N-CAM at the Vertebrate Neuromuscular Junction

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ABSTRACT We have detected the neural cell adhesion molecule, N-CAM, at nerve–muscle contacts in the developing and adult mouse diaphragm. Whereas we found N-CAM staining with fluorescent antibodies consistently to overlap with the pattern of α-bungarotoxin staining at nerve–muscle contacts both during development and in the adult, we observed N-CAM staining on the surfaces of developing myofibers and at much lower levels on adult myofibers. Consistent with its function, N-CAM was also detected on axons and axon terminals. Immunoblotting experiments with anti-N-CAM antibodies on detergent extracts of embryonic (E) diaphragm muscle revealed a polydisperse polysialylated N-CAM polypeptide, which in the adult (A) was converted to a discrete form of M, 140,000; this change, called E-to-A conversion, was previously found to occur in different neural tissues at different rates. The M, 140,000 component was not recognized by monoclonal antibody anti-N-CAM No. 5, which specifically recognizes antigenic determinants associated with N-linked oligosaccharide determinants on N-CAM from neural tissue. The relative concentration of the M, 140,000 component prepared from diaphragm muscle increased during fetal development and then decreased sharply to reach adult values. Nevertheless, expression of N-CAM in muscle could be induced after denervation: one week after the sciatic nerve was severed, the relative amount of N-CAM increased dramatically as detected by immunoblots of extracts of whole muscle. Immunofluorescent staining confirmed that there was an increase in N-CAM, both in the cell and at the cell surface; at the same time, however, staining at the motor endplate was diminished. Our findings indicate that, in muscle, in addition to chemical modulation, cell-surface modulation of N-CAM occurs both in amount and distribution during embryogenesis and in response to denervation.

Initial connections between nerve and muscle and the subsequent morphogenesis of synaptic junctions require local associations of structural proteins, enzymes, and receptors in specific temporal sequences. During embryonic development in vertebrates, for example, the acetylcholine receptor (AChR) accumulates in the postsynaptic muscle membrane adjacent to nerve terminals (reviewed in reference 1). At later stages of development, the postsynaptic membrane is characterized by junctional folds with AChR-rich crests closely apposed to the nerve terminals (2). In the synaptic regions between nerve and muscle, the basal lamina develops, containing filamentous structures that seem to be connected both to nerve and muscle membranes (3). It has been suggested that adult basal lamina can influence regrowth of severed nerves during regeneration (4), and that extracts of basal lamina can induce clustering of AChRs on myotubes in culture (5). In the embryo, however, nerves arrive at developing muscles and innervate them before AChR aggregates appear and the basal lamina is deposited, which suggests that prior AChR aggregation and lamina deposition is not essential for formation of immature synapses (reviewed in reference 6).

Although the sequences of interactive molecular mechanisms responsible for formation of the neuromuscular junction and for reinnervation are not yet known, it has been found that the initial events of nerve–muscle contact in culture require the participation of the neural cell adhesion molecule, N-CAM (7–9). N-CAM is a cell surface glycoprotein that mediates adhesion by a homophilic mechanism, i.e., an N-CAM molecule on one cell interacts with an N-CAM
molecule on an apposing cell (7, 10). N-CAM antigenic determinants have been found on central and autonomic neurons (10-12), in the myotome (12), and in developing skeletal muscle both in culture and in vivo (8, 9). Muscle cells in culture have been shown to bind vesicles reconstituted from purified N-CAM and lipids (8), and adhesion between chick embryo spinal cord neurons and muscle cells in primary cultures can be inhibited specifically by monovalent Fab' fragments of antibodies to N-CAM (9). These findings suggest that N-CAM mediates adhesion between neurons and developing muscle cells in vivo, and raise the possibility that this molecule may play a role in development and stabilization of neuromuscular junctions.

