

Agrin-induced Reorganization of Extracellular Matrix Components on Cultured Myotubes: Relationship to AChR Aggregation

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Abstract. Agrin, an extracellular matrix-associated protein extracted from synapse-rich tissues, induces the accumulation of acetylcholine receptors (AChRs) and other synaptic components into discrete patches on cultured myotubes. The appearance of agrin-like molecules at neuromuscular junctions suggests that it may direct synaptic organization in vivo. In the present study we examined the role of extracellular matrix components in agrin-induced differentiation. We used immunohistochemical techniques to visualize the spatial and temporal distribution of laminin, a heparan sulfate proteoglycan (HSPG), fibronectin, and type IV collagen on cultured chick myotubes during agrin-induced aggregation of AChRs.

Myotubes displayed significant amounts of laminin and HSPG, lesser amounts of type IV collagen, and

little, if any, fibronectin. Agrin treatment caused cell surface laminin and HSPG to patch, while collagen and fibronectin distributions were generally unaffected. Many of the agrin-induced laminin and HSPG patches colocalized with AChR patches, raising the possibility of a causal relationship between matrix patching and AChR accumulations. However, patching of AChRs (complete within a few hours) preceded that of laminin or HSPG (not complete until 15–20 h), making it unlikely that matrix accumulations initiate AChR patching at agrin-induced sites. Conversely, when AChR patching was blocked by treatment with anti-AChR antibody mAb 35, agrin was still able to effect patching of laminin and HSPG. Taken together, these findings suggest that agrin-induced accumulations of AChR and laminin/HSPG are not mechanistically linked.

FUNCTIONAL transmission across the neuromuscular junction requires the precise localization of cell surface, cytoskeletal, and extracellular components at the point of nerve–muscle contact (reviewed by Peng, 1987; Schuetze and Role, 1987; Steinbach and Bloch, 1986; Rubin and Barald, 1983; Dennis, 1981; Fambrough, 1979). It is quite remarkable that these components accumulate in such a discrete region, which represents only ~0.1% of the myofiber surface. Acetylcholine receptors (AChRs)¹, for example, are packed into the synaptic membrane at densities approaching 15–20,000 per square micron, but are virtually absent elsewhere on the myofiber surface (reviewed in Salpeter and Loring, 1985). To understand the mechanisms involved in the formation and maintenance of synaptic structure, we have treated cultured myotubes with agrin, a synaptic-organizing molecule that appears to be related to factors that function at synapses in vivo.

Studies by McMahan and colleagues demonstrated that in vivo matrix-associated factors can play a significant role in the regeneration of neuromuscular structure (Sanes et al.,

1978; Burden et al., 1979; McMahan and Slater, 1984; Anglister and McMahan, 1985; see also Bader, 1981). This led to the screening of matrix-enriched fractions of *Torpedo* electric organ for factors that affect AChR distribution (Rubin and McMahan, 1982; Nitkin et al., 1983; Godfrey et al., 1984). In this manner a proteinaceous factor, termed agrin, was identified and characterized (Nitkin et al., 1987). Agrin induces on cultured myotubes dense accumulations of AChRs, which are also associated with acetylcholinesterase (Wallace et al., 1985; Wallace, 1986) and other synaptic components (Wallace, 1989). mAbs against the active component recognize a related series of polypeptides with molecular masses of 70, 95, 135, and 150 kD (Nitkin et al., 1987; see also Godfrey et al., 1988a). These mAbs were also used to demonstrate that agrin-like molecules are concentrated at neuromuscular junctions in vivo (Fallon et al., 1985; Reist et al., 1987; Godfrey et al., 1988a). Therefore, not only is agrin a useful means of inducing “synaptic” differentiation on cultured myotubes, but it appears to be related to factors that direct synaptic development in vivo.

Several observations suggest that agrin-induced AChR patching occurs through a specific, physiological cellular mechanism. Agrin effects myotubes in a dose-dependent manner, although at higher agrin levels the number of AChR patches per myotube plateaus off (Godfrey et al., 1984; Wallace, 1989), suggesting that AChR patching may be limited

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1. *Abbreviations used in this paper:* AChR, acetylcholine receptor; HSPG, heparan sulfate proteoglycan.

by cellular constraints. The accumulation of AChRs into discrete, high density patches takes a few hours (Godfrey et al., 1984), which argues against a mere cross-linking of AChRs by agrin (by contrast, antibodies and other multivalent ligands that induce reorganization of surface components operate within minutes, to patch, cap, and rapidly internalize bound receptors). Furthermore, the AChR accumulations induced by agrin are coordinated with accumulations of synapse-specific cytoplasmic, membrane, and extracellular components (Wallace et al., 1985; Wallace, 1986; Wallace, 1989). Agrin-induced AChR aggregation appears to involve calcium, metabolic energy, and possibly phosphorylation (Wallace, 1988).

AChR accumulations can be triggered by a variety of factors including neural extracts (reviewed in Peng, 1987; Schuetze and Role, 1987), basal lamina components (Peng, 1987; Schuetze and Role, 1987), interaction with the tissue culture substratum (Bloch et al., 1985), positively charged latex beads (Peng and Cheng, 1982), and electric fields (Orida and Poo, 1978). In contrast to agrin, some of these treatments significantly increase AChR synthesis which could indirectly impact on AChR distribution. The AChR patches induced by brain extract and latex beads appear to be morphologically similar to those induced by agrin; the patches are likewise associated with esterase and basal lamina components.

As a first step toward understanding how a myotube coordinates the accumulation of cell surface components at "synaptic" sites, we examined the possibility that specific extracellular matrix components play a role in postsynaptic differentiation. Matrix components are present on developing myotubes from the earliest stages of synapse formation (reviewed by Sanes, 1989). In some developing tissues, components of the extracellular matrix have been shown to interact with specific receptors to effect cellular differentiation (Buck and Horwitz, 1987).

Embryonic myotubes have only wisps of organized basal lamina (Kelly and Zacks, 1969; Kullberg et al., 1977; Jacob and Lentz, 1979). This resembles the appearance of cultured myotubes (Burrage and Lentz, 1981; Bayne et al., 1984; Chiu and Sanes, 1984). As myotubes are innervated, matrix material accumulates at sites of nerve terminal contact, along with AChRs and other synaptic components (Weldon and Cohen, 1979; Nakajima et al., 1980; Anderson and Fambrough, 1983; Bayne et al., 1984; Buchanan et al., 1989). Likewise, matrix material and AChRs accumulate at sites induced by neuronal extracts and even latex beads (Salpeter et al., 1982; Daniels et al., 1984; Sanes et al., 1984; Olek et al., 1986; Peng and Cheng, 1982). This suggests that matrix accumulation and synaptic differentiation are part of a common developmental pathway which can be triggered by a variety of stimuli. Our studies with agrin focus on the mechanistic link between matrix accumulation and AChR clustering.

