Agrin-related Molecules Are Concentrated at Acetylcholine Receptor Clusters in Normal and Aneural Developing Muscle

Justin R. Fallon and Cecilia E. Gelfman

Neurobiology Group, Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545

Abstract. Agrin induces the clustering of acetylcholine receptors (AchRs) and other postsynaptic components on the surface of cultured muscle cells. Molecules closely related if not identical to agrin are highly concentrated in the synaptic basal lamina, a structure known to play a key part in orchestrating synapse regeneration. Agrin or agrin-related molecules are thus likely to play a role in directing the differentiation of the postsynaptic apparatus at the regenerating neuromuscular junction. The present studies are aimed at understanding the role of agrin at developing synapses. We have used anti-agrin monoclonal antibodies combined with α-bungarotoxin labeling to establish the localization and time of appearance of agrin-related molecules in muscles of the chick hindlimb. Agrinlike immunoreactivity was observed in premuscle masses from as early as stage 23. AchR clusters were first detected late in stage 25, coincident with the entry of axons into the limb. At this and all subsequent stages examined, >95% of the AchR clusters colocalized with agrin-related molecules. This colocalization was also observed in unpermeabilized whole mount preparations, indicating that the agrin-related molecules were disposed on the external surface of the cells. Agrin-related molecules were also detected in regions of low AchR density on the muscle cell surface. To examine the role of innervation in the expression of agrin-related molecules, aneural limbs were generated by two methods. Examination of these limbs revealed that agrin-related molecules were expressed in the aneural muscle and they colocalized with AchR clusters. Thus, in developing muscle, agrin or a closely related molecule (*a*) is expressed before AchR clusters are detected; (*b*) is colocalized with the earliest AchR clusters formed; and (*c*) can be expressed in muscle and at sites of high AchR density independently of innervation. These results indicate that agrin or a related molecule is likely to play a role in synapse development and suggest that the muscle cell may be at least one source of this molecule.

THE neuromuscular junction is comprised of a precisely localized array of molecular and morphological specializations. For example, the postsynaptic apparatus is characterized by high concentrations of acetylcholine receptor (AchR)[1] and acetylcholinesterase, as well as by distinctive junctional folds (for review, see Dennis, 1981; Salpeter, 1987). While much is understood about the role of these characteristic features in the function of the mature synapse, relatively little is known about the molecular mechanisms that direct the formation of this array during development and regeneration.

One approach to understanding how the neuromuscular junction is formed has been to study synapse differentiation in regenerating muscle. In this system, it has been demonstrated that information associated with the synaptic basal lamina can direct the differentiation of the postsynaptic apparatus (Burden et al., 1979; McMahan and Slater, 1984; Anglister and McMahan, 1985). Recent experiments have provided evidence that agrin, an extracellular matrix protein derived from the synapse-rich *Torpedo* electric organ, may represent at least one of the synaptic organizing molecules associated with the synaptic basal lamina. In vitro, agrin induces the formation of clusters containing several postsynaptic components including AchR and acetylcholinesterase (Wallace, 1986; Nitkin et al., 1987). In vivo, monoclonal antibodies directed against agrin also recognize a molecule that is highly concentrated in the synaptic cleft (Fallon et al., 1985). Immunohistochemical studies have demonstrated that molecules closely related if not identical to agrin remain stably associated with the synaptic basal lamina in damaged adult muscles where the myofibers and the nerves had been removed (Reist et al., 1987). Monoclonal antibodies directed against agrin bind four closely related polypeptides from

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1. *Abbreviations used in this paper*: AchR, acetylcholine receptor; MHC, myosin heavy chain; NF, neurofilament; PBST, PBS with 0.1% Triton X-100; PITB, posterior iliotibialis.

Torpedo electric organ, at least two of which possess AchR/acetylcholinesterase-aggregating activity (Nitkin et al., 1987; Godfrey et al., 1988). Agrin-related molecules have also been extracted from *Torpedo* muscle (Fallon et al., 1985). Taken together, these immunological, biochemical, morphological, and functional studies strongly suggest that agrin, or a closely related molecule, directs the differentiation of major elements of the postsynaptic apparatus at the regenerating neuromuscular junction.

The goal of the present study is to investigate the role that agrin plays in the development of the neuromuscular junction. Using monoclonal antibodies raised against *Torpedo* agrin, we have investigated the time of appearance and localization of agrin-related molecules in developing chick muscle. We find that agrin-related molecules are expressed before the first AchR clusters are detected and that, subsequently, these molecules are colocalized with >95% of AchR clusters at all times in development. We also demonstrate that agrin-related molecules are colocalized with AchR clusters on muscle cells which have never been innervated.

Materials and Methods

Experimental Animals

White Leghorn embryos (Spafas, Inc., Norwich, CT) were incubated at 39°C in a humidified forced-draught incubator. All embryos were staged according to the criteria of Hamburger and Hamilton (1951). Days in ovo are noted for reference purposes and relate to this staging paradigm, not to the actual incubation periods.

Antibodies

The antibodies used in this study have all been previously characterized. Monoclonal anti-*Torpedo* agrin antibodies 5B1 and 11D2 show identical immunohistochemical and immunochemical profiles, but recognize distinct epitopes on the agrin molecule (Nitkin et al., 1987; Reist et al., 1987). Both antibodies were used in all aspects of this study. Monoclonal antibodies C2, directed against neurofilaments (NFs; Tosney et al., 1986), and F59, specific for skeletal muscle fast myosin heavy chain (MHC; Crow and Stockdale, 1986) were generously provided by H. Tanaka (Gunma University School of Medicine, Maebashi, Japan) and F. Stockdale (Stanford University, Stanford, CA), respectively.

