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Neural cell adhesion molecule (N-CAM) accumulates in denervated and paralyzed skeletal muscles

(neuromuscular junction/synapse formation/reinnervation)

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ABSTRACT **We have used immunofluorescence and immunoblotting methods to study the amount and distribution of the neural cell adhesion molecule (N-CAM) in rat skeletal muscle; this molecule is thought to mediate adhesion of neurons to cultured myotubes. N-CAM is present on the surface of embryonic myotubes, but it is lost as development proceeds and is nearly absent from adult muscle. However, denervation of adult muscle results in the reappearance of N-CAM. In denervated muscle, N-CAM is associated both with muscle fibers and with cells in interstitial spaces between fibers. The N-CAM in interstitial spaces is concentrated near denervated endplates, which are known to be preferential sites for reinnervation. Paralysis of innervated muscle, known to mimic denervation in many respects, also induces the accumulation of N-CAM. Axons that regenerate to reinnervate muscle bear N-CAM on their terminals, and reinnervation results in the disappearance of N-CAM from muscle. Denervation induces accumulation of N-CAM in mouse and chicken, as well as in rat muscles. Thus, the expression of N-CAM in muscle is regulated by the muscle's state of innervation. In that N-CAM-rich muscles (embryonic, denervated, and paralyzed) are known to be competent to accept synapses, while N-CAM-poor muscles (normal adult and reinnervated) are refractory to hyperinnervation, N-CAM might, in turn, participate in regulating muscle's susceptibility to innervation.**

Skeletal muscle fibers regulate their ability to receive new synapses in accordance with their current state of innervation: nerves implanted in innervated muscles do not form synapses, while denervated muscles readily accept innervation (1, 2). Similarly, when muscles are partially denervated, intact axons sprout to reinnervate denervated fibers (2, 3). Thus, signals must pass both from nerve to muscle and from muscle to nerve as synapses form and are maintained. Nerves apparently use conventional processes of synaptic transmission to render muscle refractory to new innervation in that paralysis of innervated muscle induces sprouting and synapse formation, while direct stimulation of denervated muscle inhibits sprouting and synapse formation (2–4). However, it is not known how muscle fibers inform nerves of their susceptibility or refractoriness to innervation. One hypothesis is that denervation or paralysis induces the release of a soluble factor that stimulates axonal growth, synapse formation, or both. In support of this idea, several groups have shown that denervated and embryonic muscles secrete molecules that promote neuron survival and neurite extension *in vitro* (5) and that antiserum to one such molecule suppresses sprouting *in vivo* (6). A second response of muscle fibers to denervation or inactivity may be the acquisition of new surface properties that enhance their attractiveness to axons (2, 3). Here we report evidence that supports this possibility: denervation or paralysis of skeletal muscle induces the accumulation of an adhesive cell surface molecule, the neural cell adhesion molecule (N-CAM).

N-CAM is an integral membrane glycoprotein that is present on the surfaces of most peripheral and central neurons. This molecule is thought to mediate a variety of intercellular adhesive interactions in the nervous system (7–9). Antibodies to N-CAM can inhibit interactions among neurites (fasciculation) and neurons (aggregation) *in vitro* (7) and can disorder axonal growth *in vivo* (10). Although initial studies of N-CAM focused on its role in mediating interneuronal interactions, Rutishauser, Grumet, and Edelman (11, 12) have recently shown that N-CAM is present on the surface of cultured embryonic chicken myotubes, and that antibodies to N-CAM can inhibit adhesion of neurons and neurites to muscle cells *in vitro*. Our demonstration that N-CAM is induced by denervation or paralysis suggests that this molecule may also influence innervation of muscle *in vivo*.

