Distribution of N-CAM in Synaptic and Extrasynaptic Portions of Developing and Adult Skeletal Muscle

Jonathan Covault and Joshua R. Sanes
Department of Anatomy and Neurobiology, Washington University School of Medicine, St. Louis, Missouri 63110

Abstract. Previous studies of denervated and cultured muscle have shown that the expression of the neural cell adhesion molecule (N-CAM) in muscle is regulated by the muscle’s state of innervation and that N-CAM might mediate some developmentally important nerve-muscle interactions. As a first step in learning whether N-CAM might regulate or be regulated by nerve-muscle interactions during normal development, we have used light and electron microscopic immunohistochemical methods to study its distribution in embryonic, perinatal, and adult rat muscle.

In embryonic muscle, N-CAM is uniformly present on the surface of myotubes and in intramuscular nerves; N-CAM is also present on myoblasts, both in vivo and in cultures of embryonic muscle. N-CAM is lost from the nerves as myelination proceeds, and from myotubes as they mature. The loss of N-CAM from extrasynaptic portions of the myotube is a complex process, comprising a rapid rearrangement as secondary myotubes form, a phase of decline late in embryogenesis, a transient reappearance perinatally, and a more gradual disappearance during the first two postnatal weeks. Throughout embryonic and perinatal life, N-CAM is present at similar levels in synaptic and extrasynaptic regions of the myotube surface. However, N-CAM becomes concentrated in synaptic regions postnatally: it is present in postsynaptic and perisynaptic areas of the muscle fiber, both on the surface and intracellularly (in T-tubules), but undetectable in portions of muscle fibers distant from synapses. In addition, N-CAM is present on the surfaces of motor nerve terminals and of Schwann cells that cap nerve terminals, but absent from myelinated portions of motor axons and from myelinating Schwann cells. Thus, in the adult, N-CAM is present in synaptic but not extrasynaptic portions of all three cell types that comprise the neuromuscular junction. The times and places at which N-CAM appears are consistent with its playing several distinct roles in myogenesis, synaptogenesis, and synaptic maintenance, including alignment of secondary along primary myotubes, early interactions of axons with myotubes, and adhesion of Schwann cells to nerve terminals.

Materials and Methods

Animals

Sprague-Dawley rats were purchased from Chappel Breeders (St. Louis, MO). Adults were males that usually weighed 150–200 g; embryos were of either sex. The first date of pregnancy was designated embryonic day 0 (E0), and pups were born on E21 or E22. The day of birth is also referred to as postnatal day.
Antibodies

Preparation and characterization of rabbit antibodies to chicken N-CAM have been described previously (9). Affinity-purified rabbit anti-chicken N-CAM antibodies were immobilized on protein A-Sepharose (47) and used to purify N-CAM from Nonidet-40 extracts of adult rat brains; the rat N-CAM was used, in turn, to produce new antisera. New Zealand white rabbits were immunized with 100-μg aliquots of electrophoretically pure rat N-CAM (Fig. 1a). The first aliquot was emulsified in complete Freund’s adjuvant, and subsequent aliquots, injected at 1-mo intervals, were delivered in incomplete Freund’s adjuvant. Rabbits were bled 7–14 d after each boost. Immunoblotting (see reference 10 for methods) showed that these antisera recognized both the highly sialylated ~200–250-kD N-CAM found in embryonic rat brain and the less sialylated ~180-, 140, and 120-kD forms of N-CAM in adult rat brain (Fig. 1b). For immunohistochemical studies, anti-N-CAM antibodies were purified from the serum by affinity chromatography on an agarose column derivatized (25) with N-CAM from the appropriate species. Anti-α- and anti-chicken N-CAM antibodies revealed qualitatively similar distributions of N-CAM in rat muscles; results using both antibodies are therefore combined for presentation here. Chicken muscles were stained with rabbit anti-chicken N-CAM and with a monoclonal antibody, 224-1A6-A1, which recognizes chicken N-CAM but does not cross-react with rat N-CAM (9, 26).

A monoclonal antibody to embryonic rat myosin, 2B6 (15) was supplied by Neil Rubenstein (University of Pennsylvania, Philadelphia), and a monoclonal antibody to neurofilaments, 27-19 (19) was provided by Mark Willard (Washington University, St. Louis, MO). Monoclonal antibodies C24 and C29, which recognize laminin (16), were obtained as previously described (40), and a monoclonal antibody to Thy-1 (2) was a gift of Colin Barnstable (The Rockefeller University, New York). Fluorescein-, rhodamine-, and horseradish peroxidase-conjugated second antibodies (goat anti-rabbit IgG and goat anti-mouse IgG) were purchased from Cappel Laboratories (Cochranville, PA) or Atlantic Antibodies (Scarborough, ME).

Immunofluorescent Staining

Binding of antibodies to tissue sections was detected by an immunofluorescent method adapted from Sanes and Hall (42). Unfixed muscles were mounted on metal chucks with gum tragacanth (Fisher Scientific, Fair Lawn, NJ) and frozen in liquid N2-cooled isopentane. Thick sections (4–30 μm) were cut in a cryostat, air-dried on glass slides, and fixed with methanol for 2–5 min at −10°C. Thinner sections (0.5 μm) were obtained using a Sorvall MT-2 ultramicrotome (Sorvall Instruments Div., E.I. DuPont de Nemours & Co., Newtown, CT) equipped with a frozen thin sectioning system. Sections were incubated successively with affinity-purified anti-N-CAM (2–5 μg/ml) and fluorescein-conjugated goat anti-rabbit IgG, then washed and mounted in glycerol containing phenylenediamine (21). In experiments comparing the relative amount of anti-N-CAM staining during development, sections were incubated in parallel using identical dilutions of antibodies. When sections were doubly stained with anti-N-CAM and monoclonal antibodies, rhodamine-conjugated goat anti-mouse IgG was mixed with the anti-rabbit second antibody. To identify endplates, rhodamine-α-bungarotoxin (34), which binds to acetylcholine receptors (AChRs) in the postsynaptic membrane, was mixed with antibody. Sections were illuminated and photographed through filters selective for either fluorescein or rhodamine. After immunofluorescent staining and photography, some sections were counterstained with pinacynole (Eastman Kodak Co., Rochester, NY) and rephotographed.

