Motor Neurons Contain Agrin-like Molecules

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Abstract. Molecules antigenically similar to agrin, a protein extracted from the electric organ of *Torpedo californica*, are highly concentrated in the synaptic basal lamina of neuromuscular junctions in vertebrate skeletal muscle. On the basis of several lines of evidence it has been proposed that agrin-like molecules mediate the nerve-induced formation of acetylcholine receptor (AChR) and acetylcholinesterase (AChE) aggregates on the surface of muscle fibers at developing and regenerating neuromuscular junctions and that they help maintain these postsynaptic specializations in the adult. Here we show that anti-agrin monoclonal antibodies selectively stain the cell bodies of motor neurons in embryos and adults, and that the stain is concentrated in the Golgi apparatus. We also present evidence that motor neurons in both embryos and adults contain molecules that cause the formation of AChR and AChE aggregates on cultured myotubes and that these AChR/AChE-aggregating molecules are antigenically similar to agrin. These findings are consistent with the hypothesis that agrin-like molecules are synthesized by motor neurons, and are released from their axon terminals to become incorporated into the synaptic basal lamina where they direct the formation of synapses during development and regeneration.

SYNAPTIC transmission at the neuromuscular junction is dependent on the high concentration of acetylcholine receptors (AChRs)[1] and acetylcholinesterase (AChE) localized to the synaptic region of the muscle fiber's surface. It is evident from several studies (reviewed in reference 7) that molecules provided by the motor neuron's axon terminals induce the muscle fiber to form these postsynaptic specializations in the embryo, to maintain them in the adult, and to form them again during reinnervation after nerve damage. Previous studies conducted in this laboratory have shown that the portion of the muscle fiber's basal lamina sheath that occupies the synaptic cleft at the neuromuscular junction directs the aggregation of AChRs and AChE on regenerating muscle fibers (1, 21). It seems likely that the basal lamina molecules that cause the aggregates to form in regenerating muscles are similar to those released from axon terminals that mediate the nerve-induced formation and maintenance of such specializations in normal and reinnervated muscles.

We have also demonstrated that basal lamina-containing extracts of the synapse-rich electric organ of *Torpedo californica* contain a protein, called agrin, that causes the formation of patches on cultured myotubes that have a high concentration of AChRs and AChE (11, 24, 34, 35). Similar molecules have been extracted from muscle (11), but in much lower amounts, and several different monoclonal antibodies directed against agrin stain components of the synaptic cleft in muscles of *Torpedo*, frog, and chicken (9, 26). Moreover,

it has been shown for frog that the agrin-like molecules in the synaptic cleft are stably bound to the basal lamina (26). These findings have led to the hypothesis that agrin is similar to the AChR- and AChE-aggregating molecules in the synaptic basal lamina (26). If the AChR- and AChE-aggregating molecules in the synaptic basal lamina are derived from the motor neuron's axon terminals and such molecules are related to agrin, then one might expect to find agrin-like molecules in the cell bodies of motor neurons, where proteins are synthesized. Accordingly, in this study we set out to learn whether anti-agrin antibodies stain the cell bodies of motor neurons and whether preparations enriched in the cell bodies of motor neurons contain AChR- and AChE-aggregating molecules that can be immunoprecipitated with anti-agrin antibodies. A brief account of some of the experiments has appeared elsewhere (19, 31).

Materials and Methods

Tissue

For the staining experiments, we examined sections from all regions of the central nervous system (CNS) in neonatal (1 wk) *Torpedo californica*, adult frogs (*Rana pipiens*), and chick embryos (White Leghorn).

For extracts we used spinal cords of adult *Torpedos*, adult frogs, and chick embryos. We also examined the *Torpedo*'s electric lobe, which is the portion of the brain that contains the motor neurons that innervate the electric organ, and, in all species, the region of the CNS rostral to the pons (including the cerebellum), which contains few motor neurons compared to the electric lobe or spinal cord.

Staining

Light Microscopy. IMMUNOFLUORESCENCE: Frogs, *Torpedos*, and 18-d chick

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1. *Abbreviations used in this paper:* AChE, acetylcholinesterase; AChR, acetylcholine receptor; CNS, central nervous system.

embryos were perfused via the heart with 1% paraformaldehyde in Ringer's solution for 1 h (26). The brains and spinal cords were then removed and immersed in the same fixative for an additional 1-2 h. In other experiments, 5-18-d chick embryos were eviscerated and fixed by immersion in 5% acetic acid in ice-cold ethanol for 45-60 min. The ethanol-acetic acid fixation resulted in more intense staining with the anti-agrin mAbs but caused somewhat greater tissue distortion than aldehyde fixation. After fixation, the tissues were rinsed briefly in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM sodium phosphate, 1.5 mM potassium phosphate, pH 7.4), incubated overnight in 20% sucrose in PBS (4°C), and frozen. Sections (10 μm) were cut on a cryostat (Hacker Instruments, Inc., Fairfield, NJ), mounted on gelatin coated slides, stained with anti-agrin mAbs and fluorescein-labeled secondary antibodies as described in Reist et al. (26), and dehydrated in 95% ethanol at −20°C. Coverslips were mounted with Citifluor mountant medium AF1 (City University, London) and sections were viewed with a Zeiss photomicroscope equipped with epifluorescence optics.

All mAbs were routinely used as hybridoma conditioned medium diluted 1:1 in PBS containing 20% normal goat serum and 0.5% Triton X-100 (PBS-ST). To control for nonspecific staining some sections were treated with normal mouse serum (diluted 1:100), mAbs of the same subclass (hybridoma supernatant diluted 1:1) that did not immunoprecipitate agrin, or anti-agrin mAbs that did not stain the neuromuscular junctions of the species under investigation. The IgG concentration of all hybridoma supernatants ranged between 15 and 35 μg/ml (26).