α-Bungarotoxin

Rhodamine-coupled α-bungarotoxin was prepared according to the method of Ravdin and Axelrod (1977) and used at a concentration of 5×10^{-8} M. The labeling observed with this conjugate could be completely blocked by preincubation with 10^{-7} M native α-bungarotoxin (a gift of D. Berg, the University of California, San Diego).

Immunohistochemistry

All studies were carried out on the chick thigh. For quantitative studies, two muscles were examined in detail, the predominantly fast posterior iliotibialis (PITB) and the mixed iliofibularis (McClennan, 1983; Crow and Stockdale, 1986).

Frozen Sections. Unfixed hindquarters or individual muscles (depending on the age of the embryo) were immersed in freezing isopentane and mounted in Tissue-Tek (Miles Laboratories Inc., Naperville, IL). Sections 4–6 μm in thickness were cut on a cryostat (Reichert Jung, Vienna) and collected on uncoated multiwell slides (Shandon Southern Instruments Inc., Sewickley, PA). The sections were then fixed for 5 min in 1% HCHO in PBS, rinsed, and incubated for 5 min in 10% normal goat serum in PBS with 0.1% Triton X-100 (PBST). After rinsing in PBST the sections were incubated overnight at 4°C in a humid chamber with undiluted hybridoma supernatant (5B1, 11D2), or supernatant diluted to 1:100 (anti-NF) or 1:20 (anti-myosin). Staining alternate sections with anti-NF and anti-fast MHC enabled the precise monitoring of the course of nerve outgrowth and the progress of muscle differentiation in the limb. All dilutions were made in 10% normal goat serum/PBST. Control sections were incubated either with an irrelevant IgG1 monoclonal or with normal mouse serum. The sections were then washed for 1 h in PBST, incubated for 3 h at room temperature in second layer containing fluorescein-conjugated goat anti-mouse IgG1 (Caltag Laboratories, South San Francisco, CA) or goat anti-mouse Ig (Cappel Laboratories, Malvern, PA) and rhodamine-coupled α-bungarotoxin (5×10^{-8} M), and then washed as above. Slides were mounted in glycerol/1,4-diazabicyclo-[2.2.2]octane to inhibit fluorescence fading (Johnson et al., 1982) and viewed on a Zeiss Axioplan microscope equipped for dual wavelength epifluorescence and phase optics. Photographs were taken on Kodak Tri-X film and processed at 400 ASA.

Whole Mounts. To selectively label determinants on the cell surface the posterior thigh was dissected down to the PITB. After removal of the connective tissue sheath, the muscle was fixed for 5 min in 1% HCHO, rinsed in PBS, and incubated for 3 h to overnight in the primary antibody. The tissue was then washed for 1 h in three changes of PBS and incubated for 3 h in second layer as above (except that detergent was omitted from the buffer). After washing, the muscle was cut from its origin and insertion and individual fibers were teased out with tungsten needles on egg white–subbed slides. Fibers 0.5–1 mm long could be routinely isolated from stage 34 embryos using this method. The fibers were mounted and examined as above. In some experiments, the tissue was fixed only after the primary incubation and wash. The results were indistinguishable from those obtained with prefixed tissue.

Colocalization of AchR Clusters and Agrin-related Molecules

To assess the colocalization of agrin-related molecules at AchR clusters muscles were serially cryosectioned (stages 24–30) or sampled at 200-μm intervals (> stage 30) and adjacent sections labeled with monoclonal antibodies directed against agrin, anti-NF to localize nerve, or anti-MHC to assess muscle differentiation. All sections were counterstained with rhodamine-coupled α-bungarotoxin. We scored all the receptor clusters in sections taken throughout the thigh. The number of AchR clusters ≥2 μm in size in a given field were first counted using rhodamine optics with a 63× objective. The number that colocalized with agrin-related molecules was then determined by viewing the same section with fluorescein optics.

Preparation of Aneural Limbs

Spinal Cord Extirpation. This procedure was performed essentially as described by Lance-Jones and Landmesser (1980). Small windows were cut in the egg shell over the air sac of stage 17–18 embryos. At this stage, motor neuron axons have not left the spinal cord (Tosney and Landmesser, 1985). The amnion and chorion were cut, and the embryo was stained with neutral red. Sharpened tungsten needles were then used to free and remove the spinal cord from the low-thoracic through to the sacral level. Fragments of remaining cord were suctioned out using a broken off glass microelectrode. This procedure resulted in the deletion of the entire lumbosacral cord. The eggs were sealed with cellophane tape and returned to the incubator.

4–7 d after surgery, embryos with normal external thigh morphology were pinned out on sylgard dishes. The spinal cord and plexus region was exposed and viewed under a dissecting microscope. Embryos with no visible nerves entering the thigh were frozen as described above and the entire thigh was serially sectioned. Sections from intervals ≤100 μm throughout the thigh were stained with anti-NF antibody to verify the absence of nerve. 50 embryos with normal thigh morphology were generated. Of these, 20 had no grossly visible nerves entering the limb and these were serially sectioned. Three of these embryos proved to have completely aneural thighs as judged by anti-NF staining.

Limb Grafts. A second method for producing aneural limbs took advantage of the technique of grafting embryonic rudiments on to the chorioallantoic membrane. Motor axons do not enter the limb bud until stage 25 (Tosney and Landmesser, 1985; and see Results). Hind limb buds were cut off stage 18–23 embryos and were grafted onto the chorioallantoic membrane of 8–10 d host embryos. After 5–7 d, the grafts were recovered and sectioned as described above. All 20 muscle-containing grafts produced in this manner proved to be aneural. Colocalization of agrin-related molecules and AchR clusters was determined as described above except that counts were taken from photographs.