Immunohistochemical techniques were used to examine the distribution of laminin, fibronectin, type IV collagen, and a heparan sulfate proteoglycan (HSPG) on cultured myotubes. We found that agrin caused the aggregation of laminin and HSPG, but had little effect on fibronectin or type IV collagen distribution. Under these conditions, many of the laminin and HSPG accumulations colocalized with AChR patches.

To address the causal relationship between matrix accumulation and AChR patching, we compared the time courses of these agrin-induced events. AChR patching (as detected by fluorescence microscopy) was completed well ahead of laminin and HSPG patching, suggesting that matrix accumulations do not serve as precursors for AChR patching. In other experiments, AChR aggregation was prevented by treatment with anti-AChR mAbs. Under these conditions, agrin was still able to aggregate laminin and HSPG. These results suggest that agrin-induced patching of AChRs is not causally related to laminin/HSPG patching. Thus, agrin does not appear to use extracellular matrix organization to drive synaptic differentiation.

Brief accounts of this work have appeared elsewhere (Nitkin, R. M., and T. C. Rothschild. 1988. *Soc. Neurosci. Abstr.* 14:514.).

Materials and Methods

Chick Myotube Cultures

Myotubes from hindlimb muscles of 11–12-d-old White Leghorn chick embryos (Avian Services, Frenchtown, NJ) were cultured on 35-mm tissue culture dishes coated with calf skin collagen (Calbiochem-Behring Corp., La Jolla, CA) and maintained in MEM-based medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% horse serum (Gibco Laboratories) and 2% chick embryo extract (Fischbach, 1972, with minor modifications described in Godfrey et al., 1984). The experiments described here were performed on 5–9-d-old cultures.

Agrin Extracts

Agrin was partially purified from electric organ of *Torpedo californica* as previously described (Nitkin et al., 1987) except that the detergent extraction steps were omitted. To achieve maximal AChR aggregation on myotubes, 3–10 U of cibacron pool extract (sp act 5–30 U/ μ g) were used.

Localization of Extracellular Matrix Components and AChRs

To visualize the distribution of extracellular matrix components, cells were incubated with primary antibodies (concentrations listed below) for 1–2 h at 37°C, washed three times in culture medium, then incubated for an additional 1 h in fluorescein-conjugated goat anti-mouse or anti-rabbit Ig (Cappel division, Organon Teknika, Malvern, PA) diluted 1:200 in culture medium. Cultures were washed three to five times in Puck's saline (Puck et al., 1958), then fixed for 10 min at –20°C in reagent alcohol (ethanol/methanol/isopropanol, 18:1:1). After removal of alcohol, cultures were allowed to air dry, and coverslips were affixed with a drop of 50% (vol/vol) glycerol in Puck's saline, with 1 μ g/ml phenylenediamine as an antileaching agent. Incubation of cultures in fluorescent secondary antibodies alone yielded only faint uniform labeling of cells, which was well below the levels of specific labeling presented here.

For double labeling experiments, AChRs were labeled by including 2×10^{-8} M rhodamine-Bgtx (Ravdin and Axelrod, 1977) in both primary and secondary antibody solutions.

Cultures were examined through epifluorescence on an Orthoplan 2 microscope equipped with I3 fluorescein and N2.1 rhodamine filters, and 63 \times Planapo 1.4 NA oil immersion objective (E. Leitz, Rockleigh, NJ) at a total magnification of 504 \times , and photographed using TMax 400 film (Eastman Kodak Co., Rochester, NY).

Laminin was localized using mAb 31, which was raised against chick muscle laminin (Bayne et al., 1984). Optimal labeling was achieved with a primary antibody concentration of 3.7 μ g/ml. In other experiments (not shown here) cells were labeled with a rabbit antiserum (50 μ g/ml) raised against laminin from the Engelbreth-Holm-Swarm (EHS) mouse tumor and affinity purified against the P1 fragment (Yurchenco et al., 1985) of laminin; this antiserum showed no detectable cross reactivity to collagen (type I or IV) or heparin when tested in a competitive ELISA.

HSPG was localized with mAb 33, which was raised against a chick mus-

cle proteoglycan (Bayne et al., 1984). Optimal labeling was achieved with a primary antibody concentration of 8.6 $\mu\text{g/ml}$.

Type IV collagen was localized with a rabbit antiserum raised against type IV collagen purified from the EHS tumor (Yurchenco and Ruben, 1987). Cells were labeled at a 1:750 dilution of primary antibody.

Fibronectin was localized with mAb B3, which was raised against avian fibronectin (Gardner and Fambrough, 1983). Optimal labeling of cultures was achieved with a primary antibody concentration of 0.1 $\mu\text{g/ml}$. In other experiments, cells were labeled with a rabbit antiserum (1:20,000) raised against human plasma fibronectin (Bethesda Research Laboratories, Bethesda, MD).

Quantitation of AChR, Laminin, and HSPG Patches

For each assay condition, patches were counted in 10–12 evenly spaced microscopic fields (0.4 mm diam) from each of triplicate cultures (Godfrey et al., 1984). A patch was defined as a distinct, intense island of fluorescence ~ 2 – $10 \mu\text{m}$ across (e.g., Figs. 1 b, 2 b, 3 b, 4 b, 5, a–d). Control cultures had a small number of fluorescent patches as well (e.g., Fig. 1 a). These “control” patches had less distinct boundaries and tended to be less intensely fluorescent than the majority of those appearing in agrin-treated cultures. While surveying large numbers of fields, it was not practical to distinguish control patches from agrin-induced patches; thus, both types were included in all our counts. It is our feeling that similar numbers of control patches are present in agrin-treated and control cultures. Small “microclusters” ($< 0.5 \mu\text{m}$ diam) that appeared in some myotube platings (see Wallace, 1988) were not included in any of our counts.

Anti-AChR Antibody Treatment to Block AChR Aggregation

Studies reported here utilized mAb 35, a rat mAb raised against *Electrophorus* AChR (Tzartos et al., 1981), to modulate AChR number. Similar results (not shown here) were achieved with a rat antiserum raised against *Torpedo* AChR (provided by Jon Lindstrom, Salk Institute).

The ability of mAb 35 to remove AChRs from the myotube surface was examined. Antibody-treated myotubes were incubated in $2 \times 10^{-8} \text{ M}$ ^{125}I -Bgtx (DuPont-New England Nuclear, Boston, MA) for 1 h at 37°C . Cultures were washed three times in Puck's saline to remove unbound ^{125}I -Bgtx, then harvested in 0.5 ml 1 N NaOH. Cell-associated radioactivity was measured in a gamma counter. Nonspecific ^{125}I -Bgtx binding, determined in the presence of a 100-fold excess of unlabeled Bgtx, was subtracted where appropriate.

