Increased adhesiveness at sites of high acetylcholine receptor density on embryonic amphibian muscle cells cultured without nerve

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SUMMARY

In culture, myotomal muscle cells from *Xenopus laevis* embryos develop discrete patches of high acetylcholine receptor (AChR) density. To examine the relative adhesiveness of these sites, muscle cells having AChR patches on their lower surface (opposed to the culture dish) were identified and were then treated with dibucaine or potassium-Ringer in order to cause the cells to round up. More than 90% of these cells remained attached at an AChR patch after rounding up, and this was the case even when the cells had a single patch on their lower surface. When the cells were torn away from the culture dish by mechanical agitation, small cellular fragments still remained firmly attached to the dish and many of these fragments contained an AChR patch. It is concluded that AChR patches on the lower surface of the cell are often located at sites of increased adhesiveness to the culture dish. The findings are discussed in terms of the formation and maintenance of AChR patches.

INTRODUCTION

When embryonic muscle cells are cultured in the absence of nerve they develop surface patches of high acetylcholine receptor (AChR) density (Vogel, Sytkowski & Nirenberg, 1972; Fischbach & Cohen, 1973; Hartzell & Fambrrough, 1973; Axelrod et al. 1976; Anderson, Cohen & Zorychta, 1977; Powell & Friedman, 1977). This is the case even if the muscle cells have never been previously contacted by nerve (Bekoff & Betz, 1976; Moody-Corbett & Cohen, 1981). The density of AChRs within these patches can be an order of magnitude greater than elsewhere on the muscle cell and approaches that seen at the adult neuromuscular junction (Sytkowski, Vogel & Nirenberg, 1973; Anderson et al. 1977; Land, Podleski, Salpeter & Salpeter, 1977). Many of the AChR patches are also relatively stable and survive in a fixed position for several days (Anderson & Cohen, 1977; Frank & Fischbach, 1979; Moody-Corbett & Cohen, 1982). Embryonic muscle cells thus have a means of accumulating AChRs
into discrete patches and of maintaining these accumulations in the absence of innervation.

The mechanisms which participate in the development and maintenance of AChR patches and their relevance to the accumulation of AChRs at the developing neuromuscular junction are currently the focus of considerable investigation. Evidence has been obtained which indicates that the AChRs within AChR patches are much less mobile than those present at lower density elsewhere on the cell (Axelrod et al. 1976). AChRs at the neuromuscular junction also appear to be immobile (Axelrod et al. 1976; see also Fambrough, 1979). These findings have led to the notion that the formation and/or maintenance of discrete sites of high AChR density is dependent upon an anchorage of the AChRs at those sites (Axelrod et al. 1976; see also Anderson & Cohen, 1977; Orida & Poo, 1978; Bloch, 1979). Further studies have suggested that the AChRs may be anchored by components of the basal lamina on the external surface of the plasma membrane (Burden, Sargent & McMahan, 1979; Heuser & Salpeter, 1979; Heuser, 1980; McMahan, Edgington & Kuffler, 1980a; McMahan, Sargent, Rubin & Burden, 1980b) and by intracellular components which interact with the inner surface of the plasma membrane (Ellisman, Rash, Staehelin & Porter, 1976; Fertuck & Salpeter, 1976; Heuser & Salpeter, 1979; Bloch & Geiger, 1980; Heuser, 1980; Cartaud et al. 1981; Couteaux, 1981; Froehner et al. 1981; Hall, Lubit & Schwartz, 1981). In culture the occurrence of some AChR patches may also be related to direct contact with an apposing surface (Axelrod, 1981; Peng, Cheng & Luther, 1981).

