

REVIEW ARTICLE

Formation of the neuromuscular junction

Agrin and its unusual receptors

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Synapses are essential relay stations for the transmission of information between neurones and other cells. An ordered and tightly regulated formation of these structures is crucial for the functioning of the nervous system. The induction of the intensively studied synapse between nerve and muscle is initiated by the binding of neurone-specific isoforms of the basal membrane protein agrin to receptors on the surface of myotubes. Agrin activates a receptor complex that includes the muscle-specific kinase and most likely additional, yet to be identified, components. Receptor activation leads to the aggregation of acetylcholine receptors (AChR) and other proteins of the postsynaptic apparatus. This activation process has unique features which distinguish it from other receptor tyrosine kinases. In particular, the autophosphorylation of the kinase domain, which usually induces the recruitment of adaptor and signalling molecules, is not sufficient for AChR aggregation. Apparently, interactions of the extracellular domain with unknown components are also required for this process. Agrin binds to a second protein complex on the muscle surface known as the dystrophin-associated glycoprotein complex. This binding forms one end of a molecular link between the extracellular matrix and the cytoskeleton. While many components of the machinery triggering postsynaptic differentiation have now been identified, our picture of the molecular pathway causing the redistribution of synaptic proteins is still incomplete.

Keywords: acetylcholine receptor; agrin; muscle-specific kinase; receptor tyrosine kinase; tyrosine phosphorylation.

FORMATION OF THE NEUROMUSCULAR JUNCTION

The neuromuscular junction (NMJ) is a refined structure geared to ensure a rapid and efficient transmission of an action potential into depolarization of the postsynaptic target organ, the muscle. Fast synaptic transmission is achieved by the close spatial apposition of the presynaptic zone, containing synaptic vesicles filled with the neurotransmitter acetylcholine, to the postsynaptic membrane. There, a wide range of proteins is highly concentrated, most notably the nicotinic acetylcholine receptors (AChRs; [1,2]). The formation of this intricate structure is an example of the collaboration of two different cell types in forming distinct subcellular specializations. A modulatory role played by a third cell type, the Schwann cells [3,4] will not be discussed here.

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Abbreviations: AChR, (nicotinic) acetylcholine receptor; ARIA, acetylcholine receptor inducing activity; CHO, Chinese hamster ovary; CREB, cyclic AMP response element binding protein; DGC, dystrophin-associated glycoprotein complex; EGF, epidermal growth factor; G-domain, globular domain; HB-GAM, heparin-binding growth-associated molecule; MASC, myotube-associated specificity component; MuSK, muscle-specific kinase; N-CAM, neural cell-adhesion molecule; NMJ, neuromuscular junction; RATL, Rapsyn-associated transmembrane linker.

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The timing and localization of synaptic differentiation has to be coordinated and tightly controlled. This is ensured by an intensive communication between motoneurons and muscle cells through the secretion of factors that are detected by surface receptors. During the first step of synaptic differentiation, proteins initially present along the entire muscle cell surface are redistributed and concentrated at the site of contact with the motoneurone axon (Fig. 1). Subsequently, the differential distribution of synaptic proteins is enhanced by selective transcription of genes encoding these proteins from only those nuclei which are located in the vicinity of the developing synapses. In contrast, transcription of RNAs from extrasynaptic nuclei is down-regulated in response to depolarization of the myotubes caused by AChR activation [5]. The synapses, initially quite simple, are stabilized and refined (e.g. by the recruitment of cytoskeletal elements [6]). The focus of this review will be on the role which specific isoforms of the basal membrane protein agrin play in several of these processes. Retrograde signals emerging from myotubes which influence the presynaptic differentiation [7,8] will not be discussed here.

SYNAPTOGENESIS IN CULTURE

One of the advantages of the neuromuscular junction as a model system for cell–cell interaction and synaptogenesis is that the formation of synapses can be studied in tissue culture. Muscle cells in culture differentiate into myotubes upon removal of serum growth factors. Several days after fusion, synaptic proteins, most prominently the (nicotinic) acetylcholine receptor (nAChR), aggregate in discrete spots along the

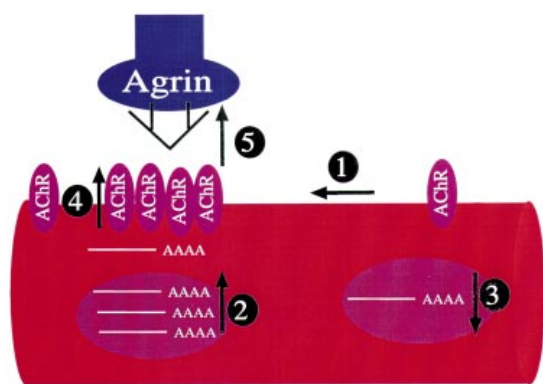


Fig. 1. Schematic representation of AChR aggregation in the postsynaptic membrane triggered by agrin. Synaptic differentiation initiated by agrin involves at least five individual steps: (1) redistribution of AChRs which are initially present on the entire myotube surface to the postsynaptic site; (2) increased transcription by synaptic nuclei of mRNAs encoding AChR subunits; (3) decreased transcription by extrasynaptic nuclei of mRNAs encoding AChR subunits; (4) rearrangement of the membrane cytoskeleton; (5) retrograde signal from the postsynaptic specialization of the myotube to the presynaptic nerve terminal.

muscle surface. In many ways, these spontaneous clusters resemble the postsynaptic specializations observed *in vivo* [9]. When motoneurons are added to these cultures, their axons make contact with myotubes at random sites and induce new aggregates of AChR [10,11]. These induced clusters are enlarged by recruitment of extrasynaptic AChRs while spontaneous clusters eventually dissolve. This and other experimental data clearly demonstrate that the machinery for postsynaptic specialization is present in myotubes but that it is triggered by signals released by motoneurons.

AGRIN – A MULTIDOMAIN PROTEIN TRIGGERING POSTSYNAPTIC DIFFERENTIATION

Early denervation experiments performed in the laboratory of J. McMahan have shown that the synaptic basal membrane plays an important role during the regeneration of synapses [12]. In these experiments, axons of motoneurons and

myotubes were destroyed and only the basal membrane was left intact. When presynaptic and postsynaptic cells were allowed to regenerate they formed synapses at exactly the position where they originally were located. These results suggested that components of the basal membrane might play an instructive role in synaptogenesis during regeneration and possibly early development. Experiments using a particularly synapse-rich tissue, the electric organ of *Torpedo californica* supported this conclusion; when extracts of this tissue were added to the medium of cultured myotubes they induced the aggregation of AChRs and other proteins [13]. A single protein called agrin, from the Greek word *agrein* (to assemble), was shown to be responsible for this activity.

Sequencing of c-DNAs coding for agrin from several species revealed that agrin is a large protein containing a number of interesting structural domains (Fig. 2; [14–16]). Some of these modules were known from other basement membrane proteins. The N-terminal part is dominated by nine follistatin repeats, each including a protease inhibitor domain of the Kazal type whose functions are unknown. This part of the molecule also contains attachment sites for glycosaminoglycans which increase the molecular mass of agrin from the calculated 200 kDa to ≈ 600 kDa [17]. The only known function of the N-terminal half of agrin is to mediate binding to other basal membrane components, in particular laminin [18], and thereby anchor agrin into the extracellular matrix. Agrin transfected into Chinese hamster ovary (CHO) cells is secreted and immediately incorporated into the extracellular matrix surrounding these cells in culture [19].