The effects of mAbs 35 on agrin-induced patching of AChRs, laminin, and HSPG were examined by exposing antibody-treated myotubes to agrin overnight, then labeling cells to visualize AChRs and laminin or AChRs and HSPG.

Results

Distribution of Extracellular Matrix Components on Cultured Myotubes

Before agrin treatment, cultured chick myotubes displayed significant amounts of laminin (Fig. 1 a) and HSPG (Fig. 2 a) immunofluorescence, ranging in distribution from punctate to mesh-like. The cells also displayed a few local accumulations of laminin and HSPG (e.g., Fig. 1 a), which may represent attachment foci. The intensity and pattern of matrix immunofluorescence among cells did not correlate with any obvious morphological characteristics of the myotubes, such as width, flatness, maturity of striations, or amount of Bgtx labeling (not shown).

In contrast to the rich laminin and HSPG immunofluorescence, most myotubes displayed only wisps of type IV collagen, although a few showed more substantial labeling (similar to the cells in Fig. 3 a). Myotubes had virtually no fibronectin immunofluorescence, although occasionally small puffs a few microns across could be detected on some myotubes (similar to those in Fig. 4 a). By contrast, fibro-

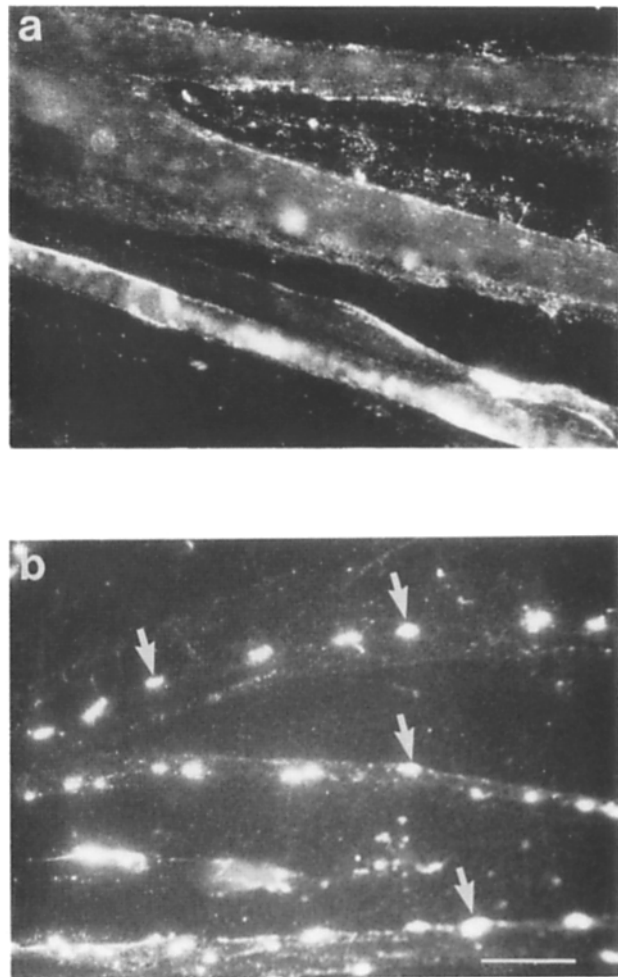


Figure 1. Laminin on chick myotubes accumulated in patches after exposure to agrin (5 U, 16 h). Laminin was labeled with mAb 31 followed by fluorescein second antibody. Untreated myotubes (a) had significant amounts of laminin. Myotubes exposed to agrin (b) had much of the surface laminin in discrete patches (arrows), along with a concomitant loss of fluorescence between patches. Similar patterns were seen when cells were labeled with an affinity-purified laminin antiserum instead of mAb 31 (not shown). Bar, 30 μm .

blasts and glial-like cells (which were present at low numbers in these muscle cultures) showed very bright, fibrous labeling. The substratum was intensely labeled by antifibronectin antibodies. This is probably due to fibronectin from the chick embryo extract which had absorbed out to the collagen-coated dish. Antifibronectin mAb B3 should not cross react with fibronectin from the horse serum (Gardner and Fambrough, 1983). Likewise, in cultures labeled with a rabbit antiserum raised against human fibronectin (data not shown), minimally labeled myotubes contrasted with brightly labeled fibroblasts and glial-like cells. The substratum was not as brightly labeled by this antiserum, although myotubes still appeared as negative images.

Effect of Agrin on Extracellular Matrix Components

After 1–2 d treatment with agrin, some of the laminin and HSPG on the myotubes appeared in discrete patches, mostly along the edges of the cells (Figs. 1 b and 2 b; see also 5,

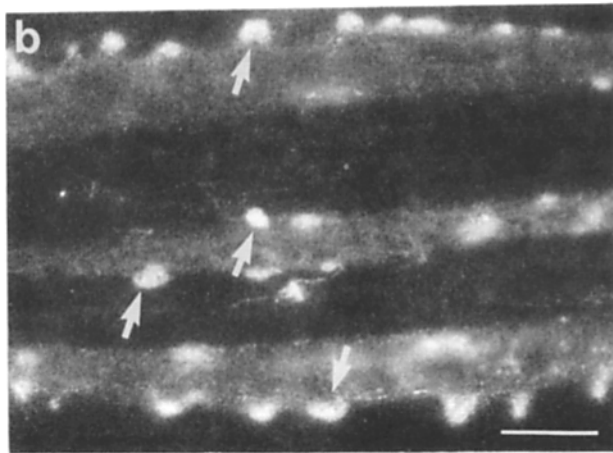
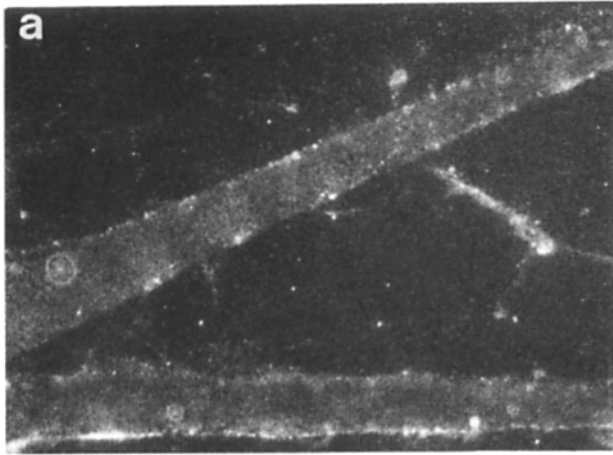


Figure 2. HSPG on chick myotubes accumulated in patches after exposure to agrin (5 U, 16 h). HSPG was labeled with mAb 33 followed by fluorescein second antibody. Untreated myotubes (*a*) had significant amounts of HSPG. Myotubes exposed to agrin (*b*) had much of the surface HSPG in discrete patches along the edge of the cell membrane (*arrows*), with a concomitant loss of fluorescence between patches. Bar, 20 μm .

a and *c*). The patching of laminin and HSPG appeared to be at the expense of other regions of the cell; that is, fluorescence in away from patched regions was below the levels typically seen on untreated myotubes. Nonetheless, it was our impression that the total amounts of myotube-associated laminin and HSPG immunofluorescence were not significantly increased by this treatment. Thus, patching of matrix in response to agrin appears to involve redistribution rather than increased local synthesis or accumulation.