· IMMUNOPEROXIDASE: The tissue was fixed, sectioned, mounted on slides, and incubated in the anti-agrin or control mAbs as described above. Sections were then rinsed for 1 h in PBS and incubated for 1 h in a 1:100 dilution of biotinylated horse anti-mouse IgG (ABC kit; Vector Laboratories, Inc., Burlingame, CA) in PBS-ST with 1% horse serum. After a 1-h wash in PBS the sections were incubated for 1 h in an avidin-biotin-horseradish peroxidase complex (ABC kit; Vector Laboratories, Inc.), rinsed 30 min each in PBS and 50 mM Tris-HCl (pH 7.4), and then incubated 5 min in Tris buffer containing 0.05% 3,3-diaminobenzidine. The reaction was begun by the addition of 0.03% H_2O_2 and was allowed to proceed for 30 min. The stained sections were rinsed in Tris buffer for 10 min, dehydrated through graded ethanols, and cleared in xylene. Coverslips were mounted with Permount (Fisher Scientific Co., Fair Lawn, NJ). Nonspecific staining was assessed as discussed above.

Electron Microscopy. Frogs were perfused 20 min with 50 ml 1% paraformaldehyde in 115 mM sodium phosphate buffer, pH 7.4. The spinal cord was removed and the brachial and lumbar enlargements were sliced with a razor blade into 3-mm-thick cross sections that were immersed in the same fixative for 3 h. The sections were processed for EM immunocytochemistry according to the procedure described by Brown and Farquhar (5), except that an ABC kit (Vector Laboratories, Inc.) was used for peroxidase staining. Nonspecific staining was assessed as discussed above.

Isolation of Embryonic Motor Neurons

Motor neurons were separated from other components of the chick spinal cord as described by Dohrmann et al. (8). The brachial and lumbar regions of spinal cords of 6-d embryos were removed and collected in Puck's saline (160 mM NaCl, 5 mM KCl, 10 mM sodium phosphate, pH 7.4) with 0.11% glucose. The tissue was incubated with 0.05% trypsin (Gibco Laboratories, Grand Island, NY) for 30 min and then placed in L15 medium (Gibco Laboratories) with 10% horse serum and 0.2% DNase (Sigma Chemical Co., St. Louis, MO). The spinal cords were gently triturated and the cells were pelleted, at 100 g, through a solution of 3.5% BSA (Fraction V, Sigma Chemical Co.) in L15. The pelleted cells were resuspended in 0.02% EDTA in PBS, layered over a 6.8% solution of metrizamide (Serva Fine Biochemicals, Inc., Garden City Park, NY) in L15, and spun at 450 g for 15 min. Cells were collected from the interface between the PBS and the metrizamide and from the bottom of the tube. Those at the interface were relatively large and phase-dark, characteristic of motor neurons (8), while most of those in the pellet were small and phase-bright.

Extracts

Spinal Cord and Brain. Tissues were homogenized in Puck's saline and the homogenates were centrifuged at 10,000 g for 20 min. After centrifugation of the supernatant at 300,000 g for 3 h, the high-speed and low-speed pellets were each extracted overnight at 4°C in 0.2 M bicarbonate buffer, 5% glycerol, 0.02% sodium azide, pH 9.0 and sonicated by placing the tubes in ice water in an ultrasonic cleaner (Sonicor Instrument Corp., Copiague, NY) for 15 min. They were then spun at 300,000 g for 3 h, and the supernatants were collected and stored frozen (−40°C). Extracts of both high-

and low-speed pellets contained AChR-aggregating activity and were routinely combined for the assays.

Isolated Motor Neurons. Motor neuron-rich and motor neuron-poor fractions made from chick spinal cords were frozen (liquid nitrogen) in 0.2 M sodium bicarbonate, 5% glycerol, 0.02% sodium azide, pH 9.0. They were thawed and frozen two more times, sonicated on ice for 15 min, and centrifuged at 15,600 g for 20 min. The supernatant was assayed for AChR-aggregating activity.

Assays

AChR and AChE Aggregation. The assays for AChR- and AChE-aggregating activities are presented in detail in references 11 and 34. We routinely only assayed for AChR-aggregating activity. 4-7-d-old triplicate cultures of chick myotubes were exposed to test solutions for 12-18 h, incubated for 1 h at 37°C with 10^{-8} M rhodamine-α-bungarotoxin to label AChRs, rinsed with Puck's saline, fixed for 5 min at −20°C in 95% ethanol, covered by coverslips mounted with glycerol, and examined by fluorescence microscopy. The level of AChR-aggregating activity in a sample was determined by counting the mean number of AChR aggregates per microscope field. AChR-aggregating activity is expressed in units; 1 U of AChR-aggregating activity is the amount of material needed to achieve a half-maximal increase in the number of aggregates. The maximal response for each experiment was determined by applying a saturating amount of partially purified agrin to triplicate cultures.

In some cases cultures were treated with extracts and labeled with rhodamine-α-bungarotoxin as described above, fixed with 1% paraformaldehyde, and labeled with a mAb against chick AChE (27) followed by fluorescein-conjugated second antibody. Cultures were examined by fluorescence microscopy and the levels of AChE-aggregating activity were determined by the same method as for AChR-aggregating activity.

Protein. Protein concentration was determined by the method of Bradford (4), using BSA as the standard.