Results

Normal Muscles

The Expression of Agrin-related Molecules at the Initial Postsynaptic Specializations in Developing Muscle. We first sought to establish the relationship between the timing of the expression of agrin-related molecules and the development of the postsynaptic apparatus. The formation of AchR clusters was taken to indicate the onset of postsynaptic differentiation. To detect reliably the earliest events in AchR aggregation, we serially cryosectioned the thighs of embryos at each stage between 24 and 30 (days 4.5–7) and double-labeled them with antibodies directed against agrin and α-bungarotoxin. AchR clusters were seen in three out of five embryos examined at stage 25 (day 4.5–5) and were observed in all embryos by stage 26 (day 5). As illustrated in Fig. 1, *c* and *d*, agrin-related molecules were concentrated at these sites of high AchR density from the first time that these postsynaptic specializations could be detected. Further, agrin-related molecules were colocalized with >95% of the AchR clusters (see Fig. 3). This high degree of colocalization was observed in sections taken along the entire proximo-distal axis of the thigh in each of the embryos examined. Thus agrin-related molecules are present at sites of postsynaptic differentiation from the onset of neuromuscular junction formation.

The observation that agrin-related molecules were colocalized with the first AchR clusters formed prompted us to examine whether the expression of these molecules preceded the appearance of postsynaptic specializations. As shown in Fig. 1, *a* and *b*, agrin-related molecules were present in the muscle masses at stage 24, before AchR clusters are observed. Agrin immunoreactivity could be detected as early as stage 23 (day 4; see below). In these early limbs, staining was restricted to the premuscle or muscle mass and the basal lamina of the skin epithelium. The intervening mesoderm was not labeled.

Agrin-related Molecules Are Highly Concentrated at Postsynaptic Specializations throughout Development of the Neuromuscular Junction. Although stage 25 marks the onset of postsynaptic differentiation, the development of the nerve–muscle synapse is not complete until several weeks later (Burden, 1977*b*; Jacob and Lentz, 1979; Smith and Slater, 1983). We therefore examined the colocalization of agrin-related molecules at junctions from stage 25 to 5 wk