Electron Microscopy

We used the cranial platysma of 4-5-wk-old females for electron microscopic immunohistochemistry; because this muscle is only a few fibers thick, antibodies penetrate readily throughout its depth, eliminating the need to section the muscle before staining it. Platysma muscles were dissected with their origin and insertion intact, and pinned out in dishes coated with Sylgard (Dow Corning Corp., Midland, MI). The muscles were incubated for 2 h at 4°C in affinity-purified anti-N-CAM (5–10 μg/ml in phosphate-buffered saline (PBS) plus 5–15% normal goat serum). The muscles were then washed for 1 h in PBS-goat serum, incubated 2 h in peroxidase-conjugated F(ab)2 fragments of goat antibodies to rabbit IgG in PBS-goat serum, washed again, and fixed with 1% glutaraldehyde, 100 mM NaPO4, pH 7.4, for 30 min. After an overnight wash in PBS, the muscles were cut into small squares, soaked in 1 mg/ml diamobenzidine, 100 mM sodium citrate, pH 6, for 30 min, and stained in diaminobenzidine-citrate plus 0.03% H2O2 for 30 min. The tissue was then rinsed, fixed in 1% OsO4 in cacodylate buffer, dehydrated in ethanol, rinsed in propylene oxide, and embedded in thin wafers of Araldite between Teflon-coated glass microscope slides. After the Araldite had polymerized, the slides were pried away; the wafer was then viewed at 160–400× with a compound microscope to identify endplate-rich areas for sectioning. Thin sections cut from the wafers were picked up on Formvar-coated slot grids and examined without further staining. After horseradish peroxidase staining but before osmium, bundles of fibers were cut from some muscles and cleared in glycerol; single fibers were teased from the bundles for light microscopy.

To test whether incubation of live muscles with anti-N-CAM affected the distribution of antigen, some muscles were fixed for 30 min at 4°C in 1% paraformaldehyde, and then rinsed with 20% normal goat serum plus 80 mM glycine, pH 9, before being stained. All results presented here were confirmed using prefixed muscle. To expose intracellular antigenic sites to antibody, muscles were treated with 0.05% saponin in PBS-goat serum for 30 min after fixation and before incubation with antibodies. Alternatively, diaphragms were fixed and cross-sectioned at 100 μm with a Vibratome before incubation with antibodies. To assess the specificity of staining, nonimmune IgG was substituted for anti-N-CAM.

For conventional electron microscopy, muscles were pinned in Sylgard-coated dishes, fixed (2% paraformaldehyde, 2.4% glutaraldehyde, 100 mM NaPO4, pH 7.4) for 60 min, rinsed, fixed in osmium, dehydrated, and embedded. Thin sections were stained with uranyl acetate and lead citrate.

Figure 1. Analysis of rat brain N-CAM and antiserum to it by gel electrophoresis and immunoblotting. (a) Silver-stained SDS gels containing 20 μg adult rat brain membranes (lane 1) and 0.3 μg of affinity-purified N-CAM from adult rat brain (lane 2). (b) Immunoblots of embryonic (lane 1) and adult (lane 2) rat brain membranes probed with anti-rat N-CAM and peroxidase goat anti-rabbit IgG. The antiserum reacts with the highly sialylated ~200–250-kD N-CAM found in embryonic brain and the less sialylated 180-, 140-, and 120-kD forms in adult brain. No staining of embryonic (lane 3) or adult (lane 4) brain is seen when nonimmune serum is substituted for anti-N-CAM. Arrowheads indicate positions of molecular mass standards (from the top: myosin, 200 kD; E. coli RNA polymerase β-subunit, 160 kD; β-galactosidase, 116 kD; E. coli RNA polymerase α-subunit, 90 kD; pyruvate kinase, 57 kD).
Results

Distribution of N-CAM in Adult Muscle

The Neuromuscular Junction. Fig. 2 shows 0.5-μm-thick frozen sections of rat diaphragm doubly stained with affinity-purified anti-N-CAM, fluorescein-second antibody, and rhodamine-α-bungarotoxin. α-Bungarotoxin binds to AChRs in the postsynaptic membrane and thus serves as a reliable marker of synaptic sites. Whereas most of the muscle was unstained by anti-N-CAM (Fig. 2,a and b), N-CAM was present in or near the toxin-stained postsynaptic membrane, within the muscle fiber beneath the postsynaptic membrane, and presynaptically in the area occupied by the nerve terminal and its Schwann cell cap (Fig. 2, c–f). None of these areas were detectably stained by nonimmune IgG (Fig. 2, i and j). Thus, in adult muscle, N-CAM is concentrated near neuromuscular junctions.

Electron microscopy of muscles incubated with anti-N-CAM and horseradish peroxidase-second antibody revealed that the most intensely stained area of the nerve terminal-Schwann cell complex was the area of apposition between these two cells (Fig. 3, a and c). In addition, the short unmyelinated preterminal portion of the motor axon (Fig. 3 b) and large portions of the Schwann cells that cap the nerve terminal (Fig. 3 c) were lightly stained. In contrast, neither myelinating Schwann cells nor myelinated portions of motor axons were detectably stained by anti-N-CAM. The gap between nerve terminal and Schwann cell is so narrow (<10 nm) that it is not possible to know whether N-CAM is associated with either membrane or with both. However, two results suggest that at least some of the N-CAM in this region is associated with the nerve terminal. First, 2 d after nerve injury, when the terminal had degenerated but the Schwann cell remained (29), anti-N-CAM no longer brightly stained presynaptic elements on cryostat sections (Fig. 2, g and h). Second, when muscles were sectioned or treated with saponin...
to expose cytoplasmic epitopes (46) before being incubated with anti-N-CAM and horseradish peroxidase-second antibody, reaction product was present within nerve terminals, even in areas where it was undetectable in the neighboring Schwann cell (Fig. 3f).

