-Asp–stained muscle fibers and nerve terminals. *A*, Ten seconds after the central portion of the middle muscle fiber was irradiated by a single laser pulse, a large vacuole appeared (*large arrow*). This is the area where a plasma bubble was briefly visible. One minute later contraction clots (*small arrows, right*) formed, which began to move away from the laser-damaged site. This is shown at low magnification in *B*. Nerve terminal *b* is on the damaged muscle fiber. One hour after laser irradiation the contraction clots on either side of the damaged site had moved about 300 µm toward the ends of the muscle fiber, leaving an empty tube (*asterisks*) behind. The debris at the site of damage (*brightly stained region*) takes somewhat longer to disappear (<24 hr). The nearby nerve terminals (*a, c–e*) are not affected by the ablation procedure; *a* and *c* are out of focus in this image.

the superficial neuromuscular junctions. A muscle fiber was then selected and positioned under a spot (visible on a monitor) that marked the position of the laser beam. After the mirror had been moved in position, the muscle fiber was irradiated with 1–15 laser pulses. Irradiation was terminated when a plasma bubble appeared at the irradiated site. For most experiments, the nerve terminal area was avoided during laser irradiation, but in one group of mice both muscle fiber and nerve terminal were intentionally damaged. To ensure that this method would damage a single muscle fiber (and sometimes its nerve terminal) without harming the neighboring fibers, we assessed the effects of laser irradiation in two ways. First, 5 min after laser treatment, in several mice (n = 10) application of propidium iodide (which selectively stains nuclei of dead cells only, Jones and Senft, 1985) stained only the nuclei of the laser-treated, damaged, and therefore leaking muscle fiber, suggesting that only one fiber had been damaged. Second, the muscles of 18 mice in which a single superficial muscle fiber had been laser irradiated, were cryosectioned and examined 1–2 d later. In all cases, only one damaged muscle fiber was found, showing loss of cytoplasm and nuclei, while none of the surrounding fibers showed any sign of degeneration.

Muscle fiber regeneration. In vivo muscle fiber regeneration was studied in two ways. First, in some mice (n = 14) the diameters of newly forming muscle fibers were measured in the living animal at three points along each muscle fiber. Second, in another group of mice (n = 30) the accumulation and addition of new acetylcholine receptors (AChRs) at nerve terminal sites on regenerating muscle fibers were studied. In these mice, the left sternomastoid muscle was exposed, and before laser damage the acetylcholine receptors were saturated for 20 min with unlabeled α-bungarotoxin (αBTx; 20 μg/ml concentration). Then a few muscle fibers (separated by 5–10 muscle fibers) were laser damaged. After survival times from 2–15 d, the newly forming muscle fibers' surfaces were screened for any acetylcholine receptors inserted since the application of unlabeled α-bungarotoxin by staining with rhodamine-conjugated α-bungarotoxin (Rh-αBTx; 20 min application, 2 μg/ml concentration, made in our laboratory according to the procedure of Ravdin and Axelrod, 1977; see also Balice-Gordon and Lichtman, 1990).

Results

Muscle fibers degenerate following laser ablation

In order to study the way in which regenerating muscle fibers become innervated, we damaged individual muscle fibers with a laser in the sternomastoid muscle of living mice. Pulsed laser irradiation focused onto a muscle fiber's membrane (1 μm spot) caused a transient plasma bubble to form, which instantly ruptured the membrane at that site (Fig. 2). Secondarily, the muscle fiber cytoplasm condensed and contraction clots (see, e.g., Schmallbruch et al., 1975) formed on both sides of the site of damage. Typically, the clots (Fig. 2*B*, small arrows) moved away from the damaged site at a rapid rate (~300 μm/hr), leaving in their wake an empty tube containing little cytoplasm (Fig. 2*B*, asterisks). Because focal damage of a muscle fiber can cause "segmental necrosis" (Schmallbruch et al., 1975; Carpenter and Karpati, 1989) in which only a small portion along the length of the muscle fiber undergoes damage and repair, we irradiated each muscle fiber at four to six separate sites along its length. In this way, a long contiguous central region (up to about 75% of the muscle fiber length) was caused to degenerate. In these first experiments, care was taken not to use the laser within approximately 300 μm of the muscle fiber's synapse. By avoiding the nerve terminal region, the effects of muscle fiber damage could be studied in the absence of direct nerve damage. Histological studies confirmed that each time only one muscle fiber was damaged using this approach (see Materials and Methods).

Muscle regeneration begins several days after laser irradiation

Muscle fiber regeneration was studied by repeated viewing of individual laser damaged muscle fibers over time. Staining with 4-Di-2-Asp, which vitally stains sarcomeres and myoblasts in addition to nerve terminals (see Figs. 2, 3), showed that for several days after laser damage, striations were absent. By the second day, however, numerous cells had aggregated in the former muscle fiber tube (Fig. 3, arrowheads). These cells closely resembled myoblasts in fixed histological material of regener-

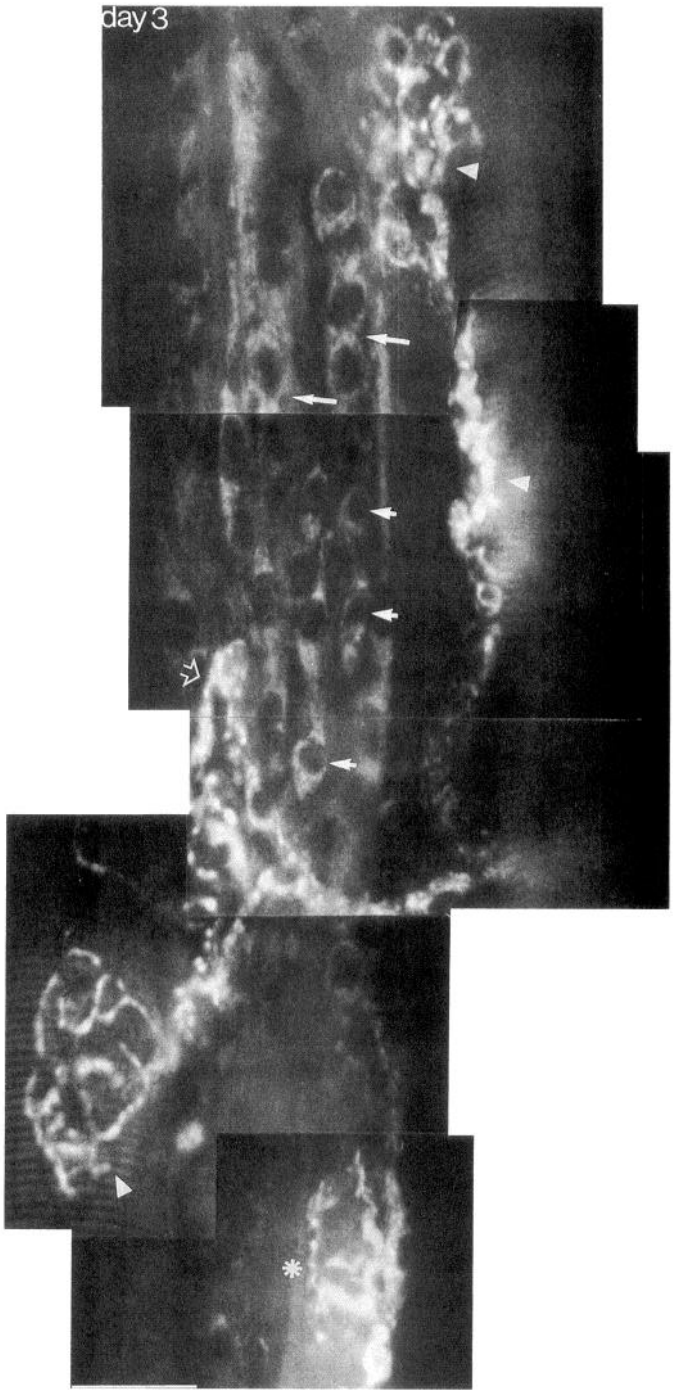

Figure 3. Several days after laser damage to a muscle fiber, signs of muscle fiber regeneration are evident. Myoblasts (*small arrows*) that aggregate at the site of the former muscle fiber can be visualized in the living mouse by staining with 4-Di-2-Asp. In some areas (*large arrows*) myoblasts are observed in the process of fusing with others along the axis of the muscle fiber. The sparsely branched nerve terminal on the regenerating muscle fiber is at the bottom of this montage (*asterisk*), the nerve terminal visible adjacent to the regenerating fiber (*open arrow*) innervates the muscle fiber beneath the damaged one. Other nerve terminals (*arrowheads*) are also visible on nearby muscle fibers. Scale bar, 40 μm.

