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THE ROLE OF AGRIN IN SYNAPSE FORMATION

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INTRODUCTION

The precise, rapid, and ordered communication between neurons and their targets is the distinguishing characteristic of the nervous system. Chemical synapses are the primary locus of this information exchange, and the structural and molecular specializations essential for their proper functioning are understood in considerable detail (reviewed in Jessell & Kandel 1993, Stevens 1993). In contrast, much less is known about how synaptic structure is regulated during development and regeneration. Such structural changes may also mediate important aspects of synaptic plasticity in learning and memory (Bliss & Collingridge 1993, Lisman & Harris 1993).

Most of our knowledge of synaptogenesis has come from studying the neuromuscular junction. A search for the mechanisms underlying differentiation at this synapse has focussed on three molecular systems: the cytoskeleton and its associated membrane proteins, trophic factors, and a specific extracellular matrix component, agrin. Recent reviews have dealt with the overall process of synaptogenesis and with the contribution of the cytoskeleton and trophic factors (Salpeter 1987, Falls et al 1993, Froehner 1993, Hall & Sanes 1993). Here we focus on agrin, which is likely to play a central role in signaling and regulating the differentiation of the postsynaptic apparatus at the neuromuscular junction (Nastuk & Fallon 1993). In addition, we also discuss the mounting evidence suggesting an analogous role for agrin in the CNS.

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THE MATURE SYNAPSE

The mature nerve-muscle synapse is comprised of pre- and postsynaptic specializations separated by a synaptic cleft. The presynaptic apparatus is characterized by ordered arrays of synaptic vesicles poised to fuse with the plasma membrane at active zones. The postsynaptic membrane is encrusted with acetylcholine receptors (AChRs), which can reach densities of >10,000 molecules/μm^2. The synaptic cleft is occupied by a discrete synaptic basal lamina, a highly specialized extracellular matrix domain that is discussed in more detail below. The localized and tightly regulated secretion of acetylcholine into the confined space of the synaptic cleft, coupled with the high AChR concentration in the postsynaptic membrane, ensures rapid and reliable synaptic transmission between cells. The signal is efficiently terminated by acetylcholinesterase (AChE), which is also localized to the synapse. Perturbation of these specializations, such as the decrease in the number of functional AChRs seen in myasthenia gravis, can lead to debilitating and often fatal clinical outcomes.

One of the most important and provocative features of synapses in both the periphery and the CNS is their restricted subcellular distribution: A given synapse typically occupies <0.1% of the cell surface. This topological restriction lies at the heart of synaptic differentiation—how to make one domain of a cell distinct from others. This arrangement offers obvious advantages for economical, efficient, and rapid signaling: All the necessary machinery is highly concentrated at the appropriate site. In neurons, this restricted distribution also facilitates the cell's ability to receive multiple (up to 20,000), diverse inputs in an ordered and segregated manner. Finally, the segregation of these components to distinct domains serves to maximize the independent regulation of synapses on the same cell, which is likely to be crucial for processes such as long-term potentiation on neurons (Bliss & Collingridge 1993).

The molecular machinery of synaptic transmission at the neuromuscular junction is understood in considerable detail. The major proteins of synaptic vesicles have been characterized, and a picture of how the docking, exocytosis, and retrieval of these vesicles is achieved and regulated is emerging (Sollner et al 1993, Sudhof et al 1993). There has been a parallel explosion in our molecular and functional understanding of the AChR (Hille 1992). In addition to the constituents directly involved in synaptic transmission, there are a host of proteins likely to be important for the structure and regulation of the synapse. The cytoskeletal proteins at the synapse can be broadly grouped into two sets: (*a*) dystrophin, utrophin (dystrophin-related protein), and their associated elements such as the 58-kD and 87-kD proteins; and (*b*) several molecules that are associated with focal adhesions, including α-actinin, vinculin, talin, paxillin, and filamin. The former group may be linked to laminin in the synaptic basal lamina via the dystrophin-associated glycoprotein complex (Bloch et al

1991, Ervasti & Campbell 1993). The latter proteins may communicate with the extracellular matrix (ECM) through integrins (Bozyczko et al 1987). Specialized forms of intermediate filament protein and β-spectrins are also localized at synapses (Burden 1982, Bloch & Morrow 1989). The postsynaptic 43-kD protein probably anchors AChRs to as yet unidentified components of the underlying cytoskeleton (Maimone & Merlie 1993, Scotland et al 1993; reviewed in Froehner 1993).

The synaptic basal lamina contains not only agrin but also high levels of laminin A chain, a heparan sulphate proteoglycan, and collagen IV (Sanes et al 1990). The asymmetric form of AChE is also localized in this structure (Anglister & McMahan 1985). These and other proteins in the synaptic ECM are likely to play diverse roles, including mediating cell-cell adhesion, directing the differentiation of pre- and postsynaptic specializations, and serving as a repository of factors regulating gene expression (Brenner et al 1992, Jo & Burden 1992).