The morphology of the laminin and HSPG patches resembled that of the agrin-induced AChR patches (bright aggregates a few microns in diameter, with distinct edges). This allowed us to quantitate laminin and HSPG patching in much the same manner as AChR patching. Occasionally, cultures were found in which laminin and HSPG did not patch in response to agrin treatment, although AChR patching appeared normal.

To demonstrate that laminin and HSPG accumulations were caused by agrin per se and not some other factor in the *Torpedo* extracts, agrin was specifically removed from par-

tially purified extracts using antiagrin mAb 11D2 (Nitkin et al., 1987). The immunodepleted extract was not able to cause AChRs, laminin, or HSPG to cluster, while an extract passed over a column of control mouse serum retained clustering activity (data not shown).

Most of the agrin-induced AChR patches coincided with patches of laminin (Fig. 5, *a* and *b*) and HSPG (Fig. 5, *c* and *d*). The high level of correlation between AChRs and laminin/HSPG (63–74%) did not improve with an additional day of agrin treatment (Table I). At some sites accumulations of laminin and HSPG were slightly more widespread than AChRs, although overall their distributions were remarkably congruent.

Agrin appears to have a selective effect on laminin and HSPG; it did not affect the distribution of type IV collagen

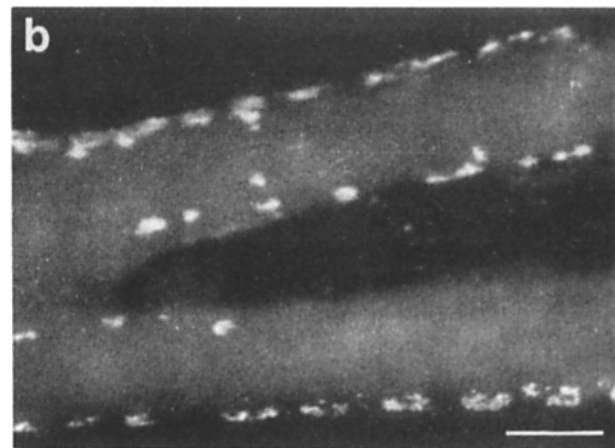
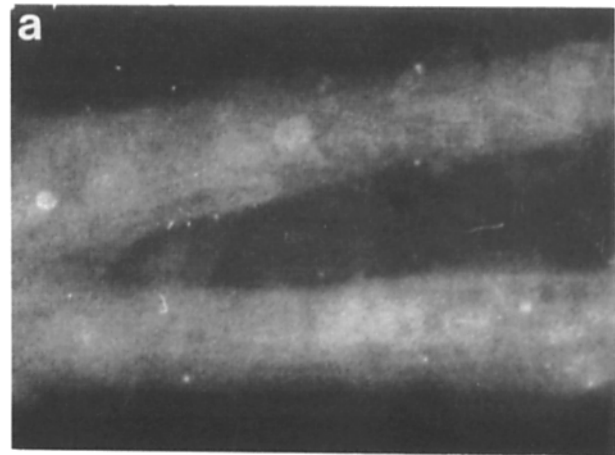


Figure 3. Agrin did not affect the distribution of type IV collagen on cultured myotubes. Myotubes treated with agrin (5 U, 48 h) were simultaneously labeled with antiserum specific for type IV collagen (followed by fluorescein second antibody) and rhodamine-Bgtx, as described in Materials and Methods. The same field was viewed through fluorescein filters to show type IV collagen (*a*) and rhodamine filters to show AChR patches (*b*). The distribution of collagen on myotubes, which varied from wispy to more dense (as shown here), showed no correspondence with AChR aggregations. A similar range of collagen distributions was found on control myotubes (no agrin), or those exposed to agrin for only 16 h (not shown). Bar, 40 μm .

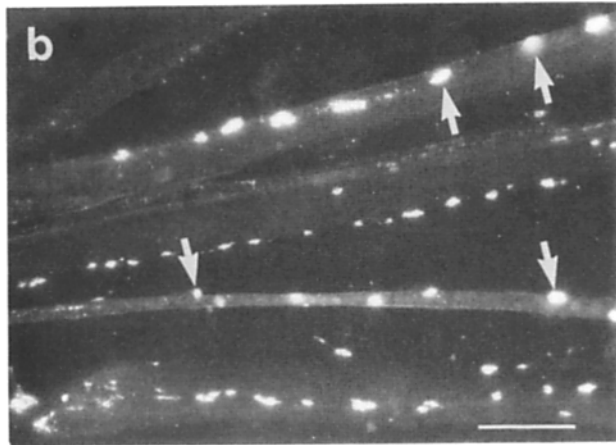
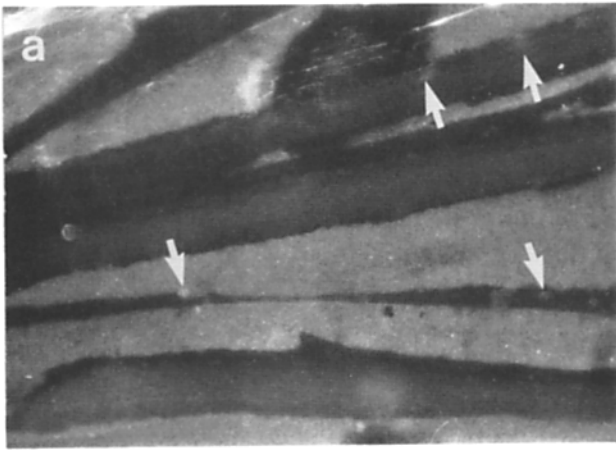


Figure 4. Myotubes had minimal amounts of fibronectin, which was largely unaffected by agrin treatment. Myotubes treated with agrin (5 U, 48 h) were simultaneously labeled with antifibronectin mAb B3 (followed by fluorescein second antibody) and rhodamine-Bgtx, as described in Materials and Methods. The same field was viewed under fluorescein optics to show the distribution of fibronectin (*a*) and under rhodamine optics to show agrin-patched AChRs (*b*). Myotubes had virtually no surface fibronectin, which caused them to appear as negative images contrasted against the brightly labeled substratum. The immunofluorescence of the substratum was probably due to fibronectin from the medium that had absorbed to the dish, as discussed in the text. Generally there was no association of fibronectin with agrin-induced AChR patches; on rare occasions (as shown here) faint patches of fibronectin could be correlated with AChRs (*arrows*). Bar, 30 μ m.

and had little, if any, effect on fibronectin. Even after 48 h of agrin treatment, collagen was still found in wispy patterns which bore no relation to the patched AChRs (Fig. 3, *a* and *b*). The amount of myotube-associated fibronectin remained minimal after agrin treatment, and generally did not correlate with AChR accumulations (Fig. 4, *a* and *b*). Occasionally, small puffs of fibronectin could be correlated with AChR patches (Fig. 4 *a*, *arrows*), but such instances were rare.