In contrast, the C-terminal half of agrin expressed behind an artificial signal sequence is released into the medium. This part of the molecule contains all the regions necessary for the interaction with receptors on the muscle surface and is capable of inducing the aggregation of AChRs with the same potency as the whole agrin molecule (Fig. 2; [20]). Its most prominent feature are three globular domains (G-domains) which can be visualized as independent domains by electron microscopy [21]. Homologous G-domains have previously been identified in α chains of laminin and are present in a variety of extracellular proteins where they often intermingle with epidermal growth factor (EGF)-repeats [22]. Only the two most C-terminally located repeats are part of a 50-kDa fragment which is the minimal fragment retaining full AChR aggregating activity [23]. Heparin, a potent inhibitor of AChR

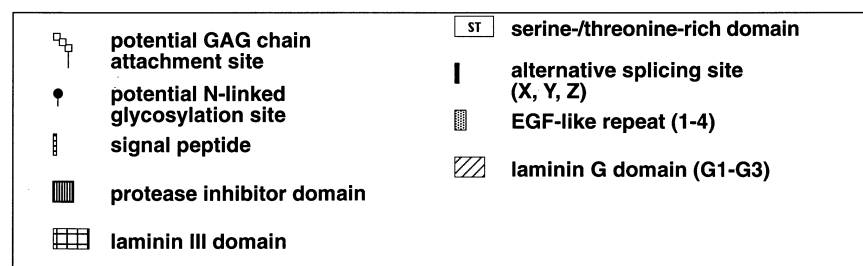
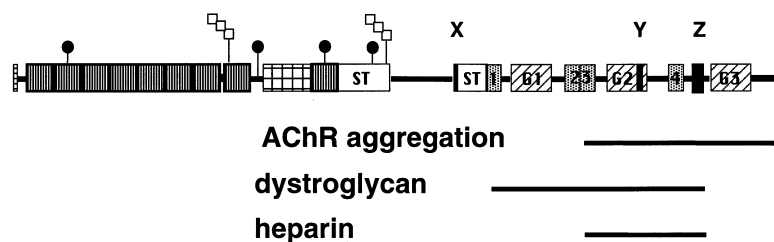


Fig. 2. Domain structure and functional regions of the agrin protein. The domains required for aggregation of AChRs and for binding to heparin as well as α -dystroglycan are indicated.

aggregation, binds to the second laminin G-domain and thereby prevents binding to the functional receptor [24]. While the first G-domain of agrin is not required for aggregation of AChRs [23,25] it is necessary for the interaction of agrin with its most abundant binding protein on the muscle surface, α -dystroglycan [24].

The central part of agrin contains two serine/threonine-rich domains, which are targets for O-linked glycosylation, and a third region, which is homologous to the domain III of laminin implicated in neurite adhesion. The role of these segments in the agrin molecule are not known.

ALTERNATIVE SPLICING OF AGRIN DETERMINES ITS FUNCTION

Expression of agrin is not restricted to motoneurons. Agrin mRNA and protein are synthesized by a large number of tissues including muscle, thus making agrin a general component of many basement membranes [26,27]. This ubiquitous distribution raises the question of how agrin can be a specific signal for synaptic differentiation. The solution to this problem lies in the existence of a number of different isoforms of agrin, which are generated by alternative splicing [15,27,28]. These agrin variants differ only in the absence or presence of small inserts of less than 20 amino acids (Fig. 2). Despite their small size, some of these insertions strongly affect the biological activity of agrin [15,23,29]. Furthermore, expression of some of them is highly tissue specific and is regulated during development [27,30–32].

An eight amino acid insert at site Z between the G-domains 2 and 3 (Fig. 2) increases the potency of agrin to induce AChR aggregation $\approx 10\,000$ fold [20] and an 11 amino acid insert ≈ 50 -fold [23]. Isoforms containing these inserts are synthesized only by neurones. Consequently nerve-derived agrin is of much greater importance for the induction of synapses than its muscle-derived counterpart. While it has been shown that strong overexpression of muscle-derived forms of agrin also induces AChR aggregation [20,33] it is unlikely that it plays such a role under physiological conditions [34–36].

Another small insertion at splicing site Y does not affect the clustering activity of the neurone-specific isoforms of agrin [20,36,37]. This highly negatively charged four amino acid peptide (KSRK) is essential for the binding of the inhibitor heparin to agrin [37–39].

AGRIN, MUSCLE-SPECIFIC KINASE (MuSK) AND RAPSIN ARE ESSENTIAL COMPONENTS FOR THE AGGREGATION OF AChRs

The identification of the pathway by which agrin induces the aggregation of AChRs proved to be a daunting task. A large number of proteins is concentrated in the synaptic region and

therefore potentially involved in the aggregation process. The still-growing list includes components of the extracellular matrix (proteoglycans, laminin) and transmembrane proteins (neuregulin, β -dystroglycan), as well as cytoskeletal proteins (utrophin). The role of most of these components in the aggregation process has not been resolved. However, three proteins are now known to be essential for AChR aggregation (Table 1). This short list includes, besides neuronal isoforms of agrin [35], a receptor tyrosine kinase, MuSK [40], which is thought to be part of an agrin receptor complex (see below), and rapsyn, a peripheral membrane protein associated with AChRs [41]. Rapsyn appears to be a central component of the clustering process containing a number of features mediating interactions with other components. These include N-terminal 34 amino acid repeats responsible for its tendency for self-aggregation, a C-terminal coiled-coil region essential for binding to AChRs and the myristoylated N-terminus which targets the protein to the plasma membrane [42].

Overexpression of each of the three proteins induces AChR aggregation. Addition of agrin-expressing cells or soluble agrin to myotubes in culture causes the formation of new AChR aggregates [15,19,29]. Similar aggregates can also be induced by injection into rat myotubes or *Xenopus* oocytes of c-DNA or RNA constructs corresponding to neuronal agrin isoforms [33,43–45].

Injection of expression constructs for MuSK into muscle cell lines also increases the number of AChR aggregates, both in the presence and absence of agrin [46,47]. Overexpression of receptor tyrosine kinases often triggers their activation by favouring their assembly into dimers even in the absence of ligand [48].

Recombinant expression of rapsyn in nonmuscle cells causes the redistribution of transfected AChRs into microaggregates on the cell surface [49,50]. These aggregates are of much smaller size than their counterparts in myotubes, suggesting that additional factors modifying the clustering process are missing in nonmuscle cells. Such factors could also explain the observation that in myotubes only moderate overexpression of rapsyn induces the number of AChR aggregates. In contrast, strong overexpression of rapsyn prevents AChR aggregation [51], an effect not observed in nonmuscle cells.

Gene-targeting experiments have demonstrated that deletion of any one of the genes encoding the same three proteins abolishes nerve-induced clustering of AChRs [35,40,41]. In each case, homozygous mice die before or at birth due to respiratory failure. Closer inspection of the myotubes in these mice revealed interesting differences. The strongest phenotype was observed in the MuSK knock-out mice, which display no AChR aggregates at all implying a central role of this protein in the clustering process [40]. In the agrin knock-out mice, AChR aggregates are still detectable. They are, however, localized randomly on the muscle surface and most of the time are not found opposite the presynaptic terminals [35]. This distribution

Table 1. Proteins essential for the formation of the neuromuscular junction.

	Agrin	MuSK	Rapsyn
Molecular weight (kDa)	600	110	43
Type of protein	Secreted	Transmembrane	Myristoylated
Location	Extracellular matrix	Plasma-membrane	Peripheral membrane
Deletion of gene	Lethal	Lethal	Lethal

suggests that the remaining aggregates are generated by a ligand-independent activation of MuSK. In the rapsyn knock-out mice no AChR aggregates are present. However, the concentration of AChRs is higher in the areas of the muscle surface near a presynaptic terminal [41]. This differential distribution is almost certainly caused by the preferential transcription of mRNAs coding for AChR subunits, a pathway, which apparently does not involve rapsyn.

MuSK – A CENTRAL PART OF THE AGRIN RECEPTOR

Investigations of second messengers that could be involved in the agrin signalling pathway demonstrated that inhibitors of tyrosine phosphorylation, such as staurosporine and herbimycin, block the aggregation of nAChRs [52,53]. This prompted a search for receptor tyrosine kinases as potential ligands for agrin. Candidate receptor genes were identified first in the electric organ of *Torpedo californica* [54] and later in mammalian muscle [55–57].

In each case efforts to demonstrate a direct binding of agrin to the recombinantly expressed receptors failed, suggesting that this subunit alone is not sufficient for ligand binding. Moreover, MuSK expressed in fibroblasts or myoblasts does not become phosphorylated upon incubation of transfected cells with agrin. In contrast, when myotubes are exposed to agrin, recombinantly expressed MuSK, as well as its endogenous counterpart, is tyrosine phosphorylated [58]. In order to explain why only MuSK present in myotubes, but not that present in myoblasts and nonmuscle cells, responds to agrin treatment in this characteristic way the existence of a myotube-associated specificity component (MASC) has been postulated [58]. This hypothetical factor is thought to form, in conjunction with MuSK, a high-affinity receptor for agrin. (Fig. 3). Binding of

agrin would trigger tyrosine phosphorylation of the cytoplasmic domain of MuSK, the prototypic response of receptor tyrosine kinases to the binding of their ligand.

Tyrosine phosphorylation of MuSK in response to agrin is very specific: it is induced exclusively by isoforms and truncation fragments which also trigger the clustering of AChRs [59]. Activation of MuSK occurs rapidly, phosphotyrosine residues can already be detected within minutes after the addition of agrin. Phosphorylation of MuSK gradually declines following 1 h of stimulation [53]. Thus, desensitization occurs with a slow time course compared with other receptors, for example the EGF-receptor.