GENERAL CONSIDERATIONS OF POSTSYNAPTIC DIFFERENTIATION

How do synapses form? Synaptic differentiation can be considered on several distinct, yet interrelated, levels. For example, the pre- and postsynaptic cells must express the specific gene products that constitute the synapse, such as synaptic vesicle proteins and neurotransmitter receptors. Recent studies have begun to reveal the mechanisms underlying the differentiation of muscle cells and motor neurons (Tapscott et al 1990, Smith 1994). Moreover, the cell needs to express these proteins in a quantitatively and temporally regulated manner. Finally, these components must be marshaled to the site on the cell where they will be used, and then they must be precisely organized into a mature synapse.

The regulation of the expression and the spatial organization of synaptic components at neuromuscular junctions are intimately related, yet are likely to be subserved by distinct mechanisms. For example, two classes of stimuli are known to regulate AChR expression: electrical activity and neuronally derived trophic factors, which apparently work in complementary fashions (Witzemann et al 1991, Simon et al 1992). In mature muscle, electrical activity causes a global decrease in AChR expression, while trophic factors provide both quantitative and qualitative regulation of AChR-subunit gene expression at the synapse. One such factor is likely to be ARIA (AChR-inducing activity), a member of the heregulin family (Falls et al 1993). [Unidentified trophic factors are likely to play an analogous role in neurons (Role 1988, Schwarz-Levey et al 1994).] However, neither electrical activity nor ARIA induce AChR clustering in the initial stages of synaptogenesis. Conversely, there is no evidence that agrin regulates AChR-subunit gene expression. However, it must

be stressed that these results stem from work on model systems that probably reflect early events in synapse formation. These factors could have different effects later in development; for example, global and local differences in electrical activity have a dramatic influence on synaptic stabilization (Lichtman & Balice-Gordon 1990).

AGRIN AND POSTSYNAPTIC DIFFERENTIATION AT THE NEUROMUSCULAR JUNCTION

Several important rules governing the early events in postsynaptic differentiation were established in pioneering nerve-muscle coculture experiments (Anderson & Cohen 1977, Frank & Fischbach 1979; reviewed in Hall & Sanes 1993). Foremost among these is that motor neurons induce the formation of the postsynaptic apparatus. Second, this induction is limited to the appropriate synaptic partners and is not a function of simple adhesion: Neurites from sensory, cerebral cortical, and other neurons form extensive myotube contacts but effect little AChR clustering (see also Lieth & Fallon 1993). Third, although motor neurons promote AChR expression at developing synapses (Role et al 1985), initial clustering occurs by lateral migration of preexisting (diffusely distributed) AChRs; new synthesis is not required. Finally, electrical activity is not necessary for nerve-induced AChR clustering. Together, these findings indicate that there are specific factors supplied by motor neurons that induce AChR aggregation at developing neuromuscular junctions.

The coculture results suggested that neurally derived factors could be distinguished on the basis of their ability to induce AChR clusters on myotubes. Indeed, neural extracts and conditioned medium were shown to contain high molecular-weight factors with such activity (Christian et al 1978, Podleski et al 1978). Although these factors have not been identified, their biochemical properties and activity profiles (Olek et al 1986, Podleski & Salpeter 1988) indicate that they are likely to be agrin. Many of these extracts also contained activities that upregulate AChR expression; two of these molecules, calcitonin gene–related peptide (CGRP) and ARIA, are synthesized by motor neurons and are good candidates for mediating neural effects on AChR gene expression (Jessell et al 1979, New & Mudge 1986, Peng et al 1989, Changeux et al 1992, Falls et al 1993). Two other factors were identified as ascorbic acid and transferrin; their role in synaptogenesis is currently unclear (Markelonis et al 1982, Knaack et al 1986, Horovitz et al 1989).

A series of elegant in vivo experiments by McMahan and colleagues showed that another, quite unexpected structure could also induce postsynaptic differentiation on regenerating myofibers: the synaptic basal lamina (Burden et al 1979, McMahan & Slater 1984; reviewed in McMahan & Wallace 1989).

When myofibers regenerate into preexisting basal lamina sheaths, postsynaptic specializations form at the site of the original neuromuscular junction. These specializations form in the absence of reinnervation and display many features of mature endplates, including high concentrations of AChRs and AChE, and postsynaptic folds (Anglister & McMahan 1985). These experiments demonstrated that molecules stably associated with the synaptic basal lamina are sufficient to induce postsynaptic differentiation. They also suggested that this specialized ECM segment offered a relatively simple source from which to purify this molecule (Rubin & McMahan 1982).