METHODS

Antibodies to N-CAM. Chicken N-CAM was purified by using a species-specific monoclonal antibody and was used to generate an antiserum that recognizes N-CAM in several vertebrate species. Monoclonal antibody 224-1A6-A1, which binds to chicken N-CAM (refs. 13 and 14; David Gottlieb, personal communication) was generously provided by D. Gottlieb (Washington University). The antibody was isolated from ascites fluid on staphylococcal protein A-Sepharose (13), coupled to Affigel-10 (Bio-Rad), and used to purify N-CAM from a Nonidet P-40 extract of embryonic day 15 chicken brain membranes (15). A New Zealand White rabbit was immunized intradermally with 50- to 100-μg aliquots of electrophoretically (16, 17) pure N-CAM. Anti-N-CAM antibodies were purified from the serum by affinity chromatography on chicken brain N-CAM-agarose. Immunoblotting (see below) showed that the antiserum and 224-1A6-A1 both recognized the highly sialylated ≈200- to 250-kDa N-CAM found in embryonic brain and the less sialylated ≈140-kDa and ≈180-kDa forms of N-CAM in adult chicken brain (Fig. 1; refs. 8 and 9). Additionally, the antiserum, unlike the monoclonal antibody, recognized the homologous N-CAM proteins from rat (Fig. 1), mouse, and frog brains.

Surgery. Rats (Sprague–Dawley; Chappel, St. Louis, MO) were anesthetized with chloral hydrate for surgery. Diaphragms were denervated by damaging the phrenic nerve intrathoracically (18); either individual muscle nerves or the entire sciatic nerve was damaged to denervate leg muscles. Nerves were severed completely when denervation alone was to be studied, or crushed with fine forceps near the muscle's edge when reinnervation was studied. Leg muscles were paralyzed for 6–9 days by implantation of a tetrodotoxin-filled capillary beneath the epineurium of the sciatic nerve as described by Mills and Bray (19).

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Abbreviation: N-CAM, neural cell adhesion molecule.

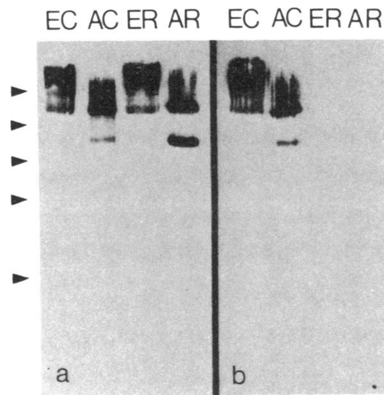


FIG. 1. Immunoblot analysis of antibodies to chicken N-CAM. Nonidet P-40 extracts of embryonic (E) or adult (A) chicken (C) or rat (R) brain were probed with anti-N-CAM antiserum (*a*) or monoclonal antibody 224-1A6-A1 (*b*) and the appropriate peroxidase-conjugated second antibody. The antiserum but not the monoclonal antibody cross-reacted with rat N-CAM. Arrowheads indicate positions of molecular mass standards (from the top: myosin, 200 kDa; *Escherichia coli* RNA polymerase β subunit, 160 kDa; β-galactosidase, 116 kDa; *E. coli* RNA polymerase σ subunit, 90 kDa; pyruvate kinase, 57 kDa).

Immunofluorescence. Cryostat sections of unfixed tissue were incubated successively with affinity-purified anti-N-CAM (5–10 μg/ml) and fluorescein-conjugated goat antibodies to rabbit IgG (Cappel Laboratories, Cochranville, PA), washed, and mounted in a glycerol/phenylenediamine mixture (details in refs. 20 and 21). To identify endplates, rhodamine-α-bungarotoxin (22) was added to the second antibody solution. To identify axons, a monoclonal antibody to neurofilaments (23) was included in the incubation with anti-N-CAM, and rhodamine-conjugated goat antibodies to mouse IgG (Cappel Laboratories) were mixed with the fluorescein-conjugated second antibody.