We present here the results of immunofluorescent staining studies in the mouse, which indicate that N-CAM is found not only on developing myofibers and nerves but also concentrated at developing and adult neuromuscular junctions. Although the distribution of N-CAM was largely co-extensive with that of AChR at the motor endplate, the CAM was also present on nerve terminal arborizations. Biochemical analyses revealed that N-CAM in muscle consisted of a major polypeptide component of Mr 140,000; this component increased in concentration during fetal development and after birth decreased to low amounts in adult tissue. After section of the sciatic nerve in adult mice, however, the concentration of this component increased sharply in the previously innervated limb muscles. These findings provide further support for the conclusion that N-CAM mediates initial interactions between nerve and muscle in mammals, and they also suggest that N-CAM probably plays an important role in subsequent stages of synapse development and stabilization.

MATERIALS AND METHODS

Animals: Adult NCS (Swiss) mice from the Laboratory Animal Research Center of The Rockefeller University were used for biochemical determinations, denervation experiments, and immunofluorescence studies. For the developmental study, we used pregnant mice of the same strain as that bearing the muscular dysgenesis mutation (13) produced by the Institut National de la Santé et de la Recherche Médicale Laboratory (Paris).

Fluorescent Studies: Mice were killed by cervical dislocation and the dissections were dissected out, pinned on a 1% agarose surface (Difco Laboratories Inc., Detroit, MI), and fixed for 1 h with neutral phosphate-buffered saline (PBS)/4% paraformaldehyde. After a 15-min incubation in PBS/0.1 M glycine/4% goat serum followed by two washes in PBS/4% goat serum (10 min each), the overlying muscular membranes were removed. Bundles of muscle fibers that had been teased apart were incubated for 2 h in the presence of 2 μg/ml fluorescein isothiocyanate-labeled α-bungarotoxin (FITC-α-BgTx; 0.8 mol FITC/mol α-BgTx; Sigma Chemical Co., St. Louis, MO) and washed three times (5 min each). Control experiments were carried out in the presence of unlabeled α-BgTx, which inhibited all staining at the motor endplates. Either before or after treatment with FITC-α-BgTx, muscles were incubated overnight in the presence of polyclonal rabbit anti-mouse N-CAM IgG (150 μg/ml) or a murine monoclonal anti-mouse N-CAM IgM (monoclonal anti-N-CAM No. 5), both of which have been described previously (11, 14). After three washes (20 min each) with PBS/4% goat serum, the muscle preparations were preincubated (4 h) with rhodamine-conjugated goat antibody to rabbit IgG (diluted 1:50 in PBS/4% goat serum; Cappel Laboratories, West Chester, PA). When monoclonal antibodies were used, the preparations were washed and then incubated for 4 h in rabbit anti-mouse IgM sera (diluted 1:50 in PBS/4% goat serum; Miles Yeda, Rehovot, Israel) before incubation (4 h) with the rhodamine-conjugated goat anti-rabbit IgG (50 μg/ml in PBS/4% goat serum). In control experiments, IgG prepared from a rabbit immunized with mouse γ-globulin and IgG from a rabbit immunized with mouse neuron-glia CAM (N-CAM) (14), were used. As a further control for specificity, anti-N-CAM IgG (150 μg/ml) was preincubcated for 2 h with N-CAM (75 μg/ml) that had been purified from mouse brain (15), and then tested for staining. The muscle bundles were washed three times with PBS/4% goat serum and teased apart into small bundles containing 5 to 50 muscle fibers. The preparations were mounted in 90% glycerol/10% PBS with 0.1% para-phenylene diamine (Sigma Chemical Co.) and observed with a Zeiss microscope equipped with appropriate filters for fluorescein and rhodamine optics. From sections were prepared as described (16).

Immunobots: Mouse tissues (10 mg wet wt) were homogenized in a Dounce homogenizer in 1 ml ice-cold PBS/0.5% Nonidet P-40/1 mM EDTA/ trisoyl (200 kIU/ml). The samples were centrifuged at 100,000 g for 1 h at 4°C, and the protein concentrations of the supernatant fractions were determined (17). Aliquots of lysates containing exactly equivalent amounts of protein were then resolved by SDS PAGE, transferred to nitrocellulose paper, and sequentially reacted with antibodies and 125I-labeled protein A (1 × 106 cpm) (14, 18). Immunobots were performed either with 50 μg rabbit IgG or with 50 μg monoclonal antibodies and then 20 μl of rabbit anti-mouse immunoglobulin antiserum (Miles Laboratories Inc., Elkhart, IN) was added. After washing was done the results were visualized by autoradiography. To quantitate N-CAM, densitometric scans of the autoradiographs were obtained and peaks corresponding to N-CAM were cut out and weighed.

