Expression of Several Adhesive Macromolecules (N-CAM, L1, J1, NILE, Uvomorulin, Laminin, Fibronectin, and a Heparan Sulfate Proteoglycan) In Embryonic, Adult, and Denervated Adult Skeletal Muscle

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Abstract. Levels of the neural cell adhesion molecule N-CAM in muscle are regulated in parallel with the susceptibility of muscle to innervation: N-CAM is abundant on the surface of early embryonic myotubes, declines in level as development proceeds, reappears when adult muscles are denervated or paralyzed, and is lost after reinnervation (Covault, J., and J. R. Sanes, 1985, *Proc. Natl. Acad. Sci. USA*, 82:4544–4548). Here we used immunocytochemical methods to compare this pattern of expression with those of several other molecules known to be involved in cellular adhesion. Laminin, fibronectin, and a basal lamina–associated heparan sulfate proteoglycan accumulate on embryonic myotubes after synapse formation, and their levels change little after denervation. L1, J1, nerve growth factor–inducible large external protein, uvomorulin, and a carbohydrate epitope (L2/HNK-1) shared by several adhesion molecules are undetectable on the surface of embryonic, perinatal, adult, or denervated adult muscle fibers. Thus, of the molecules tested, only N-CAM appears on the surface of muscle cells in parallel with the ability of the muscle cell surface to accept synapses. However, four antigens—N-CAM, J1, fibronectin, and a heparan sulfate proteoglycan—accumulate in interstitial spaces near denervated synaptic sites; regenerating axons traverse these spaces as they preferentially reinnervate original synaptic sites. Of particular interest is J1, antibodies to which block adhesion of central neurons to astrocytes (Kruse, J., G. Keihauer, A. Faissner, R. Timpl, and M. Schachner, 1985, *Nature (Lond.)*, 316:146–148). J1 is associated with collagen and other fibrils in muscle and thus may be an extracellular matrix molecule employed in both the central and peripheral nervous systems.

EMBRYONIC myotubes readily accept synapses, but motor axons cannot form new (ectopic) synapses on normally innervated adult muscle fibers. However, adult muscles regain their susceptibility to synapse formation if they are denervated or paralyzed, and denervated muscles again become refractory to synapse formation if they are reinnervated or electrically stimulated. Thus, muscles regulate their susceptibility to synapse formation in accordance with their state of innervation or activity, and considerable interest has focused on the means by which muscles inform nerves of their ability to accept innervation (reviewed in reference 39).

In investigating this problem, we recently found that muscles regulate their expression of the neural cell adhesion molecule N-CAM in parallel with their susceptibility to innervation: N-CAM is abundant in early embryonic, denervated, and paralyzed adult muscles, but present at low levels in normal and reinnervated adult muscles (3, 7, 7*a*, 7*b*). Furthermore, N-CAM (which serves as its own receptor) is present on terminals of normal and regenerating motor axons (7), and antibodies to N-CAM inhibit neurite-myotube interactions in vitro (32). Thus N-CAM may participate in regulating the susceptibility of the muscle to innervation in vivo.

These results raise a new question when considered in conjunction with recent studies on the adhesion of neurons to other cells and to external substrata. Neurons from a single source and in some cases single neurons have been shown to bear multiple adhesive mechanisms and to be able to interact with multiple ligands on other cellular or extracellular targets (e.g., references 10, 27, and 30). Furthermore, there is already evidence that muscles influence axonal behavior in several ways. For example, embryonic and denervated muscle not only accumulate N-CAM but also secrete soluble factors that promote survival and differentiation of neurons (reviewed in reference 37). It therefore seemed possible that myotubes might modulate their attractiveness to axons by coordinately regulating the expression of several adhesive macromolecules.

To test this idea we have compared the distributions, in muscle, of (*a*) four neural cell adhesion molecules—N-CAM (8, 31), L1 (28), J1 (17), and nerve growth factor-inducible large external protein (NILE)[1] (43); (*b*) a cell adhesion molecule hitherto studied in nonneural tissues, uvomorulin (14, 25); (*c*) three components of basal laminae (BL) with which neurites interact—laminin (9, 21, 30), heparan sulfate proteoglycan (18, 22), and fibronectin (2, 30); and (*d*) a carbohydrate epitope, L2/NHK-1, which is shared by several adhesive macromolecules (16). We report that, of these molecules, N-CAM is unique in appearing on muscle fiber surfaces in parallel with the susceptibility of the muscle to innervation. However, when adult muscles are denervated, interstitial spaces near synaptic sites acquire deposits of at least four adhesive macromolecules and thus may become able to influence the behavior of regenerating axons in several ways.

Materials and Methods

Animals

Sprague-Dawley rats were obtained from Chappel Breeders (St. Louis, MO). Rat embryos were obtained from pregnancies timed by the presence of sperm in vaginal smears; the first day of pregnancy was designated E0, and rats were born on E21 or 22. Based on results by Covault and Sanes (7*a*), intercostal muscles from E15 and E22 rats were chosen for detailed study here; the terms embryonic and perinatal refer to these ages, except where otherwise specified. Outbred Swiss mice (SASCO Inc., Omaha, NE) were used in studies using monoclonal antibodies that react with mouse but not rat tissue (DE1 and 324; see below).

Surgery

Rat diaphragms were denervated by cutting the phrenic nerve in the thorax or in the neck (23). Rat and mouse lower leg muscles were denervated by cutting the sciatic nerve mid-thigh. The nerve to the rat platysma was cut through an incision behind the ear. For studies of reinnervation, the appropriate nerve was crushed with fine forceps at the edge of the muscle. Rat lower leg muscles were paralyzed for 6–11 d by the implantation of a tetrodotoxin-filled capillary under the perineurium of the sciatic nerve, as described by Mills and Bray (24). The efficacy of implants was monitored using the toe spread reflex.

Antibodies

The antibodies used in this study are listed in Table I. Anti-fibronectin was provided by J. McDonald (Washington University, St. Louis, MO), DE1 by F. Jacob and N. Peyrieras (Institut Pasteur, Paris), ASCS4 by Paul Patterson (California Institute of Technology, Pasadena, CA), and HNK-1 by Tom Jessell (Harvard University, Cambridge, MA). On immunoblots, the polyclonal antibodies to N-CAM, J1, and L1 used here do not cross-react with each other's antigens (10, 17, 28, and Covault J., unpublished observation). Fluorescein-, rhodamine-, and horseradish peroxidase-conjugated second antibodies were purchased from Cappel Laboratories (Cochranville, PA), DAKO Corp. (Santa Barbara, CA), or Atlantic Antibodies (Scarborough, ME).

Immunohistochemistry

Light and electron microscopic immunohistochemical methods were performed as described by Covault and Sanes (7*a*). In brief, for light microscopy, cryostat sections of unfixed muscles were incubated successively with antibody and fluorescein-second antibody, then mounted and viewed with epifluorescence optics. Counterstains were rhodamine-α-bungarotoxin, which binds to acetylcholine receptors in the postsynaptic membrane and monoclonal antibodies to embryonic myosin (12) and to BL (38). To detect differences between innervated and denervated or between embryonic and adult muscles, two samples were mounted and frozen as a single block, and then sectioned, stained, and photographed together. For electron microscopy, rat platysma muscle was incubated live with antibody and horseradish peroxidase–second antibody, then fixed, reacted with diaminobenzidine/H_2O_2, refixed in OsO_4[2], dehydrated, and embedded in Araldite.

[1] *Abbreviations used in this paper:* BL, basal lamina(e); NILE, nerve growth factor-induced large external protein.

Table I. Antibodies to Adhesive Macromolecules

Antigen	Antibody designation and type	Source (reference)
N-CAM	Affinity-purified rabbit polyclonal	7 and 7*a*
L1	Rabbit polyclonal	28
	324; rat monoclonal IgG	19
J1	Rabbit polyclonal	17
	Affinity-purified rabbit polyclonal	
NILE	ASCS4; mouse monoclonal IgG	44
Uvomorulin	DE1; rat monoclonal IgG	15
Laminin	C24, C29; mouse monoclonal IgGs	12*a*
Fibronectin	Affinity-purified rabbit polyclonal	47
Heparan sulfate proteoglycan	B3, C17; mouse monoclonal IgGs	9*a*
CAM-associated carbohydrate	HNK-1; mouse monoclonal IgM	1
	L2 rat monoclonal IgM	16