To directly prove a role for MuSK as a central receptor component in the agrin-signalling pathway, several groups have attempted to dimerize, and thereby activate, MuSK in the absence of agrin. In many receptor tyrosine kinases, a close apposition of the cytoplasmic domains is sufficient to trigger their mutual tyrosine phosphorylation [60]. In turn, phosphorylation generates binding sites for signalling and adapter molecules. Agrin-independent MuSK had different consequences for the aggregation of AChRs, thereby revealing an important role for the extracellular domain of MuSK in its signalling. A similar feature is not known from any other member of the large family of receptor tyrosine kinases. In one approach, MuSK dimerization was achieved by incubating myotubes with antibodies directed against extracellular epitopes of MuSK [61,62]. In a second approach, a chimeric receptor consisting of the cytoplasmic domain of MuSK fused to the extracellular domain of a related receptor tyrosine kinase, TRK C, was constructed. Upon transfection into myotubes from C2C12 cells, the chimeric receptor could be activated by NT-3, the ligand for TRK C, as evidenced by its autophosphorylation [63]. Activation of the chimeric receptor did not induce the aggregation of AChRs. In contrast, antibody-induced dimerization of MuSK did trigger this aggregation. The main difference between these experimental set-ups is the absence of the extracellular domain of MuSK in the chimeric receptor. The extracellular TRK C part is able to replace the corresponding part of MuSK with respect to triggering the activation of the kinase domain. However, it does not substitute for a different signal generated by the extracellular part of MuSK. Most likely interactions of this region with other proteins are required for the concentration of AChRs in the synaptic area.

Curiously, in innervated myotubes new AChR aggregates can be induced in extrasynaptic areas by strong overexpression of the cytoplasmic domain of MuSK in the absence of its extracellular region [46,47]. Under these conditions, endogenous MuSK binds to recombinant chimeric MuSK/TRK C molecules. Apparently, very high concentrations of the kinase domain obtained by injection into muscle can activate AChR aggregation indirectly by recruiting extracellular MuSK domains from the extrasynaptic myotube surface.

Two hypothetical MuSK-binding proteins are depicted in Fig. 3. The first is the agrin-binding component MASC which has been discussed above. The second, a rapsyn-associated transmembrane linker (RATL), has been postulated to mediate an interaction between the extracellular part of MuSK and rapsyn. Such an interaction was observed in transfection experiments using the quail fibroblast cell line QT-6. Upon transfection into this line, MuSK is distributed randomly over the cell surface. In contrast, when it is cotransfected together with rapsyn, MuSK becomes concentrated in small microaggregates that also contain rapsyn [64,65]. Surprisingly, this colocalization is not mediated by an association of rapsyn with the cytoplasmic domain of MuSK. Rather it is caused by an

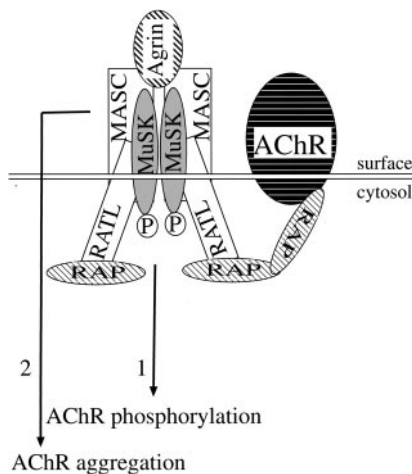


Fig. 3. Model of the agrin receptor complex. The scheme includes two hypothetical components which have been postulated according to their functional properties: (a) a myotube-associated specificity component (MASC) which binds to agrin with high affinity and mediates its association with MuSK; (b) a rapsyn-associated transmembrane linker (RATL) forming a bridge between the extracellular part of MuSK and rapsyn. In addition, rapsyn (Rap) is shown interacting with the AChR as well as with itself. MuSK is present in an activated and autophosphorylated homodimeric form. From its cytoplasmic domain, signal 1 is emerging which is sufficient for AChR phosphorylation. For AChR aggregation, both, a signal 1 and a second signal originating from the extracellular domain of MuSK are required.

indirect interaction of rapsyn with the extracellular domain of MuSK as shown by similar cotransfection experiments using truncation fragments of MuSK [65].

AGRIN-INDUCED TYROSINE PHOSPHORYLATION OF AChR

The intracellular signals triggered by phosphorylation of MuSK are still largely unknown. One important exception is a direct modification of AChRs, the tyrosine phosphorylation of its β subunit in response to agrin [52]. Phosphorylation of the AChR β subunit occurs with a similar time course and has indistinguishable requirements, with respect to various agrin fragments and isoforms, to MuSK phosphorylation and AChR aggregation [59,66]. These observations indicate that it is mediated by activation of the MuSK-containing agrin receptor. In accordance with this, AChR phosphorylation can be induced by artificial dimerization of intact MuSK molecules [61,62] or by activation of a chimeric receptor containing only the cytoplasmic domain of MuSK [63].

AChR β subunits do not seem to be phosphorylated directly by MuSK, although a small fraction of the two proteins in myotubes are so closely associated that they can be co-immunoprecipitated [67]. Evidence for an indirect effect of MuSK on AChR phosphorylation came from studies using tyrosine kinase inhibitors. The rather nonselective inhibitor staurosporine prevents phosphorylation of AChRs without abolishing the phosphorylation of MuSK [67]. Activation of MuSK most likely enables one or several soluble tyrosine kinases to phosphorylate AChRs. At least two nonreceptor tyrosine kinases, src and fyn, are associated with AChRs [68]. *In vitro*, Src can bind to AChRs and phosphorylate the β subunit, whereas fyn requires phosphorylation of AChR for binding [69].

The functional consequences of β -subunit phosphorylation are not known. Experiments with transfected β subunits, in which all potential phosphorylation sites have been mutated, suggest that phosphorylation is not required for aggregation of AChRs: endogenous receptors as well as AChRs containing the mutated β subunit become concentrated in aggregates in response to agrin [70].

Clearly, AChR phosphorylation does not trigger AChR aggregation. Activation of a chimeric receptor containing only the cytoplasmic domain of MuSK induces AChR phosphorylation but not aggregation [63]. In addition, AChR phosphorylation, but not aggregation, is induced by agrin treatment of myotubes in the presence of the calcium chelator BAPTA [71]. The lack of AChR aggregation in the presence of chelators that can rapidly bind calcium implies that a so-far unidentified calcium-sensitive intracellular step is required for AChR aggregation.

The AChR-associated protein rapsyn is an important facilitating component for tyrosine phosphorylation: For example, AChRs are not phosphorylated in response to agrin in rapsyn knock-out mice [41]. In addition, co-expression of rapsyn with AChRs in the quail cell line QT-6 induces tyrosine phosphorylation of unidentified components copurifying with AChRs [64]. In contrast to AChR phosphorylation, tyrosine phosphorylation of one or several of these components might well be necessary for AChR-aggregation. Rapsyn does not display any sequence homology to known tyrosine kinases. It is currently not understood how this protein promotes tyrosine phosphorylation. Perhaps rapsyn organizes and orients AChRs and other proteins in a way which facilitates their phosphorylation by tyrosine kinases.

AGRIN BINDING TO α -DYSTROGLYCAN

Additional agrin-binding proteins of the muscle surface, unrelated to the MuSK-containing agrin receptor, have been identified. Two examples are the neural cell-adhesion molecule (N-CAM; [72]) and the heparin-binding growth-associated molecule (HB-GAM; [73]). In both cases, the functional significance of the interaction with agrin is unclear. The most abundant and first identified agrin-binding protein, however, is the glycoprotein α -dystroglycan, which is part of a large transmembrane-spanning complex known as dystrophin-associated glycoprotein complex (DGC, Fig. 4; [74–76]). This complex comprises α -dystroglycan and β -dystroglycan, which are synthesized as a common precursor protein, a set of transmembrane proteins collectively referred to as the sarcoglycan complex [77,78], and the cytoplasmically located syntrophin [79]. The DGC generated a considerable amount of interest immediately following its discovery as a set of dystrophin-binding proteins. The bridging role of the DGC between dystrophin as an actin-binding cytoskeletal protein with the sarcolemma and the extracellular matrix appears to be important for the mechanical stability of muscle fibres [76,80]. Mutations in the dystrophin gene cause Duchenne muscle [81], while alterations in the structure of the sarcoglycans are responsible for other muscle dystrophies [82]. At the neuromuscular junction, the DGC is considerably enriched. In its synaptic form, the DGC is complexed with the dystrophin-related protein utrophin rather than with dystrophin itself [83,84].

