

Agrin-induced Clustering of Acetylcholine Receptors: A Cytoskeletal Link

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FORMATION of synaptic connections involves the localized differentiation of pre- and postsynaptic cells as the result of a bidirectional exchange of information molecules during development. At a well-studied synapse, the neuromuscular junction (NMJ)¹, agrin is thought to play an important role in the synaptic differentiation of the muscle induced by the motorneuron. Agrin is an extracellular matrix protein synthesized by motorneurons at the time of synapse formation, which can induce the clustering of synaptic molecules on the surface of culture muscle cells (for review see McMahan, 1990). Molecular analysis has shown that agrin is a family of proteins of ~220 kD that are produced by alternative splicing and that contain a number of structural motifs known from other extracellular proteins (Fig. 1; Rupp et al., 1991; Tsim et al., 1992). Full-length recombinant agrin, as well as truncated soluble forms, induce the clustering of nicotinic acetylcholine receptors (AChR) (Campanelli et al., 1991; Ruegg et al., 1992; Ferns et al., 1992, 1993). AChR clusters also form spontaneously on cultured myotubes, suggesting that the machinery for clustering is contributed by muscle. Although both motorneurons and muscles synthesize agrin, antibody inhibition experiments showed that motorneuron-derived agrin, but not the muscle-derived form, is required for synapse formation in culture (Reist et al., 1992). Here, we review the role of agrin in synaptogenesis, focusing on the structural requirements for agrin activity and implications of the recently described agrin-dystroglycan interaction for models of the mechanism of agrin-induced AChR clustering.

Structural Requirements for Agrin Activity

Multiple regions of the agrin protein contribute to clustering activity. Functional analysis of alternative splice variants reveals that insertions at two sites affect activity. Most important is the presence of an insert at splicing site Z (Fig. 1), which is found exclusively in agrin derived from neurons

(Hoch et al., 1993; O'Connor et al., 1994). An 8-amino acid insert at this position increases agrin activity ~10,000-fold (Ferns et al., 1993), whereas the presence of an unrelated 11-amino acid insert at the same position increases activity 50-fold (Hoch et al., 1994). Both inserts lead to a conformational change in the agrin protein, involving the extreme COOH terminus, which is detected by the appearance of the epitope for a monoclonal antibody, agr 86.

Insertion of a 4-amino acid sequence at the Y splice site has a modest but significant effect on activity (Ruegg et al., 1992; Ferns et al., 1993). Interestingly, the Y site splice entails the inclusion of three basic residues into the second laminin G domain. This region may mediate interactions between agrin and negatively charged cell surface proteoglycans that have been independently implicated in the activity of agrin (see below).

Antibody inhibition studies and deletion analyses of the agrin protein confirm the importance of splice site Z and the second laminin G domain, and they identify two additional regions necessary for clustering activity. Function blocking antibodies that bind the Z splice site insertions and the second laminin G domain have been identified (Hoch et al., 1994). Additionally, the third EGF-like repeat and the extreme COOH-terminal 5 kD of the protein are also required for activity (Hoch et al., 1994; Fig. 1). These studies suggest that the binding of agrin to cell surface components requires four different regions of the agrin protein contained within the COOH-terminal 50 kD (Fig. 1). It is possible that the different regions of the agrin protein required for activity form a single receptor-binding site. This would be in contrast to some other described extracellular matrix (ECM) molecule-receptor interactions that have been defined as short peptide sequences (for review see Yamada, 1991). Alternatively, the four regions of the agrin protein may each interact with different components of the muscle cell surface: the combination of these interactions might confer clustering activity.

Postsynaptic Response to Agrin, Molecules

Several studies have addressed the involvement of second messengers in the activity of agrin. A screen for possible second messengers did not identify any candidates that mimicked agrin's action (Wallace, 1988); however, several manipulations implicate phosphorylation in agrin-induced clustering. Activation of protein kinase C and energy depletion prevent agrin-induced AChR redistribution (Wallace,

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1. *Abbreviations used in this paper:* AChR, acetylcholine receptors; DGC, dystrophin-glycoprotein complex; ECM, extracellular matrix; NMJ, neuromuscular junction.