In synaptic regions of the muscle fiber, N-CAM was associated both with the cell surface and with intracellular structures (Fig. 2, c and e). At least some of the surface-associated N-CAM is externally directed in that it is stained when live muscles are incubated with antibody before being fixed and prepared for microscopy (Fig. 3a). Interestingly, reaction product was concentrated in the depths of the junctional folds that indent the postsynaptic surface; the tops of folds and primary synaptic clefts were relatively lightly stained (Fig. 3, a and d). This distribution differed from those of AChRs, which are concentrated at the crests of junctional folds (14), and synapse-specific components of basal laminae, which are present at relatively uniform density throughout the folds (40). Intracellularly, N-CAM was concentrated in T-tubules, as shown by staining and electron microscopy of saponin-permeabilized muscles (Fig. 3e).

Whereas the N-CAM of innervated muscle fibers was concentrated in synaptic areas, it was not confined to synaptic sites per se. This was best seen in single fibers teased from

Figure 3. Electron micrographs of adult neuromuscular junctions stained with anti-N-CAM and peroxidase-conjugated second antibody. a-d are from muscles stained while live. N-CAM is present in areas of contact between Schwann cells and nerve terminals (a), on immediately preterminal axons (arrows in b), and on Schwann cell surfaces (c), as well as within junctional folds (arrows in a and d; also visible in e). e and f are from muscles that were permeabilized by saponin treatment before staining. Reaction product is present within the nerve terminal (f) and in T-tubules in the subsynaptic cytoplasm (arrows in e). A, axon; M, muscle fiber; N, nerve terminal; S, Schwann cell. Bars: (a and c-f) 1 μm; (b) 0.5 μm.
muscles that had been stained with anti-N-CAM and horseradish peroxidase-second antibody: reaction product extended a few hundred micrometers on either side of the endplate, gradually declining in intensity and finally becoming undetectable (Fig. 4b). Electron microscopy confirmed the gradual decline in staining intensity and thus, presumably, in N-CAM concentration as a function of distance from the synapse (Fig. 4, c–e). N-CAM differs in this respect from a number of synapse-specific proteins such as AChRs (Fig. 2) and from glycoconjugates (Fig. 4a), which are present at far higher concentrations on the postsynaptic surface directly beneath the nerve terminal than on perijunctional surfaces just a few micrometers away (14, 39). Intracellular deposits of N-CAM were also spread along a few hundred micrometers of the muscle fiber, centered on the synapse, as shown by light and electron microscopy of saponin-treated muscles.

In summary, N-CAM has a complicated distribution at the neuromuscular junction. It is present on nerve terminals and immediately preterminal portions of axons, on terminal-associated Schwann cells, on postsynaptic and perisynaptic surfaces of muscle fibers, and in T-tubules in synaptic and perisynaptic areas. In contrast, N-CAM is undetectable on or in myelinated motor axons, myelinating Schwann cells, and portions of muscle fibers distant from synapses.

Unmyelinated Axons. Although N-CAM was not detectable on myelinated nerve fibers, it was present on unmyelinated (presumably sensory and sympathetic) nerve fibers in intramuscular nerves (Fig. 5, a, b, and d) and near blood vessels (Fig. 5, c and e). N-CAM was concentrated in the area of contact between the axon and its ensheathing Schwann cell (Fig. 5e), a disposition similar to that seen at the motor nerve terminal. Again, whereas the resolution of our methods is insufficient to localize this N-CAM to one membrane or the other, at least some portion of it is probably associated with the axon: reaction product was concentrated in axons of nerves that were sectioned before staining (Fig. 5d). It is worth noting that our inability to detect N-CAM on the surface of myelinated axons probably did not result from restricted antibody penetration as Thy-1, an axon surface glycoprotein (31), was readily detected in our cryostat sections with anti-Thy-1 monoclonal antibodies (not shown).

Mononucleated Cells. In addition to neuromuscular junctions and unmyelinated nerve fibers, N-CAM was associated with two apparently nonneural structures within adult skeletal muscles. The first were satellite cells, myogenic cells that divide and fuse to form new muscle fibers after injury (reviewed in reference 6). These mononucleated cells lie just beneath the muscle fiber basal lamina, and directly abut the surface of muscle fibers (Fig. 6). In nonsynaptic regions of normal adult muscle, satellite cells accounted for most of the N-CAM stained material detected by the immunofluorescence method. (In contrast, human satellite cells have been reported to be devoid of N-CAM [30].) N-CAM was also associated with a subpopulation of the mononucleated, fibroblast-like cells that occupy interstitial spaces between muscle fibers. The identity of these cells, and their relationship to the interstitial deposits of N-CAM that accumulate after denervation (9), will be considered elsewhere.

Distribution of N-CAM in Developing Muscle

N-CAM in the Anlage of Intercostal Muscles. Myotomes begin to form on E11; motor axons leave the spinal cord, collect
Figure 5. Anti-N-CAM-stained unmyelinated nerve fibers in intramuscular nerves and blood vessels. (a and b) Cryostat section of a mixed nerve doubly stained with anti-N-CAM (a) and a monoclonal antibody to neurofilaments (b). N-CAM is not associated with the population of large axons that includes myelinated motor axons. (c) Intramuscular blood vessel, stained in whole mount with anti-N-CAM. N-CAM is present on nerve fibers that run along the vessel wall. (d and e) Electron micrographs of unmyelinated nerve fibers in an intramuscular nerve (d) and in a blood vessel (e) stained with anti-N-CAM and peroxidase-conjugated second antibody. d is from a muscle that was fixed and sectioned before staining, to expose intracellular sites; e is from a muscle that was stained while live, restricting access of antibody to external surfaces of cells. Bar: 20 \( \mu \text{m} \) for a-c; 1 \( \mu \text{m} \) for d and e.

into ventral roots, and form spinal nerves on E12 (1). Fig. 7, b and c show a longitudinal section through a thoracic myotome in an E12 embryo, double stained with anti-N-CAM and anti-myosin. N-CAM is abundant in the myotome but undetectable in surrounding areas even though these spaces are as densely packed with cells as the myotomes themselves (1). Anti-N-CAM did, however, stain the spinal cord, ventral roots, and spinal nerves. The relationship of spinal nerves to myotomes is seen better in Fig. 7a, which shows six contiguous myotomes cut obliquely; the spinal cord is visible adjacent to rostral but not caudal intercostal areas because the latter are cut more ventrally than the former. One can thereby see, in this fortuitous ventrally, that spinal nerves have not yet grown to the most ventral portions of the myotomes (see also reference 11); nonetheless, ventral as well as dorsal portions of the myotome are rich in N-CAM. Furthermore, even in ventral areas, the nerves run 20-30 \( \mu \text{m} \) medial to N-CAM- and myosin-positive cells, and axons do not appear to contact the myotome (Fig. 7, a and b). Thus, N-CAM is present in myotomes before they are contacted by nerves.