The DGC is not only associated with cytoskeletal proteins, but also with proteins of the extracellular matrix such as laminin [78]. In fact, laminin bound to α -dystroglycan expressed on Schwann cells is used by *Mycobacterium leprae* as a receptor for entry into cells [85]. α -Dystroglycan binds to the globular domains of laminin, a region not involved in the interaction of laminin with their more well-known receptors, the integrins. Five copies of these G-domains are located in a region of the laminin molecule only found in α chains and not β chains or γ chains. Homologous G-domains are present in a number of other extracellular and transmembrane proteins

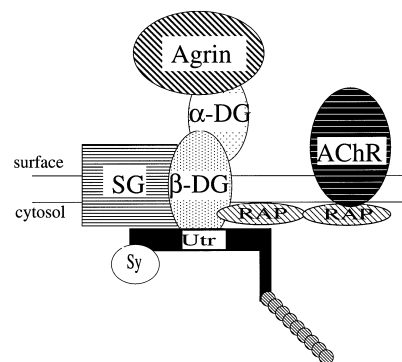


Fig. 4. Interaction of agrin with the dystrophin-associated glycoprotein complex at the neuromuscular junction. Extracellular, transmembrane and cytoskeletal proteins together form this large complex which is represented by the following symbols: α , β -DG, α β -dystroglycan; SG, sarcoglycan complex; Sy, Syntrophin. In its synaptic form, this complex is linked to utrophin (utr) rather than dystrophin. The striped circles on the bottom symbolize the actin cytoskeleton which is associated with utrophin. α -Dystroglycan is a high-affinity agrin-binding protein, whereas β -dystroglycan associates with rapsyn (Rap).

including agrin (Fig. 2) and neuroligins, and are often intermingled with EGF-repeats [22].

Similar to laminin, agrin also binds to α -dystroglycan with an affinity in the nanomolar range [86–88]. Binding is mediated by the first two of three G-domains present in the C-terminal region (Fig. 2). This part of the agrin molecule overlaps, but is not identical to, the region necessary for induction of AChR aggregation [24]. Thus an interaction of agrin with α -dystroglycan is not involved in the induction of this process. This could explain why in utrophin/dystroglycan double knock-out mice, aggregates of AChRs are still found although the density of AChR packaging is reduced slightly [89,90]. Most likely, the interaction between agrin and dystroglycan has a stabilizing function for the postsynaptic sarcolemma as has been proposed for the laminin– α -dystroglycan interaction in the extrasynaptic region.

In addition, the agrin–dystroglycan interaction could also stabilize and condense AChR aggregates [24,91]. In tissue culture, high concentrations of laminin-1 can induce AChR aggregation in the absence of agrin and enhance the number of clusters induced by agrin [92,93]. This effect might not be relevant in a physiological context or triggered by a different ligand, because laminin-1 is not expressed in the synaptic basal membrane [94]. The agrin-independent AChR aggregation does not require activation of MuSK, rather it appears to be mediated by α -dystroglycan [92]. Possibly the binding of dystroglycan to some isoforms of laminin and agrin helps to condense small AChR aggregates into larger clusters. This process may be caused by a direct interaction of β -dystroglycan with rapsyn [95,96].

ROLE OF AGRIN DURING INDUCTION OF AChR mRNA SYNTHESIS

During the formation of a new synapse between nerve and muscle, a particular site on the muscle surface is marked by the secretion of agrin isoforms and the influx of synaptic proteins such as AChRs from other parts of the myotube surface. Redistribution of existing proteins is not the only process which contributes to synapse-specific localization of proteins. Differential transcription of mRNAs coding for synaptic proteins from synaptic versus extra-synaptic nuclei is another cellular process contributing to this result (reviewed in [97]).

A number of mRNAs are known to be enriched in nuclei localized underneath the synapse [98]. At least the differential distribution of mRNAs coding for AChR subunits [99], AChR esterase [100] and utrophin [101] is the result of a higher transcription rate in the synaptic nuclei. In addition, diffusion of these mRNAs is slow enough to preserve their enrichment in the synaptic area.

An important factor involved in the up-regulation of mRNAs coding for AChR subunits is AChR-inducing activity (ARIA), also known as neuregulin [102]. ARIA, like many other splicing variants of neuregulin, binds to receptors containing two different subunits of the ErbB-family, ErbB2 and ErbB3 or ErbB4. All these receptor tyrosine kinases are close relatives of the EGF-receptor which is also called ErbB1 [103]. Activation of ErbB receptors on the myotube surface causes an increased transcription of a number of genes, for example those coding for AChR subunits [104]. Mice in which the ARIA gene has been deleted highlight the importance of this factor for synaptic AChR expression. Homozygotes die during early embryonic development, while heterozygotes remain viable. Careful analysis revealed that they contain only half the normal number

of AChRs aggregated in their postsynaptic myotube membranes [105].

Recent experiments provide evidence that agrin is involved in the pathway induced by ARIA. Cell-bound agrin induces the expression of the ϵ -subunit of the AChR [106] and utrophin [101]. This effect is most likely mediated by ARIA/ErbB, because agrin does not induce AChR expression in myotubes over-expressing a dominant-negative mutant of ErbB [44]. Agrin probably stimulates ErbB receptors indirectly by concentrating ARIA at synaptic sites [107]. This effect appears to be mediated by heparan sulfate binding sites in the ARIA molecule [108] to which agrin as a proteoglycan can bind [109]. In addition, agrin causes other heparan sulfate proteoglycans to concentrate in synaptic areas. These in turn can bind ARIA and thereby concentrate it in the synaptic region [110]. The local accumulation of ARIA should facilitate activation of ErbB receptors selectively in the postsynaptic membrane. Local restriction of ARIA signalling is further enhanced by the concentration of all ErbB subunits forming ARIA receptors in the synaptic area [103,111,112], which is caused by agrin as well. ErbB proteins are not only present in normal postsynaptic membranes but also become concentrated in the ectopic postsynaptic regions induced by local overexpression of agrin [45,107,109].

The downstream pathway of ErbB activation is far better understood than the MuSK pathway and appears to follow a more conventional pattern. It includes activation of phosphatidylinositol-3-kinase and MAP kinases [113–115] and perhaps the nonreceptor kinase LIM kinase 1 [116], and culminates in the binding of transcription factors of the ets family to a promoter element termed the N-box [117]. The N-box contains a consensus motif for binding of ets family members and is present not only in the genes encoding the δ and ϵ subunits of the AChR [118–120], but also utrophin [121] and other synapse-specific proteins [101]. Activation of common transcription factors therefore appears to increase expression of a multitude of proteins of the postsynaptic apparatus.

AGRIN AND SYNAPTOGENESIS IN THE CENTRAL NERVOUS SYSTEM

Whether or not the mechanisms directing formation of the neuromuscular junction are mirrored by similar processes during synapse formation between nerve cells in the brain is still an open question. Many of the important players of the neuromuscular junction are indeed expressed in identical or modified form in synaptic areas of neurones. The isoforms of agrin displaying high AChR aggregation activity are present not only in motoneurons but were also found in neurones from all regions of the central nervous system (CNS; [27,31,32,122]). In contrast, inactive isoforms are predominantly expressed in blood vessels and ependymal and glial cells [123]. Utrophin and dystrophin are also not muscle specific, but can both be detected in synaptic areas of neurones [124,125]. MuSK itself is not detectable in the CNS [40,56,57]. However, its closest relatives, the orphan receptor tyrosine kinases ROR 1 and ROR 2, are neuronal proteins [126].

Despite this suggestive pattern of expression, genetic experiments have so far failed to provide evidence for an essential role of these components for synaptogenesis between neurones. Neurones from mice in which the active isoforms of agrin are not produced still form synapses in culture which appear normal [127,128]. Also, the addition of agrin to neurones in either the soluble or cell-attached form does not

induce aggregation of a number of glutamate or GABA_A-receptor subunits (Leuschner and Hoch, unpublished results). These results are not completely unexpected; the high diversity of neuronal synapses requires a higher degree of specification and thus most likely more signals for the generation of new synaptic connections. One or more signals for example should specify the neurotransmitter receptor incorporated into the developing postsynaptic region out of a long list of possible receptor types and isoforms. It remains to be seen whether agrin is one part of a more complex signal.

Recently, evidence for a different signalling role of agrin has been provided: it was found that treatment of hippocampal neurones with agrin caused the phosphorylation of the transcription factor cyclic AMP response element binding protein (CREB) [129]. The effect was triggered only by a neurone-specific isoform of agrin and not by its ubiquitously expressed counterpart. As MuSK is not expressed in neurones, it remains to be seen which receptor mediates this effect. Phosphorylation of CREB, which is also triggered by neurotrophins, regulates the expression of a large number of genes and has been implicated in synaptic plasticity in various cellular systems [130]. These observations are consistent with a role of agrin during remodelling of intraneuronal synapses. Interestingly, agrin expression is influenced by changes in synaptic activity as, for example, occur during seizures [131].