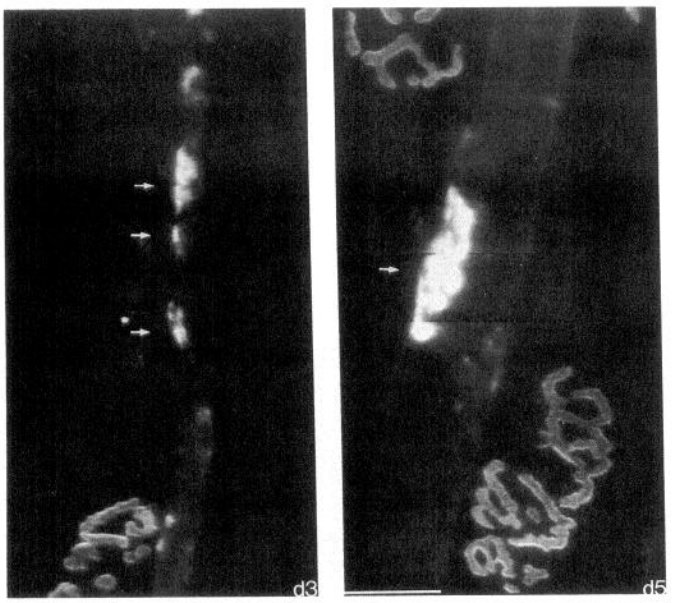

Figure 4. ACh receptor insertion rate is faster at junctions on regenerating muscle fibers than at neuromuscular junctions on normal muscle fibers. Regenerating muscle fiber differentiation was studied by looking at the production of AChRs in regenerating and normal muscle fibers. Both panels show a regenerating neuromuscular junction at its center (*arrows*), the other Rh-αBTx–stained endplates are on undamaged adjacent muscle fibers. Before laser damage at day 0, all AChRs were blocked with unlabeled α-bungarotoxin. New AChRs produced by normal and regenerating muscle fibers were visualized by staining on later days (2–21 d) with rhodaminated α-bungarotoxin. Shown here are examples of AChR accumulations observed at days 3 and 5 after muscle fiber damage. On both days the AChR areas on regenerating muscle fibers stained more brightly than surrounding endplates, owing to the large number of AChRs inserted at new junctions compared to the low insertion rate at mature junctions. By day 14 the difference in staining was much less (not shown). Scale bar, 40 μm.

ating muscle fibers during what is known as the fragmentation and myotube stages (Konigsberg, 1979; Foster and Carlson, 1980; see also Bisschof, 1975). By day 3, about 90% of the ablated muscle fibers had already been replaced by a single, thin (14.1 μm, SD = ±3.1) muscle fiber that usually contained many central nuclei, typical of regenerating muscle fibers (Schmallbruch et al., 1975).

By blocking all the AChRs in the muscle at the time of laser damage with unlabeled α-bungarotoxin and then labeling all the newly inserted receptors with fluorescent bungarotoxin at subsequent times, we could determine when the regenerating fiber began to cluster AChRs on its surface. In 22 mice, muscle fibers were laser damaged, and at that time AChRs on the surface fibers were saturated by applying unlabeled α-bungarotoxin to the muscle for 20 min. These muscles were then stained with rhodaminated α-bungarotoxin 2–21 d later. On day 2, no rhodamine-labeled AChR clusters were observed on any of the regenerating fibers, and only light AChR staining was observed at normal junctions nearby (owing to the slow turnover of AChRs at normal junctions). On day 3, in three of five mice, we observed small patches of rhodamine-labeled AChRs at the former synaptic sites beneath the remaining branches of the motor nerve terminal. On day 4, in six of six mice examined, larger AChR patches were found at the site occupied by the nerve terminal. On subsequent days the new AChR areas continued to enlarge. Early on the new AChR patches always stained much more intensely than nearby normal endplates on undamaged fibers (Fig. 4) due to more rapid accumulation of AChRs at synapses on the new muscle fibers than occurs at the normal adult junctions.

Regenerating muscle fibers have a rapid and slow phase of growth

By measuring the diameter of individual muscle fibers during regeneration, we found that muscle fibers rapidly increase in diameter from the time they were first formed (between days 2 and 3) until about 2 weeks later when their diameter was approximately 65% of that of the surrounding undamaged muscle fibers (Fig. 5). Over the next 5 weeks (as long as we measured) the diameter of regenerated fibers continued to increase but at a much slower rate, paralleling the ongoing enlargement of normal muscle fibers in these young adult mice. One regenerated muscle fiber that was followed for over 9 months after laser treatment (see Fig. 11) grew to be 73 μm in diameter, which was 87% of the adjacent 10 muscle fibers' average diameter.

Nerve terminals on both regenerating and nearby normal muscle fibers extend sprouts during the rapid phase of regenerating muscle fiber growth

Generally, the nerve terminals directly apposed to the damaged muscle fibers partially retracted (Fig. 6*A*, compare d0 with d5) after muscle fiber damage and then sprouted after a new fiber appeared (see also Rich and Lichtman, 1989b). Interestingly, however, in some of the muscles we observed that nerve terminals on muscle fibers *near* the new fiber also showed terminal sprouts (Fig. 6*B*). To test whether the sprouting from nearby nerve terminals was caused by muscle damage and regeneration rather than through inadvertent laser damage to the nerve terminals adjacent to the irradiated muscle fiber, in six mice approximately half the muscle fibers in the sternomastoid were damaged in a different way by pinching them with forceps at the ends of the fibers far away from the axons or the nerve terminals. This technique causes degeneration and regeneration of many muscle fibers (Rich and Lichtman, 1989b). Five to six days after muscle pinching, sprouting was evident from nerve terminals on several normal muscle fibers near the regenerating muscle fiber region in two of six examined muscles. Sprouting in two of six muscles examined was significant because in normal mouse sternomastoid muscles, sprouts occur very infrequently (we estimate we normally see sprouts in less than 1 in 20 muscles), indicating that muscle damage per se is sufficient to induce sprouting.

To study the temporal relation between the nerve terminal sprouting on intact muscle fibers and the regeneration of a nearby muscle fiber, one fiber was laser damaged in each of 198 muscles and groups of animals (12–13) were reviewed 1–15 d later to study the incidence of nerve terminal sprouting (Fig. 7). In each case, we chose to damage a muscle fiber adjacent to one or two superficial neuromuscular junctions. In most mice (187 of 198, 95%) the damaged muscle fiber was replaced by one muscle fiber. In the remainder, either no new muscle fiber regenerated (9 of 198) or the fiber was replaced by two new muscle fibers (2 of 198). Over the period of 1–15 d following muscle fiber ablation, we observed sprouts in about 10% of the muscles (19 of 198) on the one day each muscle was reviewed. Muscles were scanned for sprouts generally, but in all cases sprouts were seen only on muscle fibers near the new muscle fiber.

