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THE ROLE FOR PROTEOGLYCANS IN ACETYLCHOLINE RECEPTOR CLUSTERING ON CULTURED MUSCLE

by
Inhee Mook Jung

A Dissertation Submitted to the Faculty of the DEPARTMENT OF CELL BIOLOGY AND ANATOMY
In partial Fulfilment of the Requirements For the Degree of DOCTOR OF PHILOSOPHY In the Graduate College THE UNIVERSITY OF ARIZONA 1995
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ABSTRACT

The present dissertation investigated the possible role of proteoglycans (PGs) in acetylcholine receptor (AChR) clustering on cultured C2 myotubes. This system serves as a model of the molecular interactions that underlie synaptogenesis. PGs were manipulated in mutagenized cells or by chemical intervention in order to assess their roles in the clustering process. In addition, the relationship between PGs and the clustering factor agrin was investigated.

Analysis of variant muscle cell lines and their hybrid products supported the hypothesis that PGs are required for the clustering of AChRs. Three PG-defective genetic variants derived from the C2 cell line, S27, S11 and S26, all form myotubes but fail to spontaneously cluster AChRs. The three variants show different broad-spectrum defects in glycosaminoglycan (GAG) biosynthesis and are especially deficient in the synthesis of chondroitin sulfate (CS) chains that elute at high salt in ion-exchange chromatography. Formation of heterokaryon myotubes containing nuclei from two different variants spontaneously clustered AChRs and recovered synthesis of GAGs, especially of CS eluting at high salt in ion-exchange chromatography. As it is highly unlikely that defects in AChR clustering would have arisen through a chance association with three different defects in GAG biosynthesis, it strongly suggests that there is a requirement for proper GAG biosynthesis in AChR clustering.

Three lines of experimentation suggested that CS is required for the clustering of AChRs. Chlorate was found to inhibit both GAG synthesis and the clustering of AChRs in a dose-dependent manner. When extracellular calcium was raised from 1.8 to 6.8 mM in cultures of wild type C2 myotubes, both the frequency of spontaneous AChR clusters and the level of cell layer-associated CS were increased. Culture of wild type C2 myotubes in
the presence of chondroitinase ABC eliminated cell layer-associated CS and prevented the formation of AChR clusters. Treatment with chondroitinase ABC only prevented AChR clustering if begun prior to the formation of spontaneous clusters. This suggests that CS is required in the initiation but not the maintenance of AChR clusters.

Since both CS and agrin appear to be involved in the AChR clustering process, possible interactions between CS and agrin were examined. Agrin-induced AChR clustering was dramatically reduced by digestion of CS. Unlike calcium, however, agrin action on AChR clustering did not affect levels of CS. Also, agrin-induced AChR clustering was restored on S27 myotubes by adding calcium-treated C2 conditioned medium. The IIH6 monoclonal antibody against α-dystroglycan (a putative agrin receptor), laminin, and agrin all bound to a specific fraction of C2 cell extracts separated on Mono-Q ion exchange chromatography. Also IIH6, laminin, and agrin affinity-precipitations showed a smeared sulfate labelled band above 120kD which is close in molecular weight to that of α-dystroglycan. The band disappeared after chondroitinase ABC treatment. Protease-digested IIH6 immunoprecipitate eluted as two distinct peaks corresponding to CS. This results strongly suggests that CS is required for agrin activity on AChR clustering.

The present dissertation provides several lines of evidence that 1) a CS plays an essential role in AChR clustering, 2) a CS mediates agrin action, and 3) a CS is closely associated with α-dystroglycan. The simplest model for the role of CS is that agrin binds to α-dystroglycan, which is a CSPG, to induce AChR clustering.
INTRODUCTION

The neuromuscular junction is a peripheral synapse made by a motor nerve terminal on a muscle cell. Due to its peripheral localization and a relatively simple organization, the neuromuscular junction has served as a model system for the study of synapses, including their development. The mature neuromuscular junction is a highly specialized system in its morphology and biochemical organization. Both pre- and post-synaptic cells provide essential components to make specialized domains at the neuromuscular junction. At the neuromuscular junction, skeletal muscle cells produce basal lamina molecules such as laminin (Sanes, 1982), collagen IV (Sanes, 1982), heparan sulfate proteoglycans (Anderson and Fambrough, 1983; Bayne et al., 1984), and muscle agrins (Fallon and Gelfman, 1989; Lieth et al., 1992). Muscle cells also localize neural cell adhesion molecules (NCAM) (Sanes et al., 1986), acetylcholinesterase (AChE) (Sanes and Hall, 1979; Silberstein et al., 1982), and nicotinic acetylcholine receptors (AChRs) (Evans et al., 1987) which are ligand-gated ion channel receptors. On the other hand, presynaptic motor neurons provide nerve agrin (Magill and McMahan, 1988), calcitonin gene-related peptide (CGRP) (Kashihara et al., 1989), and AChR-inducing activity (ARIA) (Harris et al., 1988).

AChRs are not evenly distributed over the muscle cell membrane but are clustered adjacent to the motor nerve ending at the mature neuromuscular junction, presumably to facilitate signal transmission between motor neurons and muscle cells. Clustering of AChRs is among the earliest events in the specialization of the neuromuscular junction. It is therefore often used as a marker for the study of neuromuscular junction development. Aggregation of AChRs is induced by neurite contact onto muscle cells during development. Anderson & Cohen (1977) have shown that addition of neurons to cultured muscle cells
induces clustering of AChRs to sites of nerve-muscle contact. The aggregation process begins within hours of initial contact, and the density of receptors beneath the terminal reaches several thousand per \( \mu m^2 \) within a day or two. Before innervation, AChRs are diffusely distributed over the entire surface of the myotubes at a density of a few hundred receptors per \( \mu m^2 \) (Fertuck and Salpeter, 1976). The concentration beneath the axon terminal continues to increase up to approximately \( 10^4 \) receptors per \( \mu m^2 \) in the adult, while the density of receptors in nonsynaptic portions of the muscle fiber decreases to less than 10 receptors per \( \mu m^2 \) (Salpeter et al., 1988).

Even without innervation, skeletal muscle cells form spontaneous clusters of AChRs both in vivo and in vitro that are smaller in size than those found at the neuromuscular junction (Anderson and Cohen, 1977; Fallon and Gelfman, 1989; Fischbach and Cohen, 1973; Frank and Fischbach, 1979). When a nerve reaches the muscle, spontaneous clusters of AChRs remain for a short period of time and eventually disappear in vivo; spontaneous clusters of AChRs co-exist with nerve-induced clusters in vitro. The physiological role of spontaneous clustering, if any, is not clear at present. Initially the spontaneous clusters were proposed to serve as target regions for growth cone innervation. However, morphological and physiological experiments demonstrated that growth cones randomly contact muscle fibers ignoring pre-existing AChR clusters (Anderson and Cohen, 1977; Frank and Fischbach, 1979). One possibility is that muscle cells produce all the necessary components for AChR clustering even before innervation and are poised for nerve-induced clustering. When neurites contact muscle cells, nerve-induced clustering can happen quickly since all the necessary components already exist. Spontaneous clustering may simply be a side effect of this hair-trigger priming. Regardless of the functional roles served by spontaneous clustering, it provides a much simpler model system in which to study the molecular mechanism of AChR clustering since it involves
only post-synaptic components. The present dissertation is therefore based on experimental studies on spontaneous clustering.

The molecular mechanisms underlying AChR clustering have attracted much attention. Thanks to intensive research in this area, we are beginning to understand the molecular components of AChR clustering. In 1980, McMahan et al. (1980) identified agrin, a crucial molecule for AChR clustering. Agrin is produced both by the nerve (nerve agrin) and by the muscle cell (muscle agrin). It is now known that there are several different forms of agrin, which are RNA splicing variants from one gene (Ferns et al., 1993). There appears to be a major functional difference between nerve agrin and muscle agrin. Using a function blocking assay, Reist & McMahan (Reist et al., 1992) showed that nerve agrin, but not muscle agrin, is responsible for nerve-induced AChR clustering on chick myotubes. This is in agreement with the report that the nerve produces very active forms of agrin while muscle produces a less active form (Ferns et al., 1993). Other immunohistochemical studies suggest, however, that nerve agrin is involved in the triggering of nerve-induced clustering of AChRs whereas muscle agrin may be involved in AChR clustering at later stages of the clustering process (Lieth et al., 1992).

A crucial follow-up question is whether there exist specific agrin receptors on the cell surface. If there are, how do they interact with other basal lamina molecules to induce AChR clustering? Recent studies by several groups suggest that α-dystroglycan is a putative agrin receptor. Bowe et al. (1994) showed that the protein sequence of human α-dystroglycan has high homology with that of a protein eluted from an agrin affinity column. Campanelli et al. (Campanelli et al., 1994) have shown that CHAPS-solubilized α-dystroglycan from the C2 muscle cell line is retained by agrin agarose beads in the presence of calcium. Gee et al. (Gee et al., 1994) have shown that agrin binds to purified α-dystroglycan from rat skeletal muscle. Originally, α-dystroglycan was discovered as a member of the dystrophin-related glycoprotein complex (DGC) (Ervasti and Campbell,
While dystrophin is a cytoskeletal molecule, the DGC is a complex of membrane associated proteins: It consists of 4 integral membrane proteins, one cytoplasmic peripheral protein, and one extracellular protein. Also, α-dystroglycan has been shown to bind to laminin (Ervasti and Campbell, 1993). Ibraghimov-Beskrovnaya & Campbell (Ibraghimov et al., 1992) have postulated that the DGC may provide a bridge between extracellular matrix and the cytoskeleton inside the cell. Such a complex may provide a structural support for AChR clustering. Knowledge of DGC interactions with other cellular components may turn out to be important for understanding molecular mechanisms of AChR clustering.

Also unanswered are the molecular and cellular characteristics of the dystroglycans. α- and β-dystroglycan are produced from the same precursor protein (97kD precursor). As muscle develops, α-dystroglycan (60kD core protein) is cleaved off and becomes glycosylated to make a larger protein molecule (about 156kD). The mechanisms of the cleavage, the cleavage site and the post-cleavage connection between α- and β-dystroglycan are unknown. The nature of the glycosylation is also unknown, although it is required for the binding of both agrin and laminin (Ervasti and Campbell, 1993; Gee et al., 1994).

Experimental data indicate that many molecules other than agrin are associated with AChR clustering. For example, when electrical fields were applied to a muscle cell, other molecules aggregated to the cathode before AChRs moved to the aggregation site (Stollberg and Fraser, 1990a). Since AChRs are integral membrane proteins, they can be connected to both the outside components, such as basal lamina, and the inside components of the cell, such as cytoskeleton. Indeed, AChR clusters are colocalized with some of the basal lamina components including laminin, heparan sulfate proteoglycans (HSPGs), agrins and α-dystroglycan, and some of the intracellular cytoskeletal molecules including 43kD protein, 58kD protein, utrophin (Campanelli et al., 1994; Sealock and Froehner, 1994) and...
spectrin (Bloch and Pumplin, 1988). Therefore knowledge of the interactions between
AChRs and other extra- and intracellular components is important for understanding
molecular mechanisms underlying synaptogenesis.

The distribution of laminin was initially reported to be highly correlated with the
distribution of AChR clusters in frog, suggesting a critical role of laminin in AChR
clustering (Daniels et al., 1984). However, later studies demonstrated that the correlation
between laminin and AChR distribution is not absolute. Although the distribution of
laminin was highly correlated with AChRs a few days after myotube formation in culture,
the correlation was not so high in the beginning of AChR cluster formation; some laminin
clusters were without AChRs (Bayne et al., 1984). This result indicates that laminin may
play a role in AChR clustering, but probably additional factors are required that precede the
association of laminin with AChRs.

A 43 kD protein has received much attention as a candidate molecule essential for
AChR clustering. It is a peripheral membrane protein that binds intracellularly to AChRs in
a one to one fashion. The pivotal finding was obtained using transfection of fibroblasts.
When fibroblasts were transfected with cDNAs for AChR subunits only, no clustering was
observed. However, additional transfection with 43kD protein cDNA induced clustering of
AChRs (Phillips et al., 1991). A similar result was observed in the oocyte expression
system except that the clusters were much smaller (Froehner et al., 1990). These results
suggest that the 43kD protein may be a necessary factor for AChR clustering. However,
we cannot rule out the possibility that there are other factors for AChR clustering which are
provided by the fibroblasts or Xenopus oocytes. Indeed, the 43kD protein was present at
normal levels but AChR clustering was not observed on a mutant muscle line, S27
(Gordon et al., 1993). This finding indicates that the presence of 43kD protein is not
sufficient for AChR clustering and some other factors are required.
The above results suggest that several different molecules participate in AChR clustering and that a deficiency in any of these components may lead to a failure to form AChR clusters. One such component may be a proteoglycan (PG). PGs are distributed ubiquitously in all multicellular organisms. PGs exist in the extracellular matrix and on the surface of cell membranes. In the adult muscle, PGs form a part of the basal lamina that surrounds each muscle fiber. PGs are proteins that carry unusual carbohydrate moieties, glycosaminoglycans (GAGs). GAGs are large linear chains of repeating disaccharide units. There are several groups of GAGs which differ in the composition of the repeating disaccharides, the sugar linkages, and the degree of sulfation: heparan sulfates (including heparin), chondroitin sulfates (including dermatan sulfate), keratan sulfates, and hyaluronic acid. All GAGs except polylactosamine and hyaluronic acid contain sulfate and have strong negative charges. This electrostatic force makes it possible for GAGs to bind many substances and they appear to play a major cross-linking role in the extracellular matrix and basal lamina (Hardingham and Fosang, 1992).