Denervation Experiments: The soleus, extensor digitorum longus, and gastrocnemius muscles of adult mice were denervated by cutting out a 5-mm segment of the sciatic nerve at the upper thigh level of mice that had been anesthetized with chloral hydrate. The animals were allowed to recover from the operation, and at different intervals the animals were killed; tissues were removed and analyzed for N-CAM content by immunofluorescence staining and immunoblotting.

Analytical Procedures: For peptide mapping, detergent extracts of newborn mouse limb muscles and brain were prepared as described above and incubated at 4°C with polyclonal anti-N-CAM IgG (15) coupled to Sepharose-CL2B beads by the CNBr method (19). The beads were washed six times with the extraction buffer and the bound N-CAM was eluted by boiling (11); the eluates were resolved by SDS PAGE (20). Segments of the gels containing components of N-CAM were excised, treated with Staphylococcus aureus V8 protease, and resolved on SDS/5% polyacrylamide gels (21). For neuremsec experiments, extracts (50 μg) were run in 5% polyacrylamide gels and blotted onto nitrocellulose paper (22) and were then resolved by SDS PAGE, transferred to nitrocellulose paper, and probed with rabbit antibodies raised against N-CAM and then reacted with 125I-labeled protein A (1 × 106 cpm) (8). Bands corresponding to N-CAM were cut out and weighed.

RESULTS

N-CAM Localization in Developing Mouse Muscle

The distribution of N-CAM in whole mount preparations of diaphragm muscles of near-term (embryonic day 19) or newborn mice was studied by indirect immunofluorescence using rabbit antibodies raised against N-CAM that had been purified from mouse brain (15). Positive staining for N-CAM was seen on the surfaces of myofibers (Fig. 1). Previous experiments showed that chicken embryo myotubes in culture expressed N-CAM (8, 9), and, as expected, in immunofluorescence experiments mouse myotubes in culture also expressed N-CAM at their surface (Pinçon-Raymond, M., F. Rieger, M. Grumet, and G. M. Edelman, unpublished observations).

We observed N-CAM staining on the surfaces of myotubes in a predominantly uniform pattern which showed occasional foci of intense staining (Fig. 1 a). To determine whether these foci corresponded to neuromuscular junctions, we studied the distribution of N-CAM and AChR in developing diaphragm. Double-label staining patterns of N-CAM and of AChR were found to overlap (Fig. 1, b-e); >90% of the developing motor endplates characterized by clusters of AChRs also had a coextensive pattern of intense staining for N-CAM (Table I). It is noteworthy that at the developing motor endplate both labels were almost identical; in contrast, outside of this region the myotube surfaces were stained for N-CAM but not for AChR. Axon terminal arborizations were stained for N-CAM (Fig. 1, b* and e*), but intact axons were rarely seen in these muscle preparations because they were removed either with the muscle membranes or when the muscles were teased into fibers. In addition to these staining patterns, we observed that...
Figure 1 Immunolocalization of N-CAM in developing mouse diaphragm muscle and its co-localization with AChR. (a) Nearterm mouse diaphragm muscles were sequentially incubated with anti-N-CAM IgG and rhodamine-labeled goat anti-rabbit IgG. N-CAM staining is seen concentrated at multiple loci distributed in the motor endplate-rich region of muscle. X 430. For double-label experiments (b-e), muscles were allowed to react sequentially with anti-N-CAM IgG, rhodamine-labeled goat anti-rabbit IgG, and FITC-α-BgTx. b-e are high magnification micrographs of muscles stained with α-BgTx observed under optics for fluorescein, and b*-e*, respectively, are the same fields stained for N-CAM and visualized under optics for rhodamine. In addition to widespread staining on the surface of myofibers (b*, e*), there is also N-CAM staining of axon terminal branches at sites of motor endplates in b* (arrows) in the vicinity of distinct AChR profiles in b. X 1,250.
TABLE I. FITC-α-BgTx and N-CAM Staining at Adult and Newborn Motor Endplates (MEPs)

|                | Double-stained MEPs | α-BgTx staining only | N-CAM staining only* |
|----------------|---------------------|----------------------|----------------------|
| Newborn (n = 14) | 549 (92%)           | 22 (4%)              | 25 (4%)              |
| Adult (n = 8)   | 291 (91%)           | 21 (7%)              | 8 (2%)               |

The numbers in parentheses represent the percentage of the total MEPs counted, n, number of muscles studied.
* These presumptive MEPs had N-CAM staining patterns similar to those of authentic MEPs.