The first sprouts from nearby nerve terminals (Fig. 7*A*) were

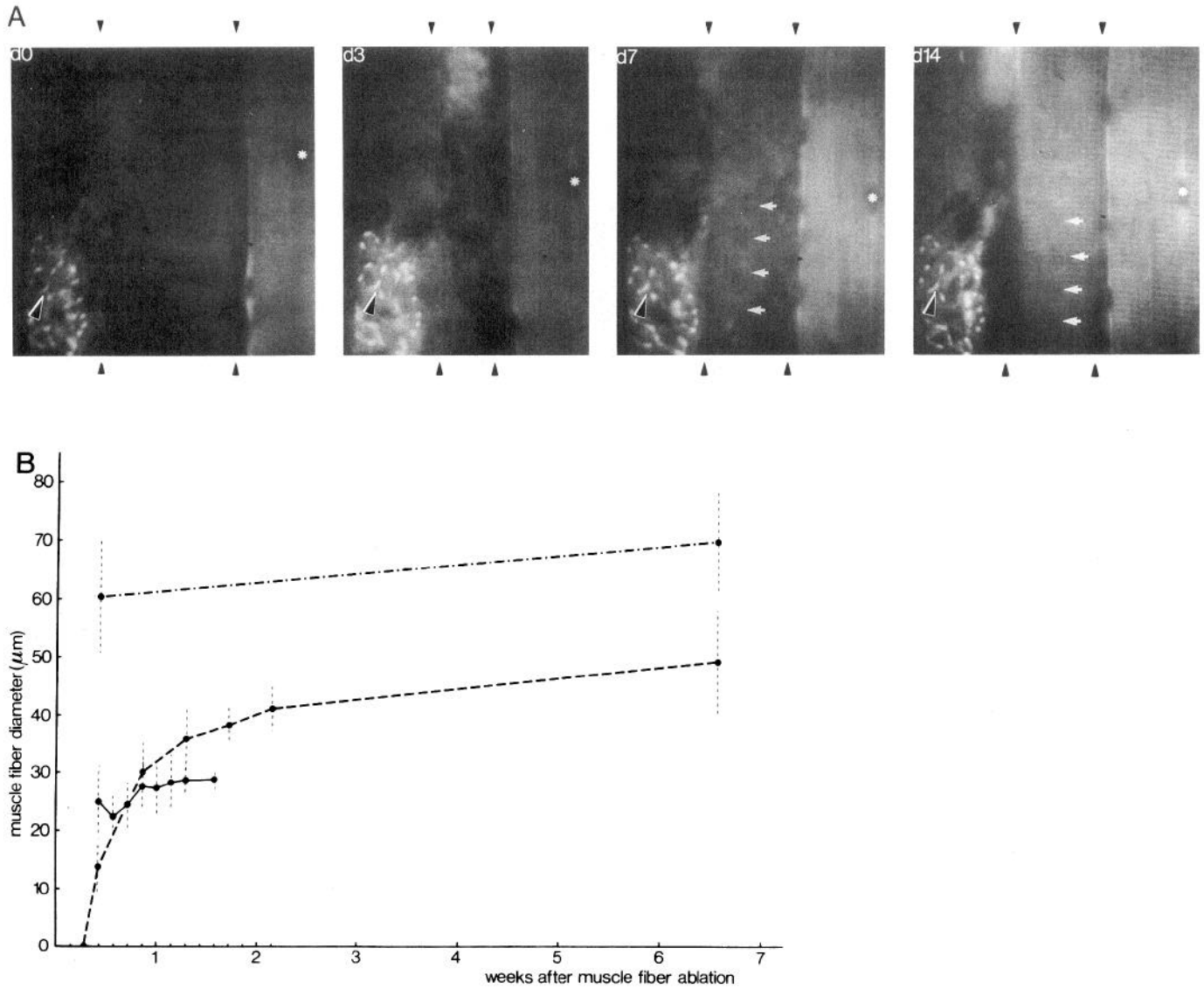

Figure 5. Muscle fiber growth during muscle regeneration has a rapid and a slow phase. *A,* Four panels show a muscle fiber on day 0 before laser damage and viewed subsequently during its regeneration on days 3, 7, and 14. The nerve terminal (*arrowhead* shows a feature present in all four views) on the muscle fiber to the left of the damaged one and a muscle fiber nucleus (*asterisk*) on the fiber to the right serve as reference for the regenerating fiber in between. Three days after laser damage a regenerating muscle fiber is visible. The new fiber could be distinguished from its neighbors because of its small diameter (*arrowheads above and below images*), the lack of striations at days 3 and 7, and the presence of central nuclei (*arrows*) at days 7 and 14. The rate at which muscle fibers regenerate was followed by measuring the diameter of the regenerating fibers at intervals ranging from 1 to 40 d after laser damage. *B,* The average diameter of 6–12 muscle fibers was plotted at each time point. Measurements were taken from all regenerating muscle fibers (*dashed line*), from undamaged muscle fibers (*dot-dashed line*) that served as a control, and from those regenerating muscle fibers (*solid line*) that induced nerve terminal sprouting from nearby undamaged muscle fibers (see, e.g., Fig. 6*B*, asterisk). The diameter of regenerating muscle fibers increased rapidly over the first 2 weeks after laser damage. Growth slowed down thereafter to match the growth rate observed in the control muscle fibers. The diameter of the regenerating muscle fibers associated with nerve sprouting was relatively constant (26.3 μm, SD = ±3.1), implying that fibers at a particular maturation stage are able to induce sprouting.

seen at day 3 and no sprouts were observed beyond day 11. The incidence of sprouts was highest on days 5 and 6 after muscle fiber damage, when 4 of 13 muscles (30.7%) showed sprouting. In the 19 mice that showed sprouting, a total of 41 sprouts was observed. All but two of these 41 sprouts originated at neuromuscular junctions on undamaged muscle fibers immediately adjacent to the regenerated muscle fiber. The two remaining sprouts came from nerve terminals on muscle fibers one and two fibers removed from the regenerating fibers.

The earliest time point at which we observed terminal sprouting (day 3), approximates the time new muscle fibers are being formed (see above), suggesting that sprouting could have been induced by the newly forming muscle fiber. In all of the 19 muscles in which sprouting was observed, a regenerating muscle fiber was adjacent to the sprouting nerve terminals. In the nine mice in which there was no muscle fiber regeneration, no sprouts were observed.

Although we only saw nerve terminal sprouting in the presence of newly forming muscle fibers, it is possible that the observed sprouting was caused by a delayed effect of degeneration products from the damaged muscle fiber. To study this possibility, 16 muscle fibers (in 10 mice) were laser damaged re-