Several lines of evidence implicate an important role for PGs in AChR clustering at the neuromuscular junction. First, heparan sulfate proteoglycans (HSPGs) are roughly colocalized with AChR clusters in *Xenopus* and chick muscles both at the adult endplate and in cultured, aneural myotubes (Anderson and Fambrough, 1983; Bayne et al., 1984). Second, PGs associate with other components of the muscle basal lamina such as laminin and agrin (Daniels et al., 1984; Ferns et al., 1993). It is intriguing that laminin is a major component of basal lamina and is roughly associated with AChR clusters, too. Third, the S27 cell line, a genetic variant defective in PG biosynthesis (Gordon and Hall, 1989), does not form AChR clusters (Gordon et al., 1993). S27 cells differentiate normally to form multinucleated myotubes, but they fail to cluster AChRs. S27 cells have the same level of the 43kD molecule as do C2 wild type cells, but the 43kD molecules are distributed all over the myotube surface (Gordon et al., 1993).
Several different approaches were used in the present dissertation to investigate a possible role of PGs in AChR clustering. First, genetic complementation analyses were performed between different genetic variants that are defective in both PG biosynthesis and AChR clustering. I examined whether AChR clustering could be rescued by genetic complementation between PG-defective mutants. While S27 cells are defective both in PG biosynthesis and AChR clustering, there is no evidence that these defects are causally related. If two phenotypes associatively change across many genetic crosses, then it is more likely that there exists a causal relationship between the two phenotypes. The second approach involved biochemical manipulations of PGs in culture. I examined whether modification of PGs induces changes in AChR clustering. This was done in several ways: 1) since it was known that deprivation of calcium in culture media causes dispersion of pre-existing AChR clusters in rat myotubes (Bursztajn et al., 1984), the effect of excess calcium on PG biosynthesis and AChR clustering was tested; 2) the effect of chlorate, a strong sulfation inhibitor, on GAG sulfation and AChR clustering was tested; and 3) enzymes that degrade specific classes of PGs were applied in the culture to test their effects on AChR clustering. Third, I examined possible interactions between PGs and agrin. The results from the above experiments indicated that a CSPG is essential for AChR clustering. Since both agrin and a CSPG appear to be involved in the AChR clustering process, it is of interest to investigate their interactions. For this, the consequence of chondroitinase ABC treatment on agrin-induced AChR clustering was tested, and the effect of agrin on overall CS synthesis was examined. Also, a PG defective genetic variant was tested for whether it can be rescued to be responsive to agrin by providing calcium treated C2 conditioned medium. Finally, I examined the relationship between α-dystroglycan and PGs. α-dystroglycan plays important roles in binding matrix elements to the muscle surface. However, neither the identity of the carbohydrate moieties on α-dystroglycan nor its role in the binding of basal lamina components is known. Since the results from the
third approach indicated that agrin action is mediated by a CSPG, it is important to understand the relationship between \(\alpha\)-dystroglycan, a putative agrin receptor, and CSPGs. Also the nature of the GAG associated with \(\alpha\)-dystroglycan was characterized. The results of this dissertation strongly suggest that a PG, especially a CSPG, is a crucial molecule for mediating AChR clustering and the effect of agrin.
CHAPTER ONE

ACETYLCHOLINE RECEPTOR CLUSTERING ASSOCIATES WITH PROTEOGLYCAN BIOSYNTHESIS IN VARIANT AND HETEROARYON MUSCLE CELLS

Abstract

Several lines of evidence suggest a possible role of proteoglycans (PGs) in AChR clustering. One such line of evidence comes from a correlation between defects in PGs and AChR clustering in the S27 cell line, a genetic variant derived from a C2 muscle cell line and isolated on the basis of a deficiency in GAG biosynthesis (Gordon and Hall, 1989). Two different approaches were used in the present study to further investigate the relationship between PGs and AChR clustering. First, the formation of AChR clusters were examined in the S11 and S26 lines isolated in parallel with the S27 line (Gordon and Hall, 1989). Results showed that S11 and S26, like S27, are defective in both AChR clustering and PG biosynthesis. Also, the biochemical composition of GAGs in C2 wild type cultures was compared to that of the S27, S11 and S26 variant lines. The results indicate that defects in GAG biosynthesis in the S11, S26 and S27 lines are all different from each other. The second approach was to examine whether or not AChR clustering can be induced by genetic complementation between two PG defective mutants that normally do not form AChR clusters. Genetic complementation by heterokaryon formation between PG defective mutants rescued AChR clustering and overall PG biosynthesis. In particular biosynthesis of a CSPG, that was defective in all of the genetic variants, was rescued in
each heterokaryon. AChR clustering was not rescued in 4 hybrid lines, but biosynthesis of the CSPG was not rescued either. These results indicate consistent correlations between defects in PGs, especially those bearing a specific CS, and the defect in AChR clustering. The present study thus corroborates the possibility that PGs play a role in AChR clustering and suggests that the important PGs for AChR clustering are CSPGs.
Introduction

Several lines of evidence suggest that proteoglycans (PGs) may play an important role in AChR clustering at the neuromuscular junction. Initial evidence was provided by an immunostaining study that demonstrated colocalization of heparan sulfate proteoglycan (HSPG) with AChR clusters in cultured frog muscle (Anderson and Fambrough, 1983). Also, nerve- or agrin-induced AChR clustering is inhibited by heparin (Hirano and Kidokoro, 1989; Wallace, 1990). Finally, the S27 genetic variant of the C2 muscle cell line is defective in PG biosynthesis, and fails to form AChR clusters (Gordon et al., 1993).

For the PG-defective cell line, Gordon and Hall (1989) created three genetic variants, S11, S26 and S27, based on deficiencies in the incorporation of radiolabeled sulfate into glycosaminoglycans (GAGs), which are long carbohydrate chains on PGs. One of the variants, S27, has been better studied than the other variants. S27 cells form normal myotubes and express more than 90% of wild type levels of AChR protein on the surface of the myotubes, but they fail to form spontaneous clusters of AChR. The consequence of the PG defect on AChR clustering has not been examined for S11 and S26 cells. This was due to a preliminary survey that suggested rare development of S11 and S26 myoblasts into myotubes (Gordon and Hall, 1989). AChR clustering cannot be examined unless cells form myotubes in the culture. Unlike the initial assessment, however, the present study shows that S11 and S26 do form myotubes (see below) even though the frequency at which myotubes form is still lower for S11 and S26 than for S27. S11 is defective in glucosamine incorporation like S27, while S26 incorporates almost the same level of glucosamine as C2 wild type cells do (Gordon and Hall, 1989). Glucosamine is a precursor for GAG chains, and thus a deficit in glucosamine incorporation results in a deficit in GAG chains. These data suggest that each variant has different defects in GAG
biosynthesis. Biochemical analysis of GAG chains in each variant would provide more direct evidence for this possibility.

The result from S27 cells shows only a correlation between PG biosynthesis and AChR clustering. It is possible that the S27 cells have multiple genetic lesions and other defects than those in PGs are responsible for the absence of AChR clustering. Like all other correlational studies, the more correlations between the defect in PGs and the defect in AChR clustering is found, the more likely becomes the possibility that the two phenotypes are causally related. Since the nature of the GAG defects in S11, S26 and S27 are likely to be different, it is of interest to test whether or not S11 and S26 also exhibit defects in AChR clustering. A strong prediction can be drawn if the defects of the genetic variants are all different: genetic complementation between the variants should rescue normal GAG biosynthesis. Furthermore, if PGs are important for AChR clustering, successful genetic complementation of PGs should result in restoration of AChR clustering.

The present study addressed the following issues: 1) Do genetic variants have different genetic defects in GAG biosynthesis? Biochemical examinations indicate that the GAG profiles of C2, S11, S26 and S27 are all different; 2) Are S11 and S26 defective in AChR clustering? They both failed to form AChR clustering; and 3) Can defects in GAG biosynthesis and/or AChR clustering be rescued by genetic complementation? Heterokaryon myotubes between two different variants recovered both AChR clustering and overall GAG biosynthesis, especially chondroitin sulfate synthesis.
**Materials & Methods**

General cell culture

C2 wild type cells were plated at 5,000 cells/cm² in growth medium (GM) which contained 20% fetal bovine serum, 2.5% chick embryo extract, 100 units/ml penicillin, and 2 mM glutamine in Dulbecco's Modified Eagle's Medium with 1 gm/liter glucose (DMEM). When cells reached 80% confluency, they were switched to differentiation medium (DM) which contained 2% horse serum and the same concentrations of penicillin and glutamine as in GM. Cultures were maintained in 100% humidified incubators with 8% CO₂.

Assay for AChR clustering

AChRs were visualized by the binding of α-bungarotoxin conjugated to rhodamine (Ravdin and Axelrod, 1977). Cultured myotubes were incubated in the toxin-containing medium for 30 min at 37°C to label AChRs. The cells were then rinsed with cold PBS and fixed with cold 2% paraformaldehyde, dehydrated in methanol at -20°C, and mounted in buffered glycerol containing p-phenylenediamine. Slides were observed and photographed on a Leitz Aristoplan fluorescence microscope. Clusters per field of view in a Leitz 100X Fluotar plan objective were determined for 25 randomly chosen fields on a 22x22 mm coverslip.

Construction of heterokaryons

Heterokaryon myotubes containing nuclei from S27 and either S26 or S11 cells were constructed using two different methods. First, the two cell lines were simply co-
cultured. Second, polyethylene glycol (PEG) was used to force fusion in order to increase the rate of fusions (Figure 1.1) (Blau et al., 1983). S27 cells were cultured on 22 mm square No.2 glass coverslips. Once the S27 cells began to form myotubes, myoblasts of either the S11 or S26 variant lines were added with 45% PEG in DM. The culture plates were spun at 600 g for 5 seconds, followed by braking. By fusing one or two days later in DM once myotubes formed and feeding the cultures only with differentiation medium, the nuclei remained distinct even in the presence of PEG. Side-by-side cultures of heterokaryon myotubes were examined for AChR clustering by fluorescence microscopy and for GAG biosynthesis by Mono-Q ion exchange chromatography.

Construction of hybrid cells

The method of Esko et al. (Esko, 1989) was used to make hybrid cells between S27 and S26 or S11 (figure 1.1). After trypsinizing the two different cell lines, the same number of each cell line was added into a centrifuge tube with 45% PEG. The cells were spun as described in the heterokaryon construction method (see above) and then plated with growth medium for one day to stabilize the fused cells. On the next day, cells were replated in a 10 cm plate at low density (300 cells per 10 cm plate) to screen hybrid cells by replica plating for incorporation of $^{35}\text{SO}_4^\text{2-}$ (Esko and Raetz, 1978).

Hybrid colonies on polyester replica cloths were incubated for 4 hours in $^{35}\text{SO}_4^\text{2-}$. The cloths were then fixed with 10% trichloroacetic acid, stained with Coomasie Blue to visualize the colonies, rinsed with PBS, dried and exposed to X-ray film overnight. Those colonies that remained defective in the incorporation of radiolabel only weakly exposed the film while hybrid colonies in which there was complementation between defects in GAG biosynthesis incorporated $^{35}\text{SO}_4^\text{2-}$ and strongly exposed the film. Meanwhile, the master plate was kept at 28°C to inhibit the proliferation of the candidate colonies. After candidate
colonies were recovered from the master plates, subcloning was performed until all colonies in the plate contained only $^{35}$sulfate-positive cells. Each of the isolated hybrid cells were examined for AChR clustering as well as for GAG biosynthesis by Mono-Q ion exchange chromatography.