A number of proteins have been identified which have at least some functional properties in common with rapsyn without sharing sequence homology. Two examples are gephyrin and PSD95, which are associated with the inhibitory glycine and the NMDA receptor, respectively. Like rapsyn, these proteins bind to the cytoplasmic domains of neuronal neurotransmitter receptors [132,133]. Many of them belong to the rapidly growing list of proteins containing PDZ-domains which mediates specific protein–protein interactions. Some members of this group can induce the clustering of the attached neurotransmitter receptors [134]. Proteins containing the PDZ-motif also bind to the C-terminus of several receptor tyrosine kinases expressed in the central nervous system as well as to MuSK [135]. These recent observations raise the interesting possibility that receptor tyrosine kinases are part of a network of proteins which regulate the synaptic expression of neurotransmitter receptors. The agrin signalling pathway at the neuromuscular junction might be just one particularly well-studied example of a recurrent theme also found in postsynaptic regions of neurones.

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REFERENCES

- Hall, Z.W. & Sanes, J.R. (1993) Synaptic structure and development: the neuromuscular junction. *Neuron* **10** (Suppl.), 99–121.
- Meier, T. & Wallace, B.G. (1998) Formation of the neuromuscular junction: molecules and mechanisms. *Bioessays* **20**, 819–829.
- Son, Y.-J. & Thompson, W.J. (1995) Schwann cell processes guide regeneration of peripheral axons. *Neuron* **14**, 125–132.
- Patton, B.L., Chiu, A.Y. & Sanes, J.R. (1998) Synaptic laminin prevents glial entry into the synaptic cleft. *Nature* **393**, 698–701.
- Duclert, A. & Changeux, J.-P. (1995) Acetylcholine receptor gene expression at the developing neuromuscular junction. *Physiol. Rev.* **75**, 339–368.
- Sanes, J.R. & Lichtman, J.W. (1999) Development of the vertebrate neuromuscular junction. *Annu. Rev. Neurosci.* **22**, 389–442.
- Noakes, P.G., Gautam, M., Mudd, J., Sanes, J.R. & Merlie, J.P. (1995) Aberrant differentiation of neuromuscular junctions in mice lacking s-laminin/laminin β 2. *Nature* **374**, 258–262.
- Connor, E.A. & Smith, M.A. (1994) Retrograde signaling in the formation and maintenance of the neuromuscular junction. *J. Neurobiol.* **25**, 722–739.
- Fischbach, G.D. (1972) Synapse formation between dissociated nerve and muscle cells in low density cultures. *Dev. Biol.* **28**, 407–429.
- Fischbach, G.D. & Cohen, S.A. (1973) The distribution of acetylcholine sensitivity over uninnervated and innervated muscle fibers grown in cell culture. *Dev. Biol.* **31**, 147–162.
- Anderson, M.J., Cohen, M.W. & Zorychta, E. (1977) Effects of innervation on the distribution of acetylcholine receptors on cultured muscle cells. *J. Physiol.* **268**, 731–756.
- Sanes, J.R., Marshall, L.M. & McMahon, U.J. (1978) Reinnervation of muscle fiber basal lamina after removal of muscle fibers. Differentiation of regenerating axons at original synaptic sites. *J. Cell Biol.* **78**, 176–198.
- Nitkin, R.M., Smith, M.A., Magill, C., Fallon, J.R., Yao, Y.-M.M., Wallace, B. & McMahon, U.J. (1987) Identification of agrin, a synaptic organizing protein from *Torpedo* electric organ. *J. Cell Biol.* **105**, 2471–2478.
- Rupp, F., Payan, D.G., Magill, S.C., Cowan, D.M. & Scheller, R.H. (1991) Structure and expression of a rat agrin. *Neuron* **6**, 811–823.
- Tsim, K.W.K., Ruegg, M.A., Escher, G., Kröger, S. & McMahon, U.J. (1992) cDNA that encodes active agrin. *Neuron* **8**, 677–689.
- Smith, M.A., Magill-Solc, C., Rupp, F., Yao, Y.-M., Schilling, J.W., Snow, P. & McMahon, U.J. (1992) Isolation and characterisation of an agrin homologue in the marine ray. *Mol. Cell. Neurosci.* **3**, 406–417.
- Tsen, G., Halfter, W., Kröger, S. & Cole, G.J. (1995) Agrin is a heparan sulfate proteoglycan. *J. Biol. Chem.* **270**, 3392–3399.
- Denzer, A., Brandenberger, R., Geesemann, M., Chiquet, M. & Ruegg, M. (1997) Agrin binds to nerve–muscle basal lamina via laminin. *J. Cell Biol.* **137**, 671–683.
- Campanelli, J.T., Hoch, W., Rupp, F., Kreiner, T. & Scheller, R.H. (1991) Agrin mediates cell contact-induced acetylcholine receptor clustering. *Cell* **67**, 909–916.
- Ferns, M.J., Campanelli, J.T., Hoch, W., Scheller, R.H. & Hall, Z. (1993) The ability of agrin to cluster AChRs depends on alternative splicing and on cell surface proteoglycans. *Neuron* **11**, 491–502.
- Denzer, A.J., Schulthess, T., Fauser, C., Schumacher, B., Kammerer, R.A., Engel, J. & Ruegg, M. (1998) Electron microscopic structure of agrin and mapping of its binding site in laminin-1. *EMBO J.* **17**, 335–343.
- Missler, M. & Südhof, T.C. (1998) Neurexins: three genes and 1001 products. *Trends Genet.* **14**, 20–26.
- Hoch, W., Campanelli, J.T., Harrison, S. & Scheller, R.H. (1994) Structural domains of agrin required for clustering of nicotinic acetylcholine receptors. *EMBO J.* **13**, 2814–2821.
- Hopf, C. & Hoch, W. (1996) Agrin binding to α -dystroglycan. Domains of agrin necessary to induce acetylcholine receptor clustering are overlapping but not identical with the α -dystroglycan-binding region. *J. Biol. Chem.* **271**, 5231–5236.
- Gesemann, M., Denzer, A.J. & Ruegg, M.A. (1995) Acetylcholine receptor-aggregating activity of agrin isoforms and mapping of active site. *J. Cell Biol.* **128**, 625–636.
- Godfrey, E.A. (1991) Comparison of agrin-like proteins from the extracellular matrix of chicken kidney and muscle with neural agrin, a synapse organizing protein. *Exp. Cell Res.* **195**, 99–109.
- Hoch, W., Ferns, M., Campanelli, J.T., Hall, Z.W. & Scheller, R.H. (1993) Developmental regulation of highly active alternatively spliced forms of agrin. *Neuron* **11**, 479–490.
- Wei, N., Lin, C.Q., Modafferi, E.F., Gomes, W.A. & Black, D.L. (1997) A unique intronic splicing enhancer controls the inclusion of the agrin Y exon. *RNA* **3**, 1275–1288.
- Ferns, M., Hoch, W., Campanelli, J.T., Rupp, F., Hall, Z.W. &