Immunoblot Analysis. Tissues were homogenized in physiological saline with or without detergent (Nonidet P-40) and centrifuged at 25,000 × *g* for 10 min. Supernatants were mixed with sample buffer [final concentrations 2% sodium dodecyl sulfate/20 mM dithiothreitol/5% (vol/vol) glycerol/20 mM Tris·HCl, pH 6.8], heated to 56°C for 10 min, and electrophoresed in 0.3-mm-thick sodium dodecyl sulfate/7% polyacrylamide gels (16). Proteins were transferred from the gels to nitrocellulose filters (BA 80, Schleicher & Schuell) in 96 mM glycine/12.5 mM Tris/20% (vol/vol) MeOH for 60 min at a field strength of 5 V/cm (24). N-CAM was detected by the sequential incubation of filters with anti-N-CAM, peroxidase-conjugated second antibody (Cappel Laboratories), and finally 0.01% diaminobenzidine/0.005% H_2O_2.

RESULTS

Denervation-Induced Accumulation of N-CAM. N-CAM was present on the surface of embryonic rat myotubes (Fig. 2*a*) but disappeared during the first 2 postnatal weeks (not shown) and was undetectable on the surface of normal adult muscle fibers (Fig. 2*b*). However, denervation of adult muscles induced a rapid and dramatic reappearance of N-CAM (Fig. 2*c*). In rat diaphragm, N-CAM appeared within 2 days of denervation, the earliest time studied. Levels of immunohistochemically detectable N-CAM increased to reach an apparent maximum 4–8 days later, and remained elevated in muscles kept denervated for up to 300 days. N-CAM levels also increased markedly after denervation of rat soleus (a predominantly slow muscle), extensor digitorum longus and intercostal (predominantly fast muscles), mouse soleus, and chicken pectoralis. Results obtained with our affinity-purified antibody to N-CAM were confirmed in mouse by using an antiserum to mouse N-CAM (25) generously provided by U. Rutishauser (Case Western Reserve), and in chicken by using monoclonal antibody 224-1A6-A1. Thus, accumulation of N-CAM is a general response to denervation of muscle.

Characterization of N-CAM in Denervated Muscles. N-CAM accumulated in three histologically distinct sites in denervated rat muscles (Fig. 2*c*). First, N-CAM was associated with the surfaces of muscle fibers. At least some of this N-CAM was externally directed, in that it was detectable when live muscles were incubated with anti-N-CAM and then washed before being sectioned and stained with fluorescein-conjugated second antibody. We do not yet know the ultrastructural relationship of surface N-CAM to the plasma membrane and to the overlying basal lamina. Second, N-CAM was present intracellularly, within muscle fibers. The intensity of intracellular staining varied greatly among fibers in a manner reminiscent of "fiber-type" staining. However, many fibers were stained in both fast (extensor digitorum longus) and slow (soleus) muscles, and staining of serial sections with type-specific anti-myosin (26) revealed that N-CAM was not uniquely associated with either fast- (type II) or slow- (type I) twitch fibers. Finally, N-CAM was present in interstitial spaces between muscle fibers. A major portion of this interstitial N-CAM was associated with mononucleated cells that typically bear one or two sparsely branched, elongated processes (Fig. 2*d*).

We used immunoblotting to determine which form(s) of N-CAM accumulated in denervated muscles. Anti-N-CAM recognized a protein with an apparent molecular mass of ≈140 kDa, in Nonidet P-40 extracts of denervated (Fig. 3, lane 1) but not innervated (lane 2) adult muscle. This protein was indistinguishable in molecular mass from the anti-N-CAM-reactive material in embryonic muscle (lane 3) and corresponded to the ≈140-kDa form of N-CAM present in adult brain (lane 4). Neither 180-kDa nor highly sialylated (≈200-kDa) forms of N-CAM were detected in late embryonic or denervated rat muscles; similar results have been reported for chicken muscle and retina (7, 12). Manyfold more N-CAM was extracted from denervated muscle with physiological saline containing the detergent Nonidet P-40 than with saline alone, suggesting that in muscle, as in brain (27), N-CAM is an integral membrane protein.