Biochemical characterization of GAGs

Three day cultured myotubes of the C2, C2 variant, or heterokaryon cells in 10 cm dishes were labeled with $\text{H}_2\text{SO}_4$ (100 μCi/ml except as noted) in sulfate-free DM for 12 hours. Cultures were then rinsed three times in cold PBS and cell layers were extracted in 3 ml of 0.1 M NaOH. 100 μl aliquots were assayed for protein (Pierce Micro assay). Nucleic acids were digested with 20 units of DNase and 20 μg RNase per ml (Sigma) with 1 mM MgCl$_2$ at pH 7 for 2 hrs at 37°C. Samples were then adjusted to pH 5.5 and 1 mg/ml shark chondroitin sulfate (Sigma) was added as carrier. They were then digested with 2 mg/ml pronase E (Sigma) for 1 day at 37°C on a shaker in order to digest the proteoglycan core proteins. Samples were bound to DEAE-sephacel beads (Sigma) and washed with 0.1 M NaCl in 20 mM piperazine, pH 5.8 to remove residual free $^{35}$SO$_4$ and other contaminants. GAGs were then eluted with 2 M NaCl in 20 mM piperazine, pH 5.8, followed by ethanol precipitation (Bame and Esko, 1989). The resulting GAGs were resuspended in 20 mM piperazine buffer containing 0.2% CHAPS (pH 5.8) and applied to a 1 ml Mono-Q column (Pharmacia). The applied sample was washed with 10 ml of buffer, and fractions were eluted with a gradient of 0.05 to 1.75 M NaCl in buffer. Pharmacia FPLC pumps, valves, and the fraction collector were controlled by a custom-built Macintosh-based chromatography controller that formed the salt gradients, insured reproducibility of all runs, and allowed for variable fraction sizes. Aliquots of fractions were counted with a scintillation counter and counts per minute of $^{35}$SO$_4$ was calculated per
ml of column eluate per mg of protein in the original culture. Counts are reported as per the day of labelling.

Digestion of heparan sulfates

Heparan sulfates were specifically cleaved at N-sulfated glucosamine residues with nitrous acid (Shively and Conrad, 1976b). GAG samples were prepared by the method of Bame and Esko (Bame and Esko, 1989) and then resuspended in 20 mM piperazine buffer and evaporated to dryness. Fresh nitrous acid (pH 1.5) was prepared by the reaction of 0.5 M H$_2$SO$_4$ and 0.5 M Ba(NO$_2$)$_2$ (Sigma) at 0°C. 150 μl were added to the dried samples of GAGs with 50 μg of heparin as a carrier for 10 min at room temperature. The deamination reaction was terminated with 25 μl of 2 M Na$_2$CO$_3$. The pH was then adjusted to 5.8 with H$_2$SO$_4$ prior to column chromatography.

Digestion of chondroitin sulfates

In order to degrade chondroitin sulfate as well as dermatan sulfate (chondroitin sulfate B), purified GAGs were incubated for 4 hr at 37°C with 2 units/ml of chondroitinase ABC from Proteus vulgaris (EC 4.2.2.4, Sigma) in a final volume of 50 μl containing 2.5 μM TrisHCl, pH 8.0, 3 μM of sodium acetate and 5 μg of BSA. After incubation, reaction mixtures were heated at 100°C for 1 min to inactivate enzymes (Yamagata et al., 1968). Also, chondroitin sulfate A and C were selectively degraded by applying chondroitinase AC from Flavobacterium heparinum (EC 4.2.2.5, Sigma) to purified GAGs in the same way as the chondroitinase ABC digestion procedure except that the pH of the solutions was 7.3.
Results

AChR clustering on S11, S26 and S27 cells

It was initially reported that levels of differentiation were low for S11 and S26 lines; apparent fusion into myotubes was very limited (Gordon and Hall, 1989). It was therefore necessary to find culture conditions in which these cells would form myotubes at higher frequencies. This was achieved by subsequent passaging of the S11 and S26 lines and the use of a more restrictive culture condition to promote differentiation (2% horse serum DM instead of the previous 5% horse serum). S11 and S26 yielded myotubes at much higher frequencies under this condition, although still at lower frequencies than that of the wild type C2 cells. AChR expression on the C2, S11, S26 and S27 myotubes appeared to be comparable by visual inspection of rhodamine conjugated α-bungarotoxin binding as well as by previous report (Gordon and Hall, 1989) even though the frequency of myotube formation varied greatly (Figure 1.2). However, no spontaneous AChR clusters were found from any of the three mutant myotubes (S11, S26 and S27, Figure 1.2). These results indicate that S11, S26 and S27 express AChRs at a similar level as C2 wild type cells, but have defects in the clustering mechanism.

GAG profiles of S11, S26 and S27 cells

Examination of the GAGs synthesized by the three genetic variants, S11, S26, and S27 on Mono-Q ion-exchange chromatography showed significant differences from those of wild type C2 cells. The most striking common difference was a deficiency in the GAGs eluting at high salt (Figure 1.3). The profiles from S26 and S27 cells were found to vary in magnitude from experiment to experiment. The experiment shown in figure 1.3
illustrates an S26 profile of the minimal magnitude and an S27 profile of the maximal magnitude. No matter what the magnitude was, all variant profiles were always deficient in labelled GAGs eluting at high salt. The prominent peak in the S27 profile was determined to be CS by degradative analysis (data not shown), but it clearly elutes at lower salt than does the CS from the wild type culture.

AChR clustering on heterokaryons

Two types of heterokaryons were constructed by inducing mixed fusions in cocultures of S27 and S26, and of S27 and S11 cells (Figure 1.4). Both heterokaryons successfully differentiated into myotubes. Examination of the AChR distribution patterns revealed that the majority of myotubes did not show AChR clusters. However, some (about 20%) myotubes did show AChR clusters, and once they showed clusters, the clustering appeared to be as extensive as that observed on wild type myotubes. These results indicate that complementation for AChR clustering has occurred in heterokaryon myotubes.

GAG profiles of heterokaryons

The biochemical analysis of GAGs from parallel cultures revealed that there was recovery of overall GAG synthesis whenever AChR clusters were observed on the heterokaryon myotubes (Figure 1.5.A), and that the profile of the heterokaryon GAGs clearly differed from those of the parent lines (Figure 1.5.B). The characteristics of the recovered peaks were determined by nitrous acid treatment and chondroitinase ABC treatment. While nitrous acid treatment on GAGs from heterokaryon myotubes did not have any effect on GAG profile, chondroitinase ABC treatment degraded most of high salt eluted
GAGs (Figures 1.5.C and D). Both S27xS11 and S27xS26 heterokaryon cultures recovered synthesis of chondroitin sulfates eluting in the same fractions as those of wild type CS (Figures 1.5.A).

Hybrids

Out of 300,000 hybrid colonies, only 4 new hybrid cell lines were isolated on the basis of a recovery in the incorporation of $^{35}$SO$_4$. However, none of the new hybrid cell lines showed AChR clustering. The biochemical analysis of GAGs revealed that hybrid GAG profiles were different from that of C2 cells in all cases (Figure 1.6). The common difference from wild type C2 cells was the peak that elutes at high salt. This is the peak that was determined to be chondroitin sulfate by enzyme digestion experiments (data not shown).
Discussion

The association of GAG and AChR clustering deficient phenotypes in three independent genetic variants of the C2 muscle cell line argues strongly that the two phenotypes are causally linked (Mook-Jung et al., 1993). The profiles of GAGs synthesized in the genetic variants suggest that the defects are each different from the others, as also suggested by earlier characterization of sulfate vs. sugar content (Gordon and Hall, 1989). It is highly unlikely that defects in GAG biosynthesis and in AChR clustering would have arisen together in three independent variant lines. The independence of the variants was tested by forming heterokaryon myotubes in two pairwise combinations. In both cases, both overall GAG biosynthesis and the ability to form spontaneous AChR clusters were recovered. Thus, none of the defects in the variants are dominant. In addition, the complementation indicates that the defect in S27 cells differs from those in S11 and S26 cells. We were unable to form heterokaryon myotubes containing S11 and S26 nuclei and so cannot distinguish their defects by complementation.

The exact mechanism of complementation in heterokaryons is not known. However, it is generally believed that newly synthesized proteins from two distinct nuclei mix together in the trans-Golgi apparatus (Ralston and Hall, 1989). In this way, functional PGs are synthesized in a Golgi apparatus to which multiple nuclei contribute gene products and are subsequently deposited to the cell surface. It is then expected that the local deposition of complemented molecules provides the local rescue of AChR clustering in heterokaryon myotubes. The pattern of rescued AChR clustering is unique in that some myotubes have high levels of AChR clusters while the rest of myotubes do not have any clusters at all. This result supports the possibility of local deposition of complemented molecules.

Even when the CS peak in heterokaryon cultures was at a level close to that of wild
type cultures, the overall number of AChR clusters was much lower compared to that of the wild type. A possible explanation for this nonlinearity is that preexisting variant GAGs competed with the newly synthesized, normal GAGs in a dominant negative fashion. Since modification on GAGs are occuring in trans-Golgi apparatus, some golgi contain the gene products from nuclei in only one variant and some golgi contain the gene products from two distinct nuclei in two variants. The golgi which contains the gene products from two distinct variants could make the rescued form of GAGs which are different from preexisting variant GAGs. This possibility is supported by the observation that heterokaryon myotubes showed increased levels of AChR clustering with longer duration of culture following fusion (data not shown).

None of the hybrid cell lines showed AChR clustering, which is somewhat unexpected. It was reasoned that if a specific enzyme that is involved in GAG biosynthesis is defective in one variant, it could be complemented by a normal gene from another variant. Unfortunately, hybrid cells lose extra chromosomes at random (Blau et al., 1983). Thus, it is likely that complementation in AChR clustering failed for the four hybrid cell lines simply due to loss of other components required for AChR clustering. So newly screened cells can increase the sulfation level while still having defective GAGs, such as short chains, which results in a defect in clustering activity.

Biochemical analysis indicates that the hybrid cell lines synthesize abnormal GAGs. This is consistent with the results from the PG genetic variants (S11, S26 and S27) and the heterokaryon experiment. The hybrid cell lines thus provide more examples in which a PG defect correlates with an AChR clustering defect. Since most glycosylation and sulfation occurs in the trans-Golgi apparatus (Poduslo, 1990), we cannot rule out the possibility that other molecules that go through the trans-Golgi are incorrectly modified. Thus, many instances of correlation between PG defect and AChR clustering defect are required to drive a strong conclusion that intact PGs are necessary for AChR clustering. The present results
demonstrate that PG defects are associated with AChR clustering defects for three PG genetic variants and four hybrid lines and that the recovery of intact PGs is associated with intact AChR clustering. Combined, they provide strong evidence that intact PGs are important for AChR clustering.

The present study further shows that intact CSPGs are associated with the ability to cluster AChRs. All three PG defective variants are deficient in GAGs eluting at high salt. All 4 hybrid cell lines, that did not show AChR clustering, were also deficient in GAGs eluting at high salt. In contrast, both heterokaryon cultures that clustered AChRs synthesized CS eluting at high salt at the same level as wild type C2 cells. No other GAGs showed such consistent correlation with AChR clustering as CS did. The results naturally lead to the possibility that it is CSPG which is important for AChR clustering.
Figure Captions

Figure 1.1. Construction of heterokaryon myotubes and hybrid cells.

Heterokaryon myotubes contain two different types of nuclei which come from two different cell lines. The nuclei deposit their own gene products into the same cytoplasm in heterokaryon myotubes. Hybrid cells are different from heterokaryon in that nuclei from two parental lines are fused and they form a new cell line.

Figure 1.2. Acetylcholine receptor (AChR) distribution in C2 and C2 variants myotubes visualized with rhodamine conjugated α-bungarotoxin (Rh-BuTx).

Spontaneous clusters of AChRs are seen on C2 wild type myotubes (A), but the distribution of AChRs is relatively uniform on the surfaces of the variant myotubes. (B, C, D) Cultures of S27, S11, and S26 myotubes, respectively. Scale=50μm.

Figure 1.3 Cell layer glycosaminoglycans (GAGs) of the C2 variants, S11, S26, and S27.

Cultures were labelled with $^{35}$SO$_4^{2-}$ and GAGs isolated as described in Materials and Methods. GAGs were were eluted from a Mono-Q column (Pharmacia) with a NaCl gradient from 0.05M to 1.75M in 20mM piperazine buffer, pH 5.8, with 0.2% CHAPS. All three variants were deficient in cell layer GAGs eluting at high salt. Profiles of both S26 and S27 were both found to vary in height although only one profile each is shown in this figure.

Figure 1.4. Heterokaryon myotubes formed between variants lines showed AChR clusters.
Heterokaryon myotubes resulting from the fusion of S27 with either S11(A) or S26 (B) showed AChR clusters when labelled with Rh-BuTx. Scale=50μm.

Figure 1.5. Characterization of cell layer GAGs in heterokaryon myotubes.

(A) GAGs in heterokaryon myotubes between S27 and either S11 or S26 were compared with those of C2 myotubes. Both heterokaryon myotube cultures expressed the main chondroitin sulfate peak at 23ml elution volume at wild type levels. (B) GAGs in heterokaryon S27×S11 myotubes were compared with those of the parent lines, S27 and S11. In order to identify the GAGs of the heterokaryon cell layer, nitrous acid treatment (C) and chondroitinaseABC digestion (D) were performed. In preparing samples for digestion, the small highest salt peak at 25ml elution volume was lost. The major GAG peak in the S27×S11 myotubes consisted predominantly of chondroitin sulfate.

Figure 1.6. Characterization of cell layer GAGs in hybrid cells.