- Scheller, R.H. (1992) RNA splicing regulates agrin-mediated acetylcholine receptor clustering activity on cultured myotubes. *Neuron* **8**, 1079–1086.
30. Smith, M.A. & O'Dowd, D.K. (1994) Cell-specific regulation of agrin RNA splicing in the chick ciliary ganglion. *Neuron* **12**, 795–804.
 31. Stone, D.M. & Nikolics, K. (1996) Tissue- and age-specific expression patterns of alternatively spliced agrin mRNA transcripts in embryonic rat suggest novel developmental roles. *J. Neurosci.* **15**, 6767–6778.
 32. Ma, E., Morgan, R. & Godfrey, E.W. (1994) Distribution of agrin mRNA in chick embryo nervous system. *J. Neurosci.* **14**, 2943–2952.
 33. Godfrey, E.W., Roe, J. & Heathcote, R.D. (1999) Overexpression of agrin isoforms in *Xenopus* embryos alters the distribution of synaptic acetylcholine receptors during development of the neuromuscular junction. *Dev. Biol.* **205**, 22–32.
 34. Reist, N.E., Werle, M.J. & McMahan, U.J. (1992) Agrin released by motoneurons induces the aggregation of acetylcholine receptors at neuromuscular junctions. *Neuron* **8**, 865–868.
 35. Gautam, M., Noakes, P.G., Moscoso, L., Rupp, F., Scheller, R.H., Merlie, J.P. & Sanes, J.R. (1996) Defective neuromuscular synaptogenesis in agrin-deficient mutant mice. *Cell* **85**, 525–535.
 36. Burgess, R.W., Nguyen, Q.T., Son, Y.-J., Lichtman, J.W. & Sanes, J.R. (1999) Alternatively spliced isoforms of nerve- and muscle-derived agrin: their roles at the neuromuscular junction. *Neuron* **23**, 33–44.
 37. Hopf, C. & Hoch, W. (1997) Heparin inhibits acetylcholine receptor aggregation at two distinct steps in the agrin-induced pathway. *Eur. J. Neurosci.* **9**, 1170–1177.
 38. Gesemann, M., Cavalli, V., Denzer, A.J., Brancaccio, A., Schumacher, B. & Ruegg, M.A. (1996) Alternative splicing of agrin alters its binding to heparin, dystroglycan, and the putative agrin receptor. *Neuron* **16**, 755–767.
 39. Campanelli, J.T., Gayer, G.G. & Scheller, R.H. (1996) Alternative RNA splicing that determines agrin activity regulates binding to heparin and α -dystroglycan. *Development* **122**, 1663–1672.
 40. DeChiara, T.M., Bowen, D.C., Valenzuela, D.M., Simmons, M.V., Poueymirou, W.T., Thomas, S., Kinetz, E., Compton, D.L., Rojas, E., Park, J.S., Smith, C., DiStefano, P.S., Glass, D.J., Burden, S.J. & Yancopoulos, G.D. (1996) The receptor tyrosine kinase MuSK is required for neuromuscular junction formation *in vivo*. *Cell* **85**, 501–512.
 41. Gautam, M., Noakes, P.G., Mudd, J., Nichol, M., Chu, G.C., Sanes, J.R. & Merlie, J.P. (1995) Failure of postsynaptic specialisation to develop at neuromuscular junctions of rapsyn-deficient mice. *Nature* **377**, 232–236.
 42. Ramaro, M.K. & Cohen, J.B. (1998) Mechanism of nicotinic acetylcholine receptor cluster formation by rapsyn. *Proc. Natl Acad. Sci.* **95**, 4007–4012.
 43. Cohen, I., Rimer, M., Lomo, T. & McMahan, U.J. (1997) Agrin-induced postsynaptic-like apparatus in skeletal muscle fibers *in vivo*. *Mol. Cell. Neurosci.* **9**, 237–253.
 44. Jones, G., Meier, T., Lichtsteiner, M., Witzemann, V., Sakmann, B. & Brenner, H.R. (1997) Induction by agrin of ectopic and functional postsynaptic-like membrane in innervated muscle. *Proc. Natl Acad. Sci.* **94**, 2654–2659.
 45. Meier, T., Hauser, D.M., Chiquet, M., Landmann, L., Ruegg, M. & Brenner, H.R. (1997) Neural agrin induces ectopic postsynaptic specializations in innervated muscle fibers. *J. Neurosci.* **17**, 6534–6544.
 46. Jones, G., Moore, C., Hashemolhosseini, S. & Brenner, H.R. (1999) Constitutively active MuSK is clustered in the absence of agrin and induces ectopic postsynaptic-like membranes in skeletal muscle fibers. *J. Neurosci.* **19**, 3376–3383.
 47. Hesser, B., Sander, A. & Witzemann, V. (1999) Identification and characterization of a novel splice variant of MuSK. *FEBS Lett.* **442**, 133–137.
 48. Heldin, C.-H. (1995) Dimerization of cell surface receptors in signal transduction. *Cell* **80**, 213–223.
 49. Froehner, S.C., Luetjens, C.W., Scotland, P.B. & Patrick, J. (1990) The postsynaptic 43 K protein clusters muscle nicotinic acetylcholine receptors in *Xenopus* oocytes. *Neuron* **5**, 403–410.
 50. Phillips, W.D., Kopta, C., Blount, P., Gardner, P.D., Steinbach, J.H. & Merlie, J.P. (1991) ACh receptor-rich domains organized in fibroblasts by recombinant 43-kilodalton protein. *Science* **251**, 568–570.
 51. Yoshihara, C.M. & Hall, Z.W. (1993) Increased expression of the 43-kD protein disrupts acetylcholine receptor clustering in myotubes. *J. Cell Biol.* **122**, 169–179.
 52. Wallace, B.G., Qu, Z. & Haganir, R.L. (1991) Agrin induces phosphorylation of the nicotinic acetylcholine receptor. *Neuron* **6**, 869–878.
 53. Ferns, M., Deiner, M. & Hall, Z. (1996) Agrin-induced acetylcholine receptor clustering in mammalian muscle requires tyrosine phosphorylation. *J. Cell Biol.* **132**, 937–944.
 54. Jennings, C.G.B., Dyer, S.M. & Burden, S.J. (1993) Muscle-specific trk-related receptor with a kringle domain defines a distinct class of receptor tyrosine kinases. *Proc. Natl Acad. Sci. USA* **90**, 2895–2899.
 55. Valenzuela, D.M., Stitt, T.N., DiStefano, P.S., Rojas, E., Mattsson, K., Compton, D.L., Nunez, L., Park, J.S., Stark, J.L., Gies, D.R., Thomas, S., Le Beau, M.M., Fernald, A.A., Copeland, N.G., Jenkins, N.A., Burden, S.J., Glass, D.J. & Yancopoulos, G.D. (1995) Receptor tyrosine kinase specific for the skeletal muscle lineage: expression in embryonic muscle, at the neuromuscular junction, and after injury. *Neuron* **15**, 573–584.
 56. Ganju, P., Walls, E., Brennan, J. & Reith, A.D. (1995) Cloning and developmental expression of Nsk2, a novel receptor tyrosine kinase implicated in skeletal muscle myogenesis. *Oncogene* **11**, 281–290.
 57. Besser, J., Zahalka, M.A. & Ullrich, A. (1996) Exclusive expression of the receptor tyrosine kinase MDK4 in skeletal muscle and the decidua. *Mech. Dev.* **59**, 41–52.
 58. Glass, D.J., Bowen, D.C., Stitt, T.N., Radziejewski, C., Bruno, J., Ryan, T.E., Gies, D.R., Shah, S., Mattsson, K., Burden, S.J., DiStefano, P.S., Valenzuela, D.M., DeChiara, T.M. & Yancopoulos, G.D. (1996) Agrin acts via a MuSK receptor complex. *Cell* **85**, 513–523.
 59. Hopf, C. & Hoch, W. (1998) Tyrosine phosphorylation of the muscle specific kinase (MuSK) is exclusively induced by AChR-aggregating agrin fragments. *Eur. J. Biochem.* **253**, 382–389.
 60. Schlessinger, J. & Ullrich, A. (1992) Growth factor signaling by receptor tyrosine kinases. *Neuron* **9**, 383–391.
 61. Xie, M.-H., Yuan, J., Adams, C. & Gurney, A. (1997) Direct demonstration of MuSK involvement in acetylcholine receptor clustering through identification of agonist ScFv. *Nat. Biotech.* **15**, 768–771.
 62. Hopf, C. & Hoch, W. (1998) Dimerization of the muscle-specific kinase induces tyrosine phosphorylation of acetylcholine receptors and their aggregation on the surface of myotubes. *J. Biol. Chem.* **273**, 6467–6473.
 63. Glass, D.J., Apel, E.D., Shah, S., Bowen, D.C., DeChiara, T.M., Stitt, T.N., Sanes, J.R. & Yancopoulos, G.D. (1997) Kinase domain of the muscle-specific receptor tyrosine kinase (MuSK) is sufficient for phosphorylation but not clustering of acetylcholine receptors: required role for the MuSK ectodomain? *Proc. Natl Acad. Sci. USA* **94**, 8848–8853.
 64. Gillespie, S.K.H., Balasubramanian, S., Fung, E.T. & Haganir, R.L. (1996) Rapsyn clusters and activates the synapse-specific receptor tyrosine kinase MuSK. *Neuron* **16**, 953–962.
 65. Apel, E.D., Glass, D.J., Moscosos, L.M., Yancopoulos, G.D. & Sanes, J.R. (1997) Rapsyn is required for MuSK signaling and recruits synaptic components to a MuSK-containing scaffold. *Neuron* **18**, 1–20.
 66. Meier, T., Gesemann, M., Cavalli, V., Ruegg, M.A. & Wallace, B.G. (1996) AChR phosphorylation and aggregation induced by an agrin fragment that lacks the binding domain for α -dystroglycan. *EMBO J.* **15**, 2625–2631.
 67. Fuhrer, C., Sugiyama, J., Taylor, R. & Hall, Z.W. (1997) Association