Association of N-CAM with Denervated Synaptic Sites. We examined the distribution of N-CAM along the length of muscle fibers in denervated rat diaphragm, a muscle in which endplates occupy a narrow central band. Denervated synaptic sites were identified by staining with rhodamine-α-bungarotoxin, which binds to acetylcholine receptors in the postsynaptic membrane. While the postsynaptic membrane itself was only slightly richer in N-CAM than adjacent extrasynaptic areas, interstitial deposits of N-CAM were strikingly associated with denervated synaptic sites (Fig. 4 *a* and *b*). Reconstructions from serial sections showed that the concentration of N-CAM-stained interstitial material was highest within a few tens of micrometers of endplates and then declined over a distance of several hundred micrometers to a low level that was present throughout the muscle (Fig. 4 *c* and *d*). In contrast, intracellular and muscle surface-associated deposits of N-CAM were present in similar abundance both near to and far from endplates. Thus, interstitial deposits of N-CAM mark endplate-containing areas in denervated muscle.

Accumulation of N-CAM in Paralyzed Muscle. Muscle inactivity mimics denervation in that it induces terminal sprouting and renders muscle fibers susceptible to hyperinnervation (2–4). We therefore asked whether N-CAM appeared in paralyzed muscles. A capillary filled with the action potential blocker tetrodotoxin was implanted in the sci-