The hybrid GAG profiles were different from that of C2 cells in all cases. 1-20-1 and 1-20-10 came from the individual fusions between S27 and S11. 2-17-d came from the fusion between S27 and S26. 3-13-1 came from the fusion between S26 and S11.
Figure 1.1.
Figure 1.3.
Figure 1.5.
Figure 1.6.
CHAPTER TWO

ACETYLCHOLINE RECEPTOR CLUSTERING IN C2 MUSCLE CELLS REQUIRES CHONDROITIN SULFATE

Abstract

Proteoglycans have been implicated in the clustering of acetylcholine receptors (AChRs) on cultured myotubes and at the neuromuscular junction. We report that the presence of chondroitin sulfate is associated with the ability of cultured myotubes to form spontaneous clusters of AChRs. Three experimental manipulations of wild type C2 cells in culture were found to affect both glycosaminoglycans (GAGs) and AChR clustering in concert. Chlorate was found to have dose-dependent negative effects both on GAG sulfation and on the frequency of AChR clusters. When extracellular calcium was raised from 1.8 to 6.8 mM in cultures of wild type C2 myotubes, increases were observed both in the level of cell layer-associated chondroitin sulfate and in the frequency of AChR clusters. Culture of wild type C2 myotubes in the presence of chondroitinase ABC eliminated cell layer-associated chondroitin sulfate while leaving heparan sulfate intact and simultaneously prevented the formation of AChR clusters. Treatment with either chlorate or chondroitinase inhibited AChR clustering only if begun prior to the spontaneous formation of clusters. We propose that chondroitin sulfate plays an essential role in the initiation of AChR clustering and in the early events of synapse formation on muscle.
Introduction

The first functional specialization in the development of the neuromuscular junction is the clustering of receptors for the neurotransmitter acetylcholine (AChRs) (Frank and Fischbach, 1977; Hall and Sanes, 1993). Nerve-associated clustering is thought to result from the release of agrin by the nerve terminal (McMahan, 1990; Nitkin et al., 1987) and the associated binding of agrin to a receptor on the muscle surface (Bowe et al., 1994; Campanelli et al., 1994; Gee et al., 1994; Nastuk et al., 1991; Sugiyama et al., 1994). Such clustering at sites of nerve contact is independent of neural activity (Cohen and Fischbach, 1973; Inestrosa et al., 1983). In addition, muscle cells are capable of forming spontaneous AChR clusters at sites not associated with neural contacts. In vivo, spontaneous clustering occurs prior to innervation or in activity-blocked muscle (Ko et al., 1977). In culture, muscle cells form spontaneous clusters of AChRs that are not associated with presynaptic elements and even do so in cultures devoid of neurons (Anderson and Cohen, 1977; Fischbach and Cohen, 1973; Inestrosa et al., 1983; Peng, 1986). The clusters defined by the aggregated AChRs also contain other components of the postsynaptic specialization including acetylcholinesterase, the 43kD protein, laminin, agrin, and α-dystroglycan (Campanelli et al., 1994; Daniels et al., 1984; Fallon and Gelfman, 1989; Froehner, 1991; Gee et al., 1994; Hoch et al., 1994a; Inestrosa et al., 1983). That the association of postsynaptic molecules occurs spontaneously in the absence of presynaptic contributions provides a reduced system in which to explore the molecular interactions that underlie synapse formation.

A central problem in synaptogenesis is to identify structural molecules that define the postsynaptic specialization by interacting with other molecular components and bringing them together into a macromolecular complex. Proteoglycans are good candidates to play
such a defining structural role. They have been implicated in the clustering of AChRs in culture, as well as in the maintenance of the neuromuscular junction (Ferns et al., 1993; Gordon et al., 1993; Hoch et al., 1994a; Mook-Jung et al., 1993). Monoclonal antibodies to heparan sulfate proteoglycans (HSPGs) localize to both spontaneous clusters and mature junctions (Anderson and Fambrough, 1983; Anderson et al., 1984; Bayne et al., 1984). Acetylcholine esterase is anchored in the junction through a non-covalent association that can be competed with heparin (Brandan and Inestrosa, 1986). And, isolation and characterization of the S27 genetic variant of the C2 mouse muscle cell line has suggested a causal relationship between biosynthesis of glycosaminoglycans (GAGs) and the clustering of AChRs (Gordon and Hall, 1989; Gordon et al., 1993).

Proteoglycans have structures that can support a role in the cross-linking of cell-surface molecules. Some core proteins, such as those of the syndecans, are transmembrane, providing an anchor to the cell surface (Bernfield and Sanderson, 1990; Hardingham and Fosang, 1992) while others are attached via phospholipids (Carey and Evans, 1989; David, 1993; McQuillan et al., 1992) or non-covalent interactions with transmembrane proteins (Hook et al., 1984). The attached glycosaminoglycan carbohydrate chains can be up to 100nm and more in length, providing the reach and multivalency to cross-link extensively other molecules of the cell surface (Lee and Lander, 1991; Paulsson et al., 1987; Yurchenco and Schittny, 1990). Laminin and collagen IV both have binding sites for heparan sulfate (Yurchenco and Schittny, 1990). Indeed, the defect in GAG synthesis in the S27 cell line is associated with a failure to bind components of the basal lamina to its surface (Gordon et al., 1993). Of the different classes of proteoglycans, heparan sulfate proteoglycans are best known to have extensive specific interactions with other molecules. HSPGs bind and present growth factors (Hardingham and Fosang, 1992), and they bind components of the basal lamina such as laminin,
collagen IV and entactin (Timpl, 1993). Such specificity as well as the known localization of HSPGs to the NMJ and their function in the localization of acetylcholine esterase has implicated them in the formation and maintenance of the neuromuscular junction.

We describe here a series of experiments which define an association of chondroitin sulfate (CS) with the ability to form spontaneous clusters of AChRs. This result was surprising given the initial circumstantial evidence for a role of HS. However, other recent reports also suggest specific roles for CS at the cell surface and in cell-cell interactions of the nervous system (Brittis and Silver, 1994; Friedlander et al., 1994; Grumet et al., 1994).
**Materials and Methods**

General cell culture

C2 wild type cells were plated at 5,000 cells/cm² in growth medium (GM) which contained 20% fetal bovine serum, 2.5% chick embryo extract, 100 units/ml penicillin, and 2 mM glutamine in Dulbecco’s Modified Eagle’s Medium with 1 gm/liter glucose (DMEM). When cells reached 80% confluence, they were switched to differentiation medium (DM) which contained 2% horse serum and the same concentrations of penicillin and glutamine as in GM. Cultures were maintained in 100% humidified incubators with 8% CO₂.

Assay for AChR clustering

AChRs were visualized by the binding of α-bungarotoxin conjugated to rhodamine (Ravdin and Axelrod, 1977). Cultured myotubes were incubated in the toxin-containing medium for 30 min at 37°C to label AChRs. The cells were then rinsed with cold PBS and fixed with cold 2% paraformaldehyde, dehydrated in methanol at -20°C, and mounted in buffered glycerol containing p-phenylenediamine. Slides were observed and photographed on a Leitz Aristoplan fluorescence microscope. Clusters per field of view in a Leitz 100X Fluotar plan objective were determined for 25 randomly chosen fields on a 22x22 mm coverslip.

Biochemical characterization of GAGs in C2 cells

C2 cells were cultured in 10 cm dishes in normal media until 12 hours prior to harvesting. At that time, the media was replaced with sulfate-free DM containing ³⁵SO₄ (100 μCi/ml except as noted). In preliminary experiments, we found that sulfate-free
media produced approximately ten-fold higher labelling efficiency than labelling in normal media but that no change was observed in the shape of chromatographic profiles from wild type GAGs. Cultures were harvested in 3 ml of 0.1 M NaOH, and 100 μl aliquots were assayed for protein content (Micro Pierce assay). Nucleic acids were digested with 20 units of DNase and 20 μg RNase per ml (Sigma) with 1 mM MgCl₂ at pH 7 for 2 hours at 37°C. Samples were adjusted to pH 5.5 and 1 mg/ml shark chondroitin sulfate (Sigma) was added as carrier. They were then digested with 2 mg/ml pronase E (Sigma) for 1 day at 37°C on a shaker in order to digest the proteoglycan core proteins. Samples were bound to DEAE-sephacel beads (Sigma) and washed with 0.1 M NaCl in 20 mM piperazine, pH 5.8 to remove free ³⁵SO₄⁻ and other contaminants. GAGs were then eluted with 2 M NaCl in 20 mM piperazine, pH 5.8, followed by ethanol precipitation (Bame and Esko, 1989). The resulting GAGs were resuspended in 20 mM piperazine buffer containing 0.2% CHAPS (pH 5.8) and applied to a 1 ml Mono-Q column (Pharmacia). The applied sample was washed with 10 ml of buffer, and fractions were eluted with a gradient of 0.05 to 1.75 M NaCl in buffer. Pharmacia FPLC pumps, valves, and the fraction collector were controlled by a custom-built Macintosh-based chromatography controller that formed the salt gradients, insured reproducibility of all runs, and allowed for variable fraction sizes. Aliquots of fractions were counted with a scintillation counter and counts per minute of ³⁵SO₄⁻ was calculated per ml of column eluate per mg of protein in the original culture. Counts are reported as per the day of labelling.

Nitrous acid deamination

Heparan sulfates were specifically cleaved at N-sulfated glucosamine residues with nitrous acid (Bame and Esko, 1989; Shively and Conrad, 1976a). GAG samples were prepared through the ethanol precipitation step, then resuspended in 20 mM piperazine.
buffer, and evaporated to dryness. Fresh nitrous acid (pH 1.5) was prepared by the reaction of 0.5 M H$_2$SO$_4$ and 0.5 M Ba(NO$_2$)$_2$ (Sigma) at 0°C. 150 μl were added to the dried samples of GAGs with 50 μg of heparin as a carrier for 10 min at room temperature. The deamination reaction was terminated with 25 μl of 2 M Na$_2$CO$_3$. The pH was then adjusted to 5.8 with H$_2$SO$_4$ prior to column chromatography.

Chondroitinase digestion

Purified GAGs were incubated with 2 units/ml of chondroitinase ABC from Proteus vulgaris (EC 4.2.2.4, Sigma) in 2.5 μM of Tris-HCl, pH 8.0 containing 3 μM of sodium acetate, 5 μg of BSA for 4 hours at 37°C to degrade chondroitin sulfate as well as dermatan sulfate (chondroitin sulfate B) in a final volume of 50 μl. After incubation, reaction mixtures were heated at 100°C for 1 min to inactivate enzymes (Yamagata et al, 1968). Also, chondroitin sulfate A and C were selectively degraded by applying chondroitinase AC from Flavobacterium heparinum (EC 4.2.2.5, Sigma) to purified GAGs in the same way as the chondroitinase ABC digestion procedure except that the pH of the solutions was 7.3.

Chondroitinase ABC treatment of C2 cultures

0.25 units/ml of chondroitinase ABC (Sigma) were added to cultures of C2 cells that had been in DM for one day. DM with chondroitinase ABC was changed once a day until the fourth day of culture. Side-by-side cultures of control and enzyme-treated C2 myotubes were examined for AChR clustering as well as for the profile of GAGs synthesized.

Chlorate and calcium treatment of C2 cultures
C2 cultures were treated with sodium chlorate (Fluka) at 5 to 30 mM or with calcium chloride (Sigma) at 5 mM beginning with the switch from GM to DM. DM with additives was changed daily for the next two days. After a total of 3 days in DM with additives, parallel cultures were either examined for AChR clusters or were extracted to analyze the GAG composition.
Analysis of GAGs synthesized by wild type C2 cultures

We found that GAGs from the cell layers of C2 cell cultures could be effectively separated and analyzed on Mono-Q resin eluted with a NaCl gradient from 0.05 to 1.75 M. GAGs from both C2 myoblast and myotube cultures eluted in two main peaks with GAGs from myoblasts eluting at lower salt (Figure 2.1). In addition, considerably more $^{35}$S$\text{SO}_4$ per mg of protein was found in the myotube culture, suggesting an upregulation in the synthesis of GAGs with differentiation. The nature of the two main peaks was examined with techniques that specifically degrade either heparan sulfates or chondroitin sulfates (Figure 2.2). Nitrous acid treatment of C2 GAGs degraded the low salt peak (Figure 2.2.A), indicating that it consisted of heparan sulfates while chondroitinase ABC treatment of a parallel sample degraded the high salt peak (Figure 2.2.B), indicating that it consisted of chondroitin or dermatan sulfates. Treatment with chondroitinase AC also degraded the high salt peak (data not shown), indicating that it did not contain appreciable pure dermatan sulfates. Successive treatment of the same sample first with chondroitinase ABC and then with nitrous acid effectively degraded both peaks (Figure 2.2.C), indicating that both procedures are efficient. Even with double digestion and extended incubations at 37°C, we were unable to completely reduce material in the CS peak to disaccharides. The failure to fully reduce CS chains may reflect either a technical problem in the enzyme digestion or a resistance to chondroitinase within specific domains of some CS chains. That chondroitinase digestions basically worked is reflected in the effective loss of the high salt peak, the reproducibility of our experiments, and the complete loss of CS when live cultures were digested with chondroitinase (see below and Figure 2.6). In the remainder of
this report, we will refer to the material eluting from Mono Q at high salt as CS, noting here the possibility of some unusual substructure.