Immunoprecipitation

100 μl of hybridoma supernatant were mixed with 100- 200 μl (∼1 U of activity) of the extract to be tested for 1-2 h at 37°C. 50 μl of goat anti-mouse IgG–conjugated Sepharose beads (e.g., reference 11) were added and the suspension was mixed at room temperature for 2-3 h. The suspension was then spun at 1,000 g for 1 min to remove the beads, bearing both immune complexes and free mouse IgG, from the supernatant. The supernatant was added to chick myotube cultures and assayed for AChR- or AChE-aggregating activity. To assess nonspecific binding to the beads, we used normal mouse serum (100 μl of 1:100 dilution) or anti-agrin mAbs that did not stain neuromuscular junctions in the species from which the extracts were made (see Results).

Results

Anti-Agrin Antibodies Stain Motor Neurons

Figs. 1 and 2 show frozen cross sections from the electric lobe of the *Torpedo* brain and from the spinal cords of *Torpedo*, frog, and chick that were incubated with anti-agrin mAbs and processed for immunohistochemistry. In each case the motor neurons are clearly stained. As illustrated, such staining was observed in embryos, neonates, and adults. A search for staining in the lumbosacral region of the chick spinal cord at early developmental stages (Fig. 2) revealed that motor neurons stained as early as embryonic day 5, the time at which motor neurons in this region begin to form neuromuscular junctions (17).

We detected staining of motor neurons with our anti-agrin mAbs only in fixed tissue. This is in contrast to the staining of the synaptic basal lamina at the neuromuscular junction with the same mAbs, which can be detected either with or without fixation (26); the immunoreactive molecules may be more stably bound to the synaptic basal lamina than to components of the motor neuron cytoplasm, or the concentration
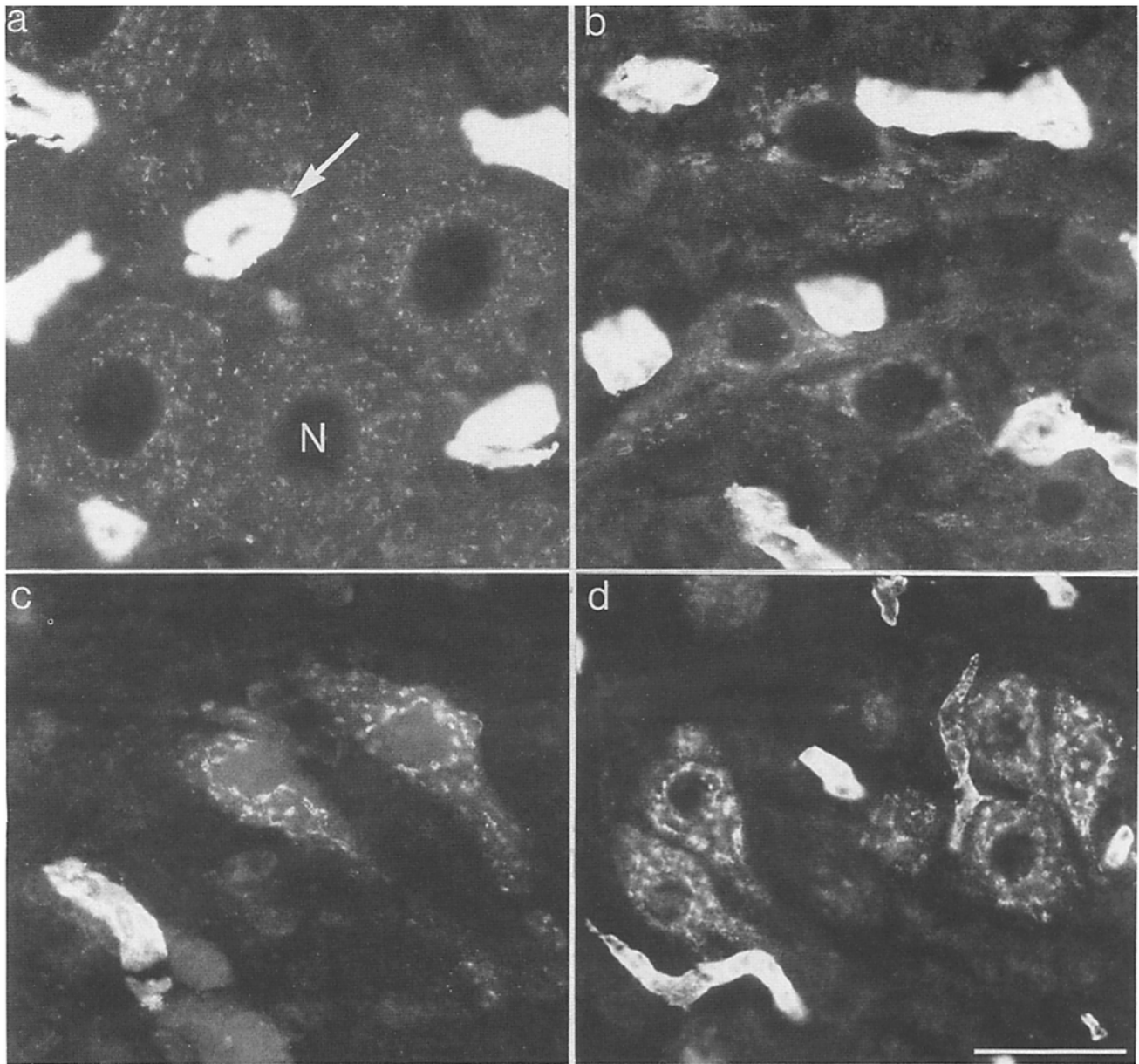
Figure 1. Anti-agrin antibodies stain the cell bodies of motor neurons and the capillaries in the CNS of *Torpedo*, frog, and chick. (*a*) Electric lobe of newborn (1 wk) *Torpedo californica*. (*b*) Ventral horn in the spinal cord of a newborn *Torpedo*. (*c*) Ventral horn in the spinal cord of an adult frog. (*d*) Ventral horn in the spinal cord of an 18-d chick embryo. *Torpedo* was stained with mAb 6D4, frog with a mixture of mAbs 3B5 and 5B1, and chick with mAb 5B1. Stain in motor neurons is distributed in patches in the cytoplasm. *N*, motor neuron nucleus; *arrow*, capillary. Bar, 50 μm.

of reactive molecules may be greater in the basal lamina than in cytoplasmic components.