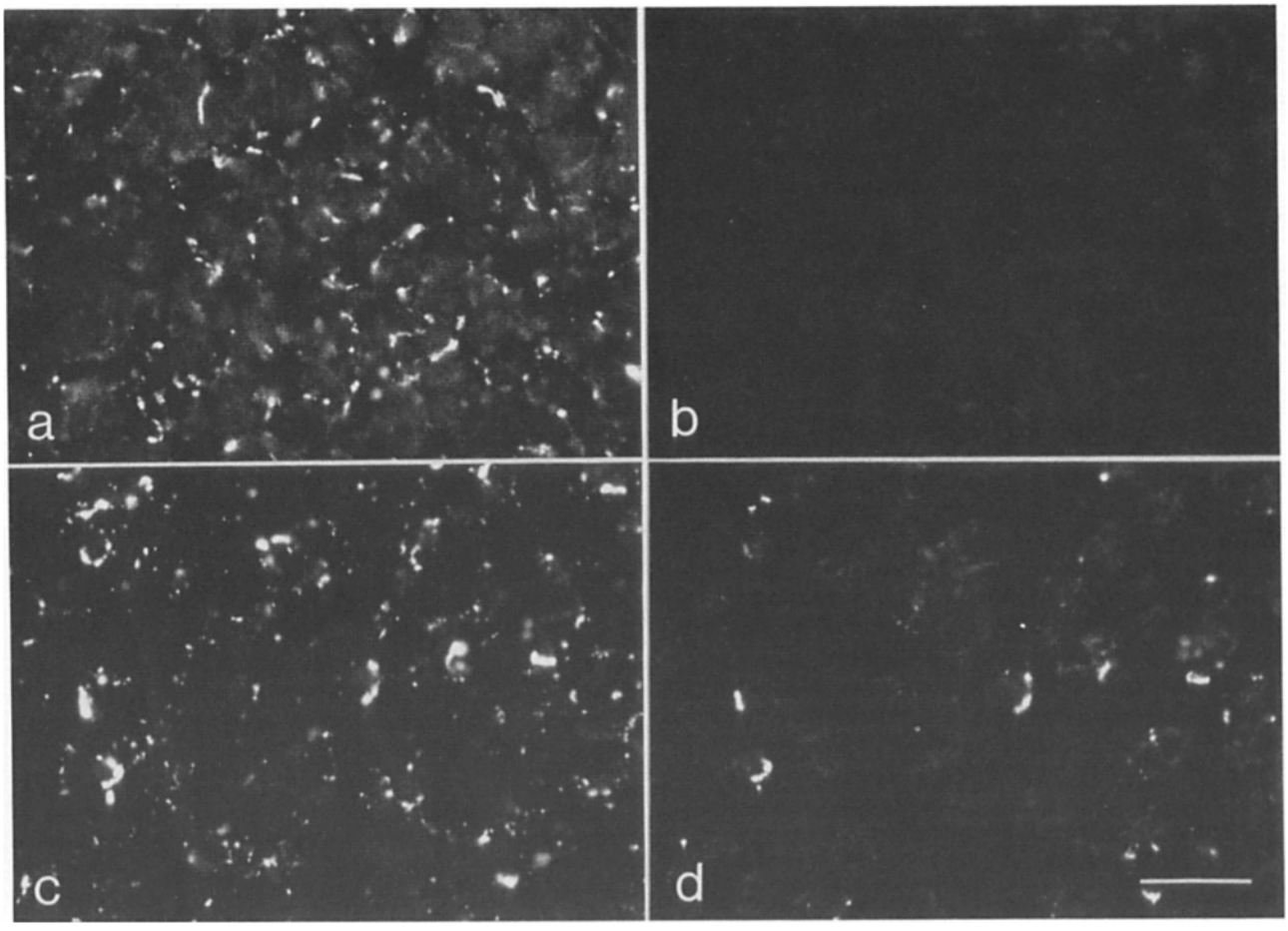

Figure 1. The expression of agrin-related molecules precedes the appearance of AchR clusters in developing muscle and is localized at the earliest-forming AchR clusters. Frozen sections of stage 24 (*a* and *b*) and stage 25 (*c* and *d*) dorsal muscle masses double-labeled with anti-agrin monoclonal 5B1 followed by a fluorescein-conjugated anti-mouse IgG and rhodamine-coupled α-bungarotoxin. The distribution of agrin-related immunoreactivity is shown in sections viewed with fluorescein optics (*a* and *c*), and the localization of AchR clusters is seen in the same sections viewed with rhodamine optics (*b* and *d*). Serial sectioning of three stage 24 hindlimbs confirmed that AchR clusters were not present at this time. The clusters seen at stage 25 are <12 h old and represent the earliest forming postsynaptic specializations in the developing thigh. Bar, 20 μm.

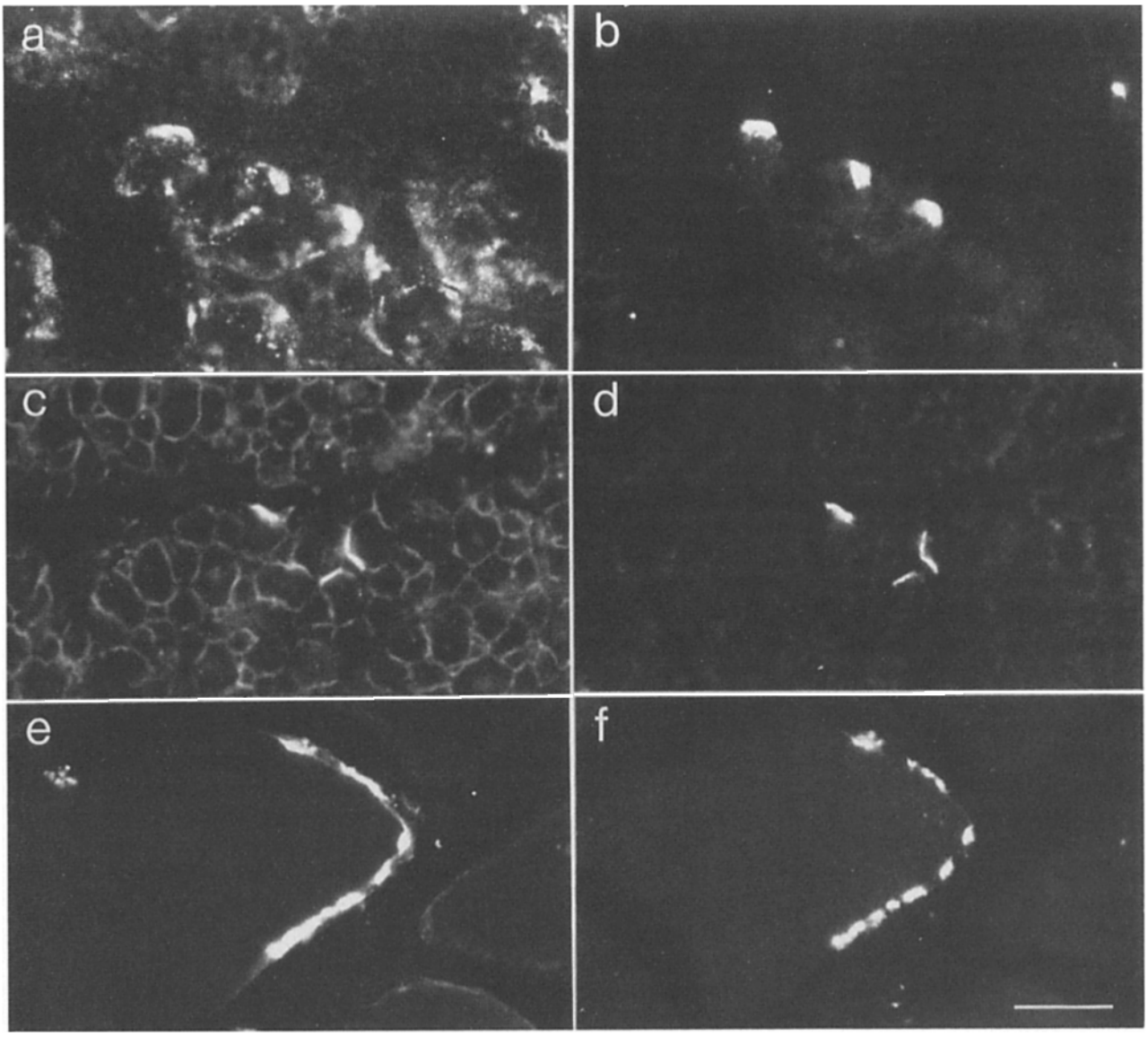

Figure 2. Agrin-related molecules are concentrated at regions of high AchR density throughout development. Frozen sections of stage 35 (*a* and *b*), stage 43 (*c* and *d*), and adult (*e* and *f*) PITB muscle double labeled with anti-agrin antibody 5B1 (*a*, *c*, and *e*) and α-bungarotoxin (*b*, *d*, and *f*) as described for Fig. 1. Note that while agrin-related molecules are concentrated at AchR clusters/postsynaptic densities at all times in development, agrin-related molecules are also present at regions of low AchR density. This expression peaks during the third week of development when the entire muscle cell surface is labeled (*c*). Bar, 20 μm.

after hatching. Fig. 2 shows that agrin-related molecules are highly concentrated at postsynaptic specializations throughout development. To quantitate the extent of colocalization of agrin-related molecules at AChR clusters, sections from throughout the limb were scored as described in Materials and Methods. As shown in Fig. 3, at all times in the development of the synapse >95% of the AchR clusters colocalized with agrin-related molecules.