Chlorate inhibits the sulfation of GAGs as well as the clustering of AChRs

We attempted to chemically manipulate the profile of GAGs synthesized in wild type C2 cells. Chlorate is an effective inhibitor of the activation of free sulfate to the high energy phospho-adenosylphosphosulfate, a required step for the transfer of sulfate to target molecules (Baeuerle and Huttner, 1986) and has proved to be a specific and non-toxic tool with which to study the role of sulfated GAGs in cellular function (Humphries and Silbert, 1988). Cultures of wild type C2 cells were treated with different concentrations of NaClO₃ beginning at the transfer to DM and continuing for three full days. Chlorate caused both reduction in the overall incorporation of label into GAGs and shifts toward elution at lower salt in a dose-dependent manner (Figure 2.3.A). Sulfated GAGs synthesized in the presence of 5 mM chlorate were found to be insensitive to chondroitinase ABC digestion (data not shown), in agreement with previous findings that chlorate preferentially affects CS (Humphries and Silbert, 1988). Nonetheless, undersulfated GAGs are still made. Chlorate also reduced the frequency of AChR clusters in a dose-dependent manner (Figure 2.3.B), indicating a requirement for proper sulfation of GAGs in the clustering of AChRs. Culture in the presence of chlorate for only the last 12 or 24 hours had no effect on the frequency of AChR clustering.

Calcium increases both AChR clustering and cell layer chondroitin sulfate
Calcium has been known to play a role in the clustering of AChRs, although the mechanism of its action has remained largely unexplored (Bloch, 1986; Bursztajn et al., 1984; Inestrosa et al., 1983; Peng, 1984). We have found that raising extracellular calcium in cultures of wild type C2 cells by 5 mM from 1.8 mM to 6.8 mM for as little as 6 hours prior to assay increased the frequency of AChR clusters. Culture in 6.8 mM calcium for 1 days raised the number of receptor clusters per field at 100X from 9.8±1.7 (s.e.m.) in controls to 36.7±3.0 (s.e.m.) in treated cultures (Figure 2.4). GAGs analyzed from cell layers of cultures incubated in the presence of 6.8 mM calcium for 1 day showed a specific increase in chondroitin sulfate content (Figure 2.5).

Chondroitinase ABC digests cell-associated chondroitin sulfate and prevents the formation of AChR clusters.

That two experimental manipulations should affect both cell layer CS and AChR clustering in concert suggested that CS might play an essential role in AChR clustering. We tested directly the hypothesis that CS is required for AChR clustering by treating cultures of differentiating wild type C2 cells with chondroitinase ABC. Cultures were induced to differentiate by incubation in DM for one day. Chondroitinase ABC was then added in fresh DM for each of the next two days. On the following day, parallel cultures were assayed for cell layer GAGs and for AChR clustering. Cultures were labelled with \( ^{35} \text{SO}_4 \) (200 μCi/ml ) in sulfate-free DM for the last 12 hours of enzyme treatment. The resulting GAGs eluted with the HS peak and virtually no label was present in the CS peak (Figure 2.6). Parallel cultures treated with chondroitinase ABC for the same 2 day period showed practically no clustering of AChRs (15.4±1.8 clusters per field at 100X in the control vs. 0.1±0.1. clusters per field for the enzyme-treated culture; Figure 2.7).
efficacy of the chondroitinase treatment could be variable, presumably depending on enzyme quality, so we always confirmed that CS was eliminated as assayed by Mono-Q chromatography before examining AChR clustering. We were unable to lower the levels of cell layer heparan sulfate with heparitinase and so could not do the complementary experiment.

Time dependence of Chondroitinase ABC inhibition of AChR clustering

In order to prevent clustering of AChRs, both chondroitinase and chlorate treatments were begun 1 day after the switch to DM and 2 days prior to assay. Treatments begun at 2 or 2.5 days had no effect on the frequency of AChR clustering. Data for a chondroitinase experiment are shown in Figure 2.8. In this experiment, all cultures were labelled with $^{35}$SO$_4$ beginning 1 day post-DM and assayed for either GAGs or clustering at 3 day post-DM. When labelled for 2 days in $^{35}$SO$_4$ rather than the 12 hours of the other experiments reported here, there was a preferential accumulation in the cell layer of CS relative to HS. CS was effectively digested at all times assayed, indicating that the entire pool of CS was accessible to the chondroitinase digestion at all times. Nevertheless, chondroitinase and chlorate treatments reduced clustering only if begun soon after differentiation of myotubes.
Discussion

Our data provide several lines of evidence for a role of chondroitin sulfates in AChR clustering. Two paradigms that manipulate GAGs, culture in chlorate or in calcium, provide correlative evidence that the nature and amount of GAGs in the cell layer are associated with the frequency of AChR clustering. While the calcium paradigm suggested a specific role for CS, a third paradigm, treatment of live cultures with chondroitinase, argues directly that chondroitin sulfates in particular are required for AChR clustering. In addition, we have found that heterokaryon myotubes containing nuclei from pairs of genetic variants defective in different aspects of GAG biosynthesis show complementation rescue of both CS synthesis and AChR clustering (see chapter 1).

C2 cultures incubated in 5 mM chlorate showed an approximately 50% reduction in the frequency of AChR clustering. Chlorate inhibits the activation of free sulfate to the high energy donor PAPS, which is the source for sulfation on GAGs (Baeuerle and Huttner, 1986; Humphries and Silbert, 1988). GAGs assayed from parallel cultures showed a profound redistribution towards lower charge. That 5 mM chlorate should have such a profound effect on the GAGs synthesized and yet reduce AChR clustering by only 50% suggests that in normal cultures there may be an excess of those GAGs and PGs required for AChR clustering. Higher concentrations of chlorate were able to further reduce sulfation of GAGs and AChR clustering. Because PAPS is a general sulfate donor, this experiment taken alone cannot rule out the possibility that effects of chlorate on the sulfation of tyrosine or glycolipids (Huttner, 1988; Mintz et al., 1994) might instead be responsible for the inhibition of AChR clustering.

Increasing extracellular calcium by 5 mM was found to increase both cell layer CS and clustering of AChRs. HS appeared to be unaffected, suggesting the hypothesis that in
both the chlorate and calcium experiments, it is the level of a CS in the cell layer that is associated with AChR clustering. Calcium has been shown previously to be required for the clustering of AChRs (Bloch, 1986; Bursztajn et al., 1984; Inestrosa et al., 1983; Peng, 1984) as well as for the binding and response to agrin (Nastuk et al., 1991). Inestrosa et al. (1983), Bursztajn et al. (1984), and Bloch (1986) found that AChR clusters on cultured rat myotubes could be dispersed by removal of calcium. In addition, the latter author reported that increasing extracellular calcium to 15 mM caused an increase in the size but not the frequency of AChR clusters. We have not measured the size of AChR clusters in our cultures of C2 myotubes, but the frequency is quickly and clearly increased in 6.8 mM extracellular calcium. It is possible that this reflects a species difference between rat and mouse in the response of myotubes to increased calcium. It is interesting that Bursztajn (Bursztajn et al., 1991) has also found that calcium causes increased deposition of extracellular matrix. Agrin, in particular, binds to the muscle cell in a calcium-dependent process (Nastuk et al., 1991), and the action of agrin requires calcium (Wallace, 1988). Calcium has not generally been associated with the regulation of glycosaminoglycans although it has been reported to regulate the turnover of heparan sulfate proteoglycans in a rat parathyroid cell line (Takeuchi et al., 1990; Takeuchi et al., 1992). Calcium may act on C2 cells either through intracellular signalling mechanisms or through an extracellular mechanism. One possibility is that calcium might stabilize cell surface CS and hence increase its presence in the cell-associated pool that we have examined. In particular, calcium might stabilize the interactions of CS with laminin, agrin, and other components of a clustering complex.

The hypothesis that CS is required for AChR clustering was tested directly by incubating live cultures in chondroitinase. We were able to achieve apparently complete and specific digestion of cell layer CS that associated with an absence of AChR clusters. That
the digestion was specific for CS is indicated by the lack of an effect on cell layer HS and the continued ability of the C2 myotubes to express AChR that bound α-bungarotoxin on their cell surfaces.

Taken together, the association of GAGs and particularly CS with AChR clustering in three experimental manipulations argues strongly for a causal link between CS and AChR clustering. The chlorate paradigm argues simply for a sulfation requirement, but the remaining two paradigms identify CS specifically, one by its selective increase in CS and the other by its selective elimination of CS. While the chlorate and calcium paradigms might directly affect molecules other than GAGs, the chondroitinase paradigm targets CS GAGs specifically.

Both chlorate and chondroitinase digestion of live cultures inhibited the production of AChR clusters when the treatments were begun soon after differentiation. It is interesting that treatments begun after 2 days of differentiation, after clusters of AChRs had formed, did not eliminate the clusters. One possible explanation is that CS is involved only in the formation of the clusters and that a secondary process stabilizes the clusters and makes them independent of the original CS. A second possibility is that normal CS synthesized prior to the intervention becomes complexed in the AChR clusters in a form that is long-lasting and resistant to chondroitinase ABC digestion. Such a population of chondroitinase-resistant chondroitin sulfates would have to be a very small fraction of the total as we were able to largely digest total CS even in cultures labelled for 2 days in $^{35}$SO$_4$. We previously reported that we were unable to disrupt AChR clusters with 20 mM chlorate (Gordon et al., 1993). With the present results and in hindsight, it appears that the timing of the chemical and biochemical intervention is crucial to obtaining an inhibition of clustering.
The clustering of AChRs on cultured myotubes has served as an important model system for early events in synapse formation. That chondroitin sulfate proteoglycans (CSPGs) play an important role in this process raises two interesting possibilities. The CSPGs may play either direct roles in the process of molecular aggregation or indirect roles by mediating or modulating the signalling response of a factor. CSPGs have the potential to provide both the reach and the multivalency to aggregate directly other molecules into a 2-dimensional complex. In particular, CS chains have a highly repetitive structure and known interactions with matrix in the nervous system (Friedlander et al., 1994) while GAGs in general can have lengths of over 100 nm (Paulsson et al., 1987). Even if CS mediates the aggregation of extracellular molecules, how could such a complex formation be involved in the aggregation of AChRs? It is well established that unclustered AChRs are mobile within the plane of the membrane (Young and Poo, 1983) and that they diffuse into traps consisting of other molecules at the muscle cell surface (Gordon et al., 1992; Kidokoro and Brass, 1985; Stollberg and Fraser, 1988; Stollberg and Fraser, 1990b). A possible explanation of our results is that CS plays an essential cross-linking role in the formation of an extracellular macromolecular complex and that calcium stabilizes the complex. The complex could then be associated with transmembrane molecules such as dystroglycan that link basal lamina components, including laminin and agrin, to the cytoskeleton (Hoch et al., 1994a; Sealock and Froehner, 1994). The resulting transmembrane complex could then trap mobile AChRs at any or all of the extracellular, membrane, and intracellular levels through as yet undescribed mechanisms. Alternatively, CSPGs could bind a factor such as agrin and play a supporting role in presenting the factor to a signal-transducing receptor, much like that known for HSPGs in the presentation of FGF to its receptor (Rapraeger et al., 1991). For example, the CS chains may be present on α-dystroglycan and could play a role in the presentation of agrin to a signal-transducing...
receptor. More complicated variations are also possible where the CS is actually on the signal-transducing receptor or on a third molecule.

Our data suggest that CS is required only in the initial events of AChR cluster formation. When treatment with either chondroitinase or chlorate was begun after cluster formation, there was no reduction in the frequency of clusters. Such a result is consistent with either direct or indirect roles of CS. In the direct hypothesis, CS may bring together other components of the cell surface that, once in close proximity, become tightly associated in a way which is independent of CS. In the indirect hypothesis, CS may simply mediate signal transduction events that give rise to AChR clusters that do not involve CS per se.

In order to establish the role(s) of CS in the process of AChR clustering, it will be important to determine whether a specific structure within the population of chondroitin sulfates is required for clustering. In this regard, it is interesting that chondroitin-6-sulfate is found over the entire muscle fiber surface while an N-Acetylgalactosaminyl transferase is localized to the neuromuscular junction (Scott et al., 1990), suggesting the possibility of specific synapse-associated modification of chondroitin sulfates. It will also be important to determine the core proteins to which the relevant CS chains are attached on muscle cells as well as the other molecules of the muscle cell surface with which they interact.
Figure Captions

Figure 2.1. Glycosaminoglycans (GAGs) accumulate in cell layers of C2 myotubes much more readily than they do in cultures of myoblasts.