Temporal Relationship of AChR and Laminin/HSPG Patching

The colocalization of laminin and HSPG with AChR patches

raised the possibility of a causal relationship between matrix accumulation and AChR patching. This was explored by comparing the rate of AChR patching with that of laminin and HSPG (Fig. 6). After treatment with agrin, increased numbers of AChR patches could be detected after only 2–3 h, reaching maximal levels by \sim 6–8 h (see also Godfrey et al., 1984). However, the increase in the number of visible laminin and HSPG patches occurred much more slowly. By the time AChR patching had reached maximal levels, the numbers of laminin and HSPG patches were only about halfway complete; they required \sim 20 h to reach maximal plateau levels. While these observations do not focus on the earliest stages of agrin-induced AChR and matrix accumulation, which may be below the level of visual detection, they show that AChR patching is completed hours before that of either laminin or HSPG. This temporal relationship makes it unlikely that extracellular matrix directs AChR patching at agrin-induced sites.

Dissociation of AChR Patching from that of Laminin and HSPG

To determine if accumulation of AChRs is necessary for agrin-induced patching of laminin and HSPG, we used the anti-AChR antibody, mAb 35, to block AChR patching. In other studies, AChR antibodies have been shown to decrease both the number and mobility of surface AChRs (Heinemann et al., 1977; Appel et al., 1977; Kao and Drachman, 1977). Direct binding tests (not shown) on alcohol-fixed myotubes (data not shown) demonstrated that mAb 35 does not prevent Bgtx from binding to available AChRs (also Tzartos et al., 1981).

The number of surface AChRs remaining after incubation in various concentrations of mAb 35 was quantitated with 125 I-Bgtx (Table II). All three concentrations of mAb 35 were effective at reducing the number of AChRs. 39 nM antibody was sufficient to achieve maximal effects (only 39% of the AChRs remained). Higher concentrations of antibody (130 nM) were no more effective (38% remained). In the presence of agrin, mAb 35 was still able to reduce the number of surface AChRs, although the effect was slightly less (45–54% of the AChRs remained). A similar percentage of AChRs were removed when the myotubes were treated with a rat antiserum raised against *Torpedo* AChR (not shown).

To examine the effect of agrin on the population of AChRs remaining after antibody treatment, myotubes were exposed to mAb 35 and agrin, then labeled with rhodamine-Bgtx. While antibody treatment reduced total AChR fluorescence (as expected due to the partial internalization of surface AChRs) most of the AChRs remaining were not patched by agrin treatment (Table II). The ability of mAb 35 to block agrin-induced patching was dose dependent. 13 nM mAb was sufficient to block the appearance of the majority of agrin-induced patches, while higher concentrations (39 and 130 nM) reduced patching to <5%. Based on these experiments, it appears that mAb 35 concentrations of 39 nM or more would be sufficient to block virtually all visually detectable agrin-induced AChR patching.

With AChR patching blocked by antibody treatment, we examined whether agrin could still induce patching of laminin and HSPG. Myotubes were incubated with 78 nM mAb 35 and/or agrin, then labeled to visualize AChRs and laminin or AChRs and HSPG (Table III).