N-CAM in Myoblasts and Newly Formed Myotubes. Intercostal muscles begin to form by migration and condensation of myotome cells on E13 and myotubes are present by E14 (1, 11). Antibody to N-CAM stained the surface of myoblasts before fusion (Fig. 8a) as well as the surface of newly formed, primary myotubes (Fig. 8b). Whereas all myosin positive cells contained N-CAM during this period (Fig. 8, b and c), ventral areas in E12 myotomes were N-CAM positive but myosin negative. Thus, expression of N-CAM by myoblasts may occur slightly before expression of myosin. Other cells within
Changes in N-CAM Distribution as Myotubes Mature. After primary myotubes are formed on E14 and E15, additional generations of myotubes form along their surface, giving rise to myotube clusters. Each cluster shares a common basal lamina sheath and contains a single large primary myotube and one or more smaller secondary myotubes (8, 32). Between E19 and birth (E21-22) both primary and secondary myotubes mature into myofibers, separate from each other, and acquire their own sheath of basal lamina (8, 23).

During this period of maturation, the distribution of N-CAM on myotubes changed dramatically. In E14–E15 intercostal, N-CAM was more or less uniformly distributed on the entire surface of primary myotubes (Fig. 9, a and c). During the next several days, N-CAM became highly concentrated in the area of contact between adjacent myotubes in clusters, whereas the outer myotube surface contained relatively little N-CAM immunoreactivity (Fig. 9 e). Perinatally as myotubes separated and matured into myofibers, N-CAM reappeared throughout the muscle fiber surface (Fig. 9 g). During this perinatal period, N-CAM was present not only on the myotube surface but also in intracellular structures that resemble the N-CAM-rich T-tubules in perisynaptic areas of adult muscle (Figs. 2 c and 3 e). Finally, levels of intracellular and surface-associated N-CAM declined gradually and in parallel over the first two postnatal weeks (Fig. 9, i and k), until N-CAM became nearly undetectable in nonsynaptic portions of adult muscle fibers.

Chiu and Sanes (8) have shown that intercostal myotubes assemble a basal lamina rich in laminin, fibronectin, heparan sulfate proteoglycan, and collagen IV during the last several days of embryogenesis. Double staining with antibodies to laminin and N-CAM revealed a striking spatial relationship between these two components of the myotube surface. On E14–15, when myotubes bore few basal laminae, they were rich in N-CAM (Fig. 9, a–d). As myotubes formed clusters, basal laminae coated the exterior, N-CAM-poor surface, and N-CAM was concentrated in basal lamina-poor areas of apposition (Fig. 9, e and f). Although some areas of overlap occurred, the accumulation of basal laminae and loss of N-CAM were generally coordinate at a micrometer level of precision on E16–19. Perinatally, however (and in denervated adult muscle [9]), N-CAM reappeared on basal laminae-coated surfaces (Fig. 9, g–j). Thus, the reciprocal relationship to these two components on E16–19 may not be obligatory.

N-CAMs on Developing Motor Axons. Ventral roots and spinal nerves are rich in N-CAMs as soon as they form, on E12 (Fig. 7). By E14, nerve branches have grown into the developing intercostals, and synaptic transmission from nerve to muscle has been established (11). On E14–15, intercostal nerves were brightly stained with anti-N-CAM antibodies (Fig. 10 a). N-CAM is presumably associated with axons since few other cellular elements are present in the nerve at this stage (Fig. 10 b). During the late embryonic period Schwann cells began to isolate motor axons from one another, first as fascicles and later as individuals. Anti-N-CAM continued to stain the intramuscular nerve intensely during this period (Fig. 10, c and d). N-CAM was lost from the nerve postnatally, as myelination occurred (Fig. 10, e and f), and was undetectable on the surface of adult motor axons (Fig. 5 a).

N-CAM at Developing Neuromuscular Junctions

Clusters of AChRs appear on intercostal myotubes on E15 (8), a day after synaptic transmission from nerve to muscle is detectable electrophysiologically (11). A single receptor cluster develops near the middle of each myotube at the site of synapse formation (4). We used rhodamine-α-bungarotoxin as a marker to examine the distribution of N-CAM at developing neuromuscular junctions.

On E15, N-CAM was present at similar, high levels in synaptic and extrasynaptic areas of the myotube surface (Fig. 11, a–d). Between E16 and E19, when the outer surface of clustered myotubes bore little N-CAM, levels were also low at synaptic sites (Fig. 11, e–h). Perinatally, as clusters disperse, N-CAM reappeared at similar levels in both synaptic and extrasynaptic areas (Fig. 11, i–l). Thus, the changes in the distribution of N-CAM between E15 and birth are unaffected by the presence or absence of developing synapses. Whereas N-CAM-rich material has been reported to be concentrated near embryonic synaptic sites (35), most of this staining is associated with nerve branches rather than with myotubes (Fig. 11, a, e, and k). The accumulation of N-CAM in

Figure 6. Anti-N-CAM-stained satellite cells. (a and b) Cryostat section of adult muscle double stained with anti-N-CAM (a) and anti-laminin monoclonal antibody (b) followed by appropriate fluorescein and rhodamine second antibodies. An N-CAM-rich satellite cell lies beneath the muscle fiber's basal lamina sheath. (c) N-CAM micrographs of muscle stained with anti-N-CAM and peroxidase-conjugated second antibody. N-CAM is associated with the surface of satellite cells. Bar: 15 μm for a–c; 2 μm for d. The muscle contained neither N-CAM nor myosin (Fig. 8, b–d); these may include myogenic precursor cells and fibroblasts.
postsynaptic areas of adult muscle fibers begins sometime around P7 (Fig. 11, m–p); junctional folds, whose depths are rich in N-CAM (Fig. 3d), form during this postnatal period (8).