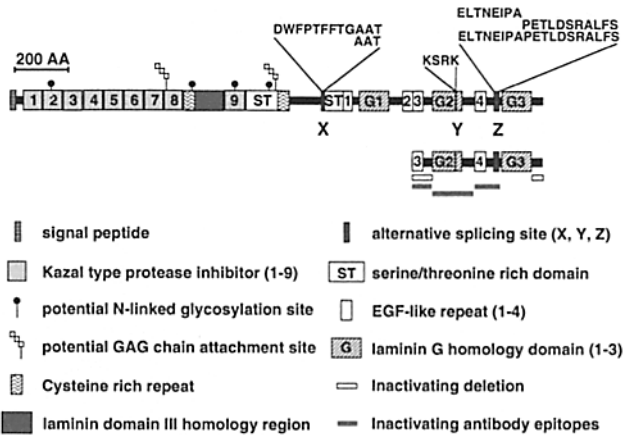


Figure 1. Structural domains of agrin. A schematic of the agrin protein (top) and the minimal fragment necessary for clustering activity (bottom) are shown. The three sites of alternative splicing are denoted X, Y, and Z with the possible insert sequences shown above the full-length protein. Domains within the 50-kD fragment that are proposed to be required for activity are shown below.

1988). Tyrosine phosphorylation of the β subunit of the AChR may be involved in agrin-induced clustering (Wallace et al., 1991). This phosphorylation occurs rapidly after treatment of chick myotube cultures with agrin. Two tyrosine kinases have recently been identified that are associated with the AChR and could be responsible for this phosphorylation (Swope and Haganir, 1993). However, during development of rat muscle, phosphorylation of the AChR was only detected postnatally, well after the formation of synapses is initiated (Qu et al., 1990). Recent experiments with inhibitors of tyrosine kinases suggest that phosphorylation is important for agrin activity, but have not yet identified the essential substrates (Wallace, 1994).

Several lines of evidence implicate proteoglycans in agrin-induced AChR clustering. Muscle cell lines defective in glycosaminoglycan synthesis are significantly less responsive to added recombinant agrin (Ferns et al., 1992, 1993). Furthermore, heparin inhibits both nerve- (Hirano and Kidokoro, 1989) and agrin- (Wallace, 1990) induced AChR clustering. Finally, agrin is a heparin-binding protein (Carnpanelli, 1993), and proteoglycans are among the subset of proteins that redistribute in response to agrin (Wallace, 1989).

An agrin-binding site has been described on the surface of cultured myotubes (Nastuk et al., 1991). This binding is Ca^{2+} dependent and blocked by heparin, as is the agrin-induced AChR clustering (Wallace, 1990). The agrin-binding site is initially distributed over the muscle surface, but it concentrates at AChR clusters after the addition of agrin. Thus, this site displays the characteristics of the proteoglycans described above, and it is likely to represent α -dystroglycan (see below).

Several cytoskeletal proteins are concentrated at AChR clusters (reviewed by Froehner, 1993). Most tightly associated with the AChR is a protein of 43 kD. The 43-kD protein forms aggregates on the surface of *Xenopus* oocytes or fibroblasts when expressed in these cells (Froehner et al., 1990; Phillips et al., 1991). When AChRs are coexpressed with the 43-kD protein, receptors are found concentrated at