Results and Discussion

N-CAM

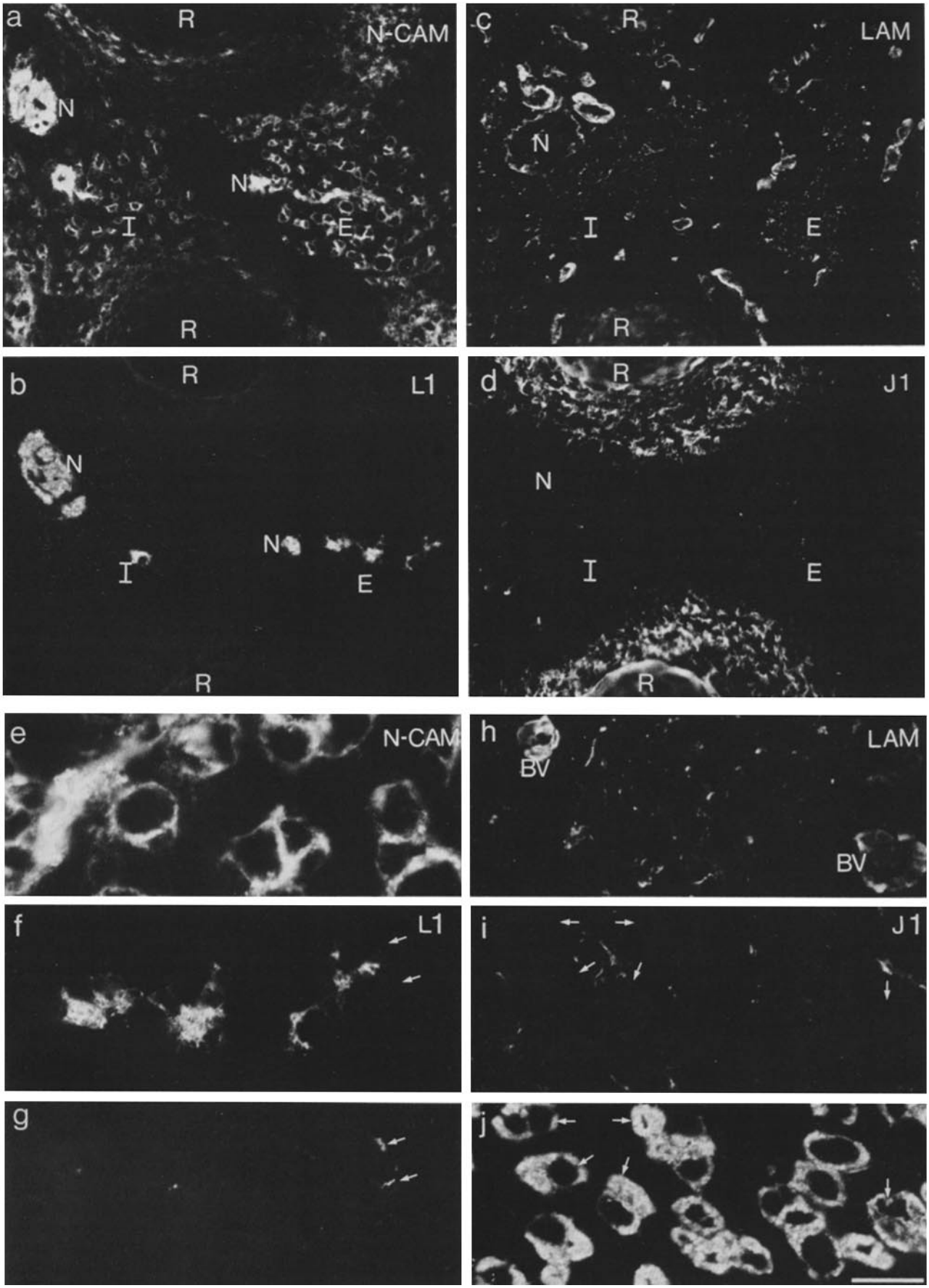
We have previously detailed the distribution of N-CAM in developing, adult, and denervated adult skeletal muscles (references 7 and 7*a*; see also reference 29). We review some of the main results here, as a basis for describing the expression of other adhesive macromolecules.

N-CAM is abundant on myotubes and intramuscular nerves in embryonic muscle (Fig. 1, *a* and *e*) but is lost from nonsynaptic areas as development proceeds (Fig. 2*a*). In adult muscle, N-CAM is concentrated near neuromuscular junctions (Fig. 2, *c* and *d*): it is present on the muscle fiber surface, intracellularly within muscle fibers, on nerve terminals, and on terminal associated Schwann cells but is undetectable on myelinated portions of motor axons, on myelinating Schwann cells, and on nonsynaptic portions of muscle fibers (7*a*). After denervation, N-CAM appears along the entire length of muscle fibers (Fig. 2*b*) and achieves comparable levels in synaptic and nonsynaptic areas. In addition, N-CAM appears in interstitial spaces between muscle fibers; these interstitial deposits are concentrated near denervated synaptic sites (Fig. 2, *e* and *f*). Paralysis mimics denervation in inducing expression of N-CAM in innervated muscle, and reinnervation results in the loss of N-CAM. In all these respects—near absence in normal and reinnervated muscle, induction in denervated and paralyzed muscles, and concentration near denervated synaptic sites—the regulation of N-CAM parallels the regulation of the ability of muscle to accept new synapses (discussed in reference 7).

L1

L1 is a glycoprotein present on the surfaces of various central neurons and on Schwann cells (10, 11, 28). Antibodies to L1 block the adhesion of neurons to each other and migration of neurons in the developing cerebellar cortex (10, 19, 27). A recently described neuron-glia adhesion molecule from chicken, Ng-CAM, resembles L1 in many respects and may be an avian equivalent of L1 (13).

L1 was not detectably associated with myotubes in embryonic (Fig. 1, *b* and *f*) or perinatal muscles, or with muscle fibers in innervated (Fig. 3*a*) or denervated (Fig. 3*b*) adult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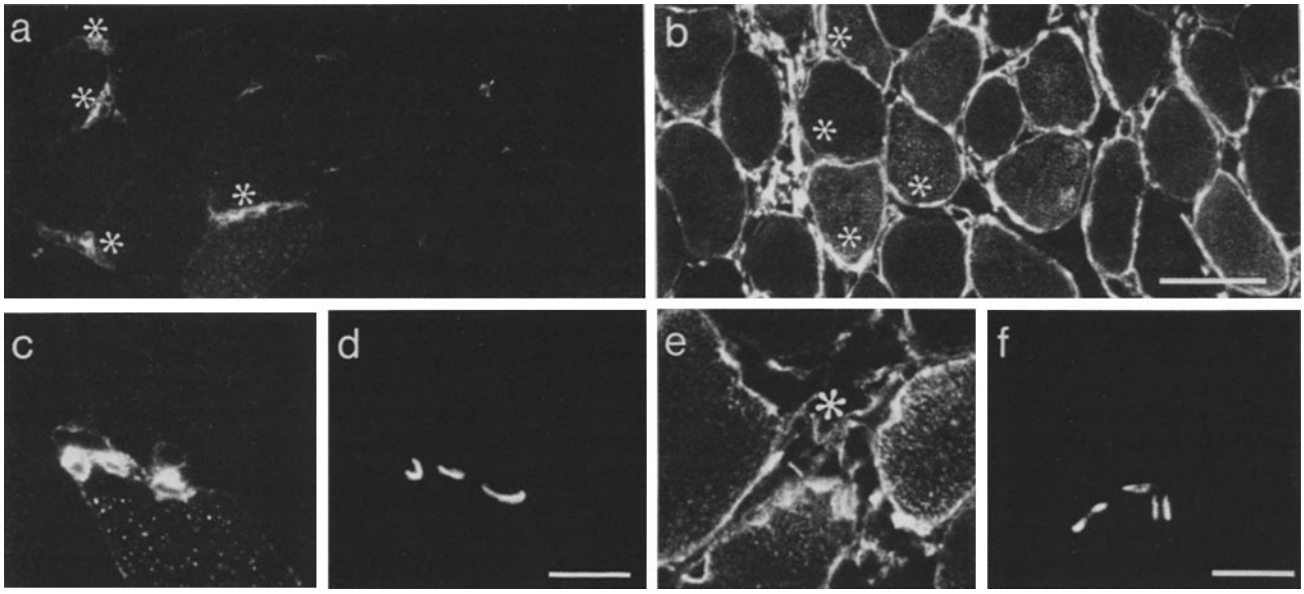

Figure 2. N-CAM in adult muscle. (*a* and *b*) Innervated (*a*) and 1-wk denervated (*b*) muscles stained with anti-N-CAM. (*c–f*) Endplates in innervated (*c* and *d*) and denervated muscle (*e* and *f*), doubly stained with anti-N-CAM plus fluorescein second antibody and rhodamine-α-bungarotoxin, then photographed with fluorescein (*c* and *e*) or rhodamine (*d* and *f*) optics. α-Bungarotoxin binds to acetylcholine receptors in the postsynaptic membrane and thereby marks synaptic sites. Asterisks in *a* and *b* mark synaptic sites, identified by counterstaining with rhodamine-α-bungarotoxin. Anti-N-CAM stains both pre- and postsynaptic structures in normal muscle but does not stain extrasynaptic areas of muscle. After denervation, presynaptic staining is decreased, but N-CAM appears extrasynaptically in muscle fibers and in interstitial spaces near endplates (asterisk in *e*). Bars: *a* and *b*, 50 μm; *c* and *d*, 15 μm; and *e* and *f*, 20 μm.

muscles. L1 was, however, present at neuromuscular junctions in adult muscle (Fig. 3, *c–f*). Electron microscopy revealed that the synapse-associated L1 in innervated muscle was concentrated in the narrow gap between nerve terminal and Schwann cell (Fig. 3*k*); N-CAM is also concentrated in this area of apposition (7*a*). We did not detect L1 on the surface of the nerve terminal that faces the muscle fiber, on the surface of the Schwann cell that faces interstitial areas, or on the muscle fiber surface. After denervation, levels of L1 decreased at synaptic sites (Fig. 3, *g–j*) and intensely L1-positive material was often displaced to the edge of the α-bungarotoxin-stained postsynaptic membrane. This pattern is consistent with the presence of L1 on Schwann cells, which are known to migrate away from synaptic sites after denervation (23).