**Distribution of N-CAM in Primary Muscle Cultures**

Embryonic muscle cells divide and fuse in culture to form contracting myotubes which accumulate patches of basal laminae and AChR clusters (43, 44). We used primary cultures of embryonic rat muscle cells to confirm and extend three observations made in situ.

First, by staining 2–3-d cultures with anti-N-CAM and anti-myosin, we confirmed that N-CAM was present on mononucleated cells as well as on myotubes. N-CAM was present on spindle-shaped, myosin-containing cells as well as on cells that were similar in shape but myosin-negative; many broad, flat mononucleated cells that resemble fibroblasts were not stained detectably with anti-N-CAM (Fig. 12, a–c). Similar results have been reported for human (30) and chick (20) muscle. Thus, as we had surmised from staining E12–15 embryos, N-CAM is present on myoblasts but absent from at least some nonmyogenic cells and may accumulate somewhat ahead of myosin in myoblasts.

Second, we stained myotubes with anti-N-CAM and anti-laminin to compare the distribution of N-CAM and basal laminae; previous electron microscope studies have shown that anti-laminin stains basal laminae on the myotube surface (43). N-CAM was present both in areas covered by basal laminae and in basal lamina-free areas (Fig. 12, d and e). Thus, the reciprocal distribution of N-CAM and basal laminae...
seen on E16–19 does not represent an obligatory relationship; rather, as seen in perinatal and adult denervated muscles, N-CAM and basal laminae can have overlapping distributions.

Finally, we stained 8–11-d cultures with anti-N-CAM and rhodamine-a-bungarotoxin to compare the distribution of N-CAM and AChRs on myotubes (Fig. 12, f and g). In vitro as in vivo, N-CAM was roughly uniformly distributed on the surface of isolated myotubes and was neither selectively concentrated at nor excluded from AChR-rich areas of the myotube surface.

**Distribution of N-CAM in Chicken Muscle**

Because N-CAM appeared at a bewildering variety of places and times in developing rat intercostal muscle, we wanted to know which features of its distribution were peculiar to this muscle and which might be generally found. We therefore studied the distribution of N-CAM in developing chicken pectoralis, using both monoclonal and affinity-purified polyclonal antibodies to chicken N-CAM. On E10, N-CAM was uniformly distributed on the surface of primary myotubes (Fig. 13a). N-CAM became concentrated on apposed myotube surfaces in clusters by E14 (Fig. 13b) but was again uniformly distributed on the myotube surface during the first week after hatching, when myotubes had separated from clusters to lie singly (Fig. 13c). Levels of N-CAM declined in extrasynaptic areas of muscle fibers during the next few weeks (Fig. 13d), but N-CAM remained associated with mononucleated cells and was concentrated at the neuromuscular junction in the adult (Fig. 13, e and f). N-CAM was abundant in embryonic intramuscular nerves but lost from motor nerves as myelination proceeds (not shown). In all these aspects of its disposition, N-CAM in chicken muscle resembled that in rat muscle.
Discussion

We (9, 45a) and others (30, 35, 49) have recently reported that N-CAM is widely distributed in embryonic skeletal muscle and concentrated near synapses in adult muscle. The present work extends these studies by providing a detailed description of the distribution of N-CAM at the neuromuscular junction and elsewhere in developing and adult muscles.
Figure 10. Association of N-CAM with developing motor axons. Cross-sections of E15 (a and b), E20 (c and d), and P3 (e and f) internal intercostal nerves. Cryostat sections were stained with anti-N-CAM and fluorescein second antibody and examined in the fluorescent microscope (a, c, and e); thin sections of conventionally prepared material were examined in the electron microscope (b, d, and f). N-CAM is lost from the axon surface as myelination occurs. S, Schwann cell processes. Bars: (e) 20 μm for a, c, and e; (f) 1 μm for b, d, and f.

Our new results permit us to draw a number of inferences about how nerve-muscle interactions influence the expression of N-CAM and how, in turn, N-CAM might influence nerve and muscle development.

Regulation of N-CAM during Neuromuscular Development

In many respects, N-CAM and AChRs are distributed similarly in developing and adult muscles. Both molecules are abundant on the surface of embryonic myotubes, become restricted to synaptic areas as development proceeds, reappear extrajunctionally when adult muscle is denervated, and disappear again when denervated muscle is reinnervated (N-CAM, Figs. 2 and 11 and references 7, 9, 10, and 35; AChRs, reviewed in reference 13). Important differences in distribution between the two molecules are (a) that AChRs become concentrated at synaptic sites during embryogenesis whereas N-CAM becomes concentrated in synaptic areas postnatally, and (b) that AChRs are highly localized to the postsynaptic membrane in adult muscle whereas N-CAM is also present perisynaptically (N-CAM, Fig. 4; AChRs, reference 14). Because the distribution and regulation of AChRs have been studied so intensively, consideration of similarities and differences between AChRs and N-CAM is helpful in understanding how the distribution of N-CAM might be controlled.

Levels of extrajunctional AChRs are regulated by nerve-evoked electrical and/or contractile activity in muscle: paralysis of innervated muscle leads to synthesis of new AChRs (13) and paralysis of embryonic muscle blocks the normal decline in AChR number (5). Similarly, paralysis of innervated adult muscle leads to the accumulation of N-CAM (9), indicating that the expression of this molecule is also regulated by activity. It therefore seems likely that the decline in N-CAM levels that we observed during the last days of embryogenesis is an activity-dependent process initiated by the establishment of synaptic transmission on E14-15. It is less clear how the transient perinatal reappearance of N-CAM could be linked to activity. However, an electrical uncoupling of myotubes from each other occurs around birth and converts the intercostal muscle from an electrical syncitium to an array of electrically independent fibers (11); it is possible that levels of electrical activity per myotube decline transiently as uncoupling proceeds.