Cultures were labelled with $^{35}$S0$_4$ (100 µCi/ml) and GAGs were isolated as described in Materials and Methods. GAGs were eluted from a Mono-Q column (Pharmacia) with a NaCl gradient from 0.05 mM to 1.75 mM in 20 mM piperazine buffer, pH 5.8, with 0.2% CHAPS. The peaks in the elution profile shift toward higher salt elutions with differentiation.

Figure 2.2. Identification of GAGs in cell layers of differentiated C2 cells.

(A) Nitrous acid specifically degraded the peak eluting at lower salt, indicating that the first major peak consists mainly of heparan sulfate. (B) Chondroitinase ABC digestion specifically degraded the peak eluting at higher salt. Chondroitinase AC digestion is not shown but had the same effect as chondroitinase ABC, indicating that the second major peak consists mainly of chondroitin sulfate. (C) Chondroitinase ABC and nitrous acid were applied successively to the same sample to confirm the reaction efficiency. The two treatments together effectively degraded both major peaks.

Figure 2.3. Chlorate inhibited the sulfation of GAGs as well as the clustering of AChRs.

(A) Cultures of C2 cells in differentiation medium were treated with different concentrations of sodium chlorate for 3 days, beginning at the switch to DM. Cells were labelled with $^{35}$SO$_4$ (100µCi/ml) for last 12 hours. Only we could detect only newly synthesized GAGs which were labelled with $^{35}$SO$_4$. Sulfation on GAGs was inhibited by chlorate in a dose-dependent manner. (B) Cells were stained with Rh-αBuTx and the
number of AChR clusters were counted per field of view in a 100X Leitz lens. Chlorate inhibited the clustering of AChRs in a dose-dependent manner.

Figure 2.4. Calcium increased the number of AChR clusters.

(A) Rh-αBuTx staining in a culture of C2 myotubes in 1.8 mM calcium. (B) 3 day treatment of C2 cells with 6.8 mM total calcium (5 mM added CaCl₂) in DM. The number of AChR clusters increased more than 3 fold. Scale = 130 μm.

Figure 2.5. Cell layer GAGs from cultures treated with or without 5 mM extra CaCl₂. Experimental cultures were treated with DM containing extra calcium for 24 hours. Calcium-treated cultures specifically accumulated chondroitin sulfate in the cell layer.

Figure 2.6. Cell layer GAGs from cultures of C2 cells treated with or without Chondroitinase ABC.

Cultures of C2 myotubes were treated with 0.25 units/ml of chondroitinase ABC for two days beginning one day after the switch to DM and labelled with $^{35}$SO₄ (200 μCi/ml) for the last 12 hours. Cell layer chondroitin sulfate is effectively removed and heparan sulfate is unaffected in this culture.
Figure 2.7. Chondroitinase ABC treatment prevents the formation of AChR clusters.

AChR distribution in parallel cultures to those examined for GAGs in Figure 2.6. (A) C2 control myotubes without chondroitinase ABC. (B) C2 myotubes treated with chondroitinase ABC as in Figure 2.8. None of the myotubes showed spontaneous clusters of AChRs.

Figure 2.8. Time dependence of chondroitinase ABC inhibition of AChR clustering.

Chondroitinase ABC digestion of C2 cultures labelled for 2 days with $^{35}$S0$_4$ for 12 hours, 1 day, or 2 day eliminated CS but only the digestion for 2 days was effective at inhibiting AChR clustering.
Figure 2.1

Elution Volume (ml)

[NaCl]

C2 myotubes

C2 myoblasts

\[ \text{cpm/1ug of total protein/ml} \]

\[ {^{45}SO}_3 \]
Figure 2.2.
Figure 2.3.
Figure 2.5.
Figure 2.8.
CHAPTER THREE

CHONDROITIN SULFATE IS REQUIRED FOR AGRIN STIMULATION OF AChR CLUSTERING

Abstract

Experimental data indicate that both agrin and chondroitin sulfate (CS) are essential components in the process of AChR clustering. However, little is known about their interactions. The present study investigated the relationship between agrin and CS on cultured muscle cells. First, the possibility that a CS mediates agrin action on AChR clustering was investigated. Chondroitinase ABC treatment dramatically reduced agrin induced clustering of AChRs. Also, S27 cells, a PG-defective line that normally is not responsive to a low dose of agrin, showed AChR clustering in response to a low dose of agrin, when cultured in medium conditioned by C2 cells cultured in extra calcium. Agrin did not change the profile of GAGs synthesized in C2 cells. These results indicate that agrin requires CS to induce AChR clustering and that agrin achieves this other than by modulating the level of CS.

Second, the relationship between CS and α-dystroglycan (a putative agrin receptor) was investigated. Monoclonal antibody IIH6 which recognizes α-dystroglycan bound to Mono-Q fractions eluting between 0.3M to 0.4M NaCl. When IIH6 immunoprecipitates of sulfate labelled C2 cell extracts were run on SDS-PAGE, a strong smeared band was found that corresponded to the molecular weight of α-dystroglycan (120kD). The band disappeared, however, after treatment with chondroitinase ABC. Similar bands were observed with laminin or agrin affinity-precipitates, but chondroitinase ABC did not abolish the bands but only lowered them by about 20kD. When the IIH6
immunoprecipitate was first digested with pronase to produce individual GAG chains, sulfate labelled materials eluted from Mono-Q as two distinct CS peaks. These results indicate that the IIH6 immunoprecipitate contains CS and suggest that α-dystroglycan may be a CSPG or associated with a CSPG.
Agrin induces clustering on muscle cells of AChR and other synaptic components such as laminin, acetylcholinesterase, and the 43kD protein (Nastuk and Fallon, 1993; Nitkin and Rothschild, 1990). Agrin is found in the synaptic basal lamina and is secreted from both motor neurons and muscles in tissue-specific isoforms (Ferns and Hall, 1992; Lieth et al., 1992; Magill and McMahan, 1988). It is a potentially key component in organizing and maintaining the specialized postsynaptic membrane. Despite its important roles, the mechanisms of agrin action are not well understood. A cascade of molecular processes probably mediates the action of agrin. Several lines of evidence suggest that proteoglycans may be one component of the action of agrin in AChR clustering. Exogenous heparin and heparan sulfate inhibit both agrin- and nerve-induced AChR clustering (Hirano and Kidokoro, 1989; Wallace, 1990). PG defective variants that fail to form spontaneous clusters of AChRs showed reduced sensitivity to exogenous agrin (Ferns et al., 1993). In addition, α-dystroglycan (see below) in PG- defective variants showed weaker binding to agrin (Sugiyama et al., 1994). Investigating the relationship between agrin and PGs may therefore shed light on the molecular mechanisms of agrin action. Considering that a CS plays an essential role in AChR clustering (see chapter 1 and 2), it is of particular interest to examine the relationship between agrin and CS. In the first part of the present study, a possible role of CS in mediating agrin action was investigated.

Agrin action is likely to be mediated by specific signal-transducing receptors. Identification and characterization of putative agrin receptors are therefore important steps toward understanding the molecular mechanisms underlying AChR clustering. Recent results strongly suggest that α-dystroglycan binds agrin: the protein sequence of isolated agrin receptors from Torpedo electric organ has high homology (more than 90% at the amino acid level) to human α-dystroglycan sequence (Bowe et al., 1994); The C-terminal
domain of agrin contains a domain with high homology to the laminin G domain. This domain binds to α-dystroglycan, and is near the active site for AChR clustering (Gee et al., 1994); purified α-dystroglycans from rabbit skeletal muscle and C2 mouse muscle cell extracts bind to 125I-agrin in a ligand blotting assay (Gee et al., 1994); and agrin binds solubilized α-dystroglycan in an agrin-conjugated beads assay (Campanelli et al., 1994). Thus, characterization of α-dystroglycan may provide crucial information regarding the mechanisms of agrin action.

Muscle-synthesized α-dystroglycan is known to bear extensive glycosylation. While it is clear that the molecule bears a single chain of polysialic acid (Ervasti and Campbell, 1993), the nature of the remaining carbohydrates has been a mystery. Consideration of the following findings suggest that the unknown carbohydrates of α-dystroglycan may actually contain a CS. The unidentified carbohydrates on α-dystroglycan are required for calcium-dependent binding of laminin (Ervasti and Campbell, 1993), and calcium increases both CS and the frequency of AChR clusters (chapter 1 and 2). Furthermore, there is a possible chondroitin sulfate attachment site on an extracellular domain of α-dystroglycan. It is thus quite plausible that α-dystroglycan is a proteoglycan that bears a chondroitin sulfate chain on it. A series of experiments was performed to investigate this possibility.
Materials & Methods

Agrin

The agrin which was used for the present study was a gift of Dr. M. Ferns (Ferns et al., 1992). It contains an 8 amino acid insert in the C-terminal half of the protein (agrin8). It is reported (Ferns et al., 1992) that this agrin is capable of inducing clusters on S27 myotubes. This agrin was expressed in transiently transfected COS cells. A 1:1000 dilution of agrin transfected COS cell supernatant with DM was used for all S27 cultures. Seven different concentrations (1:50 to 1:5000 dilution) were used for chondroitinase ABC treatment with agrin.

Cell culture for conditioned medium treatment

C2 and S27 cells were plated on 6 well plates. C2 cells were grown in DM for 3 days with or without addition of 5mM extra calcium for the last day of culture. On the third day, the supernatant from C2 culture (calcium-treated or -untreated conditioned medium) was diluted 1:1 with DM and then added to cultures of S27 myotube with or without agrin. After overnight incubation, AChRs were visualized with rhodamine conjugated α-bungarotoxin (see Materials and Methods of Chapter 1).

Cell culture for chondroitinase ABC treatment

About 2x10⁴ C2 cells were plated on 12mm round coverslips in 24 well culture plates. When cells were beginning to form myotubes (second day in DM), 2μl of chondroitinase ABC stock (Sigma, 50u/ml) were added to 300μl DM, and culture was
continued for two more days. Several different concentrations of agrin (1:50 to 1:5000 dilutions) were added to the DM one day before staining. After overnight incubation, AChR clusters were visualized with rhodamine conjugated α-bungarotoxin (see Materials and Methods of Chapter 1).

GAG isolation and column elution

GAG isolation and elution on Mono-Q ion exchange column were done as described in Materials and Methods of Chapter 1. To examine the agrin effect on GAG synthesis, high concentration of agrin (1:100 dilution) was added to DM culture on the third day. After overnight incubation, GAGs were isolated and fractionated on Mono-Q resin. To examine the relationship between α-dystroglycan and PG, cell extracts were immunoprecipitated with IIH6, a monoclonal antibody against α-dystroglycan (obtained from Drs. S. Roberds and K. Campbell), and then incubated with pronase E (sigma, 3mg/ml) overnight at 37°C to digest the core protein. The resulting material was then fractionated on Mono-Q resin with a salt gradient (0.05M to 1.75M).

Purification of total PGs and immunoblotting

C2 myotubes were sulfate-labeled by adding H$_2^{35}$SO$_4$ (100μCi/ml) to the culture medium and incubated for 16 hours. After three extensive washings with PBS, cells were extracted in a solution containing 6M urea, 1% CHAPS, 20mM piperazine, and a protease inhibitor cocktail (2μg/ml aprotinin, 2μg/ml leupeptins, 100μg/ml PMSF, 500μM bezamidine and 10mM NEM) at pH 5.8. After ultracentrifugation at 100kG for 1 hour, the supernatant was applied to a Mono-Q column and eluted with a salt gradient between...
0.05M and 2M. Each 250μl fraction was applied to nitrocellulose and immunoblotted with IIH6, laminin (90ng/ml) followed by a polyclonal antibody to laminin (E-Y laboratories, inc.), or agrin followed by a monoclonal antibody to agrin (Stressgene co.). Secondary antibodies conjugated to alkaline phosphatase (Sigma) were applied for colorimetric visualization.

Immunoprecipitation with IIH6, laminin and agrin

C2 myotubes were sulfate-labeled by adding H235SO4 (100μCi/ml) to the culture medium overnight. The culture supernatant was collected in the presence of a protease inhibitor cocktail (see above) and the pH was adjusted to 12 with 10M NaOH. Following overnight incubation at 4°C with gentle rotation, the culture supernatant was ultracentrifuged at 100kG for 1 hour. 0.7ml of the centrifuge supernatant was diluted with 0.7ml of 2x laminin binding buffer (LBB; 280mM NaCl, 2mM CaCl2, 2mM MgCl2, 20mM TEA, pH7.6) (Ervasti and Campbell, 1993) and incubated with IIH6 conjugated beads (15μl), laminin conjugated beads (15μl; obtained from Dr. K. Campbell) or agrin conjugated beads. Anti-agrin (1:1000 dilution) was attached to 50μl protein G (Pharmacia) and agrin was attached to anti-agrin by overnight incubation in 1xLBB (140mM NaCl, 1mM CaCl2, 1mM MgCl2, 10mM TEA, pH7.6). As a control, polyclonal antibody against perlecan (obtained from Dr. J. Hassell) was used following the same method as agrin preparation. Beads were washed 5 times in 1xLBB. 2μl of chondroitinase ABC stock (50u/ml) was added to the beads and incubated at 37°C for 4 hours. The materials with or without chondroitinase ABC treatment were loaded on SDS-PAGE (4-15% gradient gel) followed by autoradiography.
Effect of chondroitinase ABC on agrin-induced AChR clustering

To test whether or not CSPGs are necessary for agrin-induced AChR clustering, seven different concentrations of agrin between 1:50 and 1:5000 dilution were applied to chondroitinase ABC treated C2 cells (0.1 unit/300µl of chondroitinase ABC). Agrin increased AChR clustering in a dose-dependent manner for the control group while chondroitinase ABC treatment dramatically reduced the frequency of AChR clustering at all agrin concentrations (Figure 3.1). AChR clustering of the chondroitinase ABC treated cultures was even below that of control C2 cultures for all concentrations of agrin tested.

