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Review

Neuromuscular synaptogenesis: clustering of acetylcholine receptors revisited

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Abstract. Clustering of neurotransmitter receptors in the postsynaptic membrane is critical for efficient synaptic transmission. During neuromuscular synaptogenesis, clustering of acetylcholine receptors (AChRs) is an early sign of postsynaptic differentiation. Recent studies have revealed that the earliest AChR clusters can form in the muscle independent of motorneurons. Neurally released agrin, acting through the muscle-specific kinase MuSK and rapsyn, then causes further clustering and localization of clusters underneath the nerve terminal. AChRs themselves are required for agrin-induced clustering of several postsynaptic proteins, most notably rapsyn. Once formed, AChR clusters are stabilized by several tyrosine kinases and by components of the dystrophin/utrophin glycoprotein complex, some of which also direct postnatal synaptic maturation such as formation of postjunctional folds. This review summarizes these recent results about AChR clustering, which indicate that early clustering can occur in the absence of nerves, that AChRs play an active role in the clustering process and that partly different mechanisms direct formation versus stabilization of AChR clusters.

Key words. Neuromuscular junction; acetylcholine receptor; clustering; agrin; postsynaptic preassembly; rapsyn; MuSK.

Introduction

Cellular communication in the nervous system is mediated by synapses, the points of contact between neurons and their target cells. Here, synaptic vesicles in the nerve terminal release neurotransmitter molecules which diffuse across the synaptic cleft and activate neurotransmitter receptors in the postsynaptic membrane. These receptors must be present at high density in clusters precisely opposite sites of neurotransmitter release in the nerve ending, to ensure rapid and precise synaptic transmission. During formation of synapses, thus, clustering of neurotransmitter receptors is a key aspect of postsynaptic differentiation and is under tight temporal and spatial regulation [1, 2].

Many aspects of receptor clustering originate from studies on the vertebrate neuromuscular junction (NMJ