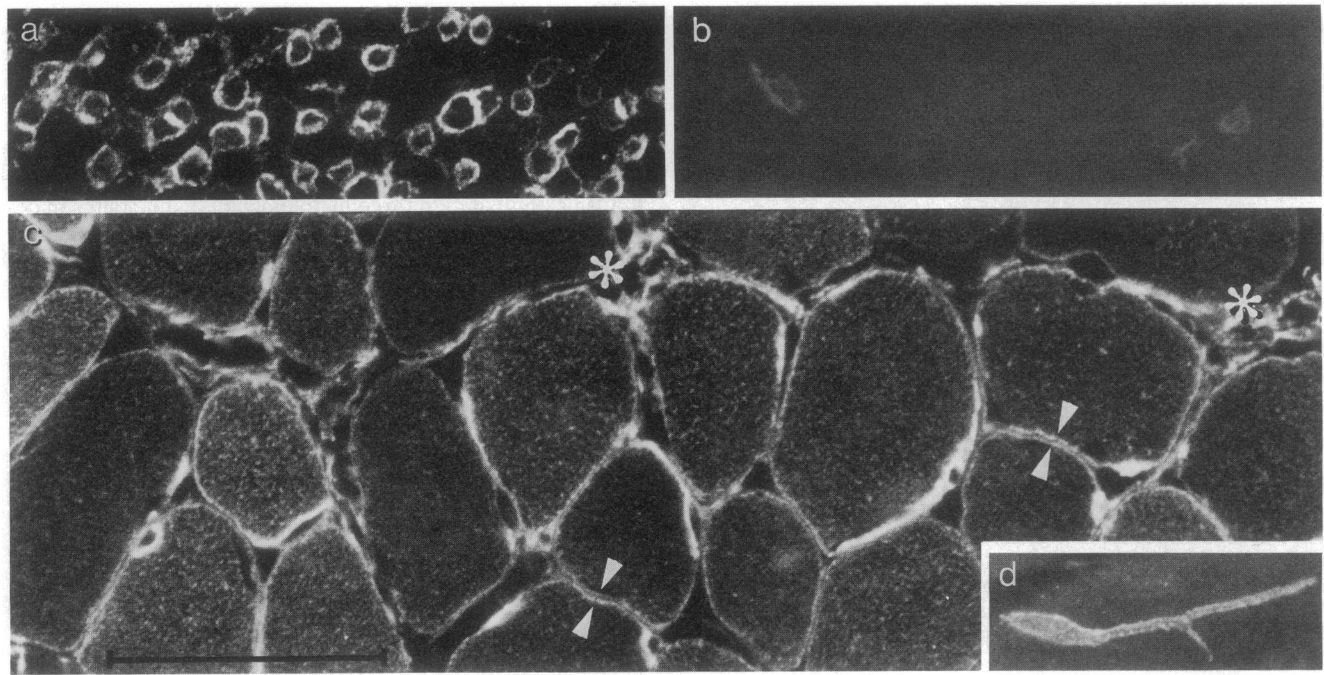


FIG. 2. Cross sections of embryonic day 15 (*a*), adult (*b*), and 2-week denervated adult (*c*) rat muscles stained with anti-N-CAM and fluorescein-conjugated goat anti-rabbit IgG. N-CAM is present on embryonic myotubes, disappears during development, and reappears after denervation. In denervated muscle, N-CAM is present within muscle fibers, on the muscle fiber surface (arrowheads), and in interstitial spaces between muscle fibers (asterisks). All staining was abolished by absorption of anti-N-CAM with N-CAM or by substitution of preimmune Ig for anti-N-CAM (not shown). (*d*) N-CAM-stained interstitial cell in a 30-μm-thick longitudinal section of a denervated muscle (*a–c* are 4-μm-thick cross sections). (Bar is 100 μm for all parts.)

atic nerve to paralyze leg muscles (19). Six to 8 days of paralysis induced accumulation of N-CAM in both soleus (Fig. 5*a*) and extensor digitorum longus muscles. In paralyzed, as in denervated muscles, N-CAM appeared within muscle fibers, on muscle fiber surfaces, and on cells in interstitial spaces between muscle fibers. Implantation of a saline-filled capillary in the sciatic nerve induced neither paralysis nor accumulation of N-CAM (Fig. 5*b*). The appearance of N-CAM in paralyzed muscle indicates that this response is not simply a consequence of nerve degeneration but is mediated, at least in part, by inactivity.

Disappearance of N-CAM from Reinnervated Muscle. Reinnervation restores denervated muscle fibers to a state in which they are refractory to further innervation (28). To ask whether N-CAM disappeared from muscles upon reinnervation, we crushed the soleus nerve to denervate the muscle in a way that favors rapid reinnervation. Reinnervation began 3–4 days after nerve crush, and was nearly complete about 10 days later (Fig. 6*a*). N-CAM appeared in the soleus during the first week after nerve crush, before reinnervation was extensive (Fig. 6*b*), but was nearly absent from muscles studied 2 weeks after nerve crush, soon after reinnervation was complete (Fig. 6*c*). In contrast, when reinnervation was prevented, by cutting the nerve instead of crushing it, high levels of N-CAM persisted (Fig. 2*c*). Similar results were obtained during reinnervation of diaphragm. Thus, reinnervation reverses denervation-induced accumulation of N-CAM.

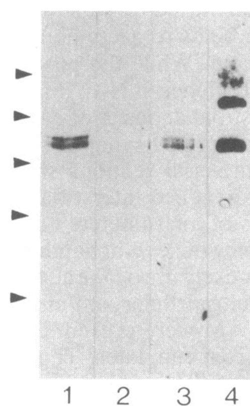


FIG. 3. Immunoblot analysis of N-CAM in Nonidet P-40 extracts of 1-week denervated adult (lane 1), normal adult (lane 2), or embryonic day 20 (lane 3) rat diaphragm or of adult rat brain (lane 4). Embryonic and denervated muscles contain a 140-kDa form of N-CAM that sometimes appears as a doublet and is undetectable in normal adult muscle. Standards as in Fig. 1.

N-CAM in Normal and Regenerating Nerves. N-CAM-mediated interactions are thought to be homophilic—that is, N-CAM molecules on adjacent cells bind directly to each other to form adhesive bonds (30). Because the ability of axons to interact with muscle N-CAM may depend on the presence of axonal N-CAM, we examined the distribution of N-CAM in normal and regenerating peripheral nerves. N-CAM was abundant in embryonic intramuscular nerves (Fig. 7*a*) but was lost as development proceeds and was undetectable on large (myelinated) axons of which adult motor axons are a subset (Fig. 7 *b* and *c*). N-CAM was present, however, on the unmyelinated terminals of motor axons at normal neuromuscular junctions (Fig. 7 *d* and *e*). After nerve crush, regenerating motor axons retained N-CAM-poor surfaces both proximal and distal to the point of injury (not shown), but N-CAM was present on terminal portions of axons at reinnervated endplates (Fig. 7 *f* and *g*). Thus, portions of normal and regenerating motor axons that contact muscle fibers are rich in N-CAM.