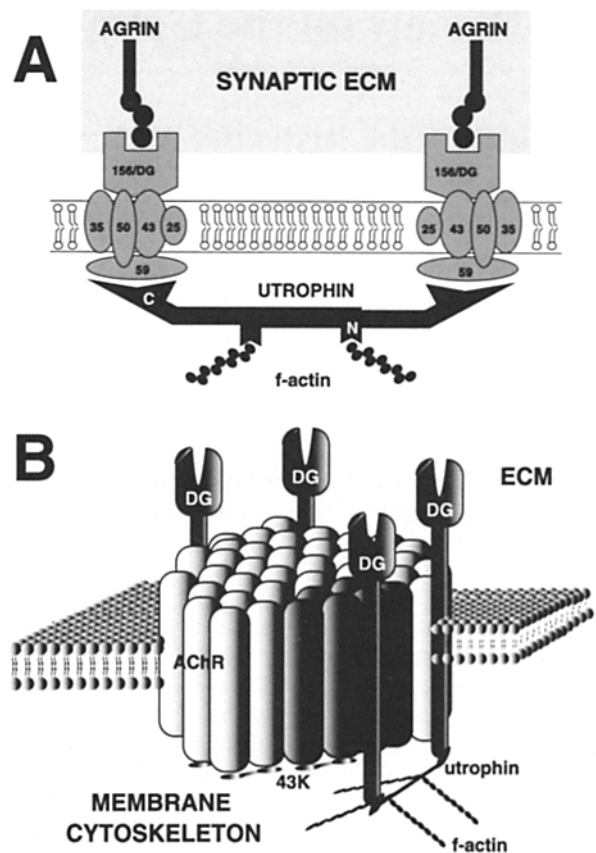


Figure 2. Models of the interaction between agrin, dystroglycan, the cytoskeleton, and AChRs. (A) The interaction of agrin with dystroglycan in the synaptic extracellular matrix, mediated by the transmembrane complex of proteins (DGC), may serve to specifically organize intracellular utrophin at synaptic sites. (B) This membrane-cytoskeleton could then organize AChRs into clusters. The clusters may be composed of groups of microclusters (darker shading) whose association may be based on interactions with the 43-kD protein.

the 43-kD protein aggregates. These microaggregates of AChRs are different from nerve-induced clusters *in vivo* or agrin-induced clusters in culture: the former are only $\sim 1 \mu\text{m}$, whereas the latter can be $>15 \mu\text{m}$.

More loosely associated with the AChR are proteins of 58, 87, and 300 kD, as well as an isoform of β -spectrin. The 58-kD protein (syntrophin) is a member of the dystrophin-glycoprotein complex (see below; Adams et al., 1993; Yang et al., 1994), the 87-kD protein is homologous to the COOH-terminus of dystrophin (Wagner et al., 1993), and the 300-kD protein is dystrophin (Sealock et al., 1991). These data suggest that a dystrophin membrane cytoskeleton may organize AChR clusters at the synapse.

Mutations or deletions in dystrophin, a major component of the sarcolemmal membrane, are responsible for Duchenne's muscular dystrophy (Hoffman et al., 1987). In rat muscle, dystrophin is expressed throughout the sarcolemma and is concentrated in the depth of the junctional folds that are rich in Na^+ channels but devoid of AChRs (Sealock et al., 1991). A homologue of dystrophin, called utrophin- or dystrophin-related protein, is concentrated at the AChR-rich crests of the junctional folds, which do not contain dystro-

phin (Man et al., 1991; Ohlendieck et al., 1991). Both dystrophin and utrophin associate with a protein complex called dystrophin glycoprotein complex (DGC) (Ervasti and Campbell, 1991; Matsumura et al., 1992), which consists of an intracellular peripheral membrane protein of 59 kD (identical to syntrophin), a transmembrane protein of 25 kD, transmembrane glycoproteins of 50 kD (adhelin), 43 kD (β -dystroglycan), and 35 kD, as well as a heavily glycosylated extracellular protein (α -dystroglycan). The DGC interacts with the COOH-terminus of dystrophin (Suzuki et al., 1994), while the NH₂-terminal region of dystrophin binds to actin (Ervasti and Campbell, 1993). An extracellular component of the DGC, α -dystroglycan, has previously been shown to bind laminin (Ibraghimov-Beskrovnya et al., 1992; Gee et al., 1993). Thus, the DGC may serve as a linkage between the cytoskeleton and the ECM (Ibraghimov-Beskrovnya et al., 1992). The importance of laminin-DGC interactions is highlighted by the observation that in several autosomal muscular dystrophies, one or several of the DGC components (Roberds et al., 1993) or merosin (a laminin A chain isoform) (Sunada et al., 1994) are reduced, whereas the dystrophin expression is not affected.