- of muscle-specific kinase MuSK with the acetylcholine receptor in mammalian muscle. *EMBO J.* **16**, 4951–4960.
68. Swope, S.L. & Huganir, R.L. (1993) Molecular cloning of two abundant protein tyrosine kinases in *Torpedo* electric organ that associate with the acetylcholine receptor. *J. Biol. Chem.* **268**, 25152–25161.
 69. Fuhrer, C. & Hall, Z.W. (1996) Functional interactions of src family kinases with the acetylcholine receptor in C2 myotubes. *J. Biol. Chem.* **271**, 32474–32481.
 70. Meyer, G. & Wallace, B.G. (1998) Recruitment of a nicotinic acetylcholine receptor mutant lacking cytoplasmic tyrosine residues in its β subunit into agrin-induced aggregates. *Mol. Cell. Neurosci.* **11**, 324–333.
 71. Megeath, L.J. & Fallon, J.R. (1998) Intracellular calcium regulates agrin-induced acetylcholine receptor clustering. *J. Neurosci.* **18**, 672–678.
 72. Burg, M.A., Halfter, W. & Cole, G.J. (1995) Analysis of proteoglycan expression in developing chicken brain: characterization of a heparan sulfate proteoglycan that interacts with the neural cell adhesion molecule. *J. Neurosci. Res.* **41**, 49–64.
 73. Peng, H.B., Ali, A.A., Dai, Z., Daggett, D.F. & Rauvala, H. (1995) The role of heparin-binding growth-associated molecule (HB-GAM) in the postsynaptic induction in cultured muscle cells. *J. Neurosci.* **15**, 3027–3038.
 74. Yoshida, M. & Ozawa, E. (1990) Glycoprotein complex anchoring dystrophin to sarcolemma. *J. Biochem.* **108**, 748–752.
 75. Ervasti, J.M. & Campbell, K.P. (1991) Membrane organization of the dystrophin–glycoprotein complex. *Cell* **68**, 1121–1131.
 76. Hemler, M.E. (1999) Dystroglycan versatility. *Cell* **97**, 543–546.
 77. Chan, Y.-M., Bönemann, C.G., Lidov, H.G.W. & Kunkel, L.M. (1998) Molecular organization of sarcoglycan complex in mouse myotubes in culture. *J. Cell Biol.* **143**, 2033–2044.
 78. Ibraghimov-Beskrovnaya, O., Ervasti, J.M., Leveille, C.J., Slaughter, C.A., Sernett, S.W. & Campbell, K.P. (1992) Primary structure of dystrophin-associated glycoproteins linking dystrophin to the extracellular matrix. *Nature* **355**, 696–702.
 79. Suzuki, A., Yoshida, H. & Ozawa, E. (1994) Molecular organization at the glycoprotein-complex-binding site of dystrophin: three dystrophin-associated proteins bind directly to the carboxy-terminal portion of dystrophin. *Eur. J. Biochem.* **220**, 283–292.
 80. Ervasti, J.M. & Campbell, K.P. (1993) A role for the dystrophin–glycoprotein complex as a transmembrane linker between laminin and actin. *J. Cell Biol.* **122**, 809–823.
 81. Monaco, A.P., Neve, R.L., Colletti-Feener, C., Bertelson, C.J., Kurnit, D.M. & Kunkel, L.M. (1986) Isolation of candidate cDNAs for proteins of the Duchenne muscular dystrophy gene. *Nature* **323**, 646–650.
 82. Campbell, K.P. (1995) Three muscular dystrophies: loss of cytoskeleton–extracellular matrix linkage. *Cell* **80**, 675–679.
 83. Ohlendieck, K., Ervasti, J.M., Matsumura, K., Kahl, S.D., Leveille, C.J. & Campbell, K.P. (1991) Dystrophin-related protein is localized to neuromuscular junctions of adult skeletal muscle. *Neuron* **7**, 499–508.
 84. Sealock, R., Butler, M.H., Kramarcy, N.R., Gao, K.-X., Murnane, A.A., Douville, K. & Froehner, S.C. (1991) Localization of dystrophin relative to acetylcholine receptor domains in electric tissue and adult and cultured skeletal muscle. *J. Cell Biol.* **113**, 1133–1144.
 85. Rambukkana, A., Yamada, H., Zanazzi, G., Mathus, T., Salzer, J.L., Yurchenco, P.D., Campbell, K.P. & Fischetti, V.A. (1998) Role of α -dystroglycan as a Schwann cell receptor for *Mycobacterium leprae*. *Science* **282**, 2076–2078.
 86. Gee, S.H., Montanaro, F., Lindenbaum, M.H. & Carbonetto, S. (1994) Dystroglycan- α , a dystrophin-associated glycoprotein, is a functional agrin receptor. *Cell* **77**, 675–686.
 87. Campanelli, J.T., Roberds, S.L., Campbell, K.P. & Scheller, R.H. (1994) A role for dystrophin-associated glycoproteins and utrophin in agrin-induced AChR clustering. *Cell* **77**, 663–674.
 88. Hoch, W., Campanelli, J.T. & Scheller, R.H. (1994) Agrin-induced clustering of acetylcholine receptors: a cytoskeletal link. *J. Cell Biol.* **126**, 1–4.
 89. Grady, R.M., Merlie, J.P. & Sanes, J.R. (1997) Subtle neuromuscular defects in utrophin-deficient mice. *J. Cell Biol.* **136**, 871–882.
 90. Deconinck, A.E., Potter, A.C., Tinsley, J.M., Wood, S.J., Vater, R., Young, C., Metzinger, L., Vincent, A., Slater, C.R. & Davies, K.E. (1997) Postsynaptic abnormalities at the neuromuscular junctions of utrophin-deficient mice. *J. Cell Biol.* **136**, 883–894.
 91. Cohen, M.W., Jacobson, C., Godfrey, E.W., Campbell, K.P. & Carbonetto, S. (1995) Distribution of α -dystroglycan during embryonic nerve–muscle synaptogenesis. *J. Cell Biol.* **129**, 1093–1101.
 92. Montanaro, F., Gee, S.H., Jacobson, C., Lindenbaum, M.H., Froehner, S.C. & Carbonetto, S. (1998) Laminin and α -dystroglycan mediate acetylcholine receptor aggregation via a MuSK-independent pathway. *J. Neurosci.* **18**, 1250–1260.
 93. Sugiyama, J.E., Glass, D.J., Yancopoulos, G.D. & Hall, Z.W. (1997) Laminin-induced acetylcholine receptor clustering: an alternative pathway. *J. Cell Biol.* **139**, 181–191.
 94. Patton, B.L., Miner, J.H., Chiu, A.Y. & Sanes, J.R. (1997) Distribution and function of laminins in the neuromuscular system of developing, adult, and mutant mice. *J. Cell Biol.* **139**, 1507–1521.
 95. Apel, E.D., Roberds, S.L., Campbell, K.P. & Merlie, J.P. (1995) Rapsyn may function as a link between the acetylcholine receptor and the agrin-binding dystrophin-associated glycoprotein complex. *Neuron* **15**, 115–126.
 96. Cartaud, A., Coutant, S., Petrucci, T.C. & Cartaud, J. (1998) Evidence for *in situ* and *in vitro* association between β -dystroglycan and the subsynaptic 43K Rapsyn protein. *J. Cell Biol.* **105**, 2471–2478.
 97. Chu, G.C., Velleca, M.A. & Merlie, J.P. (1995) Synapse-specific gene expression. *Semin. Dev. Biol.* **6**, 175–183.
 98. Moscoso, L.M., Merlie, J.P. & Sanes, J.R. (1995) N-CAM, 43K-Rapsyn, and S-laminin mRNAs are concentrated at synaptic sites in muscle fibers. *Mol. Cell. Neurosci.* **6**, 80–89.
 99. Klarsfeld, A., Besserau, J.L., Salmon, A.M., Triller, A., Babinet, C. & Changeux, J.P. (1991) An acetylcholine receptor α -subunit promoter conferring preferential synaptic expression in muscle of transgenic mice. *EMBO J.* **10**, 625–632.
 100. Jasmin, B.J.K., L.R. & Rotundo, R.L. (1993) Compartmentalization of acetylcholine esterase mRNA and enzyme at the neuromuscular junction. *Neuron* **11**, 467–477.
 101. Gramolini, A.O., Burton, E.A., Tinsley, J.M., Ferns, M.J., Cartaud, A., Cartaud, J., Davies, K.E., Lunde, J.A. & Jasmin, B.J. (1998) Muscle and neural isoforms of agrin increase utrophin expression in cultured myotubes via a transcriptional regulatory mechanism. *J. Biol. Chem.* **273**, 736–743.
 102. Falls, D.L., Rosen, K.M., Corfas, G., Lane, W.S. & Fischbach, G.D. (1993) ARIA, a protein that stimulates acetylcholine receptor synthesis, is a member of the neu ligand family. *Cell* **72**, 801–815.
 103. Altioik, N., Besserau, J.L. & Changeux, J.P. (1995) ErbB3 and ErbB2/neu mediate the effect of heregulin on acetylcholine receptor gene expression in muscle: differential expression at the endplate. *EMBO J.* **14**, 4258–4566.
 104. Jo, S.A., Zhu, X., Marchionny, M.A. & Burden, S.J. (1995) NRGs are concentrated at nerve-muscle synapses and activate ACh receptor gene expression. *Nature* **373**, 158–161.
 105. Sandrock, A.W., Dryer, S.E., Rosen, K.M., Gozani, S.N., Kramer, R., Theill, L.E. & Fischbach, G.D. (1997) Maintenance of acetylcholine receptor number by neuregulins at the neuromuscular junction *in vivo*. *Science* **276**, 599–603.
 106. Jones, G., Herczeg, A., Ruegg, M.A., Lichtsteiner, M., Kröger, S. & Brenner, H.R. (1996) Substrate-bound agrin induces expression of acetylcholine receptor epsilon-subunit gene in cultured mammalian muscle cells. *Proc. Natl Acad. Sci.* **93**, 5985–5990.
 107. Rimer, M., Cohen, I., Lomo, T., Burden, S. & McMahan, U.J. (1997) Neuregulins and erbB receptors at neuromuscular junctions and at agrin-induced postsynaptic-like apparatus in skeletal muscle. *Mol. Cell. Neurosci.* **12**, 1–15.
 108. Loeb, J.A. & Fischbach, G.D. (1995) ARIA can be released from