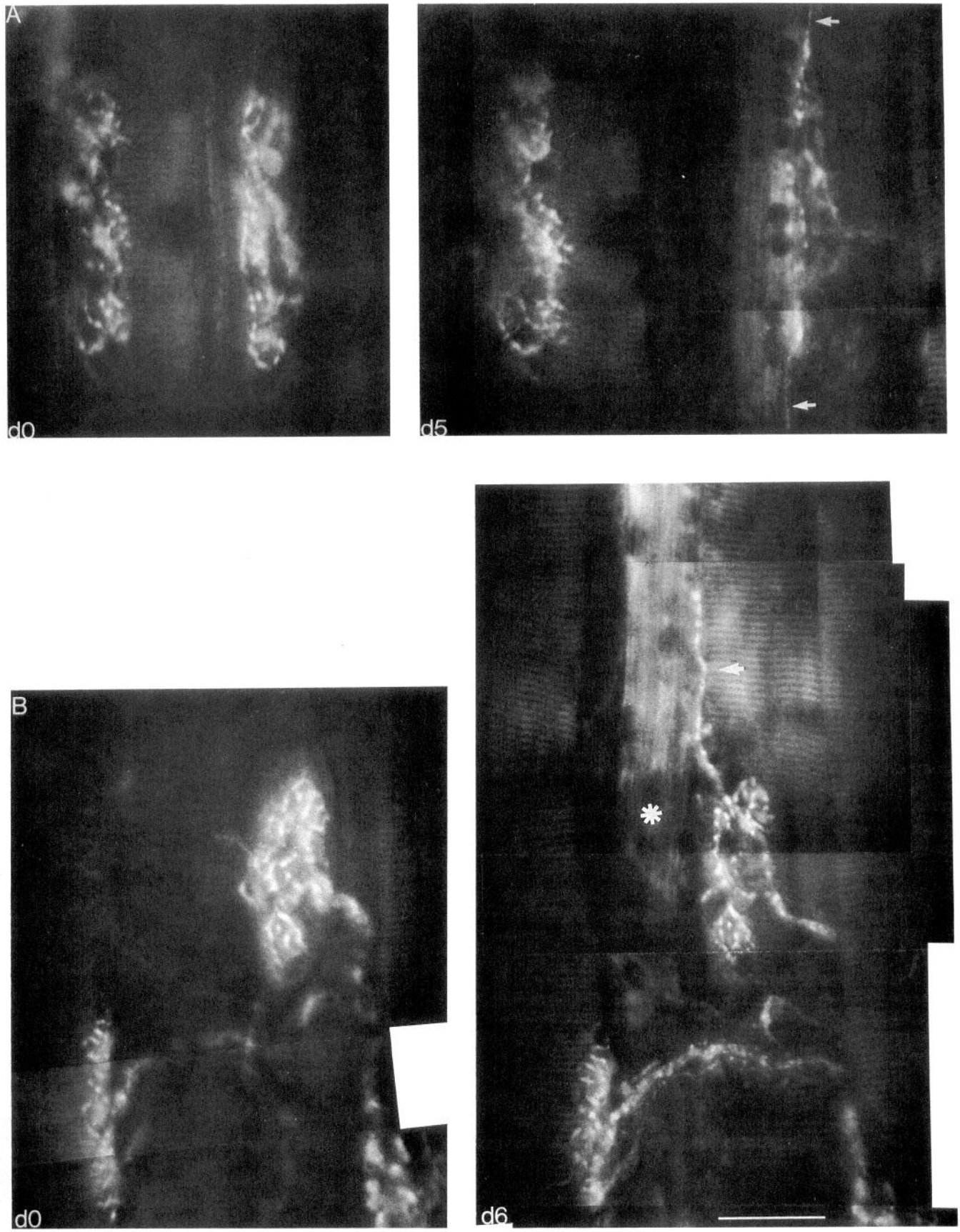

Figure 6. Nerve terminals on both regenerating and nearby normal muscle fibers sprout during the first 2 weeks of muscle fiber regeneration. Shown are two examples of living muscles 5 and 6 d after one muscle fiber was damaged. Sprouts, ordinarily very rare in the sternomastoid muscle were frequently observed in the presence of *regenerating* muscle fibers. *A,* Nerve terminal on a *regenerating* muscle fiber showing two sprouts

peatedly. As with the other laser damage experiments, we chose muscle fibers that were adjacent to one or usually two muscle fibers that each had superficial nerve terminals to allow straightforward visualization of nerve terminal sprouts. We prevented a new muscle fiber from being formed by damaging all the aggregating and sometimes fusing myoblasts in the vicinity of the "old" synaptic site about 2.5 d after the initial ablation. Reimaging the damaged regions 5–6 d after the initial laser ablation (2.5–3.5 d after second ablation), showed that none of the nerve terminals abutting the 16 regenerating muscle fibers sprouted (compared to 30.7% of the muscles that showed sprouts 5–6 d after muscle damage following one laser treatment; see Fig. 7*A*). In one mouse, the aggregating myoblasts were again irradiated at the second view. Upon reviewing this mouse at day 7 after the initial irradiation, again, no sprouting was observed. However, when the same muscle was reviewed 3 d later (day 10), a new muscle fiber had been formed and now one of the abutting nerve terminals had formed a sprout. Taken together, these results strongly suggest that sprouting is not induced by degenerating muscle fibers per se, but rather requires the presence of a newly forming muscle fiber.

Despite the range in days after ablation at which sprouting was observed (from day 3 to day 11), the diameters of the regenerating muscle fibers were relatively constant at the time sprouts were observed (from 24.6 μm at day 3 to 28.9 μm at day 11; mean 26.3 μm, SD = $\pm$2.4). On the other hand, the average diameters of all the regenerating muscle fibers (189 of 198) ranged from 14.1 μm (SD = $\pm$3.1) at day 3 to 38.8 μm (SD = $\pm$3.7) at day 12. This may mean that regenerating muscle fibers that can induce sprouting are all at about the same stage of development. As might be expected, the average size of regenerated muscle fibers on days 5–6 (when sprouting was highest) was also in this limited range (Fig. 5*B*). This further suggests that the regenerating fiber, and not the laser damaged muscle fiber, is the source of sprout-inducing factor.

Sprout growth is biased toward the regenerating muscle fiber

To obtain a better understanding of how sprouts grow in response to regenerating muscle fibers, we studied the direction of sprouts at their initiation point and during their subsequent growth (Fig. 8*A*). By taking the angle of each 10 μm segment of all sprouts we could determine the average sprout direction. From this analysis we found that sprouts begin on average without a bias toward the regenerating muscle fiber (they emerged from all sides of junctions equally; Fig. 8*B*) and begin to grow in all directions. However, every subsequent 10 μm segment had, on average, a positive (toward the regenerate) bias reaching a maximum value of +21.4° at a length of 40–50 μm (Fig. 8*C*). This average directional bias seemed significant because even when the sprouts that began by growing away from the new muscle were analyzed separately they had, as a population, changed direction and grew toward the new muscle fiber by the time they had grown 70 μm. The 19 sprouts that started out

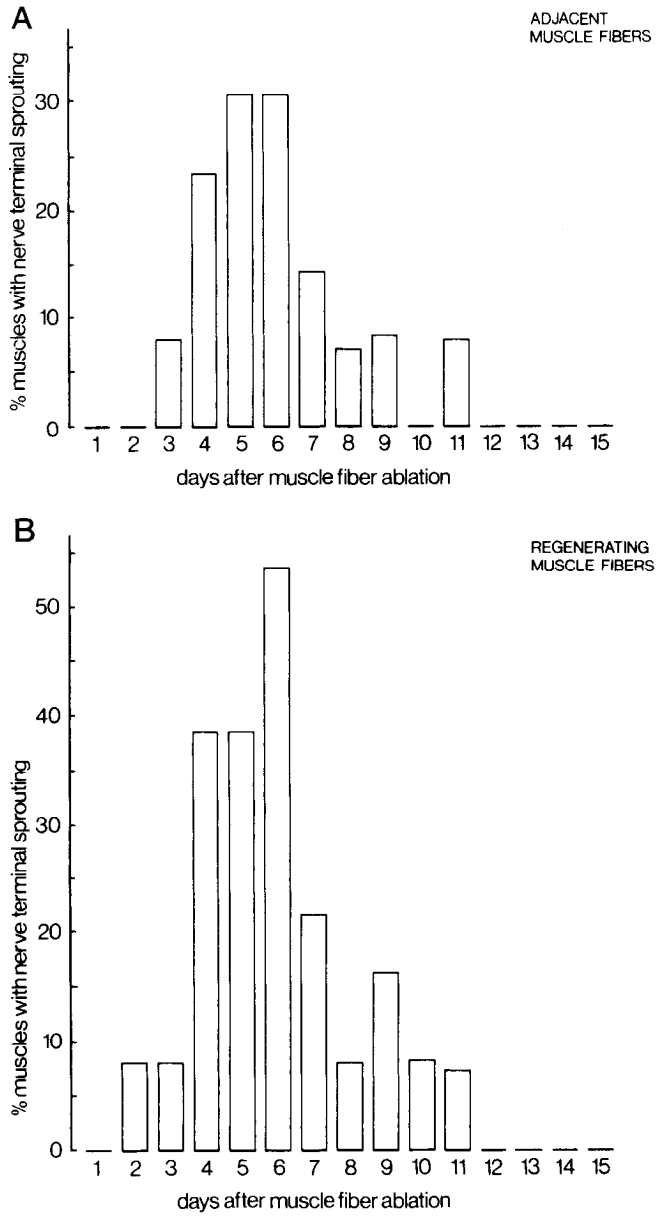

Figure 7. Sprouting from nerve terminals on regenerating and nearby undamaged muscle fibers is restricted to the first 2 weeks of muscle fiber regeneration. Data were obtained from 198 mice in which a single muscle fiber had been laser damaged. Groups of 12–13 mice were reviewed at subsequent days for the incidence of sprouting. Sprouting incidence is plotted against time in days. *A,* Nerve terminals on undamaged muscle fibers first showed sprouts at day 3. *B,* Sprouts from nerve terminals on regenerating muscle fibers were first seen 2 d after muscle damage. In both cases, sprouting peaked at days 5–6 and decreased during the second week after damage. No sprouts of either kind were observed after day 11.