Agrin-related Molecules at Regions of Low AchR Density. In addition to their localization at AchR clusters, agrin-related molecules are also found at regions of low AchR density on the muscle cell surface. Unlike the consistent expression of agrin-related molecules at sites of high AChR density, this latter distribution varies markedly during development. First, as noted above, labeling with anti-agrin monoclonal antibodies is seen throughout the premuscle mass before AchR clusters are detected (Fig. 1). When clusters first form at stage 25, agrin-related molecules are also seen at regions of low AchR density. At early stages, this labeling is patchy and punctate; subsequently the extent of labeling at regions of low AchR density increases such that by stage 39 (13 d) it completely surrounds the muscle cell. An example of such staining is shown in Fig. 2, *c* and *d*. While all muscles examined showed qualitatively the same behavior in this regard, the muscles containing slow fibers —the iliofibularis and medial adductor—displayed higher levels of labeling than did the fast muscles such as the lateral adductor and the PITB. The level of extrajunctional labeling peaks in the last week before hatching and subsequently declines until at ~5 wk after hatching when the adult configuration is attained (Fig. 2, *e* and *f*).

Agrin-related Molecules Are Expressed on the Surface

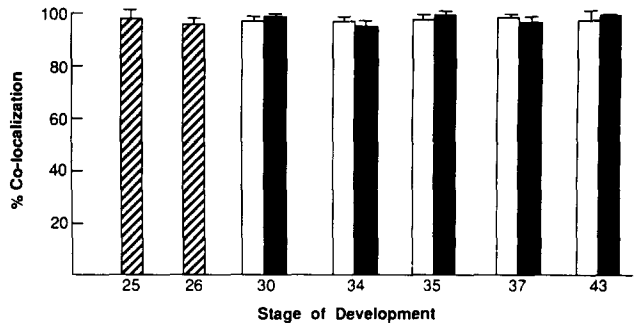

Figure 3. Colocalization of AchR clusters and agrin-related molecules during development of the neuromuscular junction. Cryostat sections of chick thigh at the stages noted were double labeled with monoclonal antibody directed against *Torpedo* agrin and rhodamine-coupled α-bungarotoxin. All the AchR clusters >2 μm in size were counted in 10–20 sections taken at equally spaced intervals along the proximo-distal axis in the iliofibularis (*solid bars*), the PITB (*open bars*), or muscle masses (*shaded bars*), and the number that colocalized with agrin-related molecules was determined. The total number of clusters scored ranged from 100 in stage 25 embryos, the time at which receptor aggregates were first detected, to over 1,000 in older limbs. In all cases >95% of the AchR clusters colocalized with agrin-related molecules.

of Developing Muscle Cells and Are in Register with AchR Clusters. The distribution of agrin-related molecules on the surface of an isolated stage 34 muscle cell is illustrated in Fig. 4. This cell, which had been labeled as part of an unpermeabilized whole mount, demonstrates the precise colocalization of the AchR cluster and the agrin-related molecules. The labeling at regions of low AchR density is also evident. Since the plasma membrane was intact during the staining period, this preparation also establishes that the agrin-related molecules are located on the surface of the muscle cell. Similar results were seen when stage 28 and 30 muscle cells were stained in an analogous fashion (not shown).

The Expression of Agrin-Related Molecules Precedes Muscle Differentiation and the Growth of Axons into the Limb. We next sought to establish the relationship between the expression of agrin-related molecules and the differentiation of muscle and nerve in the developing thigh. We used an antibody directed against skeletal muscle fast MHC to detect muscle differentiation (Crow and Stockdale, 1986) and an anti-NF antibody (Tosney et al., 1986) to reveal the progression of axons through the thigh. The results of serial section analysis of limbs from stage 23–26 are presented in Table I. The dorsal and ventral premuscle masses could be clearly distinguished at stage 23. Muscle differentiation was first observed in stage 24 embryos. At this stage, myotubes were found scattered throughout the muscle mass. Axons first left the plexus region and invaded the thigh at stage 25. At this age, the nerves were restricted to the edge of the muscle mass and did not invade between the developing muscle fibers. Agrin-related molecules could be detected in premuscle masses as early as stage 23. Thus, in the normal thigh, the appearance of agrin-related molecules in the developing muscle precedes the differentiation of muscle, the arrival of the axons, and the onset of AchR cluster formation.

Aneural Muscles

The experiments described above established that agrin-related molecules are detected before the growth of the nerve into the limb bud and the clustering of AchR. These results suggested that the target tissue, muscle, synthesizes agrin-related molecules. As discussed above, AchR clusters are first detected at the same time that axons invade the limb. Therefore, the normal developmental pathway did not allow us to determine if the expression of agrin-related molecules at AchR clusters is dependent upon the presence of nerve. To further investigate this question and the relationship between innervation and the expression of agrin-related molecules on the muscle cell surface, aneural limbs were generated by two methods as described in Materials and Methods. To verify that the limbs were aneural and contained differentiated muscle, we labeled sections taken from regions throughout the limb with anti-NF and anti-fast MHC.

Table I. Early Events in Neuromuscular Junction Differentiation in the Chick Thigh

	Stage 23	24	25	26
Agrin-related molecules	+	+	+	+
Muscle differentiation	−	+	+	+
Innervation	−	−	+	+
AchR Clusters	−	−	+ (late)	+

Appearance of agrin-related molecules, AchR clusters, and innervation were determined by examination of serial sections of the entire chick thigh at the indicated stages as described in Materials and Methods. Muscle differentiation was assessed using a monoclonal anti-fast MHC antibody and the progress of nerve growth was monitored using an anti-NF antibody. Stages 23–26 span ~ day 4–5 of development.