As previously described for mice (11), L1 was abundant in embryonic (Fig. 1, *b*, *f*, and *g*) and perinatal rat peripheral nerves but became restricted to unmyelinated fibers in adult nerves (Fig. 3*a*). Electron microscopy showed that L1 in unmyelinated fibers (like N-CAM; see reference 7*a*), was concentrated in the area of apposition between axon and Schwann cell (Fig. 3*l*). The co-existence of L1 and N-CAM in areas of axon-Schwann cell contact, both along unmyelinated fibers and at the neuromuscular junction, suggests that both molecules may participate in adhesion of axons and nerve terminals to Schwann cells.

J1

Both N-CAM and L1 are recognized by monoclonal antibodies to an unusual, sulfated glycoconjugate (5, 16). These antibodies, L2 and HNK-1 (see below), also recognize the myelin-associated glycoprotein, which may be involved in axon-myelin adhesion (16, 26). This intriguing similarity in function suggested that other proteins bearing the L2/HNK-1 epitope might also be involved in intercellular adhesion. To test this prediction, Kruse et al. (17) isolated a fourth protein from brain that bears the L2/HNK-1 epitope and used this protein, J1, to prepare monospecific polyclonal antibodies that do not cross-react detectably with N-CAM or L1. Antibodies to J1 stain astrocytes but not neurons, and interfere

Figure 1. Adhesive macromolecules in embryonic muscle. Cryostat sections of E15 (*a–h*) or E16 (*i* and *j*) intercostal were stained with antibodies to N-CAM (*a* and *e*), L1 (*b* and *f*), laminin (*LAM*; *c* and *h*), or J1 (*d* and *i*) plus fluorescein second antibodies. *a–d* are longitudinal sections through the thorax, showing single internal (*I*) and external intercostals (*E*) in the space between adjacent ribs (*R*). *e–j* show a portion of the intercostal at higher magnification. *g* is the same field as *f*, stained with rhodamine-α-bungarotoxin and viewed with rhodamine optics to show synaptic sites. *j* is the same field as *i*, stained with antimyosin and rhodamine-second antibody to show myotubes. Anti-N-CAM stains intramuscular nerves (*N*) and myotubes. Anti-L1 stains nerve trunks and nerve branches that approach synaptic sites but does not stain myotubes. Anti-laminin stains only small patches on myotube surfaces, whereas surfaces of nerve trunks and of blood vessels (*BV*) that surround (but do not yet penetrate) muscles are intensely stained. J1 is associated with the layers of fine processes that ensheath the ribs; some fine J1-stained strands are also present in spaces between myotubes. Arrows mark corresponding points on *f* and *g* and on *i* and *j*. Bars: *a–d*, 50 μm; *e–j*, 20 μm.

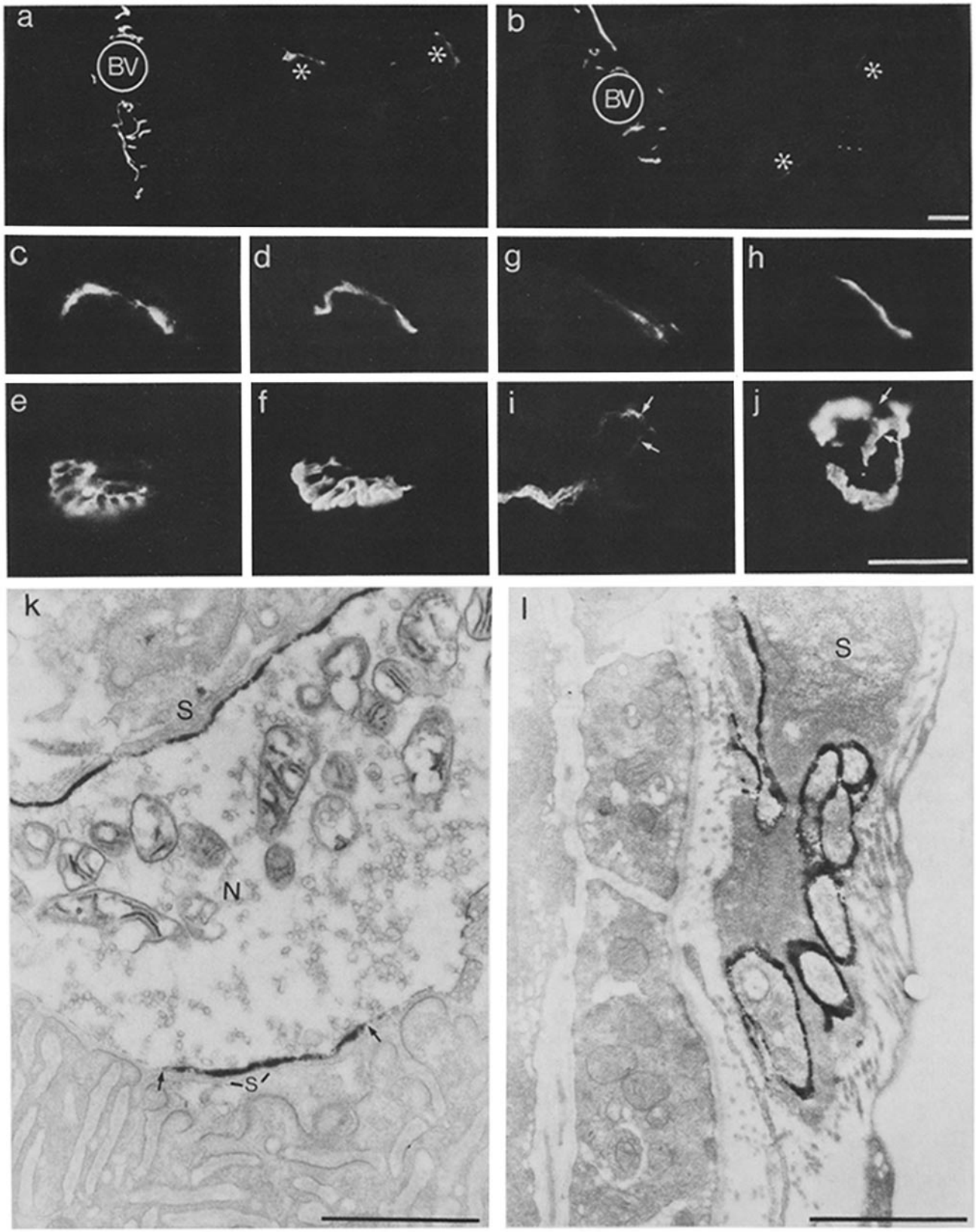

Figure 3. L1 in adult muscle. (*a* and *b*) Innervated (*a*) and 1-wk denervated (*b*) muscles stained with anti-L1. (*c–j*) Endplates in innervated (*c–f*) and denervated (*g–j*) muscle, doubly stained with anti-L1 plus fluorescein-second antibody (*c*, *e*, *g*, and *i*) and rhodamine-α-bungarotoxin (*d*, *f*, *h*, and *j*). *c* and *d* and *g* and *h* are 4-μm-thick cross-sections; *e* and *f* and *i* and *j* are 20-μm-thick longitudinal sections. In normal muscle, anti-L1 stains endplates (identified with rhodamine-α-bungarotoxin and marked by asterisks in *a* and *b*) and nerve fibers associated with blood vessels (*BV*); muscle fibers are unstained. After denervation, L1 decreases at but does not disappear from synaptic sites. Arrows mark corresponding points on *i* and *j*. (*k* and *l*) Electron micrographs of a neuromuscular junction (*k*) and an unmyelinated nerve fiber near a blood vessel (*l*), from a muscle stained with anti-L1 and HRP-second antibody. Reaction product is concentrated in the narrow gap between nerve terminal (*N*) or axon and Schwann cell (*S*) membranes. Schwann cell processes that extend between nerve terminal and muscle fiber are also stained at areas of apposition to the terminal (between arrows in *k*), but neither Schwann cell nor terminal is stained in areas of apposition to synaptic cleft or Schwann cell BL. Smooth muscles and endothelial cells of an intramuscular arteriole are visible at the left of *l*. Bars: *a* and *b*, 25 μm; *c–j*, 25 μm; *k*, 1 μm; *l*, 1.5 μm.