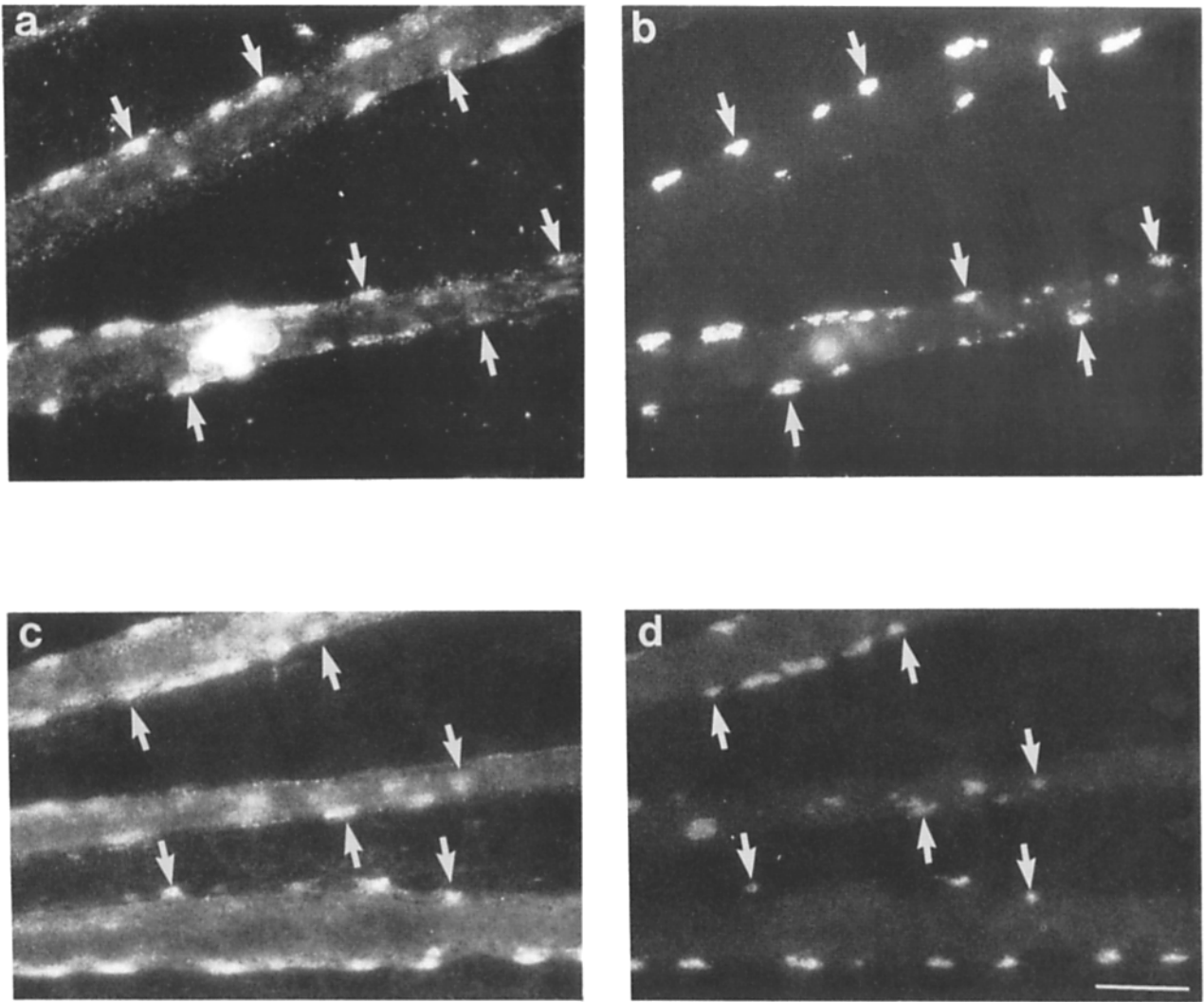


Figure 5. Laminin and HSPG accumulated at agrin-induced AChR patches. Agrin-treated (5 U, 24 h) myotubes were simultaneously labeled with mAbs against laminin or HSPG (followed by fluorescein second antibody) and rhodamine-Bgtx. In the upper pair, the same field was viewed through fluorescein and rhodamine filters to highlight the coincidence of laminin (*a*) and AChR (*b*) accumulations (*arrows*) on myotubes. In the lower pair, a field from another culture shows the coincidence of HSPG (*c*) and AChR (*d*) accumulations (*arrows*). Bar, 25 μ m.

As expected, agrin treatment alone induced significant patching of all three components; this maximal response was defined as 100%. Cells with no additions had few patches of these components; this level of patching (which probably

Table I. Appearance of Laminin and HSPG at Agrin-induced AChR Patches

Length of agrin exposure	AChR patches with laminin	AChR patches with HSPG
(h)		
20	—	223/302 (74%)
41	423/617 (69%)	64/101 (63%)
65	294/404 (73%)	—

Cultures were exposed to agrin (5–10 U) for the times indicated, then simultaneously labeled for AChRs and laminin or AChRs and HSPG. Individual fields were viewed repeatedly, first under rhodamine optics to identify AChR patches (up to three at a time), then under fluorescein optics to determine whether laminin or HSPG had accumulated at these sites. Data are presented as fractions: denominator indicates total number of AChR patches examined and numerator indicates those that had coaccumulations of laminin or HSPG.

includes attachment foci) was defined as 0%. Cultures treated with mAb 35 alone (no agrin) had even fewer AChR patches than control (<0%), suggesting that antibody treatment even prevented AChRs from accumulating at attachment foci. Anti-AChR antibody had no significant effect on the amount or distribution of laminin or HSPG immunofluorescence in nonagrin-treated cultures. In the presence of both mAb 35 and agrin, virtually no AChR patches could be seen, but significant numbers of laminin (91%) and HSPG (64%) patches were still detectable (Table III).

Long exposure photographs (10–15 s) of these cultures revealed a slightly different story (Fig. 7). The increased sensitivity of this technique showed that in mAb 35/agrin-treated cultures, low density accumulations of AChRs did occur at some laminin and HSPG patches; other patches were devoid of AChRs even at this level of scrutiny. This indicates that while mAb 35 treatment severely reduced AChR patching (so that it was not evident through simple visual inspection), it did not prevent AChR patching completely. Agrin-induced patching of laminin and HSPG was largely unaffected by the

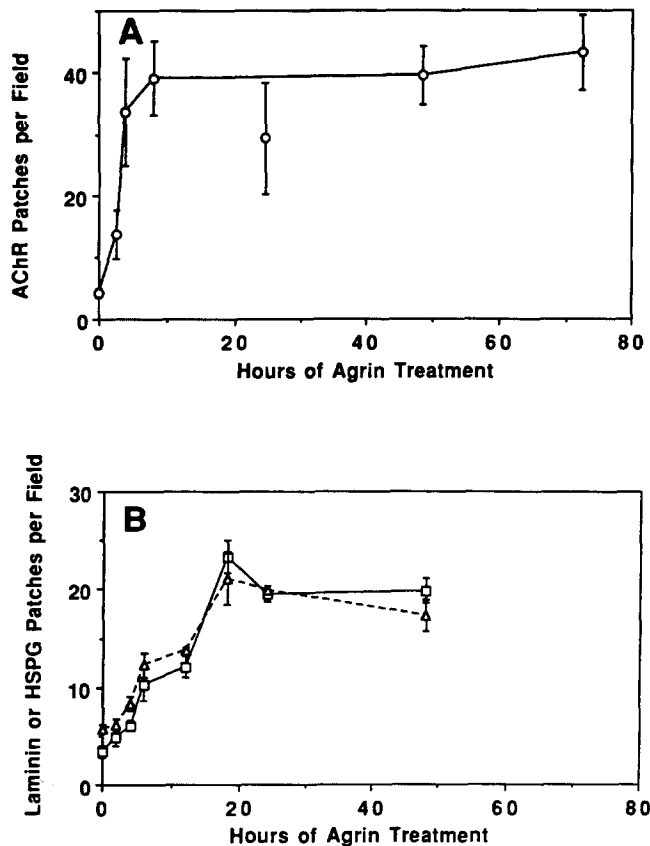


Figure 6. Agrin-induced accumulations of AChRs occurred much more rapidly than those of laminin or HSPG. Muscle cultures were exposed to agrin (5 U) for various amounts of time indicated on the abscissa, then labeled to visualize the distributions of either AChRs (a, circles/solid line), laminin (b, squares/solid line) or HSPG (b, triangles/dotted line). Each time point is the mean number of patches per microscopic field, derived from 8–12 fields per dish, using triplicate dishes; vertical error bars show SEM among those dishes. In each experiment, the addition of agrin was staggered so that all cultures were labeled and counted together; thus, all determinations were made on the same age cultures. The upper and lower graphs were derived from experiments on two different muscle platings, resulting in different plateau levels for AChR and laminin/HSPG patching.

antibody treatment (Table III), suggesting that matrix accumulations do not require the presence of dense AChR patches.

Discussion

The experiments described here focus on the role of extracellular matrix components in synaptic differentiation. We used immunohistochemical techniques to follow the distribution of laminin, HSPG, fibronectin, and type IV collagen on cultured chick myotubes. In agreement with several other immunohistochemical studies (e.g., Kuhl et al., 1982; Anderson and Fambrough, 1983; Gardner and Fambrough, 1983; Daniels et al., 1984), we found that cultured myotubes have on their surface laminin, HSPG, and type IV collagen, but little if any fibronectin.