Motor neurons were routinely identified by their position and large size. The identification was verified in frog spinal cord by severing the sciatic nerve, immersing the central stump for 4 h in rhodamine-conjugated wheat germ agglutinin (Vector Laboratories, Inc.) in 20% DMSO, and then allowing the marker to be transported retrogradely along motor axons to their cell bodies for 2 d. The rhodamine-labeled motor neurons stained with anti-agrin mAbs when processed for fluorescence immunohistochemistry.

Regardless of the age or species of animal, the stain in mo-

tor neurons, as observed by light microscopy, was concentrated in patches in the cytoplasm and excluded from the nucleus suggesting that it was associated with cytoplasmic organelles (Fig. 1 and Fig. 2, *inset*). Indeed, when we stained frozen sections of adult frog spinal cords with anti-agrin mAbs and processed the tissue for electron microscopy, we found that the stain was concentrated in the motor neuron's Golgi apparatus (Fig. 3).

We observed no neuronal staining with anti-agrin mAbs in regions of the brain and spinal cord that did not contain motor neurons (Fig. 2), nor did we observe stain in neurons of autonomic ganglia (frog) and dorsal root ganglia (frog and
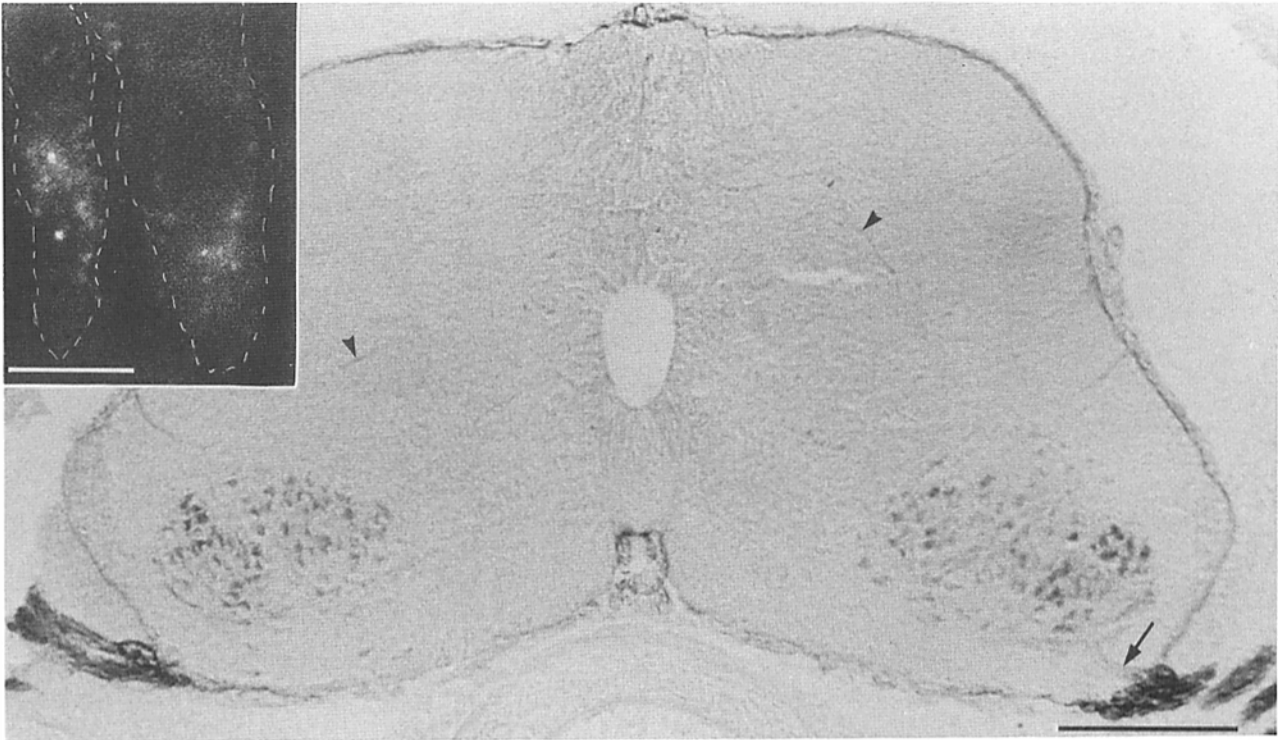
Figure 2. Selective staining of motor neurons and nonneural structures by anti-agrin mAbs. Cross section of the lumbosacral region of a spinal cord from a day 10 chick embryo incubated with mAb 5B1. Motor neurons and the pial surface of the spinal cord are intensely stained. Capillaries (arrowheads) are lightly stained; compare with intense staining of capillaries at a later stage of development in Fig. 1 d. Glial cells and other neurons are not stained. The intensely stained structures outside of the spinal cord are ventral roots; much of the stain is probably in the Schwann cell basal lamina, which is known to stain intensely in the adult (26). The lightly stained region (arrow) of the spinal cord extending from the right motor column to the right ventral root was observed at higher magnification to be composed of narrow cell processes having a nearly uniform diameter, probably motor axons. (Inset) Portions of two motor neurons labeled with mAb 5B1 in the lumbosacral region of the spinal cord of a 6-d chick embryo. Similar, though less intense, staining was seen in motor neurons of the lumbosacral region at embryonic day 5, the time at which motor neurons in this region begin to form neuromuscular junctions. Inset bar, 10 μm. Bar, 200 μm.