Effect of C2 conditioned medium on AChR clustering of S27 cells

Agrin dramatically increases AChR clustering in C2 cells, but has reduced potency on S27 cells (Ferns et al., 1993). To test whether or not the supernatant of C2 cell cultures contains materials necessary to restore agrin sensitivity on S27 cells, C2 conditioned medium (CM) (either 5 mM extra calcium-treated or -untreated) was applied to S27 cells. C-terminal agrin expressed in COS cells was diluted 1:1000 in DM and then applied to S27 cultures. The agrin dose was determined based on a test of a dose-response relationship that indicates that agrin is ineffective in inducing AChR clustering on S27 cells at or above 1:1000 dilution (data not shown). AChR clustering was observed when the calcium-treated CM and agrin (1:1000 dilution) were applied together to S27 cultures (Figure 3.2.D). Any treatment that lacked one or more of three components did not induce clustering on S27 myotubes. These include 1) CM with normal calcium alone (Figure 3.2.A), 2) agrin alone (1:1000 dilution; Figure 3.2.B), and 3) calcium alone (5mM; Figure 3.2.C). CM with
normal calcium and agrin showed few clusters on S27 myotubes (data not shown). Agrin alone induced some clustering on S27 cells, but only when applied at very high concentrations (1:100 dilution; data not shown).

Effect of agrin on GAG biosynthesis

The chondroitinase result above indicates that CSPGs are necessary for agrin-induced AChR clustering, suggesting the possibility that CSPGs might mediate the response to agrin. One possibility is that agrin might increase the expression of CS in analogy to the effect of calcium (see Chapter 2). The effect of agrin on GAG biosynthesis was therefore investigated. A very high concentration of agrin (1:100 dilution) did not change the GAG profile of C2 or S27 cell extracts on Mono-Q ion exchange column chromatography. Calcium treatment (5mM) did not change the Mono-Q GAG profile of S27 cell extracts either (Figure 3.3).

Assessment of negative charge on α-dystroglycan

If α-dystroglycan is a PG, it is expected to be negatively charged due to the presence of sulfated GAGs and should bind as an intact molecule to Mono-Q resin. After cell extracts were eluted from a Mono-Q ion exchange column, IIH6, monoclonal antibody to α-dystroglycan, laminin, and agrin binding to each fraction was tested using an immunoblotting technique. This was to find out which fractions contain α-dystroglycan, since it is known to bind laminin and agrin in a calcium dependent manner (Ervasti and Campbell, 1993). All three molecules (α-dystroglycan antibody, agrin and laminin) bound to specific column fractions eluting between 0.3M and 0.4M NaCl (Figure 3.4, horizontal bar). Also, binding of agrin and laminin was calcium-dependent (data not shown). The
results indicate that α-dystroglycan bears a negative charge, suggesting that it might be a PG.

Immunoprecipitation with IIH6

To further investigate the possibility that α-dystroglycan might be a PG, sulfate labelled cell supernatants were immunoprecipitated with the IIH6 antibody and run on SDS-PAGE. Subsequent autoradiography showed a strong smeared band for C2 cell culture conditioned medium. As shown in lane 1 of Figure 3.5.A, the strong band above 120kD is close in molecular weight to that of α-dystroglycan. The band was broad and smeared, which is the typical pattern for a proteoglycan band on SDS-PAGE, suggesting that GAG chains are likely to be contained in the band. When the IIH6 immunoprecipitate was treated with chondroitinase ABC before loading on SDS-PAGE, the band was not observed (Figure 3.5.A, lane 2). This result indicates that the band contains CS chains.

As expected, IIH6 immunoprecipitation resulted in a quite different pattern from S27 culture conditioned medium. Autoradiography showed a weak smeared band at about 80kD for S27 cells (Figure 3.5.B, lane 2). The band was much weaker than that from C2 culture supernatant. Immunoprecipitation with perlecan antibody only showed a very high molecular weight band (about 400kD, Figure 3.5.B, lane 1) indicating that immunoprecipitation of 35SO₄-labelled material around 120kD is specific to IIH6. Since perlecan contains only heparan sulfate chains on core protein, it could be seen with 35SO₄ labelling and the band still remained with chondroitinase ABC digestion (data not shown). The IIH6 35SO₄-labelled material disappeared when the immunoprecipitate was treated with chondroitinase ABC before loading on SDS-PAGE (Figure 3.5.B, lane 3).
Affinity-precipitation with laminin and agrin

Since α-dystroglycan binds to laminin and agrin, the sulfate-labeled band observed with IIH6 immunoprecipitation is expected to be present with laminin or agrin precipitation. As expected, when sulfate labelled C2 cell culture conditioned medium was affinity-precipitated with agrin (attached to agrin antibody followed by protein G) or laminin conjugated beads, a sulfate labelled band was observed at about 120kD on SDS-PAGE as in the IIH6 immunoprecipitation experiment (data not shown). Chondroitinase ABC treatment of agrin or laminin immunoprecipitates did not abolish the band but only lowered it by 20kD. A strong smeared band still existed, suggesting that CS in the band was protected by laminin or agrin binding so that it was partially digested. Binding affinities for agrin and laminin are much higher than that of IIH6 for α-dystroglycan (Kd is 10^{-9} M vs 10^{-7}M, personal communication with Dr. Campbell), so it might be expected that chondroitinase ABC is less effective in attacking CS in the agrin or laminin immunoprecipitate than in the IIH6 immunoprecipitate.

Column elution of sulfated materials in IIH6 immunoprecipitate

In order to examine the sulfated carbohydrates on α-dystroglycan, the core proteins in the IIH6 immunoprecipitate were digested with pronase E and then the remaining materials were applied to Mono-Q resin. Sulfate-labelled material was eluted as two distinct peaks that match the chondroitin sulfate peak in the C2 whole GAG profile (Figure 3.6). This result corroborates that the IIH6 immunoprecipitate contains CS chain(s).
Discussion

Two major conclusions can be drawn from the present study. First, a CSPG mediates the effect of agrin on AChR clustering. Second, α-dystroglycan is closely associated with CS. The first conclusion is supported by two separate experiments. Direct evidence is provided by the result that agrin-induced AChR clustering is blocked by chondroitinase ABC treatment of live cultures. Although indirect, the conditioned medium experiment also provides supporting evidence. The S27 cells are defective in PG biosynthesis and AChR clustering. They do not normally respond to a low dose of agrin to form AChR clusters. When S27 cells were cultured in calcium-treated C2 CM, however, AChR clustering was induced in response to a low dose of agrin. Since S27 is defective in PG biosynthesis, it is likely that PGs in the CM are responsible for the restoration of agrin responsiveness, although alternative possibilities cannot be ruled out. Interestingly, C2 CM with normal calcium was less effective. Extra calcium treatment to the C2 culture selectively increases the very high salt eluting CS that might be important for AChR clustering (Chapter 2). Hence, the key molecule in the CM is likely to be a CS. Combined, the present results argue strongly that a CSPG is necessary for agrin to be effective for AChR clustering. In addition, the present results establish a paradigm in which to investigate important molecules for AChR clustering. Unlike calcium, agrin did not affect the level of CS in C2 myotubes. This result indicates that agrin does not exert its effect on AChR clustering by modulating the level of CS.

The second conclusion is supported by converging results from several experiments. First, the α-dystroglycan-containing portion of the C2 cell extracts is negatively charged, suggesting that it may be a PG. Second, SDS-PAGE shows a typical PG sulfated band pattern (broad and smeared) for IIH6 immunoprecipitation of the C2 cell culture conditioned medium. As expected, the band was at the same molecular weight as α-
dystroglycan. The band disappeared after treatment with chondroitinase ABC. This result indicates that the sulfate-labelled band on SDS-PAGE is CS, providing direct evidence that α-dystroglycan is closely associated with CS. Third, purified GAGs from the IIH6 immunoprecipitate eluted as peaks that correspond to a CS peak on Mono-Q resin. These results indicate that the IIH6 immunoprecipitate contains CS. The simplest interpretation of the results is that α-dystroglycan is a CSPG. However, we cannot rule out the possibility that α-dystroglycan is not a CSPG but that IIH6 immunoprecipitates a CSPG in association with α-dystroglycan. Since IIH6 is a monoclonal antibody to α-dystroglycan, it is highly unlikely that IIH6 binds to a separate protein. Alternatively, a CSPG may be tightly linked to α-dystroglycan so that the immunoprecipitate contains both α-dystroglycan and a CSPG. This possibility, however, requires another assumption that the putative CSPG must have a close molecular weight to α-dystroglycan, since the sulfate labelled band was at a position (120 kD) on SDS-PAGE that corresponds to the molecular weight of α-dystroglycan.

Affinity-precipitation with agrin or laminin shows a sulfate labelled band at the same molecular weight as α-dystroglycan. However, chondroitinase ABC effects on agrin or laminin affinity-precipitates are quite different from those on IIH6 immunoprecipitates. Chondroitinase ABC treatment lowers the sulfated band by about 20kD. Laminin has much higher affinity to α-dystroglycan than does the α-dystroglycan antibody IIH6 (Kd is $10^{-9}$ M vs $10^{-7}$M respectively) (Dr. Campbell, personal communication). It is therefore possible that tight binding of agrin or laminin to α-dystroglycan somehow protects the CS chains from chondroitinase ABC attack. The simplest interpretation is that α-dystroglycan is a CSPG and a CS chain on α-dystroglycan is actually the binding site for agrin and laminin. Tight binding of agrin or laminin to α-dystroglycan may leave only part of the CS chain available for chondroitinase ABC digestion. Likewise, it is possible that α-dystroglycan itself is not a CSPG but is linked to a CSPG and that agrin or laminin binding to α-
dystroglycan induces a conformational change in the CSPG so that most of CS chain is protected.

Protease-digested IIH6 immunoprecipitate eluted as two distinct peaks within the CS profile in C2. It is uncertain why there exist two distinct peaks, but this result raises interesting questions: If α-dystroglycan is a CSPG, does α-dystroglycan contain two different CS chains? Do two different degrees of sulfation occur on one CS chain as differentiation or AChR clustering progresses? Are there two different core proteins? Are both peaks responsible for binding of agrin and laminin? The current available data suggest that the high salt peak is important for the clustering activity since the high salt peak is, like AChR clustering, regulated by calcium. Future research is required to answer these questions.
Figure captions

Figure 3.1. Effect of chondroitinase ABC on agrin-induced AChR clustering.

Agrin alone increased the AChR clustering level in a dose-dependent manner. Chondroitinase ABC treatment dramatically reduced the frequency of AChR cluster at all agrin concentrations.

Figure 3.2. Effect of C2 conditioned medium on AChR clustering of S27 myotubes.

S27 myotubes were cultured by adding (A) C2 conditioned medium (without extra calcium) alone, (B) 1:1000 dilution of agrin alone, (C) 5mM extra calcium alone, and (D) 5mM calcium treated C2 conditioned medium and agrin (1:1000 dilution). AChR clustering was observed only when the calcium treated conditioned medium and agrin were applied to S27 culture (D). AChR clusters were labelled with rhodamine conjugated α-bungarotoxin. Scale = 50µm.

Figure 3.3. Effect of agrin on GAG biosynthesis.

A very high concentration of agrin (1:100) was added to C2 or S27 culture for overnight. GAG profiles of C2 (top) or S27 (bottom) cell extracts on Mono-Q ion exchange column chromatography were not changed after treatment of agrin compared to those of control (without agrin treatment). 5mM calcium treatment did not change GAG profile of S27 cell extracts, either (bottom).

Figure 3.4. IIH6, laminin and agrin bound to specific Mono-Q column fractions.

After 6M urea cell extracts were eluted from Mono-Q ion exchange column with salt gradient (0.05M to 2M), IIH6 (monoclonal antibody against α-dystroglycan), laminin or agrin binding to each fraction was tested using immunoblotting technique. IIH6, agrin and
laminin bound to specific column fractions eluting between 0.3M and 0.4M NaCl (horizontal bar). The level of $^{35}$SO$_4$ from each fraction was counted and plotted to see the column profile of C2 PG.

Figure 3.5. Immunoprecipitation with IIH6.