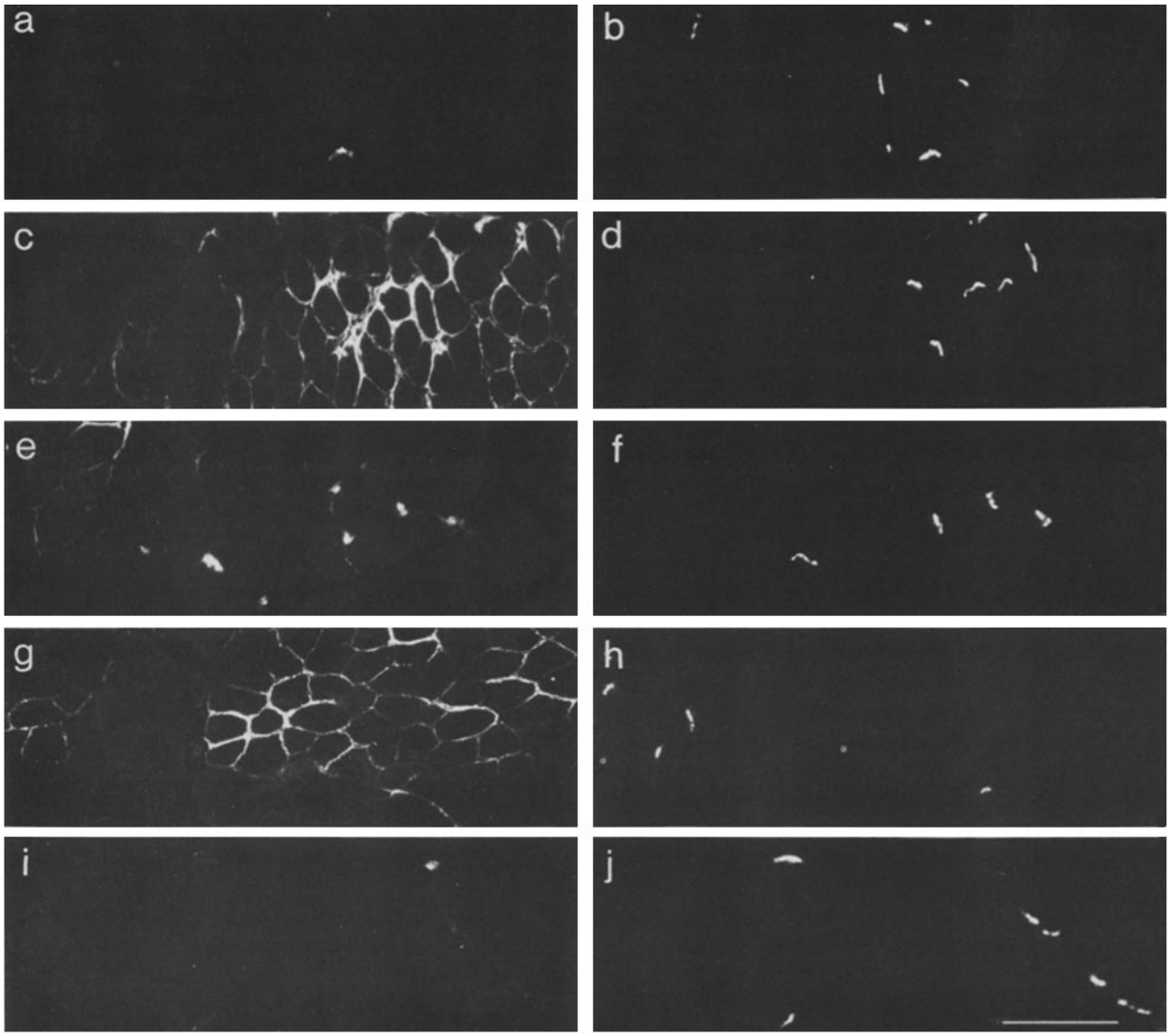

Figure 4. J1 appears in denervated or paralyzed muscle and is lost after reinnervation. Cryostat sections were doubly stained with anti-J1 plus fluorescein-second antibody (*left*) and rhodamine-α-bungarotoxin (*right*). (*a* and *b*) Normal adult diaphragm. Anti-J1 stains a few endplates lightly, most are unstained. (*c* and *d*) 1-wk denervated diaphragm. J1 appears after denervation and is concentrated in areas near endplates. (*e* and *f*) 2-wk denervated soleus. At later times after denervation, deposits of J1 are smaller and more closely associated with synaptic sites. In addition, J1 is more closely focused at synaptic sites in soleus than in diaphragm. (*g* and *h*) 11-d paralyzed soleus. Paralysis induces accumulation of J1 but not in regular association with synaptic sites. (*i* and *j*) Reinnervated soleus, 2 wk after nerve crush. Levels of J1 decrease after reinnervation. Bar, 100 μm.

with neuron-astrocyte adhesion in vitro.

In embryonic intercostal areas, anti-J1 stained material associated with developing ribs far more intensely than muscles (Fig. 1 *d*). Examination at higher power revealed that some immunoreactive material was present in muscle, in the form of small, discrete deposits occupying spaces between myotubes (Fig. 1, *i* and *j*). Deposits of J1 were observed in interstitial spaces between muscle cells from E13 until birth but were sparse at all times and disappeared after birth (Fig. 4, *a* and *b*). Intramuscular nerve fibers were stained lightly if at all in embryonic muscle and unstained in the adult, although the perineurial sheath surrounding bundles of nerve fibers was frequently J1 positive (not shown).

In light of the low levels of J1 in normal muscles, we were surprised to find that J1 accumulates in denervated muscle (Fig. 4, *c–f*). In diaphragm, considerable accumulation of J1 was apparent by 2 d after denervation, the earliest time examined, and J1 was apparent in muscles kept denervated for up to 2 mo. Staining by anti-J1 was most intense near denervated synaptic sites, but the degree of this association varied with time after denervation: J1 was most widely distributed at early times after denervation (2–4 d) and becomes focused at synaptic sites when denervation was maintained for 1–2 mo. Generally similar results—near-absence of J1 from normal adult muscle and accumulation near synapses after denervation—were also observed in soleus (Fig. 4, *e* and *f*), extensor digitorum longus, platysma, and intercostal muscles, although at similar times after denervation, J1 was more closely focused at synaptic sites in soleus (Fig. 4, *e* and *f*), extensor digitorum longus, and platysma than in diaphragm.

Thus, accumulation of J1 in synaptic regions represents a general response of rat skeletal muscle to denervation.

Three further experiments were done to study how nerves affect J1 levels in muscle. First, two diaphragms were denervated by cutting the phrenic nerve in the neck rather than in the thorax, to avoid physical contact with the muscle. Accumulation of J1 in these muscles was indistinguishable from that observed when the nerve was cut near the muscle. Thus, expression of J1 is a response to denervation per se and does not simply result from the tissue damage that accompanies surgery. Second, lower leg muscles were paralyzed for 6–11 d by implantation of a tetrodotoxin-filled capillary under the perineurium of the sciatic nerve (24). J1 appeared in paralyzed muscles (Fig. 4, *g* and *h*) but not in muscles whose nerves were implanted with a saline-filled capillary. Interestingly, the J1 in paralyzed muscle was not obviously associated with synaptic sites. Thus, accumulation of J1 is at least in part a consequence of inactivity, and does not require degeneration of axons; the accumulations of acetylcholine receptors and N-CAM in denervated muscle are similarly activity dependent (7, 20). Finally, when regeneration of axons into diaphragm or soleus muscles was facilitated by crushing the nerve rather than cutting it, J1 disappeared from the muscle soon after reinnervation was complete (Fig. 4, *i*, and *j*; see reference 7 for time course of reinnervation). Thus, axonal regeneration and/or muscle reinnervation can reverse the denervation-induced accumulation of J1.

We used light and electron microscopic methods to determine the structures in denervated muscle with which J1 was associated. Double-staining with anti-J1 and anti-BL (Fig. 5, *a* and *b*) or with anti-J1 and rhodamine-α-bungarotoxin (Fig. 5, *c* and *d*) demonstrated that most of the J1 in denervated muscle is concentrated in interstitial spaces between muscle fibers, external to the postsynaptic membrane and to BL. Electron microscopy of denervated muscle incubated with anti-J1 and HRP-second antibody revealed reaction product coating large collagen fibers (composed of type I collagen) and smaller collagen-associated fibrils near denervated synaptic sites; BL and cell membranes were lightly stained, and collagen fibrils distant from synaptic sites were unstained (Fig. 5, *e–g*). Similar results were obtained when muscles were fixed with 1% paraformaldehyde before being incubated with antibody, demonstrating that J1 had not moved to matrix from cellular sites during the staining procedure. Thus J1 is associated, at least in part, with the extracellular matrix.

In summary, J1 appears in interstitial spaces near synaptic sites after denervation of adult muscle and disappears when denervated muscle is reinnervated. The induction of J1 involves an activity-dependent step in that it is mimicked by paralysis of innervated muscle. In these respects, J1 resembles the N-CAM that appears in interstitial spaces after denervation. However, unlike N-CAM, J1 is confined to these spaces and does not accumulate on the muscle fiber surface or in muscle fiber cytoplasm. We do not yet know which cells produce J1 or even whether it is synthesized within the muscle. The association of J1 with collagen and other fibrils shows that it is a component of the extracellular matrix. Local synthesis, e.g., by Schwann cells or synaptic regions of muscle fibers, could explain the restriction of J1 to synaptic areas; alternatively, extracellular fibrils might differ in synaptic and extrasynaptic areas, with J1 binding preferentially to synapse-associated components of the matrix. In any event, the association of J1 with collagen and other fibrils in muscle raises the possibility that J1 is associated with extracellular material in the central, as well as in the peripheral, nervous system.

L2 and HNK-1

As noted above, monoclonal antibodies L2 and HNK-1 recognize a carbohydrate epitope that is shared by N-CAM, L1, J1, and myelin-associated glycoprotein. Whereas L2 and HNK-1 recognize both highly sialylated (so-called "embryonic") and less sialylated ("adult") forms of N-CAM, only a fraction of the molecules in either class bear the L2/HNK-1 epitope (16). Similarly, L2 and HNK-1 bind to subpopulations of L1 and myelin-associated glycoprotein (unpublished results). It was therefore interesting to ask whether this carbohydrate epitope was detectable in any of the sites in muscle known to be rich in N-CAM, L1, or J1.