DISCUSSION

Levels of N-CAM in muscle are regulated by innervation in a way that parallels the ability of muscle to accept synapses. N-CAM is abundant on embryonic myotubes during synap-

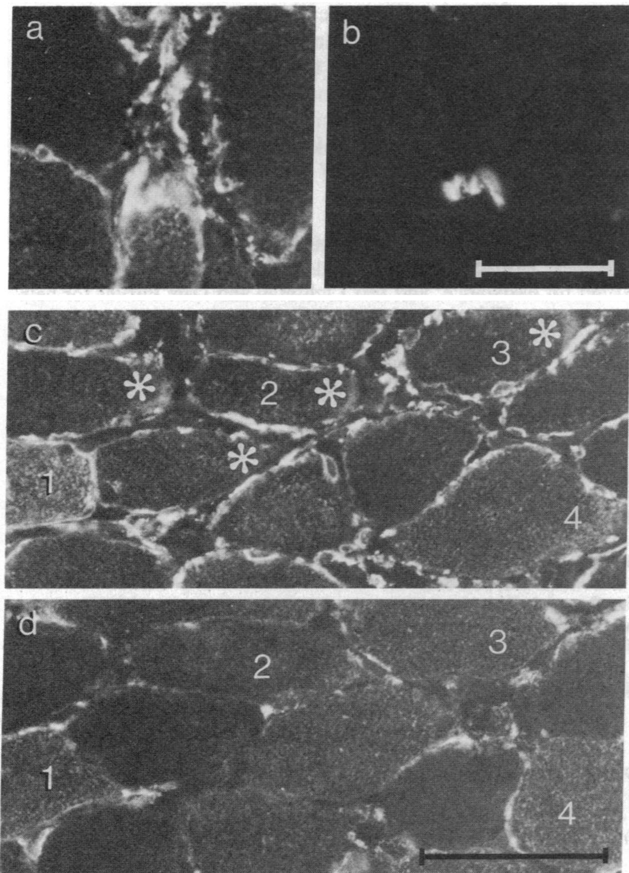


FIG. 4. (*a* and *b*) Denervated muscle doubly stained with anti-N-CAM (*a*) and rhodamine-α-bungarotoxin (*b*) to show association of interstitial N-CAM with a synaptic site. (*c* and *d*) Two sections from a serially sectioned diaphragm stained with anti-N-CAM. *c* is rich in endplates (identified with rhodamine-α-bungarotoxin and indicated by asterisks); *d* is 420 μm distant from *c* and bears no endplates. Cytoplasmic and cell surface staining are comparable in *c* and *d*, but interstitial deposits of N-CAM are more prominent in *c*. Numbers (1–4) indicate the same fibers in *c* and *d*. (Bar is 25 μm for *a* and *b*, and 50 μm for *c* and *d*.)

togenesis but disappears during development and is undetectable on adult muscles, which are refractory to hyperinnervation. Denervation induces the reappearance of N-CAM, both on muscle fiber surfaces upon which synapses form and in interstitial spaces through which regenerating axons grow. Paralysis, which mimics denervation in rendering muscles susceptible to innervation, induces accumulation of N-CAM. Reinnervation, which restores muscles to a refractory state, leads to the disappearance of N-CAM. This pattern of expression, which resembles that of acetylcholine receptors in many respects (31), takes on particular significance in light of recent experiments (11, 12) indicating that N-CAM may mediate adhesive interactions between neurons and muscle cells *in vitro*. Furthermore, N-CAM, which is thought to act as its own receptor (30), is present on the terminal portions of normal and regenerating motor axons that contact muscle fibers. Taken together, these results suggest that N-CAM could participate in regulating muscle's susceptibility to innervation *in vivo*.