chick). On the other hand, capillaries throughout the CNS were outlined by the stain, as was the surface of the brain and spinal cord (Figs. 1 and 2). In chick embryos at early stages of development there was some staining associated with a group of fibers in the dorso-lateral region of the spinal cord

(not shown). Electron microscopy of the frog's spinal cord revealed that the stain associated with capillaries was concentrated in the basal lamina that lies between the capillaries and the endfoot processes of astrocytes (Fig. 4). Thus, in the CNS, molecules antigenically similar to agrin are not confined
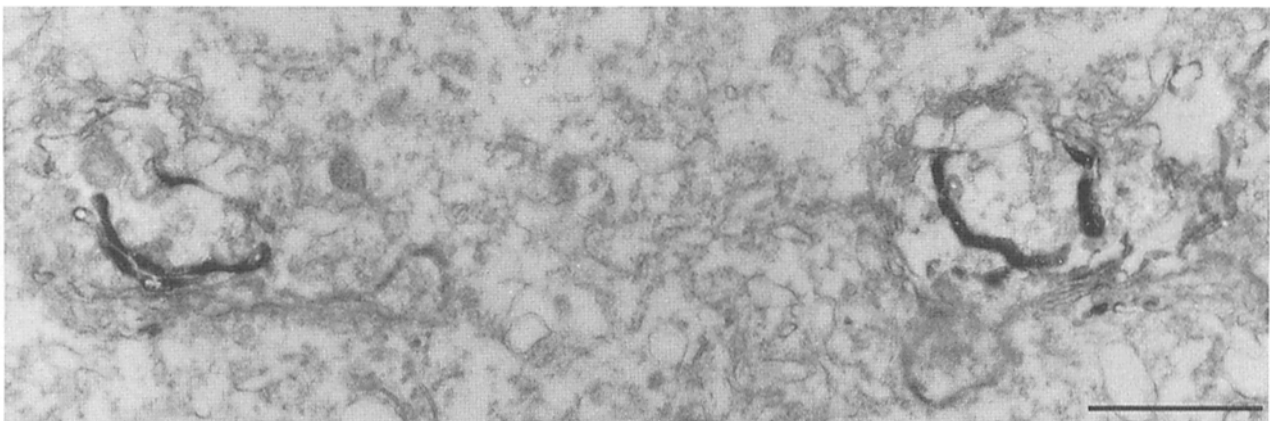

Figure 3. Anti-agrin mAbs stain stacks of flattened cisternae in motor neurons characteristic of the Golgi appartus. Two Golgi apparatus in the cytoplasm of a motor neuron in the ventral horn of a frog spinal cord treated with a mixture of mAbs 3B5 and 5B1. Bar, 1 μm.

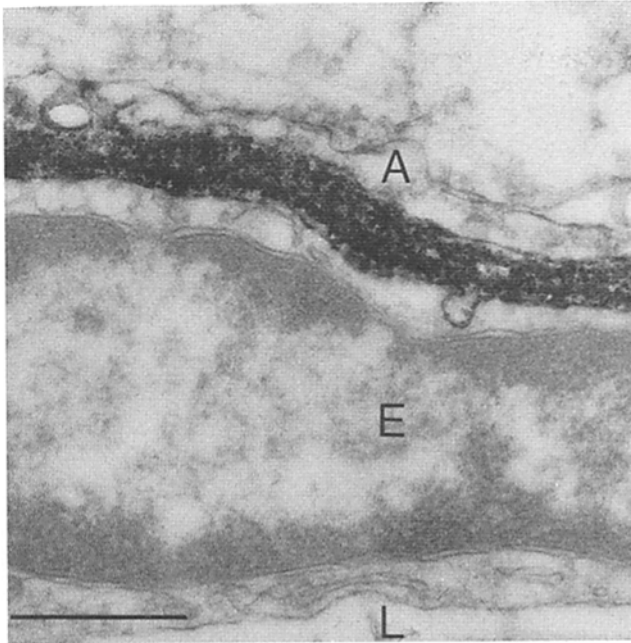Figure 4. Anti-agrin antibodies stain basal lamina between capillary endothelium (E) and endfoot processes of astrocytes (A). Frog spinal cord prepared as in Fig. 3. L, capillary lumen. Bar, 0.5 μm.

Figure 5. Extracts of motor neuron–rich regions of the CNS of *Torpedo*, frog, and chick contain AChR-aggregating activity. Similar activity is present in extracts of the region of brain containing few motor neurons (RBFM) in frog, which included cerebellum, optic lobes, mesencephalon, diencephalon, and cerebrum; little or no activity is present in the same portions from *Torpedo* and chick. The negative activity present in chick RBFM reflects the fact that cultures treated with these extracts had fewer AChR aggregates than control cultures. Data expressed as mean ± SEM; the number of observations is given within the bars.

to motor neurons, but motor neurons are distinct among neurons in that they have a high concentration of such molecules in their cell bodies.