We first prepared aneural muscle by spinal cord extirpation at stage 17, before motoneuron axons have left the spinal cord (Tosney and Landmesser, 1985). The embryos were then allowed to develop for 5–7 d after surgery. Fig. 5 shows that agrin-related molecules are expressed in these aneural limbs and that they colocalize with AchR clusters. We examined aneural embryos at stages 28, 31, and 35. At all of these stages, the number of AchR clusters was estimated to be 1–5% of that seen in innervated limbs. Nonetheless, the colocalization of agrin-related molecules and AchR clusters was observed in all muscles examined. As was the case in the normal limbs, agrin-related molecules were also seen at regions of low AchR density (Fig. 5).

Aneural muscles generated by limb bud grafting showed a similar distribution of agrin-related molecules to that seen in the spinal cord extirpation animals. In addition, the grafting technique gave a much higher yield of aneural limbs and enabled us to quantitate the colocalization of agrin-related molecules with AchR clusters. We found that ~95% of the AchR clusters had agrin-related molecules associated with them (Table II). These results show that in the absence of nerve, muscle tissue can synthesize agrin-related molecules and can express them at AchR clusters.

Discussion

The results of this paper suggest that agrin or a closely related molecule is likely to be involved in mediating some of the earliest events in the development of the neuromuscular junction. Moreover, we find that agrin-related molecules can be expressed in muscle and at AchR clusters in the absence of nerve. These results will be discussed from the

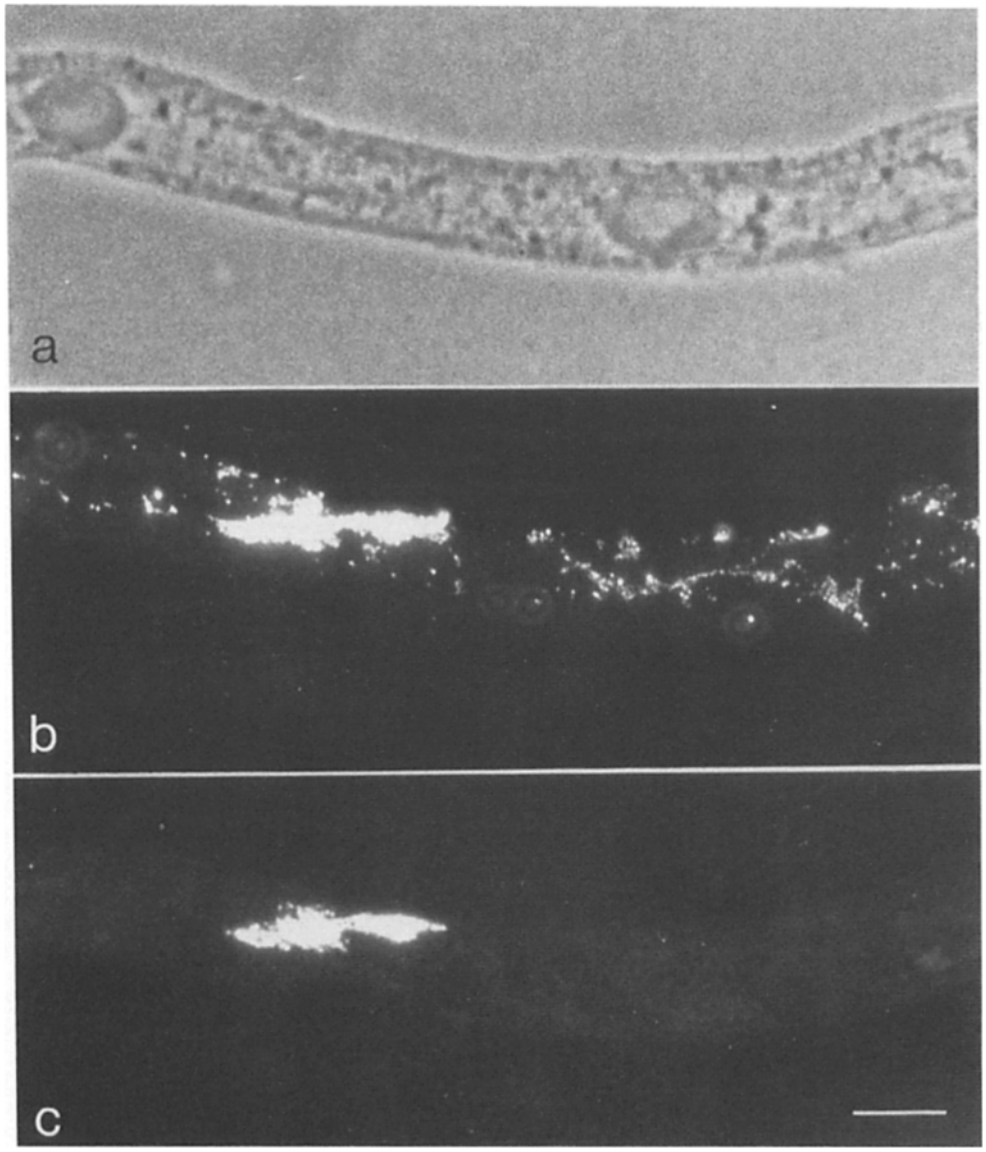

Figure 4. Agrin-related molecules are expressed at the myofiber surface and colocalize with the entire AchR cluster. In this preparation, a stage 34 PITB unpermeabilized whole mount was fixed with 1% formaldehyde and then incubated with monoclonal antibody directed against *Torpedo* agrin and rhodamine-coupled α-bungarotoxin. Individual muscle fibers were then teased out and viewed with phase (*a*), fluorescein (*b*), or rhodamine (*c*) optics to detect the antibody staining and toxin distribution, respectively. Since the plasma membrane in these cells was intact during staining, labeling is due to binding on the external surface of the cell. The agrin-related molecules are highly concentrated at the AchR clusters; in addition, labeling is also observed at regions of low AchR density. This distribution is consistent with that observed in frozen cross section (Fig. 2, *a* and *b*). Bar, 10 μm.

viewpoint of how agrin-related molecules fit into the normal development of the neuromuscular junction. In addition, we will address the question of the origin of the agrin-related molecules present at the neuromuscular junction.