ECM extracts from the synapse-rich *Torpedo californica* electric organ were used as a source to purify this factor. These extracts induced AChR aggregation on cultured myotubes (Godfrey et al 1984). Monoclonal antibodies that were raised against this clustering activity recognized an antigen stably associated with the synaptic basal lamina (Fallon et al 1985) and were used in conjunction with several biochemical methods to identify the factor, which was named agrin (from the Greek *ageirein,* meaning to assemble) (Nitkin et al 1987). Biochemically purified agrin is comprised of four polypeptides of 150, 135, 95, and 70 kD, which are stable fragments of a native 200-kD molecule (Smith et al 1992; reviewed in McMahan 1990).

Several lines of evidence support a central role for agrin in directing postsynaptic differentiation. Agrin is stably associated with the mature synaptic basal lamina (Reist et al 1987) and is present at developing synapses in vivo (Godfrey et al 1988b, Fallon & Gelfman 1989, Hoch et al 1993). Agrin is synthesized by motor neurons and released by growing neurites, where it is associated with nerve-induced AChR aggregates (Magill-Solc & McMahan 1988, Cohen & Godfrey 1992). Antibodies against agrin block the formation of motor neuron–induced AChR clusters (Reist et al 1992). Recombinant agrin induces AChR clusters on muscle cells (Campanelli et al 1991, Ruegg et al 1992). Interestingly, agrin is also synthesized by muscle cells, and this muscle agrin is concentrated at postsynaptic specializations (Fallon & Gelfman 1989, Lieth et al 1992; and see below). Finally, an agrin receptor is present on developing myotubes and has been purified from *Torpedo* electric organ postsynaptic membranes (Nastuk et al 1991, Ma et al 1993, Bowe et al 1994). Together, these data indicate that agrin directs the aggregation of AChRs at both developing and regenerating nerve-muscle synapses.

Although AChR clustering is agrin's benchmark activity, it is important to appreciate that agrin also induces the redistribution of a dozen or more proteins that are also concentrated at synapses (Table 1). Moreover, these molecules are functionally diverse, ranging from integral membrane proteins such as the agrin receptor to peripheral membrane proteins, cytoskeletal elements, and basal lamina constituents. Agrin is therefore likely to play an overarching regulatory role in postsynaptic differentiation.

Table 1 Agrin induces the redistribution of diverse components of the neuromuscular junction

Synaptic component	Phase of distribution[a]	References
Plasma membrane		
Acetylcholine receptor	Early	Godfrey et al 1984
Acetylcholinesterase (globular)	Early	Wallace 1989
Butyrylcholinesterase (globular)	Early	Wallace 1989
Agrin receptor	Early	Nastuk et al 1991
43-kD protein	Early	B Wallace, personal communication
Extracellular matrix		
Heparan sulfate proteoglycan	Late	Nitkin & Rothschild 1990, Wallace 1989
Laminin	Late	Nitkin & Rothschild 1990
Muscle agrin	Late	Lieth et al 1992
Acetylcholinesterase (A_{12} asymmetric)	?	Wallace 1989
Cytoskeleton		
α-actinin	Late	Shadiack & Nitkin 1991
Filamin	Late	Shadiack & Nitkin 1991
Vinculin	Late	Shadiack & Nitkin 1991

[a] Early: ~1–4 h; Late: >4 h

AGRIN EXPRESSION

Agrin has been detected at neuromuscular junctions in every species for which probes are available (electric ray, chick, frog, rat) (Fallon et al 1985, Reist et al 1987, Hoch et al 1993); whether agrin is expressed in invertebrates is unknown. Agrin is present at virtually all developing nerve-muscle synapses from the time they first form in embryonic chicks (Godfrey et al 1988b, Fallon & Gelfman 1989). In contrast, agrin is only detected at a subset of nerve-muscle synapses early in development in rats (Hoch et al 1993). It is unknown if this finding reflects a species difference or is the result of insufficiently sensitive immunological reagents. Agrin mRNA is expressed in motor neurons and muscle of both chick and rat from the time of initial synapse formation (Tsim et al 1992, Hoch et al 1993).

Agrin is expressed by both pre- and postsynaptic cells at neuromuscular junctions. Aneural muscle cells in vivo and in vitro express agrin (Fallon & Gelfman 1989, Lieth et al 1992). Moreover, muscle agrin is expressed at AChR clusters induced either by coculture with neurons or by incubation with purified *Torpedo* agrin (Lieth & Fallon al 1993). Muscle agrin is expressed in the late phase of agrin-induced AChR clustering (Table 1) and thus does not appear

to be involved in initial AChR aggregation. Rather, muscle agrin may play a role in the maturation or stabilization of AChR clusters.

Agrin is also detected in the basal laminae of CNS capillaries and smooth muscle cells, kidney, and cardiac cells (Godfrey et al 1988a). Expression at other sites is species dependent. For example, agrin immunoreactivity surrounds Schwann cells in myelinated nerve in *Torpedo,* chick, and rat but is restricted to nodes of Ranvier in frog (Reist et al 1987). Agrin's function outside the neuromuscular junction is unknown.