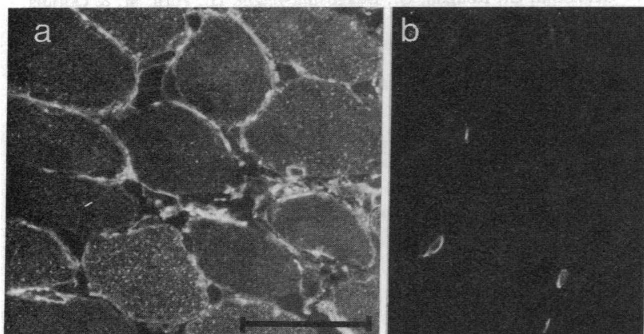
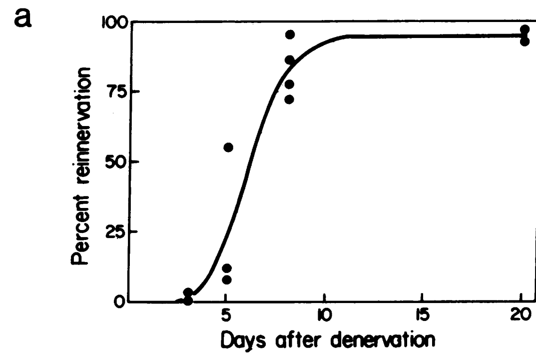


FIG. 5. Paralysis induces accumulation of N-CAM. Capillaries containing tetrodotoxin or saline were implanted in the sciatic nerve; 6 days later, the paralyzed (*a*) and control (*b*) soleus muscles were cross-sectioned and stained with anti-N-CAM. (Bar is 50 μm.)



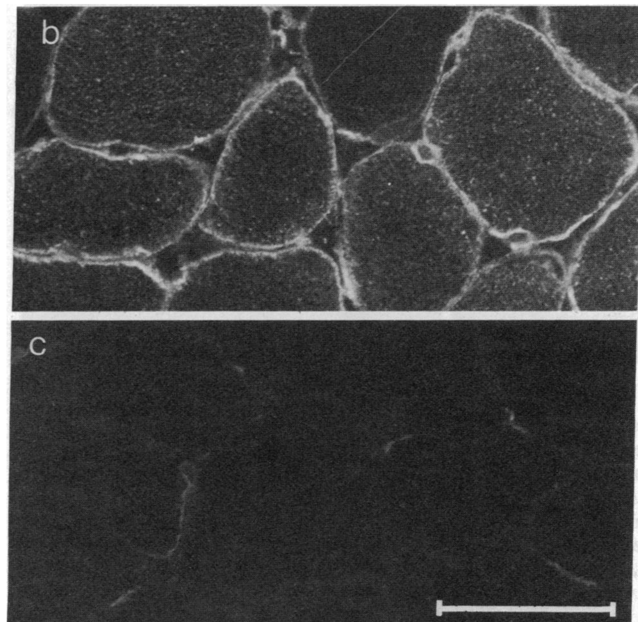


FIG. 6. N-CAM in reinnervated muscle. (*a*) Percent of original synaptic sites in rat soleus reinnervated at various times after nerve crush determined electron microscopically as described in ref. 29. Virtually all reinnervation occurred at original sites. Each point represents a single muscle. (*b* and *c*) Anti-N-CAM-stained cross sections of soleus muscles 6 days after nerve crush, during the first stages of reinnervation (*b*), and 14 days after nerve crush, when reinnervation is complete (*c*). (Bar is 50 μm.)

Recent studies on the control of reinnervation and sprouting in muscle have focused on the hypothesis that denervated or inactive muscles secrete a soluble factor, perhaps analogous to nerve growth factor, that stimulates axonal growth (5). However, the idea that denervation-induced changes in the muscle fiber surface may also influence muscle's ability to accept synapses helps to explain some hitherto puzzling observations. First, when nerves are implanted in innervated muscles, axons elongate and ramify, but form no synapses with muscle fibers until the muscle's own nerve is damaged or paralyzed (32, 33). Thus, some interaction with the mus-