To discriminate more precisely between the extents of the two patterns, we produced drawings of the contours of stained areas for both labels (Fig. 4, c and d), including the coincident (Fig. 4e) and the non-coincident (Fig. 4f) delineations. The coincident regions show the characteristic structures of the motor endplate (Fig. 4e). The regions stained by anti-N-CAM but not by α-BgTx were not organized in any clear pattern (Fig. 4f), although at least part of this difference profile (in the lower left portion of Fig. 4f) represents terminal axon branches. Drawings of motor endplate structures using several other micrographs gave similar results.

We observed a systematic co-localization of N-CAM and AChR at adult neuromuscular junctions. More than 90% of most myotendinous junctions and some interstitial tissues were stained intensely by antibodies to N-CAM (data not shown).

N-CAM Is Localized at the Adult Mouse Neuromuscular Junction

To evaluate developmental changes in N-CAM expression after morphogenesis, we performed a similar series of experiments on adult muscles. In contrast to developing myofibers, mature myofibers were very faintly labeled at their surface membrane by antibodies to N-CAM (see below, Fig. 7, a and b). Staining for N-CAM, however, was observed at nerve–muscle contacts that were also characteristically labeled by FITC-α-BgTx (Fig. 2). Structures stained with the anti-N-CAM antibodies were mostly co-extensive with the contours for motor endplates as delineated by the AChR-rich regions. Although both labels were almost coincident in the disk (en face) or arc-like (profile) structure of the motor endplate, it was clear that, as in the embryo, the axons (Fig. 3) incident upon the muscles and the terminal axon branches (Fig. 4b) were also stained by anti-N-CAM IgG. The patterns of N-CAM and AChR staining were not altered by changes in the order of staining with FITC-α-BgTx and anti-N-CAM IgG, which suggests that the observed co-localization was neither artifactual nor the result of binding of anti-N-CAM antibodies to α-BgTx. In other control experiments on developing and mature muscles, preimmune sera did not stain motor endplates that could be visualized with α-BgTx, and preincubation of the anti-N-CAM IgG with purified N-CAM was found to eliminate most of the staining at motor endplates.

FIGURE 2 Co-localization of N-CAM and AChR at the motor end-plate in adult mouse diaphragm muscle. Micrographs of staining with FITC-α-BgTx (a and c) under optics for fluorescein; the profiles delineate the classical contours of mature motor endplates. Micrographs of staining with anti-N-CAM IgG (b and d are same fields as in a and c, respectively) under rhodamine optics were obtained after exposure to rhodamine-labeled goat anti-rabbit IgG. The pattern of N-CAM staining closely corresponds to the pattern of α-BgTx staining with additional regions of N-CAM staining that, at least in part, correspond to the axon terminal branches. Bar, 10 μm. × 800.

FIGURE 3 Anti-N-CAM staining of an adult preterminal axon. Micrograph of staining with anti-N-CAM IgG and rhodamine-labeled goat anti-rabbit IgG. Axons were identified by their staining with specific antibodies to mouse neurofilament protein obtained from Dr. D. Paulin (Pasteur Institute, Paris). The axon was intensely stained and the borders of the myofibers (arrows) were faintly stained by anti-N-CAM IgG. Bar, 10 μm. × 800.
the junctions identified by α-BgTx staining were also posi-
tively stained for N-CAM; 7% of the junctions were stained
clearly with α-BgTx represented only 2% of the 320 structures
analyzed.