AGRIN STRUCTURE

Agrin is a single chain, multidomain extracellular matrix protein of ~200 kD (Figure 1). Analysis of the full-length rat and chick and partial electric ray–deduced amino acid sequences shows 60% identity and an additional 20% conservation among species (Rupp et al 1991, Smith et al 1992, Tsim et al 1992). Several domains, particularly in the carboxy terminus, show a greater than 80% identity. From both functional and structural viewpoints it is useful to consider the amino and carboxyl halves of agrin separately.

The carboxyl-terminal half of agrin (which roughly corresponds to the biochemically purified 95-kD polypeptide) (Nitkin et al 1987) binds to the agrin receptor (Ma et al 1993, Bowe et al 1994) and embodies the full range of agrin's clustering activity (Wallace 1989; and see below). Prominent in this half of the molecule are four EGF-like (epidermal growth factor–like) cysteine repeats that are similar to one another but not to those found in the amino-ter-

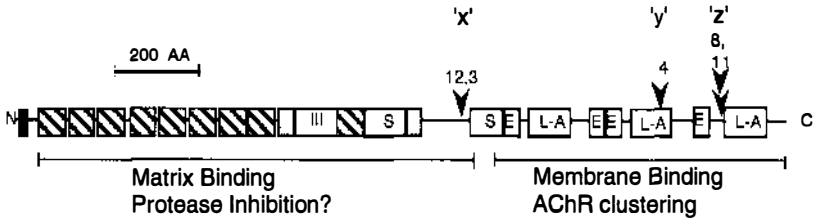

Figure 1 The structural organization of rat agrin. A 38–amino acid hydrophobic region (*filled*) at the extreme N terminus is thought to be a signal sequence. The remaining N-terminal portion of the molecule has nine Kazel-type protease inhibitor/EGF repeats (*cross hatch*), a region homologous to domain III of laminin B chains (*III*), a serine- and threonine-rich domain (*S*), and two internal cysteine-rich repeats (*stippling*). The C-terminal half, which is sufficient by itself for binding to the plasma membrane and inducing AChR clustering, has four EGF-like cysteine repeats (*E*), a serine-and-threonine-rich domains, and three regions homologous to the G-domain of the laminin A chain (*L-A*). Arrowheads indicate some sites of alternative exon splicing. At the *x* site alternative splice-acceptor-site usage results in 12– or 3–amino acid inserts; at the *y* site a 4–amino acid insert can be present; and at the *z* site there can be the 8–, 11–, 19– (both), or no amino acid insert(s). The 8– and 11–amino acid inserts are uniquely expressed in the nervous system. The organization of chick agrin is very similar (Ruegg et al 1992).

minal half. Analysis of exon structure (Rupp et al 1992) and protein sequence (Patthy & Nikolics 1993) also revealed three ~100–amino acid repeats that share significant homology to the G-domain of the laminin A chain, and to similar regions in perlecan (Noonan et al 1991, Murdoch et al 1992, Rothberg & Artavanis 1992).

The amino-terminal half of agrin is sufficient for its association with the extracellular matrix, and in some cases it modulates agrin's biological activity (Ferns et al 1993). One region of particular interest is ~40% identical to domain III of the laminin B1, B2, A, and S chains. In laminin this domain is involved in binding to the basal lamina component nidogen (entactin) (Timpl 1989). The homologous domain in agrin could play a similar role, since agrin externalized by growing neurites binds to a nidogen-rich ECM extract but not to laminin (Cohen et al 1994). The amino-terminal half of agrin also has nine tandem repeats; the carboxyl and amino portions of each are homologous to Kazel-type protease-inhibitor domains and EGF, respectively (Rupp et al 1992). Biochemical studies have confirmed that agrin has serine protease–inhibitor activity with specificity for trypsin, chymotrypsin, and plasmin (Biroc et al 1993). Interestingly, proteolysis has been implicated in synapse formation and in elimination of polyneuronal innervation (Connold et al 1986, Champaneria et al 1992). On the other hand, alternative sequence analyses have suggested that these nine repeats are homologous to follistatin, which is known to bind growth factors. This similarity raises the intriguing possibility that agrin presents trophic factors to motor neurons and/or muscle cells (Patthy & Nikolics 1993).

Agrin is encoded by a single gene located on human chromosome 1 region pter-p32 and on mouse chromosome 4 (Rupp et al 1992). There is no indication that the gene is associated with known mutations resulting in neuromuscular disorders or neurological diseases. The agrin gene consists of 37 exons. The exons and introns that have been sequenced are rather small, ranging in size from 12 to 333 and 65 to 712 base pairs, respectively.