Both HNK-1 and L2 stained intramuscular nerves in embryos (Fig. 6, *a* and *b*). Immunoreactivity was lost from nerves as development proceeded, as described above for N-CAM and L1. However, whereas N-CAM and L1 remain associated with unmyelinated fibers in adult nerves, the L2/HNK-1 epitope was undetectable in adult rat nerves. Schuller-Petrovic et al. (42) have also reported that adult rat peripheral nerves are not stained by HNK-1, although human peripheral nerves are HNK-1 positive. Although we do not know what molecule(s) HNK-1 and L2 recognize in embryonic nerve, our results are consistent with the possibility that L1 and/or N-CAM bear the HNK-1/L2 epitope in developing but not in adult peripheral nerves.

Whereas embryonic nerves bear the L2/HNK-1 epitope, embryonic myotubes are not stained by either antibody (Fig. 6, *a* and *b*). Furthermore, neither HNK-1 nor L2 detectably stained either synaptic or extrasynaptic areas of perinatal, adult, or denervated adult muscle fibers; interstitial spaces between denervated fibers were also unstained (Fig. 6, *c–f*). Immunoblotting experiments have also failed to detect binding of HNK-1 to extracts of developing or denervated muscle (not shown). These results suggest that N-CAM in developing and denervated muscle and J1 in denervated muscle do not bear the L2/HNK-1 epitope.

NILE

NILE was originally isolated from the cell line PC12 and is now known to be present on a variety of central and peripheral neurons and on Schwann cells (33, 34). Recently, Stallcup and Beasley (43) reported that antibodies to NILE inhibited axon fasciculation in cultures of embryonic brain neurons, indicating that NILE is involved in interneuronal adhesion.

A monoclonal antibody to NILE, ASCS4 (44), stained intramuscular nerves in embryonic muscle, and unmyelinated nerve fibers in adult muscle (Fig. 7, *a* and *b*). A portion of the nerve terminal/Schwann cell complex was faintly stained at the neuromuscular junction, but neither synaptic nor extrasynaptic portions of embryonic, perinatal, or adult muscle fibers were detectably stained. Levels of NILE at synaptic sites decreased upon denervation, but some NILE persisted; muscle fibers remained NILE negative. In all these respects, the distribution of NILE was similar to that described above for L1.

Uvomorulin

Uvomorulin was identified as a molecule recognized by anti-

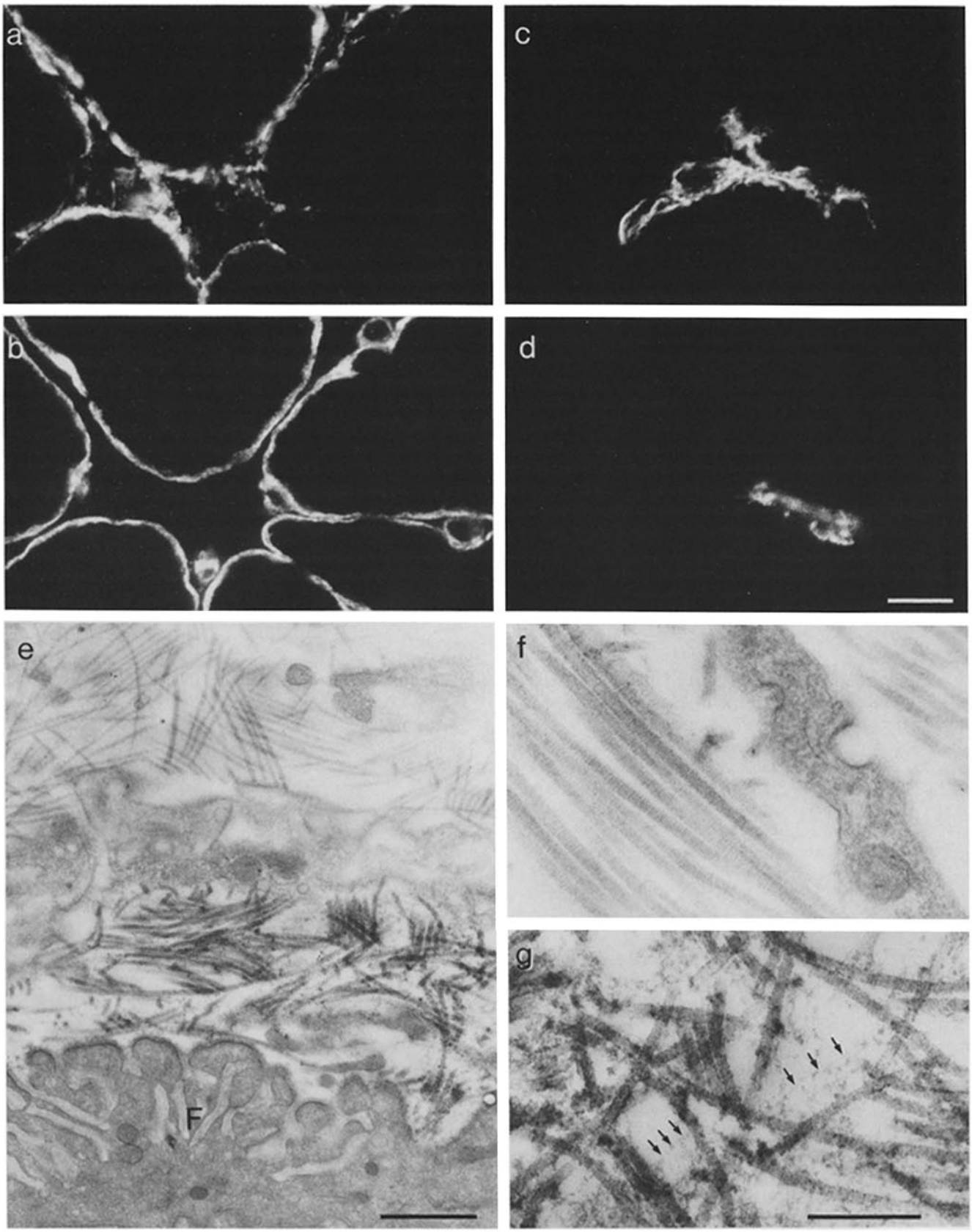

Figure 5. J1 in denervated muscle. (*a–d*) J1 (*a* and *c*) is concentrated in interstitial space between muscle fibers, as shown by counterstaining with anti-BL (*b*) or rhodamine-α-bungarotoxin (*d*). (*e*) Electron micrograph of a denervated synaptic site (identified by junctional folds [*F*] in the muscle membrane) in a muscle stained with anti-J1 and horseradish peroxidase–second antibody. Reaction product coats synapse-associated extracellular material but not cellular processes. (*f* and *g*) Extracellular matrix 5 μm (*f*) and 1 μm (*g*) from a denervated synaptic site in the same muscle shown in *e*. J1 is associated with banded collagen fibers and thinner fibrils (arrows in *g*) in areas near denervated synaptic sites. Bars: in *d*, 10 μm (*a–d*); *e*, 1 μm; in *g*, 0.5 μm (*f* and *g*).