We found that matrix accumulation at agrin-induced sites was selective. Laminin and HSPG colocalized with AChRs but fibronectin and type IV collagen distributions were not

Table II. Anti-AChR Treatment Removes Surface AChRs and Prevents Agrin-induced AChR Patching

Treatment	Number of AChRs (fmol/culture)		AChR patches (per field)	
No additions	77 ± 0.4	100%	2.3 ± 0.8	0%
13 nM mAb 35	45 ± 3.3	58	—	—
39 nM mAb 35	30 ± 1.1	39	—	—
130 nM mAb 35	29 ± 2.0	38	—	—
Agrin alone	79 ± 3.0	103	20.2 ± 2.8	100
Agrin + 13 nM mAb 35	42 ± 2.1	55	7.9 ± 4.0	27
Agrin + 39 nM mAb 35	42 ± 0.4	54	2.9 ± 1.2	3
Agrin + 130 nM mAb 35	35 ± 0.7	45	3.3 ± 0.9	5

Myotubes were incubated for 48 h with agrin (5 U) and/or mAb 35 (at concentrations indicated). ¹²⁵I-Bgtx binding was used to determine the number of AChRs on the myotube surface (nonspecific binding has been subtracted); cultures with no additions were defined as 100%. In sister cultures, cells were labeled with rhodamine-Bgtx so that AChR patches could be counted; cultures with no additions were defined as 0%, while cultures treated with agrin alone were defined as 100%. Each entry is the mean ± SEM (n = 3) of triplicate cultures.

affected (agrin-induced HSPG aggregations have also been reported by Wallace, 1989). At this time we cannot determine whether laminin and HSPG patching represent lateral migration of matrix molecules already present on the cell surface or selective placement of newly synthesized or newly bound material. It appears that agrin does not significantly alter the total amount of surface laminin or HSPG, as judged by immunofluorescence.

Molecules like agrin could be responsible for coordinating the localization of extracellular matrix material with synaptic components at sites *in vivo*, much as it does in culture. Agrin-like molecules have been detected at neuromuscular junctions from the earliest stages of development (Godfrey et al., 1988b; Fallon and Gelfman, 1989). Furthermore, initial patches of AChRs are associated with laminin, HSPG, and type IV collagen, but not fibronectin (Anderson and Fambrough, 1983; Chiu and Sanes, 1984; Bayne et al., 1984).

Table III. Although AChR Patching Is Prevented by Antibody Treatment, Agrin Can Still Cause Laminin and HSPG to Patch

Treatment	AChR patching (%)	Laminin patching (%)	HSPG patching (%)
No additions	0	0	0
Agrin alone	100	100	100
mAb 35 alone	(<0)	17 ± 9	11 ± 5
Agrin + mAb 35	10 ± 10	91 ± 20	64 ± 18

Myotubes were incubated for 48 h with 78 nM anti-AChR mAb 35 and/or agrin (3 U), then labeled to visualize the distribution of AChRs, laminin, and HSPG. Because each muscle plating had inherently different levels of response, the numbers of AChR, laminin, and HSPG patches in different experiments could not be averaged directly. Cultures (treated in quadruplicate) were normalized against controls with no additions (0%) and cultures exposed to agrin (100%) to derive percent response. Percentages pooled from five experiments are presented above (mean ± SEM, n = 5). In the case of "mAb 35 alone," antibody treatment eliminated some of the spontaneous (background) AChR patches, resulting in even less AChR patches than control (<0%). In the presence of "Agrin + mAb 35," significant patching of laminin and HSPG occurred despite the loss of AChRs. (Long exposure photographs revealed that low density accumulations of AChRs were present at some laminin and HSPG patches, see Fig. 7.)