ALTERNATIVE SPLICING OF AGRIN

Agrin mRNA is alternatively spliced at three sites, yielding several forms. Ruegg et al (1992) first recognized the importance of these forms when they observed that the AChR-clustering activity of a recombinant chick agrin depended upon the presence of an alternatively spliced 11–amino acid insert. Further analysis of rat and chick agrin showed that an eight–amino acid insert could also be included at this site, denoted *z* (Figure 1) (Rupp et al 1992, Hoch et al 1993, Thomas et al 1993). All combinations at this *z* site are expressed, yielding forms with no or 8–, 11–, or 19–amino acid inserts. Splicing at the *z* site is always accompanied by a four–amino acid insert at the *y* site, but the

latter insert can be expressed independently. A third site, *x*, can have a 12– or a 3–amino acid insert, depending upon splice-acceptor-site usage. The sequences of the *y* and *z* inserts do not show strong homology to any entries in the protein database. However, the size, location, and sequence of these inserts is conserved among species: The rat and chick 4–, 8–, and 11–amino acid inserts are 100, 75, and 45% identical, respectively.

The biological activity of recombinant rat and chick agrin alternatively spliced at the *y* and *z* sites has been assayed (Table 2) (Ferns et al 1992, 1993; Ruegg et al 1992). Both full-length, cell-attached agrin and truncated, soluble agrin (which roughly corresponds to the carboxyl half of the molecule) have

Table 2 Functional consequences of alternative agrin mRNA splicing

Agrin	Myotube type				
	Chick primary	Mouse and rat primary	C2	S26	S27
Full-length (cell-attached)					
Rat[a]					
12, 0, 0[b]	ND[c]	ND	+	–	–
12, 4, 0		+	+		
12, 4, 8	+	+	+	+	+
12, 4, 11	(+)	+	+		
12, 4, 19	+	+	+	+	+
Truncated (C-terminal)[d]					
Rat[a]					
12, 0, 0	ND	ND			
12, 4, 0	ND	ND	–	–	–
12, 4, 8	+	+	+	+	(+)
12, 0, 8	ND	ND	+	+	–
Chick[e]					
12, 0, 0		ND	ND	ND	ND
12, 4, 0	–	ND	ND	ND	ND
12, 4, 11	+	ND	ND	ND	ND
Torpedo[f]					
(?)[g]	+	+	+	ND	
Truncated (N-terminal)[h]					
Rat	ND	ND	–	–	–

[a] Campanelli et al 1992; Ferns et al 1992, 1993; M Ferns & Z Hall, unpublished observations.

[b] Numbers refer to alternatively expressed amino acid inserts at the *x*, *y*, and *z* sites, respectively (see Figure 1 and text).

[c] Symbols for AChR clustering activity: –, none; +, high activity; (+), low activity; and ND, not determined.

[d] Corresponding to carboxyl-terminal half (see Figure 1).

[e] Ruegg et al 1992.

[f] Godfrey et al 1984, Nastuk et al 1990, Gordon et al 1993.

[g] Splice form unknown.

[h] Corresponding to amino-terminal half (see Figure 1).

been used in these studies (Figure 1). All of the tested full-length molecular splice forms induce AChR clustering with equal potency when assayed on either mouse primary or C2 myotubes. However, truncated, soluble agrin was only active if at least one insert at the *y* or *z* site was present. Moreover, there were major differences in effectiveness: Truncated forms containing the eight–amino acid insert were >1000-fold more potent than those with only the four–amino acid insert. Deletion of the four–amino acid insert did not diminish the activity of the forms containing the eight–amino acid insert. In contrast, when chick agrin was assayed on chick myotubes, there was an absolute requirement for an 8– and/or 11–amino acid insert at the *z* site (Ruegg et al 1992). Soluble forms of rat agrin containing the eight–amino acid insert are also active on these cells (M Ferns & Z Hall, unpublished observations).

Further insight into the role of alternative splicing and the function of the N- and C-terminal agrin domains has come from studies using muscle cells deficient in proteoglycan expression (Table 2). These cell lines, S26 and S27, have moderate and severe proteoglycan defects, respectively. Both show nerve-induced but not spontaneous AChR clustering (Gordon & Hall 1989, Gordon et al 1993). Truncated, soluble rat agrin induces AChR clustering on S26 cells but only if the eight–amino acid insert is present. Full-length, cell-attached agrin with the eight–amino acid insert induces clustering on S27 cells, but little activity is observed with even the most potent soluble forms. These results suggest that proteoglycans may play a role in agrin's AChR-clustering activity (Ferns et al 1993).