To test whether the N-CAM staining pattern at the motor
endplates could be due simply to presynaptic neural struc-
tures, two antibodies specific for different neural antigens that
are both absent from muscle were used. Both monoclonal
antibody anti-N-CAM (No. 5), which recognizes carbohydrate
determinants on some forms of N-CAM and Ng-CAM (11,
14), and a polyclonal anti-Ng-CAM antiserum stained fixed
axons but did not stain the surfaces of muscle fibers of the
characteristic structures of the motor endplates revealed with
both FITC-α-BgTx and anti-N-CAM IgG. The fact that N-
CAM staining overlapped with AChR staining but that other
neuronal cell surface markers did not overlap suggests that
these markers are not present at the motor endplate. Thus,
N-CAM is preferentially localized at the motor endplate in
the pre- or postsynaptic membrane, basal lamina, terminal
Schwann cell, or in a combination of these regions.

A reciprocal relationship between the apparent relative
intensities of staining with anti-N-CAM antibodies and α-
Bgtx at the neuromuscular junction was found during develop-
ment. In newborn muscle, staining for N-CAM was more
intense than for α-BgTx; as the muscle matured, N-CAM
staining intensities decreased and α-BgTx staining intensities
increased (for an example, compare Figs. 1 and 2). These
results agree with previous findings that, during development,
AChR becomes concentrated at the motor endplate (1, 6);
they also suggest that at later stages of development, N-CAM,
which also becomes restricted to the motor endplate, may
even decrease in quantity at that site.

Content and Form of N-CAM in Muscle
during Development

In an initial attempt to explore expression of N-CAMs in
muscle, different antibodies raised against mouse brain N-
CAM were used in immunoblotting experiments to compare
the form and relative content of N-CAMs in detergent extracts
of diaphragm muscle and spinal cord. In developing mouse
embryos, polyclonal rabbit antibodies to N-CAM recognized
a polydisperse (Mr 140,000 to 200,000) form of N-CAM in
muscle (Fig. 5A, lane 1) and, as shown previously (11), a

![Figure 4 Co-localization of AChR and N-CAM at the adult motor
dendplate. Adult mouse diaphragm muscles were stained with FITC-
α-BgTx (a) and anti-N-CAM IgG (b) as described in the legend to
Fig. 2. The contours of staining for AChR (c) and for N-CAM (d)
were drawn. The profiles shown in c and d were superimposed,
and the common features (e) and the difference profile (f) were
drawn. e shows the characteristic features of the AChR-rich post-
synaptic apparatus. The profile in f represents primarily axon ter-

dinal branches. Bar, 10 μm. × 800.](https://example.com/figure4)

![Figure 5 Immunological and biochemical comparison of N-CAM from mouse muscle and neural tissues. (A and B) Extracts (50
μg of protein) of 14-d embryo (lanes 1 and 4), newborn (lanes 2 and 5), and adult (lanes 3, 6, and 7) diaphragm muscle (lanes 1-
3), spinal cord (lanes 4-6), and sciatic nerve (lane 7) were fractionated on 7.5% polyacrylamide gels, transferred to nitrocellulose,
and allowed to react with antibodies as described in Materials and Methods. (A) Immunoblot with polyclonal rabbit anti-
N-CAM IgG and (B) with monoclonal antibody anti-N-CAM No. 5. (C) S. aureus V8 protease digests of N-CAM components immunopre-
cipitated from mouse limb muscles that migrated at Mr 140,000 (lane 1) and immunoprecipitated from mouse brain that migrated
at Mr 180,000 (lane 2) and 140,000 (lane 3); lane 4 contained only the enzyme. The gel was stained with silver nitrate (27) and
photographed. Migration of standard proteins is indicated by their Mr × 10^-3.](https://example.com/figure5)
polydisperse (Mr 180,000 to 250,000) form in spinal cord (lane 4). The heterogeneity of N-CAM in embryonic neural tissues (7, 22, 23) and in chick muscle (9) is ascribable largely to the presence of sialic acid present as polysialic acid. After treatment of mouse muscle extracts with neuraminidase to remove sialic acid, anti-N-CAM IgG recognized primarily one component of Mr 140,000 (data not shown), which suggests that the heterogeneity of N-CAM in muscle is also due to the presence of sialic acid. The fact that N-CAM polypeptide components in neural tissues of the mouse include those of Mr 180,000 and 140,000 (11), whereas muscle does not contain the Mr 180,000 polypeptide component, is consistent with the finding that embryonic muscle does not contain N-CAM immunoreactive material in the range of Mr 200,000 to 250,000; i.e., this difference is consistent with the absence in embryonic muscle of sialylated forms of the Mr 180,000 polypeptide component.