We used three mAbs in the staining experiments, 6D4, 5B1, and 3B5, each against a different epitope on agrin (26). mAb 6D4, which stains neuromuscular junctions in *Torpedo* but not in frog and chick, stained motor neuron cell bodies and capillaries in *Torpedo* but not in the other two species. On the other hand both mAbs 5B1 and 3B5, which do stain neuromuscular junctions in frog and chick, also stained the cell bodies of motor neurons and capillaries in these species. Such findings would be expected if the agrin-like molecules
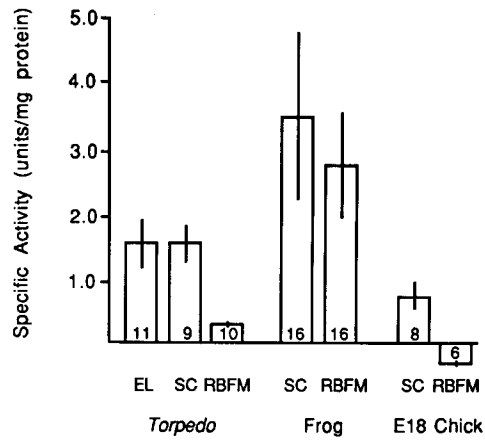
in motor neurons were identical or closely related to those at the neuromuscular junction.

Agrin-like AChR/AChE–aggregating Activity in Extracts of Spinal Cord and Brain

We made extracts of the electric lobes of adult *Torpedo* brains and the spinal cords of adult *Torpedos* and frogs and 18-d chick embryos. As shown in Figs. 5 and 6, these extracts caused the aggregation of AChRs on cultured chick myotubes. The extract from the electric lobe also caused AChE aggregation (Fig. 6 and Table I). The AChR and AChE patches had features characteristic of those induced by agrin (11, 34, 35): (*a*) they were similar in size and shape to those induced by agrin; (*b*) most were in the portion of the
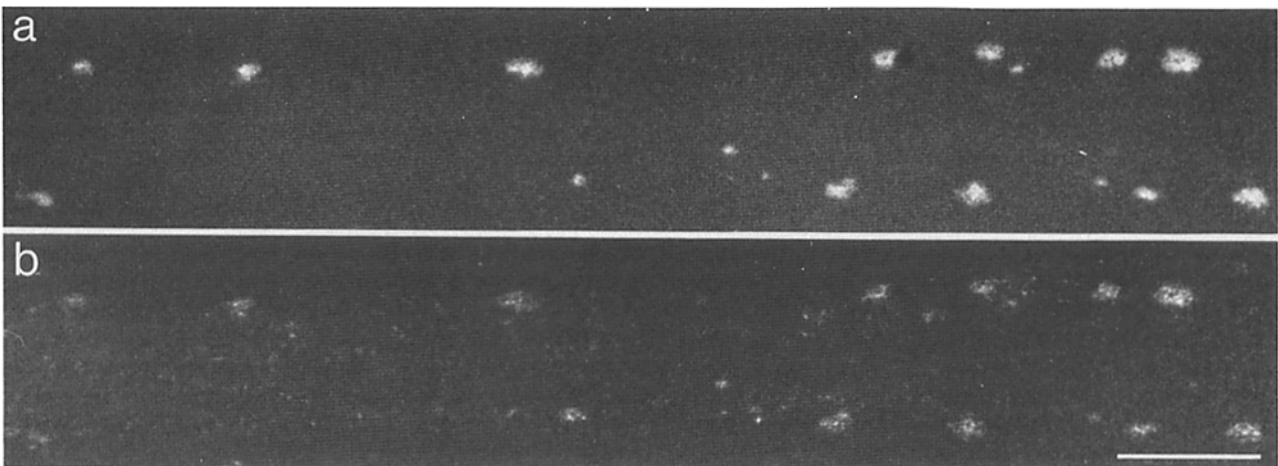

Figure 6. Aggregates of AChR and AChE induced to form on cultured chick myotubes by an extract of the electric lobe of *Torpedo* brain. *a* and *b* show the same myotube segment. (*a*) Rhodamine-α-bungarotoxin labels AChRs. (*b*) A mAb directed against chick AChE labels AChE. Bar, 30 μm.

Table I. Immunoprecipitation of AChR- and AChE-aggregating Activities

Extract	Antibody*	AChR-aggregating activity (in percent)‡	AChE-aggregating activity (in percent)‡
Torpedo			
Electric lobe	nms	100.1 ± 4.2 (38)	99.9 ± 11.7 (11)
	6D4	12.3 ± 3.7 (28)	21.5 ± 10.9 (12)
	3B5	4.3 ± 2.4 (10)	4.5 ± 11.8 (12)
	4B1	11.3 ± 1.9 (6)	
	5B1	6.8 ± 2.3 (6)	
	11D2	10.0 ± 1.7 (6)	
Electric organ§	6D4	−5.0 ± 2.0 (3)	
	3B5	5.0 ± 3.0 (16)	
	4B1	8.0 ± 3.0 (6)	
	5B1	9.0 ± 3.0 (8)	
	11D2	11.0 ± 3.0 (10)	
Spinal cord	nms	99.9 ± 4.7 (14)	
	6D4	13.1 ± 4.1 (14)	
Frog			
Spinal cord	6D4	100.1 ± 6.5 (9)	
	3B5	31.0 ± 7.3 (9)	
RBFM‖	6D4	100.0 ± 7.6 (12)	
	3B5	37.6 ± 6.9 (12)	
18-d embryonic chick			
Spinal cord	nms	113.7 ± 19.3 (6)	
	6D4	100.4 ± 8.9 (11)	
	5B1	33.2 ± 6.4 (12)	

* *nms*, normal mouse serum. All other antibodies are mAbs. Each mAb is against a different epitope on agrin and all stain *Torpedo* neuromuscular junctions. mAbs 3B5 and 5B1 also stain neuromuscular junctions in frog and/or chick (26).
‡ ± SEM. Number in parentheses is number of observations. Activity remaining in solution after immunoprecipitation expressed as percentage of control (normal mouse serum for *Torpedo*; mAb 6D4 for frog and chick).
§ from reference 26.
‖ *RBFM*, region of brain containing few motor neurons.

myofiber surface that faced the collagen substrate; and (c) the AChR patches in most cases were coextensive with the AChE patches (Fig. 6).