The formation and survival of discrete accumulations of other surface receptors, such as receptors for lectins and immunoglobulins, have been found to be markedly affected by agents such as colchicine and cytochalasin B which disrupt microtubules and microfilaments, thereby implicating these intracellular structures in the organization of the cell surface (for review, see Edelman, 1976). By contrast studies on cultures of rat myotubes have indicated that the occurrence of AChR patches is only slightly reduced by colchicine and is unaffected by cytochalasin B (Axelrod, Ravdin & Podleski, 1978; Bloch, 1979). Nor does colchicine appear to prevent the development of AChR patches on embryonic chick muscle cells in culture (Fukuda, Henkart, Fischbach & Smith, 1976). The initial aim of the present study was to determine whether the AChR patches on embryonic Xenopus muscle cells in culture are also insensitive to treatment with colchicine, cytochalasin B and dibucaine. Dibucaine was chosen because of its reported effectiveness in disrupting caps of immunoglobulin receptors presumably through disruption of both microtubules and microfilaments (Poste, Papahadjopoulos, Jacobson & Vail, 1975a; Poste, Papahadjopoulos & Nicolson, 1975b). During the course of our experiments we found that high concentrations of dibucaine caused the muscle cells to peel off the culture dish almost completely and round up. Many of these rounded up cells remained attached at the site of an AChR patch suggesting that this region
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is more strongly adhesive than other areas of the cell in contact with the culture dish.

The present study examines the latter effect in more detail and also includes a brief description of the survival of AChR patches in cultures treated with colchicine, cytochalasin B and lower concentrations of dibucaine. Our experiments confirm that AChR patches on *Xenopus* muscle cells often occur at sites of increased adhesion to the culture dish. This finding, in line with recent studies which indicate that AChR patches on rat muscle cells in culture often occur at sites of close contact with the culture dish (Axelrod, 1980, 1981; Bloch & Geiger, 1980), extends the list of similarities between AChR patches on *Xenopus* muscle cells in culture and the postsynaptic membrane at the neuromuscular junction (see Discussion).

METHODS

Muscle cultures were prepared as described previously (Anderson *et al.* 1977; Moody-Corbett & Cohen, 1981). All dissections were done under sterile conditions. Briefly, the myotomes of stage-22 to -24 *Xenopus laevis* embryos (Nieuwkoo & Faber, 1967) were isolated from surrounding tissue with the aid of collagenase and further dissociated into single cells in a calcium–magnesium-free solution of trypsin and EDTA. The muscle cells were added to culture chambers containing plating medium (67% L-15 and 5% dialysed horse serum). One day later the medium was replaced with maintenance medium (67% L-15 and 0.2 μg/ml Holmes' α-1-protein). All components of the culture medium were purchased from Grand Island Biological Co. of Canada Ltd. Sealed culture chambers were constructed as described by Anderson *et al.* (1977), their floor consisting of a collagen-coated glass coverslip. Cultures of *Xenopus* sympathetic and dorsal root ganglia were also prepared as previously described (Cohen & Weldon, 1980).

Sites of high AChR density were stained with α-bungarotoxin conjugated with tetramethylrhodamine (Anderson & Cohen, 1974; Anderson *et al.* 1977; Cohen & Weldon, 1980). For microscopy, the sealed living cultures were inverted and viewed with phase-contrast and fluorescence optics. Fine focusing readily distinguished AChR patches on the lower surface of cells (the surface apposed to the floor of the culture dish) from those on the upper surface. By photographing specific cells and utilizing the coordinates of the microscope stage the same cells could be followed before and after drug treatment. When required the cultures were restained in the presence of the drug, in order to keep the AChR patches brightly fluorescent. In experiments where successive observations were made on the same culture different portions of the culture were evaluated in order to reduce the chances of artifactual loss of AChR patches by bleaching.

The effects of dibucaine and colchicine were examined by dissolving the
Fig. 1. Phase-contrast and corresponding fluorescence views of a muscle cell in a 4-day-old culture before (A) and after (B) treatment with 0.2 mg/ml dibucaine. Two of the cell processes have ACh patches on the lower surface. After treatment with dibucaine the muscle cell rounded up but remained attached at one of the patches. Scale bar is 20 μm.
Fig. 2. Phase-contrast and corresponding fluorescence views of portions of two muscle cells in a 5-day-old culture before (A) and after (B) treatment with 0.5 mg/ml dibucaine. Each muscle cell had an AChR patch on its lower surface. After the dibucaine treatment the cells rounded up (not shown) and further agitation caused the cells to tear away from the dish. Only a small fragment (arrow) of one of the cells remained attached and this fragment contained one of the AChR patches. Scale bar is 20 μm.
Table 1. Percentage of muscle cells which remained attached at an AChR patch after rounding-up

Cells with AChR patches on their lower surface were first identified and photographed. Each of the selected cells had at least one brightly stained and relatively large AChR patch (greatest dimension: 10-25 μm). The cells were rephotographed after treatment with dibucaine or potassium–Ringer. The data include only those cases where a major portion of the cell rounded up (see Figs. 1-4.)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Category of cell</th>
<th>Rounded cells attached at an AChR patch (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dibucaine</td>
<td>More than one AChR patch on lower surface</td>
<td>93 (40)</td>
</tr>
<tr>
<td>Dibucaine</td>
<td>One AChR patch on lower surface</td>
<td>93 (42)</td>
</tr>
<tr>
<td>Potassium–Ringer</td>
<td>One AChR patch on lower surface</td>
<td>100 (17)</td>
</tr>
</tbody>
</table>

Numbers in parentheses indicate total number of cells.

RESULTS

Increased adhesion at AChR patches

Previous studies on endothelial cells in culture have revealed that dibucaine can cause the cells to round up and even detach from the culture dish (Nicolson & Poste, 1976; Nicolson, Smith & Poste, 1976). Treatment of Xenopus muscle cultures with a high concentration of dibucaine (0.2-1.0 mg/ml) also caused the muscle cells to round up within 5-30 min. Usually the rounded-up cells remained attached to the culture dish in a region which contained an AChR patch on the lower surface (Fig. 1). In some cases, especially with further agitation (see Methods), the rounded-up cells tore away completely from the dish leaving behind small adherent cellular fragments which often contained an AChR patch of approximately similar size (Fig. 2). As summarized in Table 1, in a sample of 40 identified cells which rounded up after exposure to dibucaine,
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Table 2. Percentage of processes with or without lower surface AChR patches which remained attached after the cell rounded up

Only processes which extended for at least 10 μm beyond the main margin of the cell were considered (see Figs. 1 and 4). Processes without AChR patches were considered attached if any part of the process was still in contact with the dish after the cell rounded up. Processes with AChR patches were considered attached only if an AChR patch was still in contact with the dish after the cell rounded up.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Processes without AChR patches</th>
<th>Processes with AChR patches</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dibucaine</td>
<td>8 (119)</td>
<td>58 (76)</td>
</tr>
<tr>
<td>Potassium–Ringer</td>
<td>19 (16)</td>
<td>80 (10)</td>
</tr>
</tbody>
</table>

Numbers in parentheses indicate total number of processes. From the same experiments as Table 1.

93% either remained attached at an AChR patch (33 cells) or left behind a cell fragment which contained an AChR patch (4 cells). Since AChR patches on the lower surface of these muscle cells occupy less than 3% of the surface area (Moody-Corbett & Cohen, 1981) it is unlikely that these results are due to chance. Rather they indicate that the region of the AChR patch is more resistant to detachment by dibucaine than the remainder of the cell.

AChR patches on the lower surface of muscle cells in these cultures are usually located at the ends of the cells or their processes (Anderson et al. 1977) and one might imagine that these sites would be more adherent to the culture dish even if they did not contain AChR patches. This possibility was examined in two ways. Muscle cells having prominent processes, extending at least 10 μm beyond the main border of the cell, were identified and their processes were characterized according to whether or not they had AChR patches on their lower surface (see Fig. 1). After treatment with dibucaine most of the processes without AChR patches on their lower surface withdrew completely from the culture dish; in only 8% of the cases was some portion of a process still adherent to the culture dish (Table 2). By contrast, the corresponding value for processes with AChR patches on their lower surface was 58% (Table 2). In a second set of experiments the effect of dibucaine was examined on muscle cells which had only a single AChR patch on their lower surface. Ninety-three percent of these cells (n = 42) remained attached at the AChR patch (Table 1). As shown in Fig. 3 this was the case even for cells whose AChR patch was located in the central region of the cell. All of these observations indicate that regions of high AChR density are preferentially more resistant to detachment by dibucaine.

Since in the above experiments the AChRs were stained with fluorescent
Fig. 3. Phase contrast and corresponding fluorescence views of two muscle cells in a 2-day-old culture before (A) and after (B) treatment with 0.4 mg/ml dibucaine. Each cell had a single AChR patch on its lower surface. After treatment with dibucaine the cells rounded up and remained attached in the region of the AChR patches, even in the case where the patch was in the central region of the cell (lower muscle cell). Scale bar is 20 μm.
Fig. 4. Phase contrast and corresponding fluorescence views of a muscle cell in a 5-day-old culture before (A) and after (B) the medium was changed to a Ringer solution with a high concentration of potassium. The potassium–Ringer caused the muscle cell to contract and round up. However, the cell remained attached to the dish at the two sites with AChR patches. Scale bar is 20 μm.
toxin before exposure to dibucaine it was possible that the toxin somehow increased adhesiveness at AChR patches. In order to examine this possibility six cultures were first treated with dibucaine and were exposed to the fluorescent toxin only after the cells had rounded up. Randomly chosen rounded-up cells were then examined (mean sample size per culture: 52). In these six cultures 60 ± 10\% (mean and standard error) of the rounded-up cells were attached at an AChR patch. In six control cultures (mean sample size per culture: 52) which were stained first and then treated with dibucaine 59 ± 11\% of the rounded-up cells were attached at an AChR patch. These results indicate that prestaining with the fluorescent toxin did not enhance the adhesiveness of the lower surface at AChR patches. The reason why the percentages in these experiments are less than those in Table 1 may be related to the fact that some muscle cells in these cultures have no AChR patches on their lower surface and others have lower surface patches which are relatively small and/or faintly stained. The cells in Table 1, however, were selected because they had relatively large (greatest dimension: 10–25 \( \mu \)m) and brightly stained AChR patches on their lower surface.

To check whether preferential attachment at AChR patches following dibucaine treatment is peculiar to this drug, additional experiments were made in which muscle cells were caused to detach partially from the culture dish by eliciting contraction with a Ringer solution containing a high concentration of potassium (see Methods). Seventeen cells with single AChR patches on their lower surface were followed in five cultures before and after changing the medium and, even though major portions of these cells detached from the culture dish and rounded up, all of the cells remained attached at the region of the AChR patch (Table 1). In addition, as with dibucaine treatment, muscle cell processes with AChR patches were much less susceptible to detachment than muscle cell processes without AChR patches (Table 2, Fig. 4). Finally, after staining one muscle culture with fluorescent toxin almost all of the cells were removed by extensive agitation so that only tightly adherent cell fragments remained attached to the culture dish. The fragments were first identified with phase-contrast optics and then examined for AChR stain. Fifty-three percent of the fragments had AChR stain. In view of the fact that AChR patches occupy less than 3\% of the lower cell surface (Moody-Corbett & Cohen, 1981), and the possibility that some of the cell fragments were derived from non-muscle cells, these findings further emphasize that AChR patches on the lower surface of the muscle cell are sites where the cell is more firmly attached to the culture dish.

**Survival of AChR patches in the presence of colchicine, cytochalasin B and dibucaine**

Colchicine and cytochalasin B, unlike dibucaine, did not cause muscle cells to detach from the culture dish. Accordingly, it was possible to examine the effects of these drugs on the survival of AChR patches. Dibucaine was also
Fig. 5. Phase contrast and corresponding fluorescence views of two muscle cells in a 4-day-old culture 16 h after the addition of 100 \( \mu \text{g/ml} \) colchicine to the medium. The upper cell had a normal appearance in phase contrast and also had an AChR patch on its lower surface. The lower cell appeared degenerated and had no AChR patches. Scale bar is 20 \( \mu \text{m} \).
Table 3. Occurrence of AChR patches on the lower and upper surface of muscle cells whose appearance in phase contrast was normal or deteriorated after drug treatment

Bracketed data were obtained from the same culture. Values with an asterisk (*) were obtained by dividing the total number of cells into the total number of patches.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Culture age (day)</th>
<th>Time after adding drug</th>
<th>Normal</th>
<th>Deteriorated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>No. of cells</td>
<td>Cells with AChR patches (%)</td>
</tr>
<tr>
<td>Colchicine (100 µg/ml)</td>
<td>1</td>
<td>0 h</td>
<td>35</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16 h</td>
<td>60</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 days</td>
<td>56</td>
<td>93</td>
</tr>
<tr>
<td>Cytochalasin B (20 µg/ml)</td>
<td>3</td>
<td>16 h</td>
<td>60</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 days</td>
<td>100</td>
<td>85</td>
</tr>
<tr>
<td>Cytochalasin B (100 µg/ml)</td>
<td>4</td>
<td>0 h</td>
<td>25</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 h</td>
<td>25</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 h</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>Dibucaine (100 µg/ml)</td>
<td>4</td>
<td>0 h</td>
<td>25</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 h</td>
<td>25</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11 h</td>
<td>19</td>
<td>89</td>
</tr>
<tr>
<td>1 % Dimethyl-sulphoxide</td>
<td>3</td>
<td>16 h</td>
<td>60</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 days</td>
<td>100</td>
<td>94</td>
</tr>
</tbody>
</table>

N.D. denotes cases where the relevant values were not determined.
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tested at half the minimum concentration which caused muscle cells to round up. For these experiments the drug was added to the culture medium of 1 to 4-day-old cultures and the cultures were then followed for up to 2 days. At the concentrations tested (see Table 3) each of the drugs caused some of the muscle cells to show signs of deterioration which became more prominent with time. The deterioration was characterized by a loss of the smooth appearance of the cell surface and a partial disruption of the cell striations (Fig. 5). At these concentrations the drugs were also effective in abolishing, within 2 h, growth-cone activity in cultures of *Xenopus* dorsal root ganglia and sympathetic ganglia. Table 3 summarizes the frequency of occurrence of AChR patches in the presence of the drugs. Those muscle cells which appeared normal when viewed with phase contrast optics usually had AChR patches (Fig. 5), even after 2 days in colchicine, 2 days in cytochalasin B or 11 h in dibucaine. Furthermore in cases where the number of AChR patches per cell was measured, the value before and after treatment did not change markedly (Table 3). By contrast the frequency of occurrence of AChR patches was reduced on those cells which had a degenerated appearance, but even some of these cells still had well-defined AChR patches (Table 3). Dimethylsulphoxide (1%), which was used for dissolving cytochalasin B (see Methods), did not by itself cause any cell degeneration or loss of AChR patches (Table 3). From these results it appears that colchicine, cytochalasin B and dibucaine do not disrupt AChR patches independent of other cytotoxic effects.

**DISCUSSION**

The present study indicates that AChR patches on the lower surface of embryonic *Xenopus* muscle cells in culture are located at sites where the cells are more strongly adherent to the culture dish. A similar conclusion has been reported for AChR patches on the lower surface of rat myotubes in culture (Bloch & Geiger, 1980). This increased adhesiveness at AChR patches bears a certain resemblance to the situation at the adult neuromuscular junction where there is a strong adhesion between the nerve terminal and the postsynaptic membrane which contains a high density of AChRs (Betz & Sakmann, 1971, 1973). Our finding thus extends the list of similarities between AChR patches on cultured *Xenopus* muscle cells and the postsynaptic membrane at the neuromuscular junction. In addition to being sites of high AChR density and of adhesion, many of the AChR patches, like the postsynaptic membrane, are also sites of localized cholinesterase (ChE) activity (Moody-Corbett & Cohen, 1981). In turn the ChE is located at sites where the sarcolemma has basal lamina-like material on its outer surface and fine filamentous material on its inner surface (Weldon, Moody-Corbett & Cohen, 1981; see also Weldon & Cohen, 1979). Similar ultrastructural specializations are found associated with the outer and inner surface of the postsynaptic membrane at the neuromuscular junction.
(Ellisman et al. 1976; Fertuck & Salpeter, 1976; Kullberg, Lentz & Cohen, 1977; Heuser, 1980; Couteaux, 1981) and with AChR patches on embryonic chick muscle cells in vivo and in culture (Jacob & Lentz, 1979; Burrage & Lentz, 1981). The association of basal lamina material with the AChR patches on cultured *Xenopus* muscle cells has also been established by employing appropriate antibodies (Anderson & Fambrough, 1981) and may account for the increased adhesiveness to the culture dish at these sites. This would be akin to the situation at the neuromuscular junction where it has been established that the basal lamina acts as an adhesive material between the nerve terminal and the postsynaptic membrane (Betz & Sakmann, 1971, 1973).

The development of adhesive material at sites of high AChR density may have important relevance for the development of the neuromuscular junction. The most obvious possibility is that this material participates in the strong adhesion that develops between the pre- and postsynaptic membranes. Furthermore, if the adhesive material is indeed a component of the basal lamina at AChR patches it might have other functional capabilities similar to those of the basal lamina at the adult neuromuscular junction. For example, adult synaptic basal lamina can induce a local differentiation of regenerating motor axons into synaptic terminals (Sanes, Marshall & McMahan, 1978; McMahan et al. 1980a, b) and it can also act as a focus for the accumulation of AChRs in the sarcolemma (Burden et al. 1979; McMahan et al. 1980a, b). For the adhesive material on embryonic muscle cells in culture to act as a focus for AChR accumulation, it would, of course, have to appear on the cell surface before the AChR patches. In the present study the increased adhesion at AChR patches was already apparent in 2-day-old cultures (see Fig. 3) thereby indicating that both properties develop in close temporal relationship. However our data do not permit any conclusion concerning the sequence of development of the increased adhesion and the AChR patches.

The occurrence of AChR patches at sites of strong adhesion with the culture dish is consistent with recent studies which have found that AChR clusters on rat myotubes in culture occur at sites of close contact with the culture dish (Axelrod, 1980, 1981; Bloch & Geiger, 1980). In culture, such adhesion plaques on other cell types occur at sites of the cell margin which are associated with the internal support system involving filamentous material (Grinnell, 1978). It may be that the sites where AChR patches and their associated specializations form are determined by how the internal support system becomes organized during cell spreading and growth. Such a mechanism could also apply to AChR patches which form on the upper surface in the apparent absence of any contact with a solid substrate (Fischbach & Cohen, 1973; Hartzell & Fambrough, 1974; Anderson et al. 1977; Bloch & Geiger, 1980). In *Xenopus* muscle cells upper surface AChR patches and their associated specializations tend to be located in the central regions where the cells are thicker (Anderson et al. 1977; Moody-Corbett & Cohen, 1981; Weldon et al. 1981). An internal support system
involved in the development of such cell geometry might act as a focus for the accumulation of AChRs and related specializations. However, our attempts in the present study to disrupt AChR patches with drugs which are known to disrupt cytoskeletal elements have not been successful. Cases where colchicine or cytochalasin B reduced the incidence of AChR patches on muscle cells were accompanied by cell degeneration. It is unclear, therefore, whether the drugs acted by disrupting any of the specializations associated with AChR patches or whether their action on AChR patches was secondary to other cytotoxic effects. Other studies using colchicine, cytochalasin B and dibucaine have likewise revealed little disruption of AChR accumulations (Axelrod et al. 1978; Orida & Poo, 1978; Bloch, 1979). It may be that the internal structural and molecular specializations which are associated with sites of high AChR density (e.g. Heuser & Salpeter, 1979; Bloch & Geiger, 1980; Cartaud et al. 1981; Couteaux, 1981; Froehner et al. 1981; Hall et al. 1981) do play a role in AChR patch survival but are unaffected by these drugs. An alternative possibility which also remains to be investigated is that the associated basal lamina can itself maintain the survival of AChR patches.

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