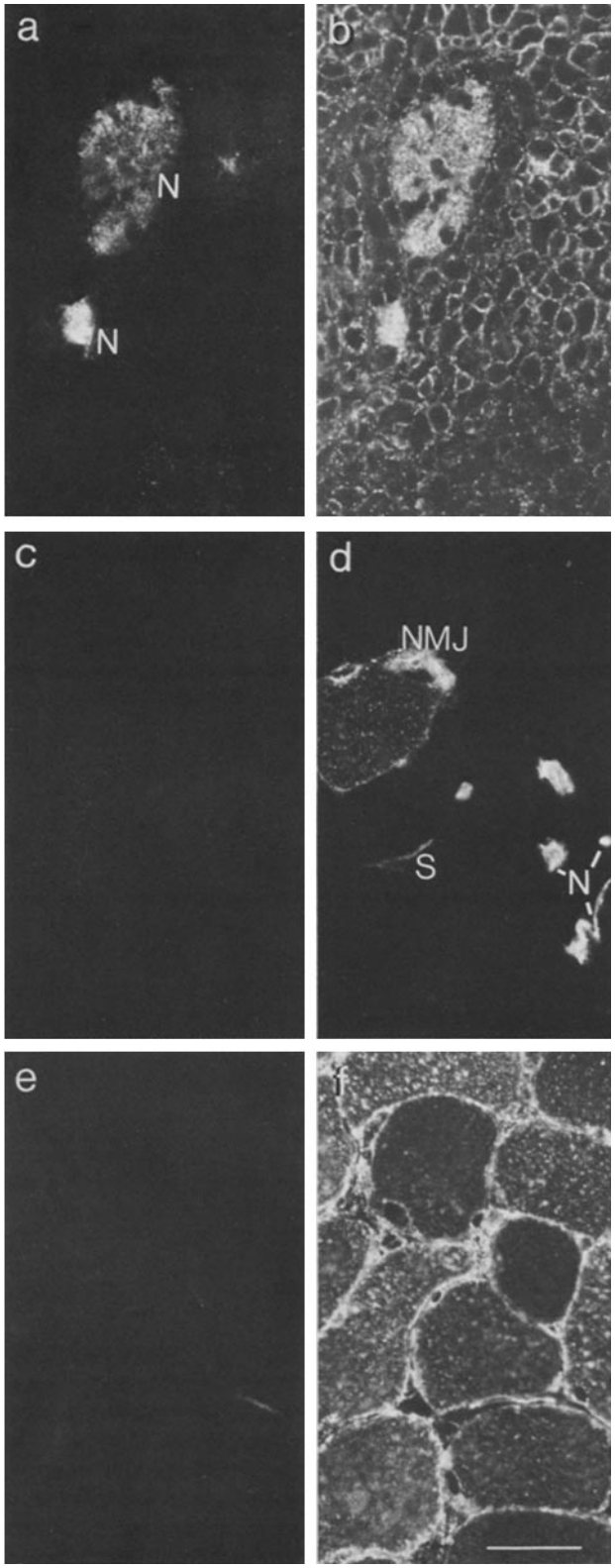

Figure 6. Embryonic (*a* and *b*), adult (*c* and *d*), and denervated adult (*e* and *f*) muscles doubly stained with L2 (plus fluorescein goat anti-mouse IgM; *a*, *c*, and *e*) and anti-N-CAM (plus rhodamine swine anti-rabbit IgG; *b*, *d*, and *f*). L2 stains embryonic intramuscular nerves (*N*) but does not detectably stain embryonic myotubes or innervated or denervated muscle fibers. Neuromuscular junction (*NMJ*), satellite cells (*S*), and unmyelinated nerve fibers associated with a blood vessel are marked in *d*. Bar, 30 μm.

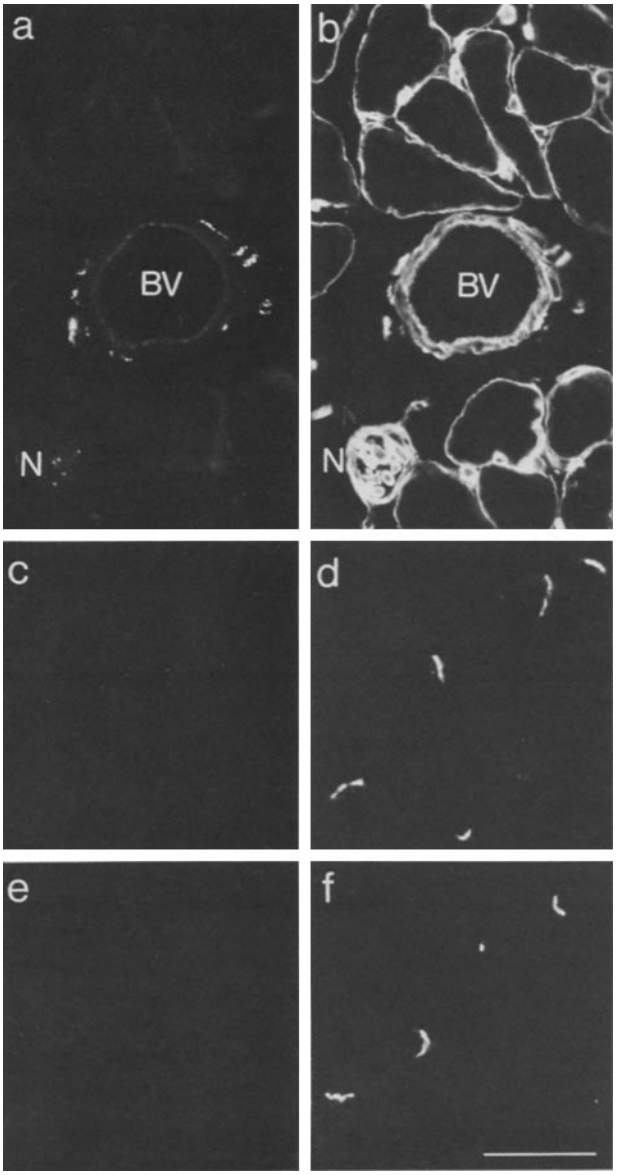

Figure 7. NILE and uvomorulin in muscle. (*a* and *b*) Adult muscle doubly stained with monoclonal antibody to NILE (plus fluorescein goat anti-mouse IgG; *a*) and antiserum to BL (plus rhodamine swine anti-rabbit IgG; *b*). Anti-NILE stains unmyelinated nerve fibers near blood vessels (*BV*) and within intramuscular nerves (*N*) but does not stain muscle fibers. (*c–f*) Normal (*c* and *d*) and 10-d denervated (*e* and *f*) mouse muscles, doubly stained with anti-uvomorulin (*c* and *e*) and rhodamine-α-bungarotoxin (*d* and *f*). No uvomorulin is detectable in innervated or denervated muscle. Bar: 40 μm for *a* and *b*, 100 μm for *c–f*.

bodies that block the calcium-dependent compaction of the mouse embryo morula (14, 25). Subsequent studies have shown that uvomorulin is present in various adult tissues and that anti-uvomorulin blocks calcium-dependent aggregation of several cell types (45, 46).

A monoclonal antibody to uvomorulin, DE-1 (15), stained liver in a pattern identical to that previously reported for anti-uvomorulin serum (45). However, we could not detect uvomorulin in either synaptic or nonsynaptic regions of embryonic, adult, or denervated adult muscles (Fig. 7, *c–f*).

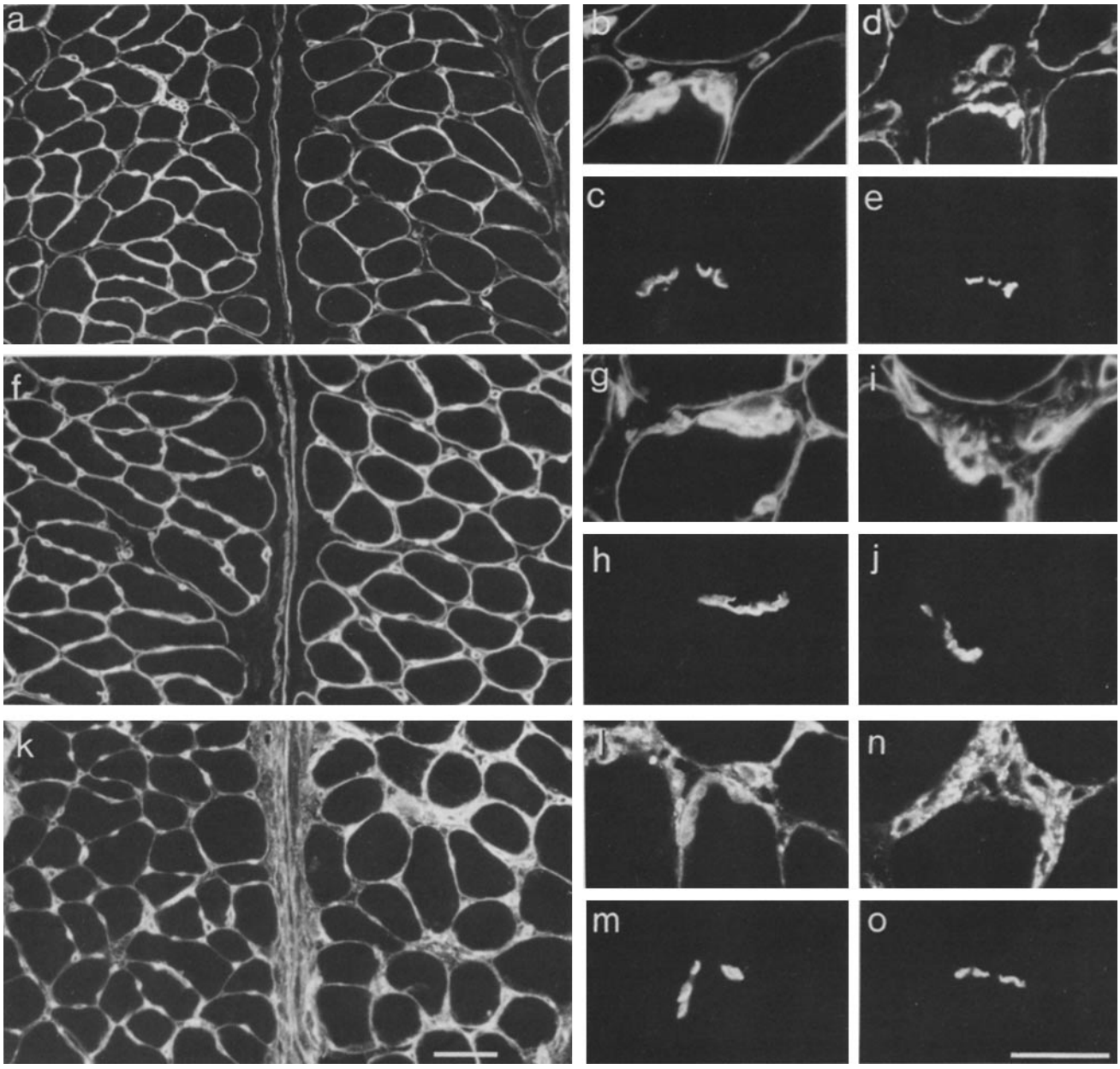

Figure 8. Laminin (*a–e*), a heparan sulfate proteoglycan (*f–j*) and fibronectin (*k–o*) in innervated and denervated adult muscle. (*a*, *f*, and *k*) Innervated (*left*) and denervated (*right*) diaphragms were mounted as a single block, then sectioned and stained. Laminin (*a*) and a heparan sulfate proteoglycan (*f*) are present at similar levels in the two samples, whereas anti-fibronectin (*k*) stains denervated muscle slightly more intensely. Denervated diaphragm muscle fibers hypertrophy transiently and are somewhat larger, on average, than the innervated fibers. (*b–e*, *g–j*, and *l–o*) Endplates in innervated (*left*) and denervated (*right*) counterstained with rhodamine-α-bungarotoxin (*c*, *e*, *h*, *j*, *m*, and *o*). All three antigens are present in both synaptic and extrasynaptic surfaces of both innervated and denervated muscle fibers. Laminin is confined to these surfaces. Heparan sulfate proteoglycan appears in interstitial spaces following denervation. Fibronectin is present in interstitial spaces in normal muscle, but becomes highly concentrated there after denervation. Bars: *a*, *f*, and *k*, 50 μm; 25 μm for all other parts.