To determine whether the AChR- and AChE-aggregating activities in the electric lobe extracts were antigenically related to agrin, we assayed the ability of anti-agrin antibodies to immunoprecipitate the activities. For these experiments we used five mAbs, each of which is directed against a different epitope on agrin (26). As shown in Table I, each of the mAbs immunoprecipitated nearly all of the AChR-aggregating activity from the extracts of electric lobe of *Torpedo* brain as they did agrin from electric organ extracts. Two of these mAbs (the only ones tested) also immuno-precipitated electric lobe AChE-aggregating activity, as expected (Table I). Thus the AChR- and AChE-aggregating molecules in extracts of the electric lobe are antigenically similar to agrin.

Most of the AChR-aggregating activity in extracts of the spinal cords of *Torpedos*, frogs, and chicks was also immunoprecipitated by anti-agrin antibodies (Table I). Of the mAbs tested, only those that stained components of the synaptic cleft at the neuromuscular junction of frog and chicken immunoprecipitated the activity from the spinal cord extracts of those species. Thus extracts of the spinal cord also contain AChR-aggregating molecules which are antigenically related to agrin, and their immunoreactivity varies between species in a way similar to that of the agrin-like molecules that stain in the cell bodies of motor neurons and in the synaptic cleft of the neuromuscular junction.

Extracts were also made from portions of the chick, frog, and *Torpedo* brain that contain few motor neurons. In frog, such extracts contained nearly as much AChR-aggregating activity as those from the spinal cord, while in *Torpedo* they contained much less activity than extracts from spinal cord and electric lobe, and no activity was observed in such extracts from chick brain (Fig. 5). Activity in extracts of regions of the frog brain containing few motor neurons was immunoprecipitated by the anti-agrin mAbs (Table I). The amount of activity detected in the extracts from *Torpedo* was too low to determine whether or not it could be immunoprecipitated with anti-agrin antibodies (Fig. 5). However, since our initial accounts of agrin-like AChR-aggregating molecules in the CNS of *Torpedo* (19, 31), others have provided evidence suggesting that AChR-aggregating molecules in regions of *Torpedo* brain containing few motor neurons can be isolated by immunoaffinity purification techniques using anti-agrin mAbs (12). It may be that extracts of the regions of the brain containing few motor neurons from *Torpedo* and chick have more AChR-aggregating activity than we detect but that the activity is inhibited by other components of the extracts.

Agrin-like AChR-aggregating Activity in Extracts of Motor Neurons

To learn whether any of the AChR-aggregating activity detected in spinal cord extracts is derived from motor neurons, we separated motor neurons from other cellular components
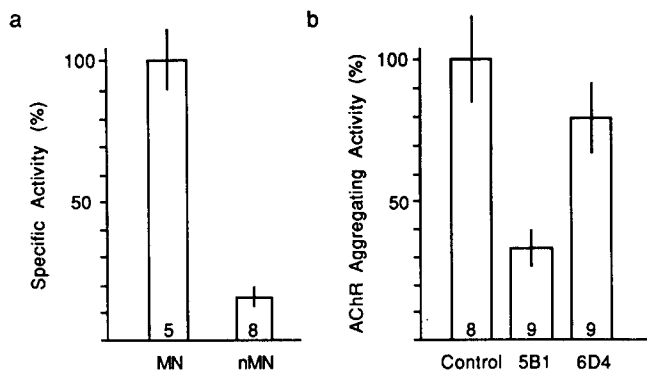
Figure 7. Preparations of dissociated chick spinal cord cells enriched in motor neurons are enriched in agrin-like AChR-aggregating activity. (*a*) The AChR-aggregating activity in extracts of motor neuron–enriched preparations (*MN*) have 7 times more AChR-aggregating activity than extracts of preparations enriched in nonmotor neurons (*nMN*). The specific activity of the nonmotor neuron preparation is plotted as a percentage of that of the motor neuron preparation. (*b*) The AChR-aggregating activity in an extract of the motor neuron–enriched preparation (*control*) is immunoprecipitated by anti-agrin mAb 5B1 which stains neuromuscular junctions in both chick and *Torpedo* ($P < 0.01$). Little or no activity is immunoprecipitated by mAb 6D4, which stains neuromuscular junctions in *Torpedo* but not chick ($P > 0.2$). Data is expressed as mean $\pm$ SEM; the number of observations is given within the bars.

in the spinal cord of 6-d chick embryos. The separation procedure results in two cellular fractions: one in which >95% of the cells are motor neurons and one which is enriched in nonmotor neurons (8). Extracts of each fraction were made and tested for AChR-aggregating activity. We found that the specific activity of the motor neuron–enriched fraction was sevenfold greater than that of the nonmotor neuron–enriched fraction (Fig. 7 *a*). Moreover, ∼60% of the activity in the motor neuron–enriched fraction was specifically immunoprecipitated by an anti-agrin antibody that stains chick neuromuscular junctions and motor neuron cell bodies (5B1; Fig. 7 *b*). Thus, motor neurons contain AChR-aggregating molecules antigenically related to agrin, and they are present at the time neuromuscular synapses are beginning to form.