Although agrin is present in many tissues, expression of the 8– and 11–amino acid inserts is restricted to the nervous system (Ruegg et al 1992, Smith et al 1992, Biroc et al 1993, Hoch et al 1993). The pattern of alternative splicing at the *z* site changes during development (Hoch et al 1993, Thomas et al 1993). Splicing of the four–amino acid insert occurs in the CNS, PNS, skeletal and cardiac muscle, and to a lesser extent in kidney. A recent elegant study using electrophysiological recording followed by RT-PCR (reverse translation–polymerase chain reaction) analysis of individual cells has shown that in ciliary ganglia, over 70% of the neurons express agrin with the 8– and 11–amino acid inserts (Smith & O'Dowd 1994). In contrast, agrin expressed by glial cells was devoid of inserts at the *z* site. Further, individual neurons varied in their patterns of alternative agrin mRNA splicing and often expressed more than one splice type. Interestingly, over one third of these neurons expressed forms with no splicing at the *z* site. These results indicate that although alternative splicing at the *z* site is a property of neurons, it may not be necessary for all the functions of neuronally expressed agrin.

How these inserts regulate agrin's activity is unknown. They apparently do not form part of agrin's binding site for its receptor, since excess synthetic peptides corresponding to these inserts neither inhibit nor stimulate AChR

clustering. It seems that these inserts cause a conformational change that augments agrin's activity or renders it independent of accessory molecules such as proteoglycans (Yayon et al 1991). The differential activity of these forms could indicate the existence of multiple agrin receptors, but only a single receptor has been detected to date (see below). These studies also reveal that the biological activity of the agrin forms is target dependent. Myotubes may therefore vary in response to different forms depending, for example, on their developmental stage or physiological state (e.g. during synapse elimination or after denervation). Finally, these observations suggest that there may be more than one intracellular signaling pathway for agrin-induced AChR clustering. The existence of such multiple pathways is consistent with some of the findings concerning agrin-induced tyrosine phosphorylation of the AChR (discussed below).

AGRIN'S MECHANISM OF ACTION

There are at least two distinct phases of agrin-induced molecular redistribution (Table 1). The early stage begins 1–2 h after agrin treatment of cultured myotubes. At that time there is clustering of several membrane proteins, including AChRs, globular cholinesterases (Wallace 1989), agrin receptors (Nastuk et al 1991), and the 43-kD protein (BG Wallace, personal communication). The aggregation of these molecules is contemporaneous and does not require new protein synthesis.

A second set of molecules becomes concentrated at these induced AChR aggregates after several hours of agrin treatment. These late-phase elements include cytoskeletal molecules and basal lamina components such as laminin and muscle-derived agrin (Wallace 1989, Nitkin & Rothschild 1990, Shadiack & Nitkin 1991, Lieth et al 1992). The appearance of the basal lamina molecules requires protein synthesis. The expression of the late-phase molecules correlates well with increased AChR cluster stability, suggesting that these molecules may be important for synaptic maturation. This biphasic cellular response also suggests that agrin initiates a cascade of signaling events, with both short- and long-term consequences.

Agrin's mechanism of action has been studied most with regard to AChR redistribution. Agrin induces AChR clustering at concentrations in the 0.1–1 pM range (Nitkin et al 1987, Ferns et al 1993). Signaling is initiated by its calcium-dependent binding to a high affinity plasma membrane receptor (see below). Formation of AChR aggregates requires metabolic energy and is inhibited by activators of protein kinase C (Wallace 1988). On the other hand, altering cyclic nucleotide levels or inhibiting protein synthesis, glycosylation, or calmodulin function does not affect agrin's activity (Wallace 1988). Further, the stoichiometry of agrin receptors to AChRs is in the range of 1:50–1:100

(Bowe et al 1994). These results indicate that intracellular signal-transduction mechanisms mediate agrin's effects.

Several lines of evidence indicate that tyrosine phosphorylation is involved in agrin-induced AChR clustering. Junctional AChRs are phosphorylated on tyrosine (Qu et al 1990). Agrin elicits an increase in tyrosine phosphorylation on AChR β-subunits in cultured myotubes (Wallace et al 1991). In addition, agrin-induced AChR clustering is temporally correlated with tyrosine phosphorylation of AChRs and is blocked by tyrosine kinase inhibitors (Wallace et al 1991, Wallace 1992, Qu & Huganir 1993). Further, AChR clustering induced by overexpression of the 43-kD protein is accompanied by tyrosine phosphorylation of unidentified non-AChR proteins (Dai et al 1993). These results raise the possibility of a link between agrin's signaling pathway and the 43-kD protein. The tyrosine kinase(s) involved in any of these cases have not been identified, but one transmembrane and two cytoplasmic tyrosine kinases present in *Torpedo* electric organ are candidates (Jennings et al 1993, Swope & Huganir 1993).

Other pathways may also mediate agrin's activity. For example, unlike in chick, tyrosine phosphorylation of AChRs in rat is not detected until after birth, several days after synapses form and agrin accumulates at them (Qu et al 1990). This observation suggests alternative or cooperative mechanisms of agrin-induced AChR clustering or a biphasic nature of agrin's action. The presence of more than one such mechanism could also account for the target-dependent bioactivity of agrin isoforms.