Agrin and Agrin-Related Molecules

The nature of antigens revealed by the immunohistochemical staining is an important consideration in this study. Several lines of evidence indicate that the molecules recognized by the anti-*Torpedo* agrin monoclonal antibodies in chick muscle are closely related if not identical to agrin. First, the pattern of labeling in normal and aneural limbs was identical when two monoclonal antibodies, 5B1 and 11D2, recognizing distinct epitopes on the agrin molecule were used (Nitkin et al., 1987; Reist et al., 1987). Second, as is the case with *Torpedo* agrin (Fallon et al., 1985), the agrin-related molecules described here are expressed on the surface of the muscle cell and are concentrated at sites of high AchR density (Fig. 4). Third, these two antibodies are part of a library of thirteen monoclonal antibodies generated against *Torpedo* agrin. The twelve members of this panel that can be used for immunohistochemistry all show identical staining patterns on adult *Torpedo* muscle; moreover, this distribution is very similar to that seen in adult chicken muscle (Reist et al., 1987). Fourth, all the antibodies, including 5B1 and 11D2, immunoprecipitate the same polypeptides from electric organ preparations and bind the AchR-aggregating molecules from muscle extracts (Fallon et al., 1985; Nitkin et al., 1987; Reist et al., 1987). Finally, anti-agrin antibodies with similar

Table II. Localization of Agrin-related Molecules at AchR Clusters in Aneural Muscle

Graft No.	Donor stage*	Colocalization (± SEM)
		%
1	18	95.3 ± 5.9
2	21	96.7 ± 4.1
3	23	93.0 ± 3.2

* Grafts were analyzed 4–5 d after transplant. Colocalization was determined from micrographs of 10 sections from each graft. 30–50 AchR clusters were scored for each point.

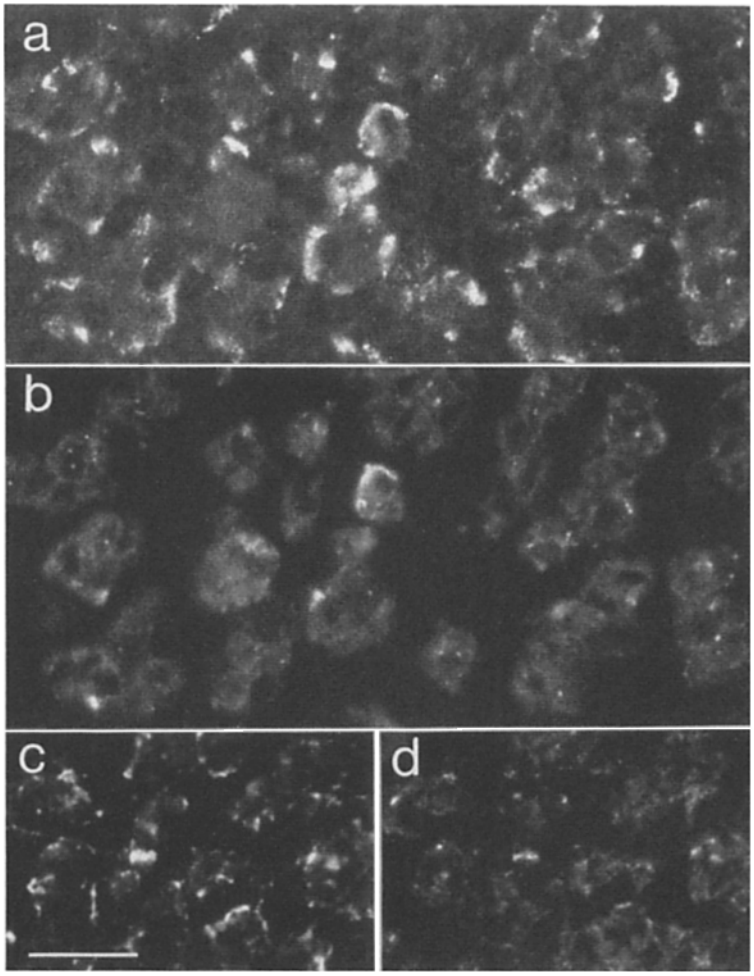

Figure 5. Agrin-related molecules are colocalized with AchR clusters in aneural muscles. (*a* and *b*) Frozen section of femorotibialis muscle from aneural stage 35 hindlimb prepared by extirpation of the spinal cord at stage 17. (*c* and *d*) Frozen section of aneural muscle from stage 31 limb bud graft. Sections were double labeled with anti-agrin antibodies (*a* and *c*) and α-bungarotoxin (*b* and *d*) as described for Fig. 1. Agrinlike immunoreactivity is seen both at AchR clusters as well as at regions of low AchR density. Bar, 20 μm.

specificities to those used here bind polypeptides from chick tissue in the same molecular weight range as *Torpedo* agrin (Godfrey et al., 1988).

Agrin-related Molecules at the Developing Neuromuscular Junction

Our results demonstrate that agrin-related molecules are present before the appearance of AchR clusters on the muscle cell surface. In addition, we find that from the onset of AchR aggregation, agrin-related molecules are colocalized with these specializations. We examined five stage 25 embryos by serial sectioning, and three were found to display AchR clusters. In no case did we observe <95% colocalization of agrin-related molecules at AchR clusters. Since stage 25 represents <12 h of embryonic development (Hamburger and Hamilton, 1951; and our unpublished observations), the clusters observed must be among the first formed in the developing limb. However, from our present data, we cannot determine if AchR clusters form at preexisting domains of agrin-related molecules on the muscle cell surface. We are currently approaching this question in tissue culture.