Recent experiments demonstrate that α -dystroglycan also interacts with agrin (Bowe et al., 1994; Campanelli et al., 1994; Gee et al., 1994; Sugiyama et al., 1994). This binding is Ca²⁺ dependent and it is disrupted by heparin, similar to agrin-induced clustering of AChRs (Wallace, 1990). The observation that dystrophin-null mdx mice form apparently normal NMJs suggests that agrin may serve to link a utrophin cytoskeleton to the ECM at the synapse (Fig. 2 A), consistent with the localization of utrophin to agrin-induced clusters in culture (Campanelli et al., 1994) and AChR-rich regions in vivo (Man et al., 1991; Ohlendieck et al., 1991).

Postsynaptic Response to Agrin, Potential Mechanisms

The accumulated data are consistent with the suggestion that α -dystroglycan is the cell surface proteoglycan implicated in agrin activity, and that it corresponds to the previously described agrin-binding site on cultured myotubes (Nastuk et al., 1991). The agrin- α -dystroglycan interaction may play an instructive role in synaptic differentiation. This possibility is supported by the observation that the blocking of this interaction by an anti- α -dystroglycan mAb alters agrin-induced AChR clustering, although the effects of this mAb remain controversial. One group observed a reduction in the number of clusters after addition of the mAb (Gee et al., 1994). A second group detected changes in the appearance of the agrin-induced clusters; instead of the large relatively homogeneous clusters normally induced by agrin, loose aggregates of microclusters were visible in cultures treated with mAb (Campanelli et al., 1994). These microclusters look similar to those induced by coexpression of the 43-kD protein with the subunits of the AChR in *Xenopus* oocytes (Froehner et al., 1990) and COS cells (Brennan et al., 1992). They also appear to correspond to immature clusters observed in vivo, and the early stages of agrin-induced aggregates in culture (Steinbach, 1981; Wallace, 1992). Under normal conditions, these microaggregates consolidate into larger clusters as development proceeds (Bevan and Steinbach, 1977). Interestingly, utrophin is associated with large clusters but not microaggregates (Phillips et al., 1993). These data suggest that an interaction of microclusters with the agrin-dystrogly-

can-utrophin complex leads to their consolidation into larger aggregates, either by directly anchoring them to the cytoskeleton or by indirectly corralling them (Fig. 2 B).

A testable model is that agrin discriminates between DGC bound to utrophin vs dystrophin. Alternatively, agrin binding to DGC might change the affinity of the complex for these cytoskeletal proteins. In either case, agrin binding could initiate or stabilize a rearrangement of the cytoskeleton that could serve as a template for the concentration of synaptic molecules. Whether agrin also acts at an earlier stage of the aggregation process (i.e., the formation of microclusters) by an independent mechanism remains an open question. Because utrophin has not yet been documented as a component of microclusters, other proteins may be necessary to initiate agrin action.

Another important question is whether agrin and the DGC are important for the formation of interneuronal synapses in the central nervous system. While expression of dystrophin is highest in muscle, low amounts can be detected in the brain, where the protein is found in synaptic areas (Lidow et al., 1990) and cofractionates with postsynaptic densities (Kim et al., 1992). Utrophin is expressed in much higher amounts in the brain, and it has been localized to the postsynaptic membrane of dendrites by immunoelectron microscopy (Kamakura et al., 1994). Finally, agrin expression in the brain coincides with the periods of synaptogenesis. Thus, the molecular machinery implicated in NMJ formation is present in brain, and it may serve similar functions in the elaboration of interneuronal synaptic connections.

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