←

(*arrows*) 5 d after the underlying fiber was damaged. The nearby nerve terminal on the left serves as reference. The *left panel* shows muscle appearance just prior to laser irradiation. Note that the nerve terminal on the regenerating fiber at day 5 is much smaller than it was prior to laser irradiation on day 0, indicating that during muscle degeneration many of the nerve terminal branches were retracted (for a fuller description see Rich and Lichtman, 1989b). *B,* Sprouting (*arrow*) from an adjacent nerve terminal on an *undamaged* muscle fiber 6 d after laser irradiating the muscle fiber to the left of it. *Left panel* shows the muscle just prior to laser irradiation of the fiber between the two visible neuromuscular junctions. In the *right panel* a lengthy sprout can be seen growing first toward and then along the adjacent regenerating muscle fiber. Scale bar, 40 μm.

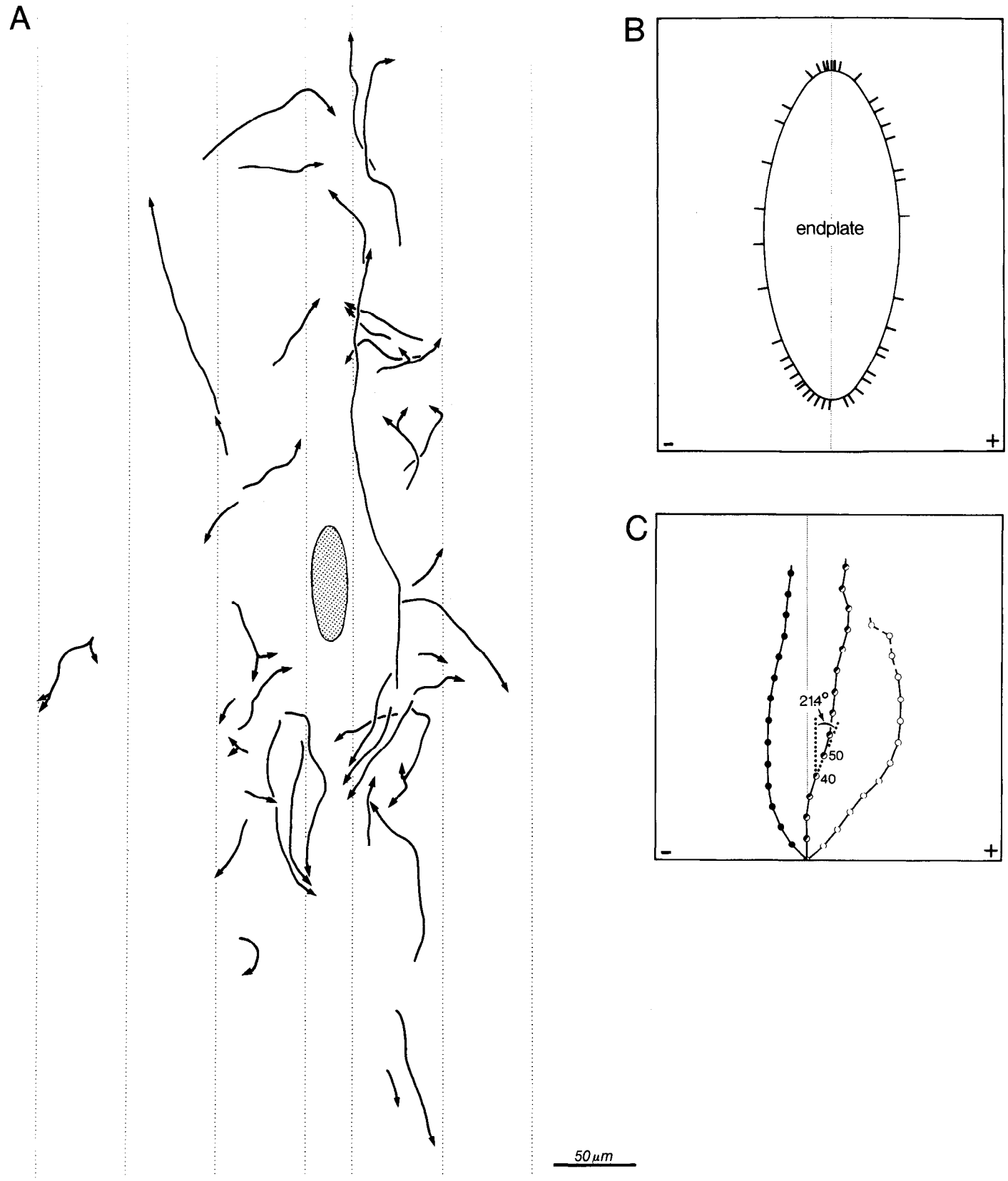

Figure 8. Directional sprouting is observed from nerve terminals adjacent to a regenerating muscle fiber. *A*, Schematic drawing showing 41 sprouts observed in 19 of 198 mice in which a single muscle fiber had been damaged. Sprouts are drawn as solid lines from their points of origin to their tips (marked with *arrowheads*). Their position relative to the regenerating muscle fiber and its endplate (*shaded area*) are shown. Most sprouts emerged from nerve terminals on adjacent muscle fibers; only two originated from nerve terminals more than one muscle fiber away from the regenerating muscle fiber. Note the absence of sprouts growing toward the site of the junction on the regenerating fiber. Sprouts were initiated from all sides of the endplates, as is shown in *B*. Each sprout origin is shown with a *short line*. About half originated from the side of the junction facing