THE AGRIN RECEPTOR

A primary function of agrin is to convey information from the neuron to the muscle cell, information that instructs the muscle cell to form a postsynaptic apparatus in a specific location. The muscle cell in turn secretes agrin at the site of nerve contact, perhaps forming a feedback loop to amplify or stabilize this signal (Lieth & Fallon 1993). A crucial component in these interactions is the agrin cell surface receptor. Using ligand-binding techniques, our laboratory characterized agrin receptors expressed on cultured myotubes and on postsynaptic membranes from *Torpedo* electric organ. Agrin binding to these receptors occurs in physiological buffers, is saturable, and is of high affinity; half maximal binding occurs at ~10^{-10} M (Nastuk et al 1991, Ma et al 1993). Significantly, like nerve- and agrin-induced AChR clustering, ligand binding requires extracellular calcium (Henderson et al 1984, Wallace 1988). Additionally, both agrin binding to postsynaptic membranes and agrin-induced AChR clustering on cultured myotubes exhibit a similar pH dependence; they are inhibited at pH < 7 (Wallace 1988, Ma et al 1993). Ligand binding is unaffected by pretreatment of membranes with chondroitinase, heparatinase,

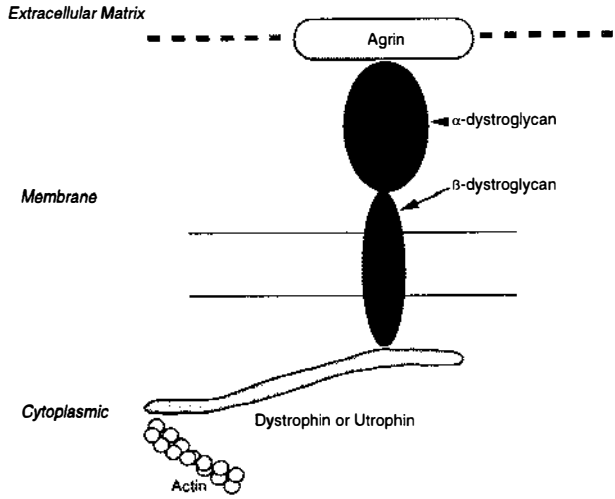

Figure 2 A schematic representation of the agrin receptor (α- and β-dystroglycan) from *Torpedo* postsynaptic membranes. The purified receptor is a complex of α-dystroglycan, which is sufficient for ligand binding, and β-dystroglycan (Bowe et al 1994). The cytoplasmic portion of β-dystroglycan can bind to dystrophin (Suzuki et al 1994). In muscle the dystroglycans are part of a larger complex consisting of adhalin, the syntrophins, and 25- and 35-kDa dystrophin-associated glycoproteins (Ervasti & Campbell 1994, Peters et al 1994; see Fallon & Hall 1994 for review).

keratinase, neuraminidase, O-glycanase, and N-glycanase (Nastuk et al 1991, Ma et al 1993, Bowe et al 1994). Neither peptides containing the RGD (arginine-glycine-aspartic acid) sequence, which inhibit many integrin-based interactions, nor the LRE (leucine-arginine-glutamic acid) tripeptide, which is involved in S-laminin interactions, inhibit agrin binding (Hunter et al 1991, Hynes 1992, Ma et al 1993, Bowe et al 1994).

Ligand-binding studies also provided the first evidence that the agrin receptor is distinct from the AChR. As described above, agrin receptors coaggregate with AChRs on agrin-stimulated myotubes. However, anti-AChR antibody-induced clustering of AChRs does not cause aggregation of agrin receptors, and AChR internalization driven by these antibodies does not deplete surface agrin receptors (Nastuk et al 1991). Further, AChRs can be separated from agrin receptors in solubilized postsynaptic membranes (Ma et al 1993).

These ligand-binding studies laid the groundwork for the identification and purification of an agrin receptor (Bowe et al 1994) (Figure 2). Monocolonal antibodies were raised against *Torpedo* electric organ postsynaptic membrane proteins and screened for their ability to immunoprecipitate solubilized agrin-binding proteins. Biochemical and immunoaffinity purification methods, together with agrin-affinity chromatography, demonstrated that the agrin receptor is a complex of two membrane glycoproteins of 190 kD and 50 kD.

Microsequencing of the purified material yielded the surprising result that the 190-kD and 50-kD polypeptides correspond to α- and β-dystoglyan, respectively. α-Dystroglycan is a highly glycosylated extrinsic peripheral membrane glycoprotein. Both proteins were originally characterized as dystrophin-associated glycoproteins (Ervasti & Campbell 1993; reviewed in Fallon & Hall 1994). When solubilized membranes were size-fractionated by gel filtration, all agrin-binding activity coeluted with the dystroglycan complex in a single symmetrical peak, indicating that this complex is the major agrin receptor in these membranes. A ligand-overlay method revealed that α-dystroglycam is sufficient to bind agrin in a calcium-dependent fashion. Moreover, α-dystroglycan was the only agrin-binding protein detected with this method. Together, these date indicate that agrin binding to α-dystroglycan is a fundamental event in the induction of postsynaptic differentiation. The associated β-dystroglycan, which has several phosphotyrosine consensus sequences and proline-rich regions in its cytoplasmic tail, could mediate the signal-transduction cascade initiated by ligand binding to α-dystroglycan.