The extreme concentrations of AChRs in the postsynaptic membrane (AChR levels fall >99% within 3 μm of the edge of the endplate although some AChRs are detectable perisynaptically [14, 33]), may result from the combined action of several factors. One is that AChRs are preferentially synthesized near synaptic sites: levels of AChR mRNAs are several-fold higher near the synapses than away from them.
Figure 11. Cross-sections of developing neuromuscular junctions double stained with anti-N-CAM (left panel of each pair) and rhodamine-a-bungarotoxin (right panel of each pair). Arrows mark corresponding points on the two panels of a pair. (a–d) E15, N-CAM is present in similar amounts in junctional and non-junctional areas. (e–h) E16, N-CAM is absent from most junctions. (i–l) PO, N-CAM is present but not concentrated at junctions. (m–p) P7, N-CAM is concentrated in some junctional areas. N, nerve branches. Bar, 10 μm for a–h; 15 μm for i–p.

We have speculated that nuclei near neuromuscular junctions preferentially transcribe a set of genes that encode synapse-specific proteins (28), and N-CAM might be a member of this hypothetical set. In addition to their local synthesis, AChRs probably become and remain concentrated in the postsynaptic membrane as a result of interactions with synapse-specific components of the cytoskeleton and extracellular matrix (reviewed in reference 48). It could be the lack of such highly localized interactions that accounts for the wider distribution of N-CAM than of AChRs in synaptic regions.

One intermolecular interaction in which N-CAM does participate is that of self-association: external domains of N-CAM molecules in adjacent membranes interact homophilically to bind the membranes together (38). A consequence of this homophilic interaction is that N-CAM might become stabilized and thereby concentrated in areas of apposition between N-CAM-bearing cells. The local concentrations of N-CAM in contacting surfaces of clustered myotubes (Fig. 9e) and in axon-Schwann cell contacts (Figs. 3 and 5) might arise in this way. It is interesting to realize that such local concentrations of N-CAM may be thought of as either the cause or the effect of such close appositions.

**Regulation of Nerve and Muscle Development by N-CAM**

As noted above, N-CAM can act as its own receptor (i.e., homophilically). It is not yet known whether N-CAM has other receptors as well, in which case N-CAM-poor membranes might also be involved in N-CAM-dependent adhesion; or whether other molecules modulate N-CAM interactions, in which case N-CAM-rich membranes would not inevitably adhere to each other. Nevertheless, enumeration of apposed cell surfaces that do or do not bear N-CAM provides a means of categorizing intercellular interactions as more or less likely to be mediated by N-CAM.

**Myoblasts.** N-CAM is present on mononucleated myogenic cells in vivo (Fig. 8) and in vitro (Fig. 12). The fusion of myoblasts to form myotubes is thought to be preceded by steps involving intercellular recognition and adhesion (50); N-CAM could participate in these prefusion steps. The observation that little N-CAM is present in spaces surrounding myotomes (Fig. 7) suggests that N-CAM on myoblasts might also play a role in the compaction of myotomes and their segregation from other, nonmuscle structures; an analogous role for N-CAM has been postulated in gangliogenesis of peripheral neurons (36). Grumet et al. (20) reported that anti-N-CAM could not strongly inhibit adhesion of cultured chick myoblasts to each other. However, it is now clear that myoblasts bear multiple adhesive systems, and that different systems can dominate the adhesion process in vitro, depending on how the cells are prepared (18). Thus, it may be that anti-N-CAM has not been tested on appropriately prepared myoblasts, and it remains possible that N-CAM is involved in interactions among myoblasts in vivo.

**Myoblasts and Myotubes.** Some myotubes are present in intercostal muscles by E14; these are called primary myotubes.
Figure 12. Anti-N-CAM stains myoblasts and myotubes in culture. (a–c) 4-d-old primary culture of E19 rat muscle, doubly stained with anti-N-CAM (a) and a monoclonal antibody to embryonic myosin (b). c is the same field photographed with Nomarski optics. Anti-N-CAM stains myosin-positive myoblasts; other cells (arrow) are unstained. (d and e) 8-d-old culture doubly stained with anti-N-CAM (d) and anti-laminin (e). N-CAM is present on both basal lamina-covered and basal lamina-free portions of the myotube. (f and g) 8-d-old culture, double stained with anti-N-CAM (f) and rhodamine-α-bungarotoxin (g). N-CAM is neither concentrated at nor depleted from patches of AChRs. Bar, 20 μm for a–g.

Subsequently, myoblasts line up along the sides of these myotubes and fuse to form a discrete class of secondary myotubes (23, 32). It has been postulated that recognition of primary myotubes by secondary myoblasts may regulate the alignment of the latter (22). In that N-CAM is present on the surfaces of these cells (Fig. 8) it may be one of the recognition molecules involved in this interaction. Adhesion of satellite cells to myofibers in adult muscle (Fig. 6) may similarly be mediated by N-CAM. Furthermore, Kelly and Rubenstein (22) have speculated that loss of an adhesive quality later in development might limit the number of late-generation myotubes that can form. It is interesting that N-CAM is lost from the external faces of clustered myotubes and becomes concentrated on apposing myotube surfaces within the clusters (Fig. 9) once secondary myotubes have formed. Thus, N-CAM is appropriately regulated both to mediate alignment of myotubes and to limit formation of new myotubes.

Motor Axons. Antibodies to N-CAM disrupt fasciculation of axons in vitro and in ovo (36). Anti-N-CAM stains intramuscular nerves brightly at a time when axons are abundant and Schwann cell processes rare within the nerves (Fig. 10, a and b); N-CAM is therefore almost certainly present on embryonic motor axons and could mediate interactions among them that result in the formation and maintenance of nerve trunks. In addition, by constraining late-arriving motor axons to pre-existing pathways, N-CAM-mediated adhesion could participate in limiting axonal contacts to orginal syn-
aptic sites on myotubes during the transient period of poly-
nervous innervation that occurs late in embryogenesis (see refer-
ence 8 for further discussion).