Laminin, Heparan Sulfate Proteoglycan, and Fibronectin

Regenerating peripheral axons frequently grow along BL to reach and reinnervate their targets (41). Three components of BL that have been shown to influence axonal elongation in vitro are laminin, fibronectin, and heparan sulfate proteoglycan. Neurites adhere to and elongate on substrata coated with laminin or fibronectin (2, 9, 21, 30). Neurons are not known to adhere to heparan sulfate proteoglycan alone, but neurite outgrowth-promoting material secreted by a variety of cultured cells consists of a heparan sulfate proteoglycan–laminin complex (18). Furthermore, a monoclonal antibody (22) that blocks neurite outgrowth on this material recognizes the complex but not laminin alone; antisera to pure laminin recognize the complex but do not block outgrowth on it (reviewed in reference 37). Heparan sulfate may also be involved in the adhesion of retinal neurons to each other and to extracellular substrata (6). Thus heparan sulfate proteoglycans seem to be important for neuronal adhesion, whether or not they are adhesive macromolecules per se.

Newly formed embryonic myotubes bear little BL and are

poor in laminin (Fig. 1, *c* and *h*), fibronectin, and a BL-associated heparan sulfate proteoglycan. As development proceeds, myotubes accumulate a BL that is rich in all three of these antigens (4). Double-labeling experiments have shown that the accumulation of BL on and the disappearance of N-CAM from the myotube surface occur coordinately from E16–E19 (7*a*). To seek effects of denervation on the amount or distribution of these BL components, innervated and denervated hemidiaphragms were mounted in a single block, and then sectioned, stained, and photographed together. Both laminin (Fig. 8*a*) and a heparan sulfate proteoglycan (Fig. 8*f*) were present at indistinguishable levels in innervated and 4–30-d denervated muscles. Each antibody stained the surfaces of muscle fibers as well as surfaces of intramuscular capillaries, blood vessels, and nerve trunks. Anti-fibronectin also stained these BL-coated surfaces and, in addition, stained interstitial spaces between muscle fibers in both innervated and denervated muscles (Fig. 8*k*). Staining by anti-fibronectin was somewhat more intense in denervated than in innervated muscle, but the difference was small and inconsistent. Thus, laminin, heparan sulfate proteoglycan, and fibronectin are all retained at high levels after denervation.

In adult muscle, laminin, fibronectin, and heparan sulfate proteoglycan are all present in both synaptic and extrasynaptic portions of muscle fiber BL, as well as in Schwann cell BL (Fig. 8, *b*, *c*, *g*, and *h*; and references 9*a* and 35). After denervation, laminin remained restricted to areas known to be occupied by BL (Fig. 8, *d* and *e*), whereas levels of fibronectin increased markedly in interstitial spaces near synaptic sites (Fig. 8, *n* and *o*). Heparan sulfate proteoglycan displayed an intermediate pattern: staining remained concentrated in BL, but some immunoreactive material appeared in interstitial spaces near endplates (Fig. 8, *i* and *j*).

Conclusions

Table II compares the distribution of several adhesive macromolecules on embryonic (E15), adult, and denervated adult muscle fiber surfaces and in interstitial spaces near adult endplates. Two main conclusions can be drawn from these results.

First, of the eight adhesive molecules that we have studied, N-CAM is the only one whose regulation in muscle parallels the susceptibility of muscle to innervation: early embryonic, denervated adult, and paralyzed adult muscles are rich in N-CAM and susceptible to innervation, whereas innervated and reinnervated adult muscles are poor in N-CAM and refractory to innervation. In contrast, laminin, heparan sulfate proteoglycan, and fibronectin accumulate on embryonic myotubes after synapses form, are abundant on adult myotubes, and change little after denervation. L1, NILE, J1, and uvomorulin are undetectable on muscle fibers at all stages examined. Although these results do not show that N-CAM is involved in mediating the susceptibility of muscle to innervation, they are consistent with the suggestion that N-CAM plays an important role in regulating innervation of muscle (7).

Second, several adhesive macromolecules—N-CAM, J1, fibronectin, and a heparan sulfate proteoglycan—accumulate in interstitial spaces near synaptic sites after denervation. This response is intriguing in view of the fact that regenerating axons preferentially reinnervate original synaptic sites (39). Although the BL of the synaptic cleft is particularly attractive to regenerating axons and contains components that induce the differentiation of regenerating nerve terminals (40), axons must use more widely distributed cues to reach these sites. Denervated nerve trunks provide some guidance in this regard, but axons growing outside of pre-existing pathways also preferentially reinnervate original synaptic sites (discussed in references 39 and 40). The localized accumulation of several adhesive molecules in interstitial spaces near denervated endplates may be an important factor in guiding such axons back to the particularly attractive but highly localized synaptic BL.

The accumulation of J1 in interstitial spaces near denervated synaptic sites is interesting for several reasons. First, although our attention was drawn to these areas in the course of studies on N-CAM (7), anti-J1 stains them more selectively and may therefore be a more useful marker in further studies of their structure and function. Second, J1 is present on cultured astrocytes and mediates adhesion of neurons to these cells, presumably via a neuronal J1 receptor (17). Regenerating motor axons may bear a similar receptor and thereby interact with J1 during reinnervation. Third, the distribution of J1 is intermediate in extent between that of components that are highly concentrated in the synaptic BL (38, 40) and others, such as N-CAM (7), that are widely distributed in denervated muscle. Acting together, these molecules may form an adhesive hierarchy that renders the terrain increasingly attractive to axons with decreasing distance from former synaptic sites.

Table II. Distribution of Adhesive Macromolecules in Embryonic, Adult and Denervated Adult Muscles

	Muscle fiber surface			Interstital spaces near endplates	
Antigen	Embryo	Adult	Denervated adult	Adult	Denervated adult
N-CAM	+	−	+	−	+
L1	−	−	−	−	−
J1	−	−	−	−	+
NILE	−	−	−	−	−
Uvomorulin	−	−	−	−	−
Laminin	−	+	+	−	−
Fibronectin	−	+	+	±	+
Heparan sulfate proteoglycan	−	+	+	−	±
L2/HNK-1	−	−	−	−	−

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Note Added in Proof: Bock, E., C. Richter-Landsberg, A. Faissner, and M. Schachner (*EMBO [Eur. Mol. Biol. Organ.] J.* 11:2765–2768) have recently reported that L1 and NILE are immunochemically identical.

References

1. Abo, T., and C. M. Balch. 1981. A differentiation antigen of human NK and K cells identified by a monoclonal antibody (HNK-1). *J. Immunol.* 127:1024–1029.

2. Akers, R., D. Mosher, and J. Lilien. 1981. Promotion of retinal neurite outgrowth by substratum-bound fibronectin. *Dev. Biol.* 86:179–188.

3. Cashman, N. R., J. Covault, R. L. Wallman, and J. R. Sanes. 1985. Neural cell adhesion molecule (N-CAM) accumulates in diseased (denervated) human muscle fibers. *Neurosci. Abstr.* 11:1302.

4. Chiu, A. Y., and J. R. Sanes. 1984. Differentiation of basal lamina in synaptic and extrasynaptic portions of embryonic rat muscle. *Dev. Biol.* 103:456–467.

5. Chou, E. H., A. A. Ilyas, J. E. Evans, R. H. Quarles, and F. B. Jungalwala. 1985. Structure of a glycolipid reacting with monoclonal IgM in neuropathy and with HNK-1. *Biochem. Biophys. Res. Commun.* 128:383–388.

6. Cole, G. J., D. Schubert, and L. Glaser. 1985. Cell-substratum adhesion in chick neural retina depends on protein-heparan sulfate interactions. *J. Cell Biol.* 100:1192–1199.

7. Covault, J., and J. R. Sanes. 1985. Neural cell adhesion molecule (N-CAM) accumulates in denervated and paralyzed skeletal muscle. *Proc. Natl. Acad. Sci. USA.* 82:4544–4548.

7*a*.Covault, J., and J. R. Sanes. 1986. Distribution of N-CAM in synaptic and extrasynaptic portions of adult and developing skeletal muscles. *J. Cell. Biol.* In press.