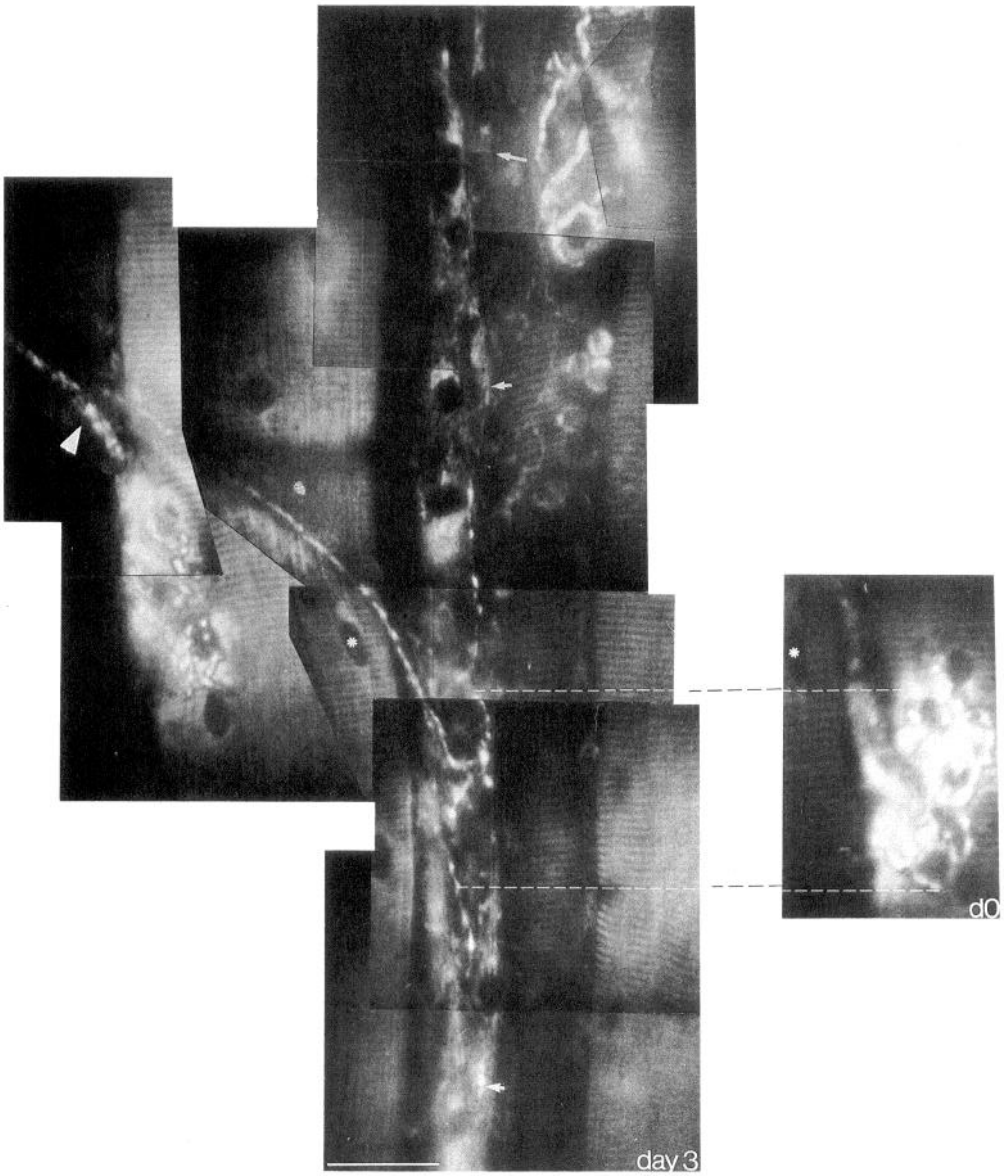

Figure 9. Regenerating axons form long sprouts on regenerating muscle fibers. Neuromuscular junctions and the underlying muscle fibers were simultaneously damaged to delay the innervation of the new muscle fiber (in this way the original axon had less of an advantage compared to sprouts from nearby nerve terminals in making contact with the regenerate). Upon making contact with the regenerating muscle fiber the regenerating axon (which is thin because it is not yet myelinated; compare with the adjacent intact myelinated axon, *arrowhead*), typically (as shown in this montage) formed sprouts (*small arrows*) along the fiber. These sprouts were far longer than those seen when an undamaged nerve terminal sprouted to innervate the regenerating muscle fiber underneath it (Fig. 6*A*). *Right panel* shows the size of the original neuromuscular junction shortly before laser irradiation. Scale bar, 40 μm.

growing toward the new muscle fiber (open circles) only reoriented upon reaching the regenerating muscle fiber, at which time they began to grow along but never off the fiber (see Fig. 8*A*). Because these results are based on single time point analysis, these data do not directly reveal whether sprouts actually orient toward the new muscle fiber or alternatively that the lifetime of a sprout is greater if it happens to orient in the direction of the new muscle fiber (see Discussion). Nonetheless, the result of this directional bias is clear, many sprouts (16 of 41, 39%) managed to reach the new muscle fiber. This suggests that the regenerating muscle fiber can induce sprouts to make contact with it.

Nerve terminal sprouts can establish synapses with a regenerating muscle fiber

We did not observe any sprouts that contacted the endplate site of the new muscle fiber. Despite the directional bias toward the newly forming muscle fiber, in all but 6 of the 41 sprouts studied, the sprouts were directed away from the endplate site on the new muscle fiber (Fig. 8*A*). This suggested that either the original endplate site itself or, more likely, the original motor terminal remaining and regrowing at that site, was inhibiting sprout growth to that area. To explore this possibility, in a group of 21 mice the overlying nerve terminal as well as the muscle fiber it in-

←

(+) the regenerating muscle fiber. Shown in *C* are the average directions of all sprouts, plotted in three groups. Sprouts that started with a negative angle with respect to the regenerating muscle fiber (*solid circles*), sprouts that started with a positive angle (*open circles*), and the average direction of all sprouts (*half-shaded circles*). Sprouts were divided into 10 μm segments, and the angle of each sprout segment was measured, with respect to a line (*dotted*) parallel to the axis of the regenerating muscle fiber, and averaged (− angle, away; + angle, toward the regenerating muscle fiber). The sprout initiation point for all groups is at the bottom of the graph. *Dashed parts of curves* are not average because number of sprouts for these lengths had decreased to one.

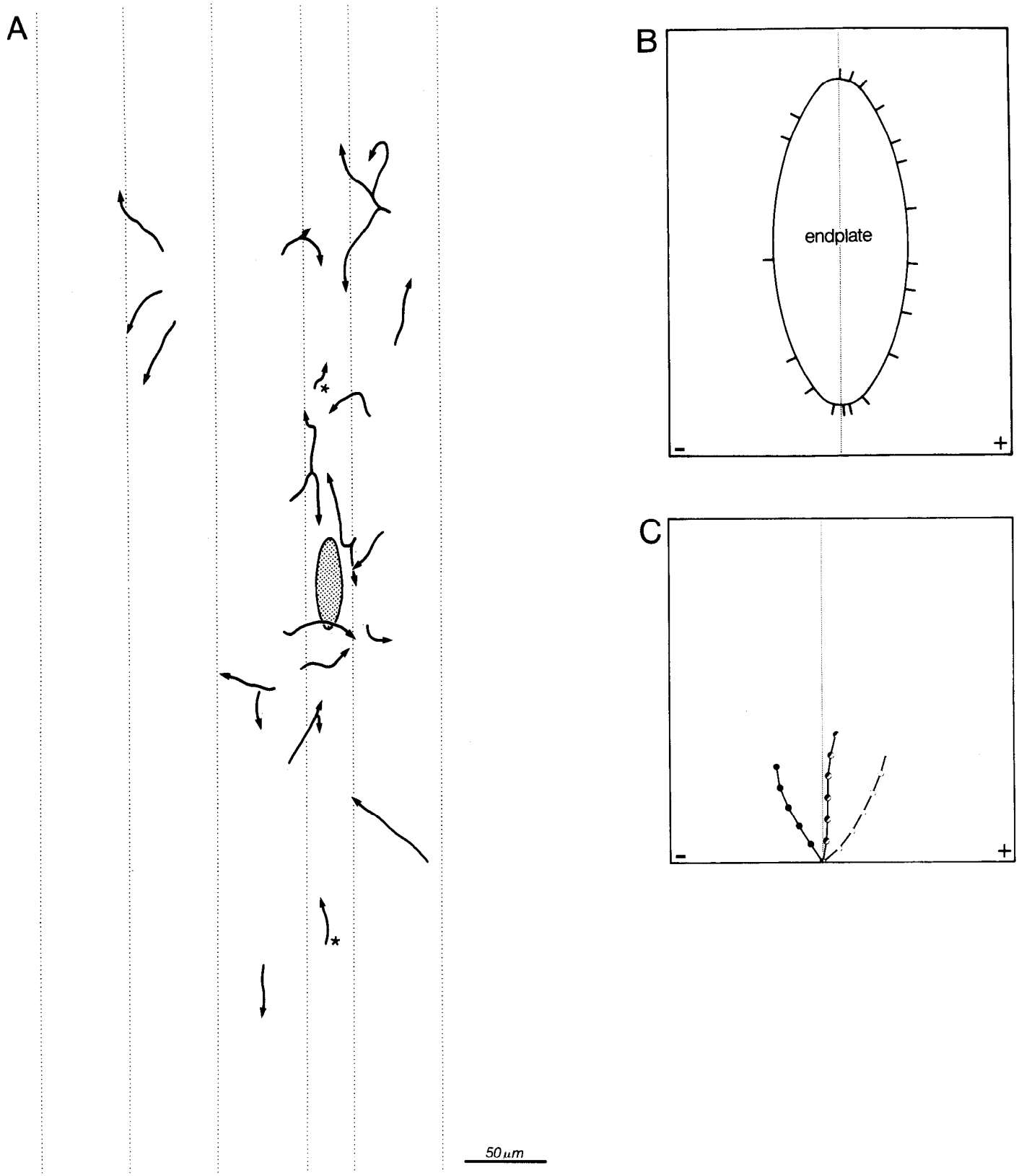

Figure 10. Adjacent nerve terminal sprouting is abundant after a muscle fiber and its nerve terminals are damaged. *A*, Schematic drawing showing 20 sprouts observed 5–6 d after a single muscle fiber and its nerve terminal had been damaged. In contrast to sprouting observed when muscle fibers were damaged without damage to the overlying nerve terminal (see Fig. 8), the incidence of sprouting more than doubled when the nerve was also damaged. Note that several sprouts came very close to the site of the endplate on the regenerating muscle fiber. The two sprouts marked with an *asterisk* originated from nerve terminals underneath the regenerating fiber. *B*, Sprout initiation was directionally biased with 70% of the sprouts initiating on the endplate side facing the regenerating muscle fiber. *C*, As in muscles without nerve damage, the orientation of sprouting axons was biased toward the regenerate.

nervated were intentionally damaged by laser irradiation. We reviewed these muscles at days 5–6 after irradiation, the same time at which we found the highest incidence of sprouting (30.7%) in muscles in which only muscle fibers were ablated (see Fig. 7*A*). Whereas undamaged nerve terminals on regenerating muscle fibers typically formed small sprouts (Fig. 6*A*), the regenerating damaged axons in these muscles sprouted extensively (Fig. 9). We also observed a higher incidence of sprouting (15 of 21 muscles, 71.4%) from the adjacent nerve terminals on undamaged fibers. In total, we found 20 sprouts in 15 muscles. In contrast to sprouts seen when the original nerve terminal was not damaged, these sprouts did not seem to avoid the region of the former endplate (Fig. 10). Moreover, the sprouts showed greater directional bias at their initiation (Fig. 10*B*). Their subsequent trajectories measured by the angle of each 10 μm segment were also biased toward the regenerating fiber.