In adult mice, the forms of N-CAM in muscle and neural tissues also differed; in contrast to the spinal cord, which contained a major component of Mr 120,000 and minor components including those of Mr 180,000 and 140,000 (11; Fig. 5 A, lane 6), the muscle contained only one major component of Mr 140,000 (lane 3). Most experiments were focused on comparing muscle and spinal cord because it was not feasible to dissect sufficient amounts of developing peripheral nerves for extensive biochemical analysis. Nevertheless, sciatic nerves from adult mice were found to contain N-CAM components of Mr 140,000 and 120,000 (Fig. 5 A, lane 6). The heterogeneity of N-CAM in embryonic neural tissues of the mouse includes those of Mr 180,000 and 140,000 (11), whereas muscle does not contain N-CAM components in neural tissues of the mouse include those of Mr 180,000 and 140,000 (11; Fig. 5 A, lane 6), the muscle contained only one major component of Mr 140,000 (lane 3). Most experiments were focused on comparing muscle and spinal cord because it was not feasible to dissect sufficient amounts of developing peripheral nerves for extensive biochemical analysis. Nevertheless, sciatic nerves from adult mice were found to contain N-CAM components of Mr 140,000 and 120,000 (Fig. 5 A, lane 6).

During development of the central nervous system, N-CAM is converted at different rates in different neural regions from a polydisperse embryonic (E) form rich in sialic acid to several distinct adult (A) forms containing much less of this sugar (11, 22); the loss of sialic acid in this so-called E-to-A conversion is associated with an increase in the rate of N-CAM-mediated binding (24). In diaphragm muscle, N-CAM was also found to be converted from a polydisperse E form to a sharply resolved A form (Fig. 5 A, lane 3). Quantitative analysis of the immunoblots in Fig. 5 A indicated that the concentration of N-CAM in extracts of diaphragm muscle increased 6-fold from the 14-d embryo to the newborn and then decreased 3-fold in the adult (Table II). The amount of the Mr 140,000 component in adult diaphragm muscle was found, however, to vary among experiments and in some experiments it was barely detected. Note that the decrease in relative concentration of N-CAM in adult muscle may reflect the fact that the data have been normalized to total detergent-extracted protein, and that during maturation, muscle contains an increasing proportion of intracellular proteins and a decreasing proportion of plasma membrane proteins. Nevertheless, the methods used here are internally consistent: for example, they confirmed the previous observation (11) that the relative amount of N-CAM in spinal cord increased twofold from the 14-d embryo to the newborn and then decreased fourfold in the adult.

Because it recognizes N-linked oligosaccharide determinants of N-CAM, we used also monoclonal antibody anti-N-CAM No. 5 in immunoblotting experiments. This monoclonal antibody recognized components in neural tissues but not in the muscle (Fig. 5 B), which suggests that, at least in their carbohydrate composition, some forms of N-CAM found in muscles differ from those in neural tissues.

To determine further whether significant amounts of nerve were present in the muscle preparations and thereby contributed to the N-CAM found in muscle extracts, we tested these extracts for immunoreactivity with a polyclonal antibody to Ng-CAM that specifically stains neuronal plasma membranes (14, 16). Ng-CAM components were identified both in extracts of spinal cord and sciatic nerve but not in extracts of muscle (data not shown). The results both with monoclonal antibody anti-N-CAM No. 5 and with anti-Ng-CAM IgG suggest that contamination of the muscle extracts with fragments of nerve is minimal, and, therefore, we conclude that the N-CAM detected in muscle extracts is derived mainly from muscle cells.