7*b*.Covault, J., J. P. Merlie, C. Goridis, and J. R. Sanes. 1986. Molecular forms of N-CAM and its mRNA in developing and denervated muscles. *J. Cell. Biol.* In press.

8. Edelman, G. M. 1983. Cell adhesion molecules. *Science (Wash. DC).* 219:450–457.

9. Edgar, D., R. Timpl, and H. Thoenen. 1984. The heparin-binding domain of laminin is responsible for its effects on neurite outgrowth and neuronal survival. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:1463–1468.

9*a*.Eldridge, C. F., J. R. Sanes, A. Y. Chiu, R. P. Bunge, and C. L. Cornbrooks. 1986. Basal lamina-associated heparan sulfate proteoglycan in the rat peripheral nervous system; characterization and localization using monoclonal antibodies. *J. Neurocytol.* In press.

10. Faissner, A., J. Kruse, C. Goridis, E. Bock, and M. Schachner. 1984. The neural cell adhesion molecule L1 is distinct from the N-CAM related group of surface antigens BSP-2 and D2. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:733–737.

11. Faissner, A., J. Kruse, J. Nieke, and M. Schachner. 1984. Expression of neural cell adhesion molecule L1 during development, in neurological mutants, and in the peripheral nervous system. *Dev. Brain Res.* 15:69–82.

12. Gambke, B., and N. A. Rubinstein. 1984. A monoclonal antibody to the embryonic myosin heavy chain of rat skeletal muscle. *J. Biol. Chem.* 259:12092–12100.

12*a*.Gatchalian, C., A. Y. Chiu, and J. R. Sanes. 1985. Monoclonal antibodies to laminin that distinguish synaptic and extrasynaptic domains in muscle fiber basal lamina. *J. Cell. Biol.* 101(5, Pt. 2): 89a. (Abstr.)

13. Grumet, M., S. Hoffman, C. Chuong, and G. M. Edelman. 1984. Polypeptide components and binding functions of neuron-glia cell adhesion molecules. *Proc. Natl. Acad. Sci. USA.* 81:7989–7993.

14. Hyafil, F., D. Marello, C. Babinet, and F. Jacob. 1980. A cell surface glycoprotein involved in the compaction of embryonal carcinoma cells and cleavage stage embryos. *Cell.* 21:927–934.

15. Hyafil, F., C. Babinet, and F. Jacob. 1981. Cell-cell interactions in early embryogenesis: a molecular approach to the role of calcium. *Cell.* 26:447–454.

16. Kruse, J., R. Mailhammer, H. Wernecke, A. Faissner, I. Sommer, C. Goridis, and M. Schachner. 1984. Neural cell adhesion molecules and myelin-associated glycoprotein share a common carbohydrate moiety recognized by monoclonal antibodies L2 and HNK-1. *Nature (Lond.)* 311:153–155.

17. Kruse, J., G. Keilhauer, A. Faissner, R. Timpl, and M. Schachner. 1985. The J1 glycoprotein—a novel nervous system cell adhesion molecule of the L2/HNK-1 family. *Nature (Lond.)* 316:146–148.

18. Lander, A. D., D. K. Fujii, and L. F. Reichardt. 1985. Laminin is associated with the "neurite-outgrowth-promoting factors" found in conditioned medium. *Proc. Natl. Acad. Sci. USA.* 82:2183–2187.

19. Lindner, J., F. G. Rathjen, and M. Schachner. 1983. L1 mono- and polyclonal antibodies modify cell migration in early postnatal mouse cerebellum. *Nature (Lond.).* 305:427–430.

20. Lømo, T., and R. H. Westgaard. 1976. Control of ACh sensitivity in rat muscle fibers. *Cold Spring Harbor Symp. Quant. Biol.* 40:263–274.

21. Manthorpe, M., E. Engvall, E. Ruoslahti, F. M. Longo, G. E. Davis, and S. Varon. 1983. Laminin promotes neuritic regeneration from cultured peripheral and central neurons. *J. Cell Biol.* 97:1882–1890.

22. Matthew, W. D., and P. H. Patterson. 1983. The production of a monoclonal antibody that blocks the action of a neurite outgrowth-promoting factor. *Cold Spring Harbor Symp. Quant. Biol.* 48:625–631.

23. Miledi, R., and C. R. Slater. 1968. Electrophysiology and electron microscopy of rat neuromuscular junctions after nerve degeneration. *Proc. R. Soc. Lond. B Biol. Sci.* 169:289–306.

24. Mills, R. G., and J. J. Bray. 1979. A slow-release technique for inducing prolonged paralysis by tetrodotoxin. *Pfluegers Arch. Eur. J. Physiol.* 383:67–70.

25. Peyrieras, N., F. Hyafil, D. Louvard, H. L. Ploegh, and F. Jacob. 1983. Uvomorulin: a nonintegral membrane protein of early mouse embryo. *Proc. Natl. Acad. Sci. USA.* 80:6274–6277.

26. Quarles, R. H. 1984. Myelin-associated glycoprotein in development and disease. *Dev. Neurosci.* 6:285–303.

27. Rathjen, F. G., and U. Rutishauser. 1984. Comparison of two cell surface molecules involved in neural cell adhesion. *EMBO (Eur. Mol. Biol. Organ) J.* 3:461–465.

28. Rathjen, F. G., and M. Schachner. 1984. Immunocytochemical and biochemical characterization of a new neuronal cell surface component (L1 antigen) which is involved in cell adhesion. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:1–10.

29. Rieger, F., M. Grumet, and G. M. Edelman. 1985. N-CAM at the vertebrate neuromuscular junction. *J. Cell Biol.* 101:285–293.

30. Rogers, S. L., P. C. Letourneau, S. L. Palm, J. McCarthy, and L. T. Furcht. 1983. Neurite extension by peripheral and central nervous system neurons in response to substratum-bound fibronectin and laminin. *Dev. Biol.* 98:212–220.

31. Rutishauser, U. 1984. Developmental biology of a neural cell adhesion molecule. *Nature (Lond.)* 310:549–554.

32. Rutishauser, U., M. Grumet, and G. M. Edelman. 1983. Neural cell adhesion molecule mediates initial interactions between spinal cord neurons and muscle cells in culture. *J. Cell Biol.* 97:145–152.

33. Salton, S. R. J., C. Richter-Landsberg, L. A. Greene, and M. L. Shelanski. 1983. Nerve growth factor-inducible large external (NILE) glycoprotein: studies of a central and peripheral neuronal marker. *J. Neurosci.* 3:441–454.

34. Salton, S. R. J., M. L. Shelanski, and L. A. Greene. 1983. Biochemical properties of the nerve growth factor-inducible large external (NILE) glycoprotein. *J. Neurosci.* 3:2420–2430.

35. Sanes, J. R. 1982. Laminin, fibronectin and collagen in synaptic and extrasynaptic portions of muscle fiber basement membrane. *J. Cell Biol.* 93:442–451.

36. Sanes, J. R. 1984. More nerve growth factors. Nature (*Lond.*) 307:500.

37. Sanes, J. R. 1985. Laminin for axonal guidance? *Nature (Lond.).* 315:714–715.

38. Sanes, J. R., and A. Y. Chiu. 1983. The basal lamina of the neuromuscular junction. *Cold Spring Harbor Symp. Quant. Biol.* 48:667–678.

39. Sanes, J. R., and J. Covault. 1985. Axon guidance during reinnervation of skeletal muscle. *Trends Neurosci.* In press.

40. Sanes, J. R., L. M. Marshall, and U. J. McMahan. 1978. Reinnervation of muscle fiber basal lamina after removal of myofibers. Differentiation of regenerating axons at original synaptic sites. *J. Cell Biol.* 78:176–198.

41. Scherer, S. S., and S. S. Easter. 1984. Degenerative and regenerative changes in the trochlear nerve of goldfish. *J. Neurocytol.* 13:519–565.

42. Schuller-Petrovic, S., W. Gebhart, H. Lassmann, H. Rumpold, and D. Kraft. 1983. A shared antigenic determinant between natural killer cells and nervous tissue. *Nature (Lond.)* 306:179–181.

43. Stallcup, W. B., and L. Beasley. 1985. Involvement of the nerve growth factor-inducible large external glycoprotein (NILE) in neurite fasciculation in primary cultures of rat brain. *Proc. Natl. Acad. Sci. USA.* 82:1276–1280.

44. Sweadner, K. J. 1983. Post-translational modification and evoked release of two large surface proteins of sympathetic neurons. *J. Neurosci.* 3:2504–2517.

45. Vestweber, D., and R. Kemler. 1984. Rabbit antiserum against a purified surface glycoprotein decompacts mouse preimplantation embryos and reacts with specific adult tissues. *Exp. Cell Res.* 152:169–178.

46. Vestweber, D., C. Ocklind, A. Gossler, P. Odin, B. Obrink, and R. Kemler. 1985. Comparison of two cell adhesion molecules, uvomorulin and cell-CAM 105. *Exp. Cell Res.* 157:451–461.

47. Villiger, B., D. G. Kelley, W. Engelman, C. Kuhn, and J. A. McDonald. 1981. Human alveolar macrophage fibronectin: synthesis, secretion and ultrastructural localization during binding of gelatin coated latex particles. *J. Cell Biol.* 90:711–720.