- extracellular matrix through cleavage of a heparin-binding domain. *J. Cell Biol.* **130**, 127–135.
109. Meier, T., Masciulli, F., Moore, C., Schoumacher, F., Eppenberger, U., Denzer, A.J., Joner, G. & Brenner, H.R. (1998) Agrin can mediate acetylcholine receptor gene expression in muscle by aggregation of muscle-derived neuregulins. *J. Cell Biol.* **141**, 715–726.
 110. Loeb, J.A., Khurana, T.S., Robbins, J.T., Yee, A.G. & Fischbach, G.D. (1999) Expression patterns of transmembrane and released forms of neuregulin during spinal cord and neuromuscular synapse development. *Development* **126**, 781–791.
 111. Moscoso, L.M., Chu, G.C., Gautam, M., Noakes, P.G., Merlie, J.P. & Sanes, J.R. (1995) Synapse-associated expression of an acetylcholine receptor-inducing protein, ARIA/hereregulin, and its putative receptors, ErbB2 and ErbB3, in developing mammalian muscle. *Dev. Biol.* **172**, 158–169.
 112. Zhu, X., Lai, C., Thomas, S. & Burden, S.J. (1995) Neuregulin receptors, erbB3 and erbB4, are localized at neuromuscular synapses. *EMBO J.* **14**, 5842–5848.
 113. Si, J.T., Luo, Z.J. & Mei, L. (1996) Induction of acetylcholine receptor gene expression by ARIA requires activation of mitogen-activated protein kinase. *J. Biol. Chem.* **271**, 19752–19759.
 114. Altioek, N., Altioek, S. & Changeux, J.P. (1997) Heregulin-stimulated acetylcholine receptor gene expression in muscle. Requirement for MAP kinase and evidence for a parallel inhibitory pathway independent of electrical activity. *EMBO J.* **16**, 717–725.
 115. Tansey, M.G., Chu, G.C. & Merlie, J.P. (1996) ARIA/HRG regulates AChR ϵ -subunit gene expression at the neuromuscular synapse via activation of phosphatidylinositol 3-kinase and RAS/MAPK pathway. *J. Cell Biol.* **134**, 465–476.
 116. Wang, J.Y., Frenzel, K.E., Wen, D. & Falls, D.L. (1998) Transmembrane neuregulins interact with LIM kinase 1, a cytoplasmic protein kinase implicated in development of visuospatial cognition. *J. Biol. Chem.* **273**, 20525–20534.
 117. Koike, S., Schaeffer, L. & Changeux, J.P. (1995) Identification of DNA element determining synaptic expression of the mouse acetylcholine receptor α -subunit gene. *Proc. Natl Acad. Sci. USA* **92**, 10624–10628.
 118. Sapru, M.K., Florance, S.K., Kirk, C. & Goldman, D. (1998) Identification of neuregulin and protein tyrosine phosphatase response element in the nicotinic acetylcholine receptor ϵ -subunit gene: regulatory role of an Ets transcription factor. *Proc. Natl Acad. Sci. USA* **95**, 1–6.
 119. Schaeffer, L., Duclert, N., Huchetdymann, M. & Changeux, J.P. (1998) Implication of a multisubunit ETS-related transcription factor in synaptic expression of the nicotinic acetylcholine receptor. *EMBO J.* **17**, 3078–3090.
 120. Fromm, L. & Burden, S.J. (1998) Synapse-specific and neuregulin-induced transcription require an ETS site that binds GABP α /GABP β . *Genes Dev.* **12**, 3074–3083.
 121. Khurana, T.S., Rosmarin, A.G., Shang, J., Krag, T.O.B., Das, S. & Gammeltoft, S. (1999) Activation of utrophin promoter by heregulin via the ets-related transcription factor complex GA-binding protein α/β . *Mol. Biol. Cell* **10**, 2075–2086.
 122. Cohen, N.A., Kaufmann, W.E., Worley, P.F. & Rupp, F. (1997) Expression of agrin in the developing and adult rat brain. *Neuroscience* **76**, 581–596.
 123. Lieth, E. & Fallon, J.R. (1993) Muscle agrin: neural regulation and localisation at nerve-induced acetylcholine receptor clusters. *J. Neurosci.* **13**, 2509–2514.
 124. Kamakura, K., Tadano, Y., Kawai, M., Ishiura, S., Nakamura, R., Miyamoto, K., Nagata, N. & Sugita, H. (1994) Dystrophin-related protein is found in the central nervous system of mice at various developmental stages, especially at the postsynaptic membrane. *J. Neurosci. Res.* **37**, 728–734.
 125. Kim, T.-W., Wu, K., Xu, J.-L. & Black, I.B. (1992) Detection of dystrophin in the postsynaptic density of rat brain and deficiency in a mouse model of Duchenne muscular dystrophy. *Proc. Natl Acad. Sci. USA* **87**, 11642–11644.
 126. Masiakowski, P. & Carroll, R.D. (1992) A novel family of cell surface receptors with tyrosine kinase-like domain. *J. Biol. Chem.* **267**, 26181–26190.
 127. Serpinskaya, A.S., Feng, G., Sanes, J.R. & Craig, A.M. (1999) Synapse formation by hippocampal neurons from agrin-deficient mice. *Dev. Biol.* **205**, 65–78.
 128. Li, Z., Hilgenberg, L.G.W., O'Dowd, D.K. & Smith, M.A. (1999) Formation of functional synaptic connections between cultured cortical neurons from agrin-deficient mice. *J. Neurobiol.* **39**, 547–557.
 129. Ji, R.-R., Böse, C.M., Lesuisse, C., Qiu, D., Huang, J.C., Zhang, Q. & Rupp, F. (1998) Specific agrin isoforms induce cAMP response element binding protein phosphorylation in hippocampal neurons. *J. Neurosci.* **18**, 9695–9702.
 130. Deisseroth, K., Bito, H. & Tsien, R.W. (1996) Signaling from synapse to nucleus: postsynaptic CREB phosphorylation during multiple forms of postsynaptic plasticity. *Neuron* **16**, 89–101.
 131. O'Connor, L.T., Lauterborn, J.C., Smith, M.A. & Gall, C.M. (1995) Expression of agrin mRNA is altered following seizures in adult rat brain. *Mol. Brain Res.* **33**, 277–287.
 132. Prior, P., Schmitt, B., Grenningloh, G., Pribilla, I., Multhaup, G., Beyreuther, K., Maulet, Y., Werner, P., Langosch, D., Kirsch, J. & Betz, H. (1992) Primary structure and alternative splice variants of gephyrin, a putative glycine receptor tubulin-linking protein. *Neuron* **8**, 1161–1170.
 133. O'Brien, R.J., Lau, L.-F. & Huganir, R.L. (1998) Molecular mechanisms of glutamate receptor clustering at excitatory synapses. *Curr. Opin. Neurobiol.* **8**, 364–369.
 134. Sheng, M. & Wyszynski, M. (1997) Ion channel targeting in neurons. *Bioessays* **19**, 847–853.
 135. Torres, R., Firestein, B.L., Dong, H., Staudinger, J., Olson, E.N., Huganir, R.L., Brecht, D.S., Gale, N.W. & Yancopoulos, G.D. (1998) PDZ proteins bind, cluster, and synaptically colocalize with Eph receptors and their ephrin ligands. *Neuron* **21**, 1453–1463.