These sprouts showed another important difference as well. In a separate experiment in which we applied rhodaminated α-bungarotoxin to the muscle 5–7 d after laser damaging a muscle fiber and its nerve terminal (n = 20 mice), we found in five cases that the newly forming muscle fiber contained multiple distributed AChR patches. In contrast, in nine mice in which the muscle fiber only had been damaged we did not find any new AChR patches when we stained with rhodaminated α-bungarotoxin. Staining with 4-Di-2-Asp showed that in each case the patches were either beneath sprouts from nearby junctions or the regenerating original nerve fiber. We presume that the associations of a sprout with AChR clusters in the muscle are nascent synaptic contacts.

In one muscle, in which the nerve terminal and muscle fiber were damaged, we followed the fate of these new synaptic contacts by reviewing the same muscle fiber 11 times over 9 months (Fig. 11). In this case, the regenerated muscle fiber was contacted by sprouts at three different sites 7 d after laser damage (each site had AChR clusters associated with it); one site was contacted by the original nerve (sprout 1, Fig. 11) and two additional sites by sprouts that originated from each of the adjacent nerve terminals (sprouts 2 and 3, Fig. 11). The AChR clusters contacted by sprout 3 were located about 300 μm away from the other two sites (Fig. 11, lower middle panel). Three weeks later, however, the original nerve's sprout (1) had regressed. Two weeks after that (day 42), the muscle fiber was singly innervated as sprout 3 from the junction on muscle fiber a (see Fig. 11, lower right panel) had also retracted. Interestingly, the AChR patches beneath sprout 3 could no longer be stained with rhodaminated α-bungarotoxin. By comparing the AChR labeling before and after adding a new dose of rhodaminated α-bungarotoxin, we could decide whether the loss of AChRs was due to the lack of insertion of new AChRs only or, in addition, to a more rapid loss of the previously stained AChRs (already inserted in the membrane) in that region. Prior to adding new rhodaminated α-bungarotoxin the other AChR patches on this muscle fiber under sprouts 2 and 4 were still labeled from the rhodaminated α-bungarotoxin applied 2 weeks previously (at day 28). However, the site associated with sprout 3 was not visible showing that the AChRs previously stained had disappeared more rapidly from this site than elsewhere. Because after adding a new dose of rhodaminated α-bungarotoxin no new staining was seen at that site either, we conclude that AChR depletion was due to loss of previously inserted AChRs that were not replaced by newly inserted AChRs. Our time resolution, however, was not sufficient to tell which (the sprout or the AChRs) had been removed first. It is probably true that the AChRs beneath sprout 1 were also eliminated. However, a new sprout (4) from the junction on muscle fiber c (Fig. 11) occupied AChRs near that former site making it difficult to determine the fate of the AChRs under sprout 1. The remaining sprouts (2 and 4), both originating from the same neuromuscular junction, persisted throughout the last view 9 months after laser damage. Sometime between days 28 and 42 sprout 2 became myelinated (determined by the thickening of the sprout characteristic of myelinated axons), followed by the thickening of sprout 4 between days 42 and 72, suggesting that they had become the permanent innervation of the regenerated muscle fiber. Over the last several months of viewing the only change we detected was enlargement of the diameter of the sprouts and the muscle fiber they innervated. Thus, sprouts can be transformed into large axonal branches that are long lived.

Discussion

We have damaged individual muscle fibers and followed their regeneration and reinnervation over time *in situ*. Our aim was to study if and how newly forming muscle fibers become innervated in living animals. We have found that when a muscle fiber is damaged by laser irradiation, away from its neuromuscular junction (to spare the motor axon from damage), the regenerated muscle fiber is innervated by the same axon and at the same site as the previous muscle fiber (see also Rich and Lichtman, 1989b). We also observed that muscle fiber regeneration is accompanied not only by sprouting from the nerve terminal on the regenerating muscle fiber but, interestingly, also sprouting from immediately adjacent neuromuscular junctions.

Several previous studies in which muscle fibers were damaged also reported sprouting (Huang and Keynes, 1983; Brown and Lunn, 1988). In those studies, in which muscle fibers were destroyed either with a chemical cocktail or mechanically, signs of sprouting were seen exclusively on the damaged fibers and was said to begin as early as 4 hr following damage. This interpretation was based on the rapid change in the shape of the neuromuscular junctions on degenerating muscle fibers. Our experiments, however, suggest that this change is due to the mechanical deformation of the muscle membrane that occurs shortly after the muscle is damaged. Those authors interpreted the sprouting to be due to a local disinhibition of nerve growth induced by killing the muscle fiber underlying the nerve terminal. However, our attempts to induce sprouting in repeatedly damaged muscle fibers failed; indeed, we found just the opposite, a significant retraction of nerve branches when the muscle fiber died (see also Rich and Lichtman, 1989b). Furthermore, the incidence of sprouting increased in our studies (and in the previous work) over several days, reaching a maximum at 5–6 d when in every case we found that the nerve terminal was once again sitting on a healthy muscle fiber. Lastly, the sprout initiation we observed from nearby muscle fibers argues that diffusible signals stimulate sprouts to grow even on normal muscle fibers. We thus believe that sprout growth is a response to new muscle fiber formation.

Because the sprouting we observed from adjacent nerve terminals showed a directional bias toward the regenerating muscle fiber, and was largely confined to the muscle fibers abutting the regenerate, it seems likely that the regenerating fiber is emitting a short range signal that is sensed by nearby intact nerve fibers. Whatever the signal is, it is transitory. When the original motor axon reestablishes its junction with the regenerating muscle fi-