Discussion

The neuromuscular junction is composed of the muscle fiber, the axon terminals of a motor neuron and the Schwann cells that cap the axon terminals. Any or all of these cells might produce the agrin-like molecules that are bound to the basal lamina in the synaptic cleft. Here we show that the cell bodies of motor neurons stain with anti-agrin mAbs and that the staining is concentrated in the Golgi apparatus, which processes proteins for secretion. We also provide evidence that motor neurons contain agrin-like AChR/AChE–aggregating molecules. Taken together, these findings support our hypothesis, as proposed by Nitkin et al. (24), that motor neurons synthesize agrin-like AChR/AChE–aggregating molecules, release them at their axon terminals to become incorporated into the basal lamina of the synaptic cleft, and that these molecules account for the synaptic basal lamina's ability to induce AChR and AChE aggregation on regenerating muscle fibers. Our evidence that such molecules are present in the cell bodies of motor neurons in embryos and normal

adults suggests further (24) that they also account for the motor neuron's ability to cause the formation of postsynaptic specializations on developing myofibers during embryogenesis and the maintenance of such specializations on mature muscle fibers.

This hypothesis does not exclude the possibility that some of the molecules in the synaptic basal lamina that stain with anti-agrin mAbs are provided by Schwann cells and muscle fibers. Indeed, our previous studies have demonstrated that the basal lamina sheaths of both the terminal Schwann cells and the extrajunctional regions of certain muscle fibers stain with anti-agrin mAbs (26), suggesting that both myofibers and Schwann cells produce molecules antigenically related to agrin. It seems unlikely such molecules would cause the aggregation of AChRs or AChE; neither AChRs nor AChE is aggregated on the surface of Schwann cells or in the extrajunctional region of those muscle fibers bound by basal lamina that is stained by anti-agrin antibodies (26). Even in our electric organ extracts anti-agrin mAbs recognize at least two polypeptides that do not cause AChR/AChE aggregation (24). Molecules antigenically related to agrin that are produced by muscle fibers might, for example, be those in the basal lamina known to cause the formation of active zones in regenerating axon terminals (28). Our hypothesis also does not rule out a role for other neuron-derived factors in the formation of the postsynaptic apparatus, such as regulating the levels of AChRs and AChE (see below and reference 24).

An alternative interpretation of our observation that motor neurons contain agrin-like molecules is that such molecules are produced and secreted by their target cells, the muscle fibers, and/or Schwann cells and are taken up by the motor neurons. Certain plant lectins that bind tightly to membrane glycoproteins have been shown to appear in the Golgi apparatus of neurons after internalization (for example 13). However, the only protein produced by Schwann cells and target cells and internalized by neurons that we know of, nerve growth factor, appears in several types of organelles within the neurons but not the Golgi apparatus (2, 6, 30).

It seems unlikely that the amount of agrin-like AChR-aggregating activity detected in portions of the frog and *Torpedo* brains that contained few motor neurons is due to the motor neurons themselves. The fraction of brain occupied by the oculomotor and trochlear nuclei, the only groups of motor neurons included in these portions, is negligible compared to the fraction of spinal cord occupied by the motor neuron columns or the amount of electric lobe occupied by the electromotor neurons. This raises the possibility that agrin-like molecules are made by neurons other than motor neurons and that they play a role in the formation and maintenance of postsynaptic specializations at neuron-to-neuron synapses; there is now good evidence that receptors at neuron-to-neuron synapses are aggregated in the postsynaptic membrane as at the neuromuscular junction (14, 15, 16, 18, 20, 33). We have not yet detected anti-agrin staining in either the cell bodies of nonmotor neurons or at neuron-to-neuron synapses. Our inability to see such staining may be owing to a lower concentration of agrin-like molecules in these structures than in the cell bodies of motor neurons and at neuromuscular junctions. Alternatively, agrin-like AChR-aggregating molecules in extracts of regions containing few motor neurons may be derived from the basal lamina be-

tween capillaries and endfoot processes of astrocytes, which stains with anti-agrin antibodies. These molecules might play a role in directing the aggregation of proteins in the cell membrane of the astrocyte endfeet, such as K^+ channels (23), or in capillary endothelial cells and resemble agrin closely enough to induce AChR-aggregation on myofibers in culture. We have already demonstrated that agrin-like molecules are highly concentrated at nodes of Ranvier along frog axons, sites of a high concentration of sodium channels in the axonal plasma membrane, and we have suggested that agrin-like molecules may function at many places throughout the body where plasma membrane proteins are aggregated (26). Indeed, molecules that are recognized by anti-agrin mAbs and cause aggregation of AChRs on cultured myotubes have been extracted from *Torpedo* heart and gut (12) as well as neural tissue and skeletal muscle.

Polypeptides that cause AChR aggregation on cultured myotubes have been identified in central nervous tissue by others. These include a 42-kD polypeptide (ARIA) extracted from chick brain (32) and calcitonin gene–related peptide (CGRP) that is 23 kD and is present in motor neurons (10, 22). As discussed in Nitkin et al. (24), the molecular mass of each clearly differs from that of agrin (agrin's two forms: 150 and 95 kD), they are much less effective in causing AChR aggregation than agrin, and unlike agrin, they cause a marked increase in AChR insertion into the plasma membrane of myotubes. Thus, these polypeptides are distinct from agrin and are very likely different from the agrin-like AChR-aggregating polypeptides in our CNS extracts. Our anti-agrin mAbs did not immunoprecipitate all of the AChR-aggregating activity from our motor neuron extracts; perhaps the residual activity was due to molecules such as ARIA and CGRP. On the other hand, the agrin-like AChR-aggregating molecules in our extracts may be similar to the as yet unidentified AChR-aggregating factors others have extracted from rat brain (25), cultured embryonic rat neurons (29), and neuronal cell lines from rat (3) that have apparent molecular masses of >50 kD. We are currently conducting studies aimed at generating anti-agrin antibodies that cross react with mammalian tissue components which will enable examination of such possibilities.

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