Stage 25–26 marks the inception of neuromuscular junction formation in the thigh as judged by (*a*) the entry of motoneuron axons into the limb (Table I; Tosney and Landmesser, 1985); (*b*) the first appearance of AchR clusters, and (*c*) the onset of nerve-evoked muscle contraction (Landmesser and Morris, 1975). At these early stages, the majority of the AchR clusters were detected within a 100-μm radius of the nerve trunks and branches. Thus, while the AchR clusters were evidently induced by the presence of nerve, a minority of the clusters appeared to be in contact with nerve processes as judged by anti-NF staining (our unpublished observations). Similar findings have been reported from the study of whole mount preparations of these same muscles (Dahm and Landmesser, 1988). It is therefore not clear what proportion of these early AchR clusters represent the postsynaptic apparatus. However, as discussed above, >95% of these AchR clusters do colocalize with agrin-related molecules.

Over the next several weeks of development, there are several major events in the development of both the muscle cell and the synapse including muscle mass cleavage (~stage 28–30; Tosney and Landmesser, 1985); motoneuron cell death (~stage 30–34; Pittman and Oppenheim, 1979); formation of secondary myotubes (~stage 35–40; Crow and Stockdale, 1986); synapse elimination (~stage 42–45; Pockett, 1981; Brown et al., 1976); and metabolic stabilization of AchR (3–5 wk after hatching; Burden, 1977*b*). At all times in development, we found that agrin-related molecules are localized at >95% of the AchR clusters/postsynaptic densities (Fig. 3). Similar findings have also been reported by Godfrey et al. (1988). These results suggest that agrin-related molecules are likely to be fundamental to the organization of the postsynaptic apparatus.

Agrin-related Molecules at Regions of Low AchR Density

While the expression of high levels of agrin-related molecules at developing postsynaptic specializations was expected in light of the distribution of this molecule in the adult, the substantial levels of agrin-related molecules detected at regions of low AchR density on the muscle cell surface was surprising. The role of the agrin-related molecules at sites of low AchR density is not known. It is of interest, however, that Burden (1977*a*) has shown that in the posterior latissimus dorsi muscle the density of the extrajunctional AchR reach peaks at day 16 and subsequently declines. In the present studies, we found that the peak expression of extrajunctional agrin-related molecules occurred late in the third week of development (16–20 d; Fig. 2). The development of the posterior latissimus dorsi precedes that of thigh muscles by at least 2 d. It is possible then that the expression of extrajunctional agrin-related molecules and AchR might be related. It is also of interest that an AchR-aggregating factor isolated from mammalian muscle is also expressed both junctionally and extrajunctionally (Barald et al., 1987).

Muscle-derived Agrin Is Localized at AchR Clusters

A major goal of this study was to determine the timing of agrin-related molecule expression as compared to innervation. The timing of innervation and muscle development established in this study confirmed the earlier work of Tosney and Landmesser (1985) and Crow and Stockdale (1986). The normal course of chick development, where the differentiation of muscle precedes the growth of axons into the hindlimb by at least one stage (Table I), allowed us to establish that the expression of agrin-related molecules in muscle is not dependent upon the presence of nerve. These results indicate that there is a muscle-derived form of agrin. To determine if this muscle-derived agrin could also be expressed at AchR clusters, we turned to aneural limb preparations. In these experiments, muscles were allowed to develop for several days in the complete absence of innervation. We found that agrin-related molecules were colocalized with the AchR clusters in aneural muscles prepared by two independent methods (Fig. 5). It will be of interest to examine the level of agrin-related molecules in paralyzed but innervated developing muscle to determine the relative contribution of the muscle activity and the presence of the nerve in regulating the expression of agrin-related molecules at the neuromuscular junction.

These aneural experiments suggest that muscle is likely to contribute a form of agrin to the basal lamina at the developing postsynaptic apparatus. These results also raise the possibility that the agrin-related molecules present at the mature neuromuscular junction are synthesized at least in part by the muscle cells. Moreover, in recent experiments we have found that muscle-derived agrin is present at nerve-induced AchR clusters in culture (Lieth et al., 1989). These results obviously do not preclude that the agrin-related molecules at the neuromuscular junction might also be derived from other cells that constitute the synapse, such as Schwann cells and motoneurons. Indeed, agrin-related molecules have been localized in the motoneuron cell body. It has been proposed that this neuron-derived agrin is secreted at the nerve terminal and induces the formation of the postsynaptic apparatus (Magill-Solc and McMahan, 1988). The present results raise the possibility that the agrin-related molecules present at the neuromuscular junction are derived from muscle as well as nerve. Given the close antigenic similarity between the agrin-related molecules localized in muscle and neurons, and the observation that AchR clustering activity is associated with the agrinlike molecules extracted from many tissue sources (Godfrey et al., 1988), it is reasonable to propose that this muscle-derived agrin will also prove to be important in the differentiation of the synapse. It is also possible that the presence of more than one form of agrin in the synaptic basal lamina reflects its functional diversity. For example, muscle-derived agrin could be involved not only in organizing AchR on the postsynaptic membrane, but also in directing the differentiation of the presynaptic apparatus (Sanes et al., 1978).

In summary, our results indicate that agrin is likely to play an important role in the development of the postsynaptic apparatus. Further, taken together with other findings, it is likely that the function, cellular origin, and regulation of the agrin-related molecules at the neuromuscular junction is complex. We are currently developing in vitro systems and more refined markers that will allow us to sort out the role of agrin in the organization of the developing and adult nerve–muscle synapse.

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