To compare the polypeptide structures of N-CAMS from mouse muscle and neural tissues, the component of Mr 140,000 isolated from muscle and the components of Mr 180,000 and 140,000 isolated from brains were subjected to one-dimensional peptide mapping after digestion with S. aureus V8 protease (Fig. 5 C). Most of the major peptide fragments derived from the muscle component of Mr 140,000 were similar to the peptides derived from the brain components of Mr 140,000 and 180,000, which suggests extensive homology, and possibly, near-identity between most of the polypeptide portions of the CAM forms.

Increases in Muscle N-CAM after Denervation

In addition to the changes that occur during normal deval-
FIGURE 7 Prevalence of N-CAM in muscle after denervation. 2 wk after section of the sciatic nerve in one thigh of the mouse, the gastrocnemius muscles from the unperturbed side (a and b) and the denervated side (c and d) were dissected, frozen, and cut in cross-section. The sections were stained with 150 μg/ml anti-N-CAM IgG as described in Materials and Methods. Note (by comparison to the unperturbed muscle) the atrophy in the denervated muscle. Bar, 10 μm. × 800.

opment of the neuromuscular junction, dramatic changes in the muscle and at the motor endplate also occur upon denervation (5). 1 wk after severing of the sciatic nerve of adult mice, the form and relative content of N-CAM in extracts prepared from the limb muscles was determined by immunoblotting experiments. The concentration of the M, 140,000 component of N-CAM in extracts of denervated soleus, extensor digitorum longus, and gastrocnemius muscles was found to be increased dramatically (Fig. 6). By comparison, the concentration of N-CAM in the proximal segment of the sciatic nerve was unchanged; the increase in the proportion of the M, 120,000 component of N-CAM in the severed nerve may reflect changes induced by the perturbation. Immunofluorescence staining experiments on cross-sections of gastrocnemius muscles confirmed that, after denervation, the amount of N-CAM present both intracellularly and at the myofiber membrane was increased (Fig. 7). The intensity of intracellular staining, however, varied among muscle fibers. In contrast, in whole mounts it was found that the amount of N-CAM staining present at motor endplates was decreased (Fig. 8). In a statistical study, only 14% of the denervated motor endplates retained detectable N-CAM staining whereas most of the normal contralateral motor endplates were double stained for AChR and N-CAM (Table III). It is interesting that after denervation of the muscle, the distributions of AChR and N-CAM changed in parallel in whole muscle; both molecules increased dramatically in extrajunctional regions of the muscle cell.

DISCUSSION

The major findings of this paper are that N-CAM is concentrated in motor endplates and that it is modulated in amount and distribution in muscle during development and after denervation. In the fetus, we found N-CAM both on the surface of developing myotubes and in clusters that co-localized with AChRs. During development, the pattern of N-CAM distribution on muscle became mostly restricted to the regions of the motor endplates. This redistribution of N-CAM was accompanied by a dramatic decrease in the relative prevalence of the molecule on the muscle surface. These findings, in conjunction with the known mechanism of cell-cell binding mediated by N-CAM (reviewed in reference 10), suggest several possible roles for N-CAM in the stabilization and maturation of nerve-muscle contacts and motor endplates. Before we discuss these possibilities further, we should consider the origin and structural characteristics of N-CAM in muscle.

As demonstrated previously for different neural regions (11, 23), N-CAM in mouse muscle was also found to undergo E-to-A conversion. Although both N-CAMs identified in neural and muscle tissues were found to be in polydisperse forms in embryos and in discrete forms in adults, the cell adhesion molecules differed in molecular form and amount at all times (Fig. 5). Moreover, N-CAM from skeletal muscle never showed a polypeptide component of M, 180,000. Based on peptide mapping experiments, N-CAMs from brain and mus-
expressed N-CAM on their surface (8, 9), and that denervated
guinea pig muscle showed detectable levels of N-CAM (b). Bar, 10 μm. × 1,030.