Axons and Schwann Cells. N-CAM is concentrated in re-
ions of axon-Schwann cell apposition at mature neuromus-
cular junctions (Fig. 3) and in unmyelinated nerve fibers (Fig. 5). Our results suggest, although they do not prove, that both cells contribute N-CAM to these deposits, raising the possibility that N-CAM participates in mediating the adhesion between these cells. The presence of N-CAM between the Schwann cell cap and nerve terminal implies a role for this molecule in maintaining the integrity of the adult neuromuscular junction. In contrast, the apparent absence of N-CAM from areas of contact between motor axons and their myelin sheaths indicates that N-CAM is not involved in maintenance of nonterminal portions of the motor nerve.

Axons and Myotubes. Rutishauser et al. (37) reported that antibodies to N-CAM disrupt interactions of neurites with myotubes in culture, and suggested that N-CAM could mediate some of the initial nerve-muscle interactions that lead to synapse formation. Our results are consistent with this suggestion: N-CAM is present on motor axons before they enter the intercostal muscles, and on myotubes as soon as they form (Figs. 7 and 8). However, N-CAM does not appear to be concentrated on the growing tips of axons, nor is it restricted to specific areas of the myotube surface. N-CAM is therefore unlikely to determine the sites at which synapses will form. It might, however, serve as a nonspecific “glue” whose action is prerequisite to other, more specific transactions between the synaptic partners.

On the other hand, there is little evidence that nerve-muscle interactions subsequent to the initial formation of the synapse are mediated by N-CAM. N-CAM is not concentrated in the postsynaptic membrane during late embryonic or early post-
natal life; if anything, N-CAM is more abundant in non-
synaptic than in synaptic areas (Fig. 11). Furthermore, in the adult, even though N-CAM is concentrated at the neuromuscular junction, it is depleted at areas of nerve-muscle contact: more N-CAM is present in the depths of junctional folds than in the primary synaptic cleft, and more N-CAM is present on the Schwann cell-coated “rear” surface of the nerve terminal than on the surface that faces the muscle (Fig. 3). We have previously shown that motor axons recognize the basal laminae of the synaptic cleft (45) and that synapse-specific components of the basal laminae accumulate after synapse formation (8). It is possible that an initial, N-CAM-dependent adhesion of nerve to muscle is replaced by a basal lamina-
dependent, N-CAM-independent adhesion system as synap-
togenesis proceeds.

We thank D. Dill, S. Eads, and J. Mosher for assistance, F. Bacou for help with muscle cultures, C. Barnstable, D. Gottlieb, N. Rubinstein, and M. Willard for antibodies, and D. Purves (Washington University, St. Louis, MO) for helpful comments.

This work was supported by grants from the National Institutes of Health and the Muscular Dystrophy Association. J. Covault received a postdoctoral fellowship from the Muscular Dystrophy Association; J. Sanes is an Established Investigator of the American Heart Asso-
ciation.

Received for publication 13 September 1985, and in revised form 23 November 1985.

References

1. Altman, J., and S. Bayer. 1984. The development of the rat spinal cord. Adv. Anat. Embryol. Cell Biol. 85:32-46.

2. Barnstable, C. J., and U. C. Drager. 1984. Thy-1-antigen, a ganglion cell specific marker in rodent retina. Neuroscience. 11:847-855.

3. Bennett, M. R. 1983. Development of neuromuscular synapses. Physiol. Rev. 63:913-1048.

4. Bevan, S., and J. H. Steinbach. 1977. The distribution of a-bungarotoxin binding sites on mammalian skeletal muscle developing in vivo. J. Physiol. (Lond.) 267:195-213.

5. Burden, S. J. 1977. Development of the neuromuscular junction in the chick embryo: the number, distribution and stability of acetylcholine receptors. Dev. Biol. 57:317-329.

6. Carlson, B. M., and J. A. Faulkner. 1983. The regeneration of skeletal muscle fibers following injury: a review. Med. Sci. Sports Exercise. 15:187-198.

7. Cashman, N. R., J. Covault, R. L. Wallman, 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.

8. Covault, J., J. P. Merlie, C. Goridis, and J. R. Sanes. 1986. Molecular forms of N-CAM and its RNA in developing and denervated skeletal muscles. J. Cell Biol. 102:731-739.

9. Dennis, M. J., L. Ziskind-Conhaim, and A. J. Harris. 1981. Development of neuromuscular junctions in rat embryos. Dev. Biol. 81:266-279.

10. Edelman, G. M. 1983. Cell adhesion molecules. Science (Wash. DC). 219:450-457.

11. Fambrough, D. M. 1979. Control of acetylcholine receptors in skeletal muscle. Physiol. Rev. 59:165-227.

12. Fertuck, H. C., and M. Salpeter. 1976. Quantitation of junctional and extrajunctional acetylcholine receptors by electron microscope autoradiogra-
phy after 125I-alpha-bungarotoxin binding at mouse neuromuscular junctions. J. Cell Biol. 69:144-158.

13. Gambhe, 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.

14. Gatchalian, C. A., Y. Chiu, and J. R. Sanes. 1985. Monoclonal antibodies to laminin that distinguish synaptic and extrasynaptic portions of embryonic rat muscle. J. Cell Biol. 102:731-739.

15. Gennarini, G., G. Rougon, H. Deagostini-Bazin, M. Hirn, and C. Goridis. 1984. Studies on the transmembrane disposition of the neuronal cell adhesion molecule N-CAM: A monoclonal antibody recognising a cytoplasmic domain and evidence for the presence of phosphoserine residues. Eur. J. Biochem. 142:57-64.

16. Gilbarker, D., and D. C. Turner. 1986. Dual adhesive systems of chick myoblasts. Dev. Biol. In press.

17. Glicksman, M. A., and M. Willard. 1985. Differential expression of the three neurofilament polypeptides. Annu. NY Acad. Sci. 455:479-491.