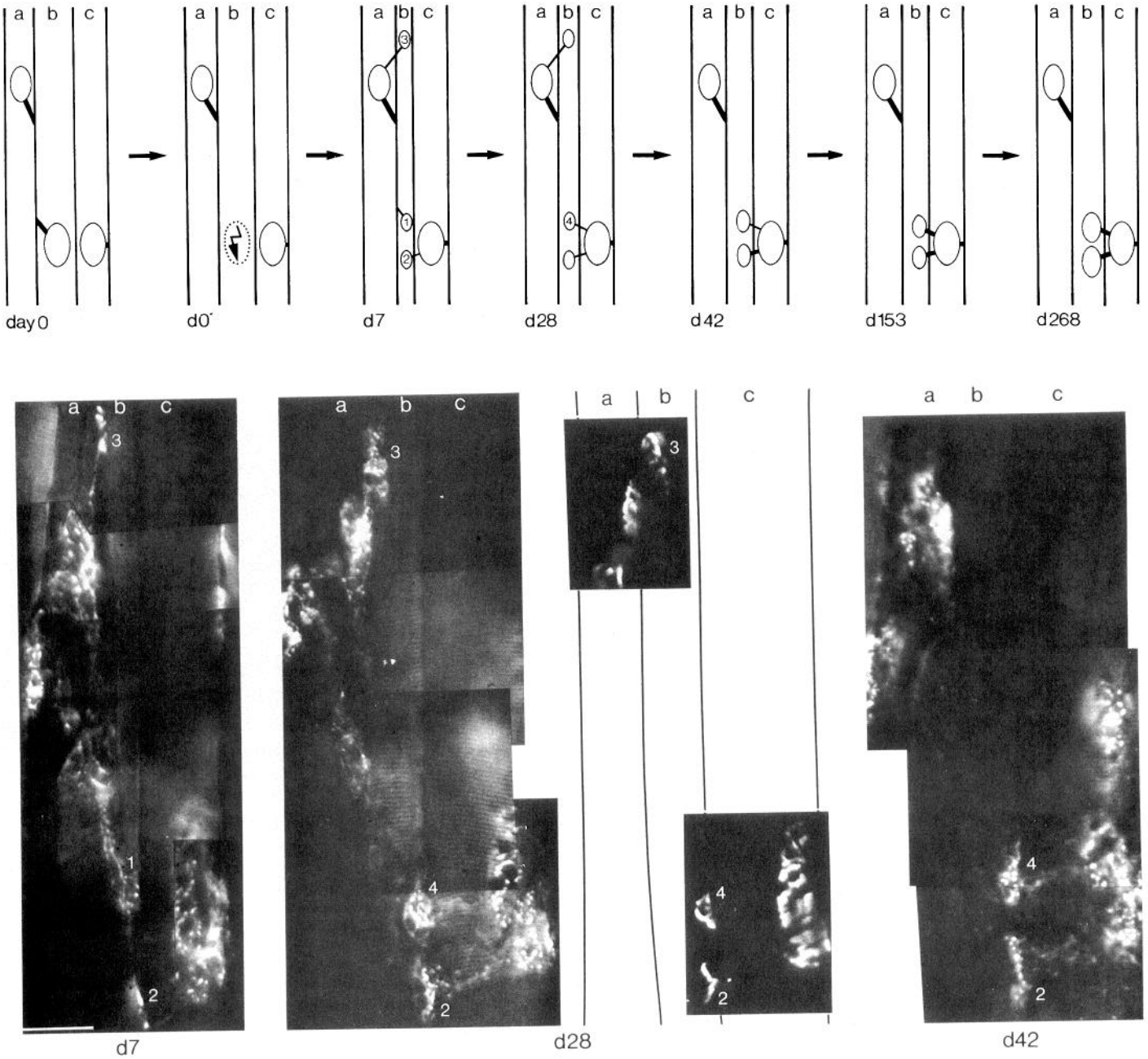

Figure 11. Some regenerating muscle fibers are temporarily innervated by sprouts and the original nerve fiber. One mouse was viewed 11 times over 9 months after a single muscle fiber and its nerve terminal were damaged. In addition to nerve terminal staining with 4-Di-2-Asp, the muscle was also labeled with rhodaminated α-bungarotoxin to show AChR clusters. The diagram (*top*) shows the innervation pattern over this time. Between days 42 and 153 the mouse was reviewed three more times (not shown), and between days 153 and 262 two additional times (not shown). Each ellipse in the upper drawing represents a nerve overlying an AChR cluster. Below are photographs taken at several of these times. At day 7 following damage, the regenerating muscle fiber (*b*) appeared to be innervated by its former nerve fiber (*1*) and sprouts *2* and *3* from nerve terminals on adjacent muscle fibers *a* and *c* (shown with 4-Di-2-Asp staining in *lower left panel*). However, at day 20 this situation had changed in that axon *1* had retracted and a second sprout (*4*) had appeared from the nerve terminal on muscle fiber *c* (4-Di-2-Asp and rhodaminated α-bungarotoxin staining are shown in the *bottom middle two panels*). The correspondence between sprouts and AChRs indicate that the new fiber had several functional synaptic sites. Between day 28 and 42 the situation had changed again; now muscle fiber *b* had lost its innervation from sprout *3* (*lower right panel*) and when we stained with rhodaminated α-bungarotoxin we found that the AChRs underlying that sprout had also disappeared (and did not return at any subsequent view). This state of innervation appeared to be final. Over the subsequent 226 days and seven views (five not shown) the pattern of innervation remained unchanged. Between view 3 and 4 (days 28 and 42) sprout *2* from the junction on muscle fiber *c* began to myelinate. Sprout *4* myelinated between view 4 and 5 (days 42 and 70). Both sprouts remained myelinated through the 11th and last view on day *268*. Scale bar, 40 μm.

ber, sprouts disappear. We believe that the reexpanding nerve terminal on the new muscle fiber may be responsible for this suppression because in the one long-term case we followed in which both the nerve terminal and muscle fiber were damaged, sprouts were permanently maintained. Presumably, this maintenance occurred because the sprouts were able to establish a synaptic foothold on the regenerating muscle fiber (as evidenced by the AChR patches beneath them). With time, these sprouts increased in diameter and eventually became myelinated. In this particular case (Fig. 11), sprouts from more than one axon made synapses on the same regenerating muscle fiber, provoking a competition that ended with all but one axon being eliminated. This scenario has similarities to synapse elimination as occurs during development (Balice-Gordon and Lichtman, 1993; Balice-Gordon et al., 1993) and reinnervation (Rich and Lichtman, 1989a) in the sternomastoid muscle. In each case, the loosing motor axons and their underlying AChRs are removed and in each case, AChR removal is due both to a more rapid disappearance of previously inserted receptors as well as a lack of new AChR insertion. One difference, however, is that in this experiment the distance between competing synaptic sites was much larger than occurs during synapse elimination in normal development. This argues that the competitive mechanisms that operate within an endplate causing pre- and postsynaptic loss also can be called into play over much longer distances. Thus, the competitive elimination of ectopic synapses (see Brown et al., 1976; Kuffler et al., 1977), which occurs over distances of a millimeter or more may be due to a similar or identical process as that causing synapse elimination at individual neuromuscular junctions. Thus, the rapid retraction of sprouts that have not made synaptic contact is probably different from the much slower process that permits elimination of synapses originating from sprouts.

Because many of the sprouts that were rapidly resorbed had not yet reached the regenerating muscle fiber, their survival was presumably dependent on a continual supply of agents were now present in the extracellular matrix. On the other hand, the slower removal of sprouts overlying AChRs may either be because synaptic sites are a chronic source of trophic support or that these sites provide an adhesive anchor that holds the sprout in place.

The directional bias of growing sprouts we observed may be due to a chemical gradient that attracts sprouts to grow toward the regenerating muscle fiber. One alternative possibility is that sprouts grow with no directional bias but those that happen to grow in the proper direction are longer lived than sprouts growing away from the regenerated fiber. Because sprouts were rarely seen more than once when the same muscle was viewed twice over several days, the second possibility must be taken seriously. Based on the present data we think, however, that the results more support directional growth because even the sprouts whose early trajectories were directed away from the regenerating muscle clearly corrected their trajectories rather than be eliminated. Obviously, time-lapse viewing of sprouts *in situ* over 12–24 hr should give direct information about this question (see van Mier et al., 1991).

The sprouting we observed nearby regenerating muscle fibers is probably related to the phenomenon of terminal sprouting that occurs after partial denervation (Brown et al., 1980; Slack and Pockett, 1981; Pockett and Slack, 1982; Slack et al., 1983) or inactivation of muscles (Betz et al., 1980; Holland and Brown, 1980). In some of the cases, the sprouting also seemed to be confined to junctions in close proximity to the affected muscle fibers (Slack and Pockett, 1981; Pockett and Slack, 1982). Although the restriction of sprouting to adjacent motor terminals has been used as an argument of a contact-mediated sprouting stimulus (Brown et al., 1978), the directional growth we saw implies a gradient of some factor either associated with the extracellular matrix or cellular elements therein (Reynolds and Woolf, 1992) or the factor is freely diffusing.

In summary, the ability of nerve terminals to rapidly respond by sending a neurite toward a regenerating fiber in their midst shows that regenerating fibers can efficiently cause nerves to reinnervate them. The fact that all but one axon are eliminated by sprout withdrawal and synapse elimination indicates that the same program in operation in development and reinnervation is used to organize the innervation on regenerating fibers.

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