Independently, three other laboratories used a monoclonal antibody against α-dystroglycan to show that it is the major agrin-binding protein on muscle cells and *Torpedo* membrane (Campanelli et al 1994, Gee et al 1994, Sugiyama et al 1994). They all reported that agrin binding to an α-dystroglycan, like nerve- and agrin-induced AChR clustering, is calcium dependent and is blocked by heparan. Moreover, the monoclonal antibody blocked agrin binding to α-dystroglycan on Western blots. The next step in these studies is a direct demonstration of the dystroglycan complex as the receptor that signals agrin-induced AChR clustering.

Dystroglycan as an agrin receptor has intriguing implications for our understanding of how agrin works to cluster AChRs. In the muscle cell membrane, α- and β-dystroglycan are likely to form a critical transmembrane link between dystrophin in the cytoskeleton and agrin and/or laminin in the basal lamina (Ervasti & Campbell 1993). Since dystrophin in turn binds actin, the dystroglycans could be part of the long-sought cytoskeletal mechanism underlying AChR clustering. An earlier finding by Burden and colleagues that dystrophin can be chemically cross-linked to the AChR-associated 43-kD protein (Burden et al 1983) supports an attractive model, whereby agrin binding to dystroglycan is coupled to the immobilization of AChRs.

Finally, these results provide a new way of thinking about the pathogenesis of Duchenne Muscular Dystrophy (DMD). It has been postulated that the dystrophin-associated protein complex serves a purely structural role in linking the basal lamina to the cytosekelton. Disruption of this complex following dystrophin loss may thus lead to a weakening of the cell membrane and subsequent rupture (Matsumura & Campbell 1994). The placement of the dystroglycans in a signal-transduction pathway raises the possibility that the

loss of dystrophin or dystrophin-associated proteins could also lead to defective signaling in DMD or other neuromuscular disorders.

AGRIN IN THE CNS

Although neuronal synapse formation and regulation is far more complex than that observed at neuromuscular junctions, these events share important similarities. For example, postsynaptic densities, high concentrations of neurotransmitter receptors, extracellular material in the synaptic cleft, and submembranous cytoskeletal networks are characteristic of synapses in both the PNS and the CNS (McMahan & Kuffler 1971, Fertuck & Salpeter 1976, Peters et al 1976, Triller et al 1985, Jacob et al 1986, Bekkers & Stevens 1989, Killisch et al 1991, Craig et al 1993, Petralia et al 1994). Synaptic conservation at the molecular level is manifested in the observation that all the ligand-gated ion channels characterized to date, whether in the CNS or PNS, are members of the same gene superfamily (Olsen & Tobin 1990, Sargent 1993, Seeburg 1993). The key structural and functional attributes shared by these receptors suggest that widely conserved mechanisms underlie their organization in the postsynaptic membrane.

Consistent with the functional conservation of synapses across the nervous system, evidence suggests that agrin's role in directing synaptic differentiation at neuromuscular junctions may be recapitulated in the CNS. Agrin mRNA and protein are present in the brain (Rupp et al 1991, Ruegg et al 1992, Smith et al 1992, Hoch et al 1993). Agrin mRNA expression is ontogenetically regulated such that peak levels coincide with periods of maximal synaptogenesis among young neurons (Hoch et al 1993, Thomas et al 1993). In situ hybridization shows that agrin mRNA is present in subpopulations of cortical and subcortical neurons (O'Connor et al 1994). It is notable that the hippocampus and olfactory bulb, two regions distinguished by lifelong synaptic plasticity, contain neurons expressing some of the highest levels of agrin mRNA in the adult brain. Moreover, agrin message levels in the hippocampus and several linked brain regions are altered following induced epileptiform seizures (O'Connor et al 1992). Taken together, these findings suggest the involvement of agrin in synaptic differentiation and regulation in the brain.

Recent findings indicate that neurons express agrin receptors. For example, agrin binds to dendrites and cell bodies of cultured hippocampal neurons and is concentrated at some of the synapses on these cells (M Nastuk, G Banker & J Fallon, unpublished observations). Agrin also binds to isolated rat brain synaptosomes. Moreover, as observed on myotubes, agrin also induces the redistribution of its receptor on cultured neurons (Nastuk & Fallon 1991). The study of neuronal agrin receptors promises to yield important insights into the

molecular mechanisms underlying synapse formation and plasticity in the brain.

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