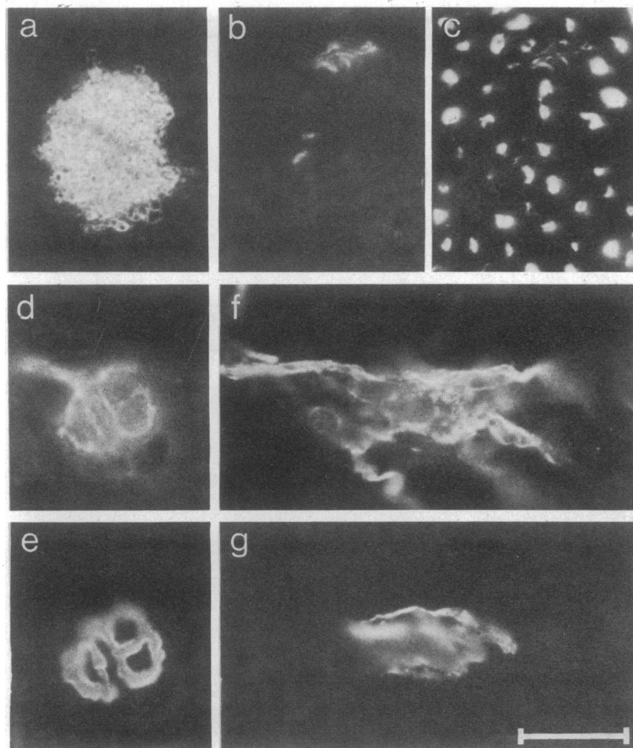


FIG. 7. N-CAM in normal and regenerating axons. (*a–c*) Cross sections of embryonic day 15 (*a*) and normal adult (*b* and *c*) motor nerves. The adult nerve was doubly stained with anti-N-CAM (*b*) and a monoclonal antibody to neurofilaments (*c*) to show axons. N-CAM is abundant in embryonic nerve, but is restricted to small (presumably unmyelinated sympathetic and sensory) fibers in the adult nerve. (*d–g*) Synaptic sites in normal (*d* and *e*) and reinnervated (12 days after nerve crush; *f* and *g*) muscles, doubly stained with anti-N-CAM (*d* and *f*) and rhodamine-α-bungarotoxin (*e* and *g*). N-CAM-rich nerve terminals abut acetylcholine receptor-rich postsynaptic membrane at normal and reinnervated endplates. N-CAM-rich terminal sprouts frequently extend beyond reinnervated endplates. (Bar represents 25 μm for *a–g*.)

cle fiber surface rather than axonal growth may be prevented in innervated muscle. Second, after partial denervation, only terminals within 100–200 μm of a denervated muscle fiber form sprouts (34, 35), indicating that some signal emanating from denervated fibers has a short effective range and may not be freely diffusible. These results are perhaps best interpreted by hypothesizing that cell surface molecules such as N-CAM, as well as soluble factors, are involved in signalling muscle's susceptibility to innervation.

When axons reinnervate skeletal muscles, they preferentially form synapses at original synaptic sites; however, axons can form new (ectopic) synapses in previously extrasynaptic areas if they are implanted far from (or denied access to) original sites (2). Recent experiments have suggested that some of the cues that axons recognize at original synaptic sites are associated with the basal lamina of the synaptic cleft (29, 36). To reach these sites, regenerating axons often grow through surviving nerve sheaths; however, axons growing outside of sheaths can also find original synaptic sites (discussed in ref. 29). In this regard, the interstitial deposits of N-CAM that appear after denervation are intriguing. These deposits, which may be associated in part with cells that proliferate after denervation (37, 38), are particularly abundant near endplates. Unlike acetylcholine receptors or synaptic components of the basal lamina (39), the synapse-associated N-CAM is not concentrated in the postsynaptic surface *per se*; instead it extends several hundred micrometers from denervated endplates and thus may play a role in guiding axons in endplate regions to synaptic sites. Axons that did not encounter this region would receive no such guidance and might be more likely to form ectopic synapses. Thus, interstitial deposits of N-CAM might help to account, along with nerve trunks and synaptic basal lamina, for the topographic features of reinnervation.

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