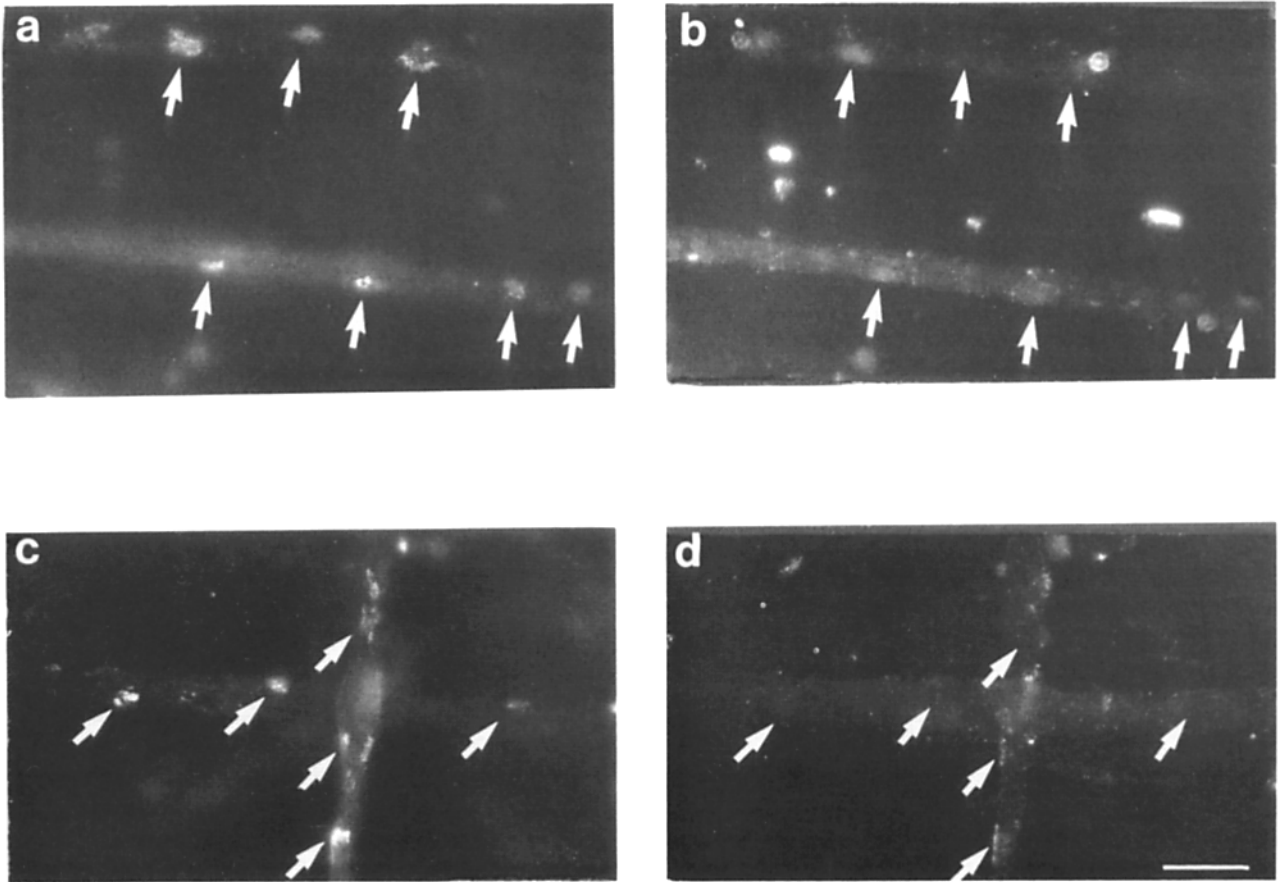


Figure 7. Laminin and HSPG accumulated at agrin-induced patches even though AChRs were blocked with antibodies. Myotubes were incubated for 48 h with 78 nM anti-AChR mAb 35 (to remove or immobilize AChRs) and agrin (3 U). The cultures were simultaneously labeled to visualize laminin or HSPG, and AChRs. In the upper pair, the same field was viewed through fluorescein and rhodamine filters to contrast the distribution of laminin (a) and AChRs (b), respectively. In the lower pair, a field from another culture contrasts HSPG (c) with AChRs (d). Because of the mAb 35 treatment, only minimal accumulations of AChRs appeared at laminin or HSPG patches (arrows). To achieve adequate contrast in b and d, film exposures were about two times longer compared to other rhodamine-labeled cultures (e.g., Figs. 3 b, 4 b, 5 b, and d); adjustments in the developing process were also made. While surveying large numbers of fields by direct visual observations alone (e.g., Table III), faint AChR patches such as those shown above (b and d) lack sufficient contrast to be detected. Bar, 40 μ m.

In this study, AChR accumulations and matrix differentiation were followed by fluorescence microscopy. While this technique is effective for monitoring relative distributions of components, it provides only a qualitative description of actual site density. Indirect immunofluorescence makes quantitative comparisons even more difficult, although we have tried to optimize binding of primary and fluorescein secondary antibodies. The earliest stages of AChR/matrix accumulations could go undetected by these techniques. Agrin itself appears to function catalytically in the sense that only a few hundred molecules are sufficient to induce a patch containing tens of thousands of AChRs (Nitkin et al., 1987).

On agrin-treated myotubes, AChR patching was completed quite a few hours ahead of laminin/HSPG patching (Fig. 6). While it is possible that small amounts of laminin and/or HSPG actually precede AChRs at some agrin-induced sites, our results indicate that the distribution of AChRs matures into dense discrete patches hours before that of either laminin or HSPG. Thus, unless matrix accumulation is a multi-step process, it is unlikely that it directs the placement of AChRs.

Conversely, we considered the possibility that agrin-induced patching of AChRs is necessary to attract extracellular matrix components (AChRs do have extensive extracellular domains which could potentially interact with matrix material). We developed a means of specifically blocking agrin-induced AChR aggregation using the anti-AChR antibody, mAb 35. The reduction in AChR number is probably due to accelerated removal of AChRs from the myotube surface through antibody-mediated internalization. This process involves cross-linking of mobile surface receptors by a multivalent ligand which rapidly leads to patching, capping, and ultimately internalization of bound receptors. mAb 35 was able to internalize AChRs without secondary antibodies; this may be due to the fact that each of the two alpha subunits of the AChR offers a potential site to allow for antibody cross-linking (Tzartos et al., 1981). AChRs were likewise internalized by treatment with an antiserum raised against *Torpedo* AChR (not shown).

The AChRs that remain on the myotube surface after antibody treatment, $\sim 40\%$ in our studies (Table II), may represent a population of less mobile receptors. Even on myotubes

not exposed to antibodies, a significant number of AChRs did not participate in agrin-induced patching (note diffuse rhodamine fluorescence between AChR patches, especially in Figs. 3 b and 4 b). The presence of a population of less mobile AChRs has been suggested by photobleaching studies (e.g. Axelrod et al., 1976). Thus, it is not surprising that the population of AChRs remaining after antibody treatment was not readily patched by agrin treatment (Table II).

Long exposure photographs (Fig. 7, b and d) revealed that while AChR patching is significantly reduced by antibody treatment, a small number of AChRs still appear at agrin-induced sites. Over the 48-h antibody/agrin treatment, it is possible that some AChRs were not bound by mAb 35 or bound in a configuration that did not allow for cross-linking. These AChRs would then avoid immobilization/internalization and would be able to migrate into patches. We have not investigated whether AChRs that reach agrin-induced patches are immune to subsequent mAb treatment.

Although mAb 35 had dramatic effects on AChR patching, it had much less effect on the number of agrin-induced matrix patches (Table III). The data pooled from five experiments indicate that laminin and HSPG were decreased only 9 and 36%, respectively, with significant variation among individual experiments. In some experiments, the full complement of agrin-induced matrix patches were present despite the virtual elimination of dense AChR patches by mAb35. The reason for this variability is unclear; it could be that prolonged exposure to antibodies (48 h) and subsequent AChR patching and capping begins to affect the distribution of other membrane receptors. Alternatively, it could suggest that there is some minimal direct connection between AChRs and laminin/HSPG localization. In vivo, AChR accumulations can be uncoupled from matrix differentiation. As muscles get innervated, AChRs are recruited away from matrix-associated regions to new sites beneath developing nerve terminals (Weinberg et al., 1981; Chiu and Sanes, 1984). Similarly, in culture AChRs that are initially associated with HSPG (and probably other matrix components) migrate to developing neuromuscular junctions, leaving much of the HSPG behind (Anderson et al., 1984; Anderson, 1986).

Redistribution of extracellular matrix components could be brought about by a variety of mechanisms. Our results suggest that the accumulation of laminin and HSPG at agrin-induced sites is not stoichiometrically linked to the accumulation of AChRs (Table III). It is possible that matrix components accumulate in response to the recruitment of specific cell surface matrix receptors (reviewed by Buck and Horwitz, 1987). For example, the myotube could localize integrins or other laminin receptors, causing extracellular laminin to follow. HSPG could be attracted to these sites because of specific proteoglycan-binding domains on laminin (Sakashita et al., 1980). This would account for the striking similarity between the time courses of laminin and HSPG accumulations (Fig. 6 b).

AChR patching is completed before that of laminin or HSPG, suggesting that matrix accumulation does not initiate "synaptic" differentiation. Nonetheless, the matrix may have an important role in the maintenance of synaptic structure (Nitkin et al., 1987). The accumulation of matrix material at developing synapses could attract additional material (including more agrin), which would further enhance synaptic differentiation in that region. Such a reinforcing mechanism

could be used to stabilize developing synapses as well as strengthen especially active synapses. This could have important implications for the process of polyneuronal synapse elimination. Furthermore, if analogous mechanisms operate in the central nervous system, it could serve to reinforce specific pathways during learning.

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