TABLE III. α-BgTx and Anti-N-CAM Stainings at Adult Control and Denervated Motor Endplates (MEPs)

|                | Double-stained MEPs | α-BgTx staining only | N-CAM staining only |
|----------------|---------------------|----------------------|---------------------|
| Control (n = 22) | 18 (82%)            | 0 (0%)               | 4 (18%)             |
| Denervated (n = 49) | 7 (14%)             | 38 (78%)             | 4 (8%)              |

n, number of MEPs examined.

cules were indistinguishable, but the molecules were distin-
guished by monoclonal antibody anti-N-CAM No. 5. Al-
though the conclusion that unique forms of N-CAM are
synthesized in the muscle remains to be proven, the findings
that trypsinized myoblasts fused in culture into myotubes that
expressed N-CAM on their surface (8, 9), and that denervated
muscle expressed increased quantities of the Mr 140,000
component of N-CAM, strongly suggest that the molecule
can be synthesized in muscle. Note, however, that the bio-
chemical analysis of N-CAM was performed on whole muscles and
therefore does not necessarily represent the N-CAM at the
motor endplate. The existence of biochemical difference
between N-CAM in extrajunctional and junctional regions
requires further investigation.

The complete lack of correspondence at motor endplates
between the distributions of Ng-CAM and AChR emphasizes
the significance of the finding of extensive morphological
similarities between the distributions of N-CAM and AChR.
When combined with the immunochemical observations that
muscle contains N-CAM antigenic determinants but not other
neuronal cell surface antigenic determinants, the anatomical
results indicate that N-CAM is concentrated at motor end-
plates. The precise ultrastructural localization and quantita-
tion of N-CAMs in muscle, on pre- and postsynaptic mem-
branes, and in basal lamina, will require detailed study at the
electron microscopic level.

Our finding that N-CAM is abundant during development
in the region of AChR clusters is consistent with the previous
conclusion, based on neuron–myotube interactions in culture
(8, 9), that N-CAM is involved in initial binding between
nerve and muscle. The key observation that N-CAM becomes
mostly restricted in its distribution during development to the
motor endplate on the surface of myofibers raises the addi-
tional possibility that N-CAM is functionally involved in
several stages of synaptogenesis. It is particularly interesting
that the temporal schedule of N-CAM modulation by modi-
fication in sialic acid content and prevalence (11) is concor-
dent with the schedule of synapse elimination and stabilization
at nerve–muscle contacts (6). This correlation is consistent
with the hypothesis that an increased rate of N-CAM binding
associated with E-to-A conversion may selectively help to
stabilize certain synapses while most others are eliminated.

Increases and changes in compartmentalization of N-CAM
and after section of the nerve innervating muscle occur at the
same time that AChR increases and acetylcholinesterase de-
creases (25). Thus, selective regulation of the distribution of
N-CAM occurs in response to denervation. N-CAM and
AChR may be regulated coordinately or may interact with
each other. In any event, the persistence of N-CAM at mature
motor endplates and the changes in N-CAM induced by
denervation suggest the possibility that N-CAM function is
also necessary for maintenance and reformation of mature
neuromuscular junctions.

The present observations and the fact that N-CAM is a
homophilic ligand whose rate of binding is faster after E-to-A
conversion (10, 24) suggest a number of potential functions
of N-CAM in muscle: (a) initial binding of motor and sensory
neurons to muscle cells early in fetal development; (b) inter-
actions with itself, with other cell-surface molecules (e.g., the
AChR), or with elements of basal lamina to organize AChRs
directly or indirectly; (c) selective strengthening of certain
cell–cell bonds as a result of E-to-A conversion during regres-
sion of polyneuronal innervation leading to synapse stabili-
zation, and (d) reformation of neuromuscular connections
after injury to the nerve. Determination of particular func-
tions for each of the different forms of N-CAMs will require
the application of specific immunological and genetic (26)
probes for individual molecular forms combined with assays
for various stages of nerve–muscle interaction.

We thank Dr. M. Pincon-Raymond for fruitful discussions and help
in denervation experiments, Ms. Monique Murawsky for help in
immunofluorescence experiments, and Ms. Erica Weineger for excel-
 lent technical assistance.

This work was supported by U. S. Public Health Service grants
HD-09635, HD-16550, AI-11378, and AM-04236, and by the Institut
National de la Santé et de la Recherche Médicale and the Ministère
de l’Industrie et de la Recherche.

Received for publication 21 February 1985, and in revised form 29
March 1985.

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