18. Grumet, M., U. Rutishauser, and G. M. Edelman. 1982. Neural cell adhesion molecule is on embryonic muscle cells and mediates adhesion to nerve cells in vitro. Nature (Lond.). 295:693-695.

19. Johnson, G. D., and G. M. de C. Araujo. 1981. A simple method of reducing the fading of immunofluorescence during microscopy. J. Immunol. Methods. 43:349-350.

20. Kelly, A. M., and N. A. Rubinstein. 1985. Muscle histogenesis and muscle diversity. UCLA (Univ. Calif. Los Ang.) Sympl. Biol. New Series. 29 In press.

21. Kelly, A. M., and S. I. Zacks. 1969. This histogenesis of rat intercostal muscle. J. Cell Biol. 42:135-153.

22. Kelly, A. M., and S. I. Zacks. 1969. The fine structure of motor endplate morphogenesis. J. Cell Biol. 42:154-169.

23. Kohn, J., and M. Wilchek. 1982. A new approach (cyano-transfer) for cyanogen bromide activation of sepharose at neutral pH, which yields activated resins, free of interfering nitrogran derivatives. Biochem. Biophys. Res. Com-
mun. 107:878-884.

24. Lemmon, V., E. B. Staros, H. E. Perry, and D. I. Gottlieb. 1982. A monoclonal antibody which binds to the surface of chick brain cell and myotubes. Dev. Brain Res. 3:349-360.

25. Merlie, J. P., and J. R. Sanes. 1985. Acetylcholine receptor mRNA is concentrated in synaptic regions of adult muscle fibers. Nature (Lond.). 317:86-88.

26. Merlie, J. P., and J. R. Sanes. 1986. Regulation of synapse-specific genes. In: Molecular Aspects of Neurobiology: International Symposium. E. R. Kandel and R. Levi-Montalcini, editors. Fondazione Giovanni Lorenzini. Piccin Medical Books, Padua, Italy. In press.

27. 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.

729 Covault and Sanes N-CAM at the Neuromuscular Junction
30. Moore, S. E., and F. S. Walsh. 1985. Specific regulation of N-CAM/D2-CAM cell adhesion molecule during skeletal muscle development. EMBO (Eur. Mol. Biol. Organ. J.) 4:523–630.
31. Morris, R. J., P. C. Barber, J. Beech, and G. Raisman. 1983. The distribution of Thy-1 antigen in the P.N.S. of the adult rat. J. Neurocytol. 12:1017–1039.
32. Ontell, M., and K. Kozeka. 1984. The organogenesis of murine striated muscle: a cytoarchitectural study. Am. J. Anat. 171:133–148.
33. Pestronk, A. 1983. Intracellular acetylcholine receptors in skeletal muscles of the adult rat. J. Neurocytol. 5:1111–1117.
34. Ravdin, P., and D. Axelrod. 1977. Fluorescent tetramethyl rhodamine derivatives of α-bungarotoxin: preparation, separation and characterization. Anal. Biochem. 80:585–592.
35. Rieger, F., M. Grumet, and G. M. Edelman. 1985. N-CAM at the vertebrate neuromuscular junction. J. Cell Biol. 101:285–293.
36. Rutishauser, U. 1984. Developmental biology of a neural cell adhesion molecule. Nature (Lond.), 310:549–554.
37. 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.
38. Rutishauser, U., S. Hoffman, and G. M. Edelman. 1982. Binding properties of a cell adhesion molecule from neural tissue. Proc. Natl. Acad. Sci. USA. 79:685–689.
39. Sanes, J. R., and J. M. Cheney. 1982. Lectin-binding reveals a synapse-specific carbohydrate in skeletal muscle. Nature (Lond.), 300:646–647.
40. Sanes, J. R., and A. Y. Chiu. 1983. The basal lamina of the neuromuscular junction. Cold Spring Harbor Symp. Quant. Biol. 48:667–678.
41. Sanes, J. R., and J. Covault. 1985. Axon guidance during reinnervation of skeletal muscle. Trends Neurosci. 8:523–528.
42. Sanes, J. R., and Z. W. Hall. 1979. Antibodies that bind specifically to synaptic sites on muscle fiber basal lamina. J. Cell Biol. 83:357–370.
43. Sanes, J. R., and J. C. Lawrence. 1983. Activity-dependent accumulation of basal lamina by cultured rat myotubes. Dev. Biol. 97:123–136.
44. Sanes, J. R., D. H. Feldman, J. M. Cheney, and J. C. Lawrence. 1984. Brain extract induces synaptic characteristics in the basal lamina of cultured myotubes. J. Neurosci. 4:464–473.
45. Sanes, J. R., L. M. Marshall, and U. J. McMahan. 1978. Rennervation of muscle fiber basal lamina after removal of myofibers: differentiation of regenerating axons at original synaptic sites. J. Cell Biol. 78:176–198.
46. Sargent, P. B., B. E. Hedges, L. Tsavaler, L. Clemmons, S. Tzartos, and J. M. Lindstrom. 1984. Structure and transmembrane nature of the acetylcholine receptor in amphibian skeletal muscle as revealed by cross-reacting monoclonal antibodies. J. Cell Biol. 98:609–618.
47. Schneider, C., R. A. Newman, D. R. Sutherland, U. Asser, and M. F. Greaves. 1982. A one-step purification of membrane proteins using a high efficiency immunomatrix. J. Biol. Chem. 257:10766–10769.
48. Steinbach, J. H., and R. J. Bloch. 1986. The distribution of acetylcholine receptors on vertebrate skeletal muscle cells. In Receptors in Biological Systems. R. M. Gorczynski, and G. B. Price, editors. Academic Press Inc., New York. In press.
49. Tosney, K. W., M. Watanabe, L. Landmesser, and U. Rutishauser. 1986. The distribution of N-CAM in the chick hind limb during axon outgrowth and synaptogenesis. Dev. Biol. In press.
50. Wakelam, M. J. O. 1985. The fusion of myoblasts. Biochem. J. 228:1–12.