Sulfate labelled materials were immunoprecipitated with the IIH6 antibody and run on SDS-PAGE. Subsequent autoradiography showed a strong smeared band at about 120kD for C2 culture supernatant (A, lane 1). IIH6 immunoprecipitate with S27 culture supernatant showed a weak smeared band at about 80kD (B, lane 2). When the IIH6 immunoprecipitates were treated with chondroitinase ABC before loading on SDS-PAGE, the band was not observed for both C2 (A, lane 2) and S27 (B, lane 3). As a control, perlecan antibody immunoprecipitate only showed the high molecular weight band at about 400kD (B, lane 1).

Figure 3.6. Mono-Q column elution of sulfated materials in IIH6 immunoprecipitate of C2 culture supernatant.

The proteins of the IIH6 immunoprecipitate were digested with pronase E and then remaining materials were applied on Mono-Q column. Sulfate material was eluted as two distinct peaks that match the chondroitin sulfate peak in the C2 whole GAG profile.
Figure 3.1.
Figure 3.3.
Figure 3.4.
Figure 3.5.
Figure 3.6.
DISCUSSION

Proteoglycans (PGs) can interact with a large variety of macromolecules through their long carbohydrate glycosaminoglycan (GAG) chains. PGs are synthesized by most eukaryotic cells and are present in virtually all mammalian tissue. PGs exist in diverse structures in different tissues, each reflecting its unique biological property. The well-known functions of PGs include acting as co-factors for basic fibroblast growth factor and transforming growth factor, inhibiting neurite outgrowth, and participating in the formation of the extracellular matrix. Considering their ubiquitous distribution, diversity of core proteins, and potential for carbohydrate substructure, PGs are likely to have many more as yet unknown functions. The present dissertation focuses on the possible role of PGs in AChR clustering and identification and characterization of the PGs that are important for AChR clustering. Two conclusions can be drawn from the present study: 1) a chondroitin sulfate proteoglycan (CSPG) plays an important role in AChR clustering, and 2) CS mediates the effect of agrin on AChR clustering.

CSPGs are important for AChR clustering

Several lines of evidence indicate that CSPGs play an important role in AChR clustering. First, a consistent correlation was found between the level of CSPGs and the degree of AChR clustering. When CS biosynthesis was rescued by forming heterokaryons between C2 PG defective variants, AChR clustering resulted. However, when genetic complementation by forming hybrids failed to rescue CS biosynthesis, AChR clustering was not rescued either. Furthermore, treatments that changed the AChR clustering level (calcium and chlorate enhance and reduce clustering, respectively) changed CS biosynthesis in the same direction as AChR clustering. Second, AChR clustering is
blocked by chondroitinaseABC. Thus, both correlational and interventive approaches provide converging evidence that CSPGs are essential for AChR clustering.

CSPG mediates the effect of agrin on AChR clustering

It is now well accepted that a neural form of agrin is released by the motor nerve terminal thereby inducing clustering of AChR receptors at synaptic sites. Agrin treatment of muscle culture increases AChR clustering dramatically even in the absence of the nerve. Thus agrin appears to be an important molecule for AChR clustering. The results of the present study suggest that the effect of agrin on AChR clustering may be mediated by a CSPG. First, Case treatment of live cultures blocks agrin stimulated AChR clustering. Second, S27, a genetic variant that is defective in both PG biosynthesis and AChR clustering, forms AChR clusters in response to small doses of agrin when cultured in calcium treated C2 conditioned medium. It is not known which molecule in the conditioned medium contributed to AChR clustering; however, since S27 was selected based on a defect in PG biosynthesis and calcium increases a CSPG biosynthesis, one likely candidate molecule is a CSPG. Combined, these results suggest that intact CSPG is required for agrin to be effective for AChR clustering.

Interaction between CSPG and agrin

How does CSPG mediate agrin activity? Agrin treatment does not increase the total amount of GAGs. This result indicates that, unlike calcium, the effect of agrin on AChR clustering is not due to an increase in the expression level of CSPG. Rather, it suggests that CSPG somehow mediates agrin activity through molecular interactions.
It would be reasonable to assume that an early step in agrin action is binding to a signalling receptor. The stoichiometry of agrin:AChR is about 1:125 (Bowe et al., 1994), suggesting no direct interaction between agrin and AChR. Also, agrin induces tyrosine phosphorylation on the beta subunit of the AChR prior to the onset of AChR clustering (Wallace, 1992), suggesting that the action of agrin on AChRs is mediated by intracellular signalling.

What role would CSPG play in mediating responsiveness to agrin? CSPG could play a role in the process of signalling or downstream in the pathways from signalling through clustering. In terms of signalling, CSPG could act to facilitate binding of agrin to a signalling receptor by analogy to the basic fibroblast growth factor (bFGF) receptor (see below), or the agrin receptor itself could be a CSPG. Alternatively, CSPG could act downstream, interacting with the agrin-receptor complex participating directly in the clustering process. Some of the present data indicate that CSPG is closely related to agrin receptors. Immunoprecipitation of cell extracts with antibody to \( \alpha \)-dystroglycan shows a sulfate labelled band at the same molecular weight as \( \alpha \)-dystroglycan. After treatment with chondroitinaseABC, the band disappeared. Also, purified GAGs from the \( \alpha \)-dystroglycan antibody immunoprecipitate eluted from Mono-Q resin as two peaks corresponding to chondroitin sulfate. These results allow two possible interpretations. The most parsimonious explanation is that \( \alpha \)-dystroglycan (a putative agrin receptor) is itself a CSPG. This possibility is supported by the presence of a potential CS attachment site within \( \alpha \)-dystroglycan (DLIASSGDIIKVSAAGKE). Another possibility is that \( \alpha \)-dystroglycan is not a CSPG but is tightly linked to CSPG, so that \( \alpha \)-dystroglycan antibody immunoprecipitate contains both \( \alpha \)-dystroglycan and a CSPG.

Molecular basis of AChR clustering
The previous arguments suggest strongly that an early step in agrin action is binding to its receptor and that the receptor is a CSPG or tightly linked to a CSPG that is essential for agrin to be effective. The molecular processes following agrin binding to its receptor are not known. In the following, possible molecular steps for the spontaneous as well as nerve- and agrin-induced AChR clustering will be discussed with the focus on possible interactions among agrin, α-dystroglycan, and other extracellular matrix molecules.

α-dystroglycan is a highly glycosylated extracellular protein. It is a component of the dystrophin related glycoprotein complex (DGC). One function of DGC in skeletal muscle is to link the extracellular matrix to the cytoskeleton. It links to the actin cytoskeleton via dystrophin or utrophin depending on location. DGC interacts with utrophin at the neuromuscular junction but with dystrophin at the extrasynaptic membrane. Utrophin is a dystrophin homolog but encoded by a separate gene. In both mdx mice and Duchenne muscular dystrophy patients which lack dystrophin, DGC is lost in the extrasynaptic membrane, but preserved at the neuromuscular junction, since DGC forms a complex with utrophin at the synaptic sites. Recent studies suggest that the utrophin and α-dystroglycan complex may mediate agrin-induced AChR clustering (Bowe et al., 1994; Campanelli et al., 1994; Gee et al., 1994).

It is already known that α-dystroglycan binds to laminin or agrin extracellularly. α-dystroglycan presumably binds to agrin at the neuromuscular junction and to laminin at the extrasynaptic sites. Intracellularly, the agrin and α-dystroglycan complex binds to utrophin via transmembrane DGC, and the laminin and α-dystroglycan complex binds to dystrophin via DGC (Campanelli et al., 1994). One important question is how α-dystroglycan binds to a specific protein in a specific region. One speculative possibility is that the binding specificity is controlled by the relative expression of α-dystroglycan binding proteins; each region may dominantly express, by unknown mechanisms, one of two types of protein.
(agrin and laminin). Once α-dystroglycan forms a complex with one of these proteins, it may activate a distinct intracellular signal pathway to bind an appropriate cytoskeletal protein to stabilize the complex.

Another important issue is the way that the agrin and α-dystroglycan complex transduces signals intracellularly. One possibility is that the DGC contains kinase activity. Two (β-dystroglycan and adhalin) out of the four transmembrane proteins in the DGC are cloned, and they do not have any kinase activity. The 25kD and 35kD proteins are yet to be cloned, so it is not known whether or not they contain any kinase domain. Another possibility is that there exists another type of high affinity agrin receptor which is in low abundance and so would not be detected with the previous binding experiments. This model is analogous to that for the basic fibroblast growth factor (bFGF) receptor. Heparin or heparan sulfate proteoglycan acts as a cofactor to change the conformation of bFGF to bind to their high affinity receptors. By analogy, α-dystroglycan might bind to agrin through a CS chain and the inserts in splicing site Z which is responsible site for conformational change in agrin (Hoch et al., 1994b) could undergo a conformational change which in turn could allow agrin binding to high affinity signalling receptors. According to this model, the kinase domain in the high affinity receptor initiates intracellular signaling in order to choose appropriate cytoskeletal proteins, such as utrophin, which in turn interact with AChRs.

Spontaneous and nerve-induced clustering: a model

Since muscle agrin and nerve agrin bind to α-dystroglycan with similar affinity (Sugiyama et al., 1994), the mechanisms of spontaneous clustering and nerve- or agrin-induced clustering might be similar. One possible model for the overall clustering
mechanism is presented below. Before the nerve contacts the muscle, only muscle agrin exists and binds to \( \alpha \)-dystroglycan. When the nerve contacts the muscle, the nerve secretes nerve agrin at a high concentration into the synaptic cleft. Newly synthesized \( \alpha \)-dystroglycans at the synaptic site then have a higher chance to bind to the nerve agrin over the muscle agrin. The inserts in splicing site \( Z \), which exist only in the nerve agrin, induce a conformational change for preferential binding to the high affinity receptor. Faster intracellular signaling happens at the synaptic site than extrasynaptic sites, which locally uses up the components required for clustering activity. Hence, spontaneous clusters co-exist with nerve-induced clusters until previously formed spontaneous clusters are degraded. New spontaneous clusters cannot be formed due to the limited availability of clustering components. The physiological role of spontaneous clustering may be the preparation of components which are required for nerve-induced clustering. It can give a fast response to make AChR clusters as soon as nerve contacts muscle.

Future work

Several issues remain outstanding. First, do the two remaining transmembrane DGC proteins (25kD and 35kD DGC) have the kinase activity? Since two transmembrane DGC proteins (\( \beta \)-dystroglycan and adhalin) do not have kinase activity domains, it is important to know whether the remaining proteins contain a kinase activity domain. Second, does the hypothesized high affinity receptor of agrin really exist? If the hypothesis is correct that a CS chain on \( \alpha \)-dystroglycan acts as a cofactor for agrin to present to a high affinity receptor, the \( \alpha \)-dystroglycan/agrin complex might be used for affinity purification of the high affinity receptor. Third, what is the mechanism of action of calcium for clustering activity? If calcium acts outside of the cell, it is important to understand the
interactions among CS, calcium and agrin. Preliminary data indicate that only one specific CS peak elutes at different salt concentrations depending on the calcium concentration in solution, suggesting that only a specific form of CS may interact with calcium. Then, it is interesting to know how calcium recognizes a specific CS. If calcium acts inside the cells, what is activated by calcium for clustering activity? This may be investigated with intracellular calcium chelators such as BAPTA-AM. Fourth, gene blocking with antisense oligonucleotides may provide more direct evidence for the role of α-dystroglycan in AChR clustering. Fifth, if α-dystroglycan is a CSPG, genetic manipulations to induce mutations in its CS attachment can provide a tool to investigate interaction of CS with other basal lamina molecules. Sixth, since it is known that agrin causes phosphorylation on AChR, it would be interesting to know whether or not CS mediates the agrin effect on phosphorylation of AChR. Finally, since the AChR clustering defect on S27 was rescued by calcium treated C2 conditioned medium and agrin, it is important to identify the responsible molecule in the conditioned medium. One approach would be to eliminate candidate molecules from the CM. For example, it would be interesting to examine the effect of case-treated CM and agrin on AChR clustering of S27 cells. These issues require future investigation.

Summary

The present study suggests strongly that 1) a CSPG is essential for AChR clustering at the neuromuscular junction, 2) its role in AChR clustering is to mediate the action of agrin, and 3) the CSPG is α-dystroglycan itself, a putative agrin receptor, or tightly linked to α-dystroglycan. Thus, the present study provides strong constraints as well as insights for elucidation of molecular mechanisms underlying AChR clustering at the neuromuscular junction.
In A and B, α-dystroglycan (α-DG) has a chondroitin sulfate (CS) chain that is the binding site for agrin, whereas α-DG is linked to a CS chain of a separate molecule in C and D. In A and C, agrin binding to α-DG initiates intracellular signalling by activating a kinase domain within the dystrophin associated glycoprotein complex (DGC). In B and D, the agrin/α-DG/DGC complex binds to a functional high affinity agrin receptor (AgR) which initiates intracellular signalling while unbound agrin cannot bind to the high affinity agrin receptor. In each case the intracellular signal activates the association of utrophin, AChR and the 43 kD protein.
Figure 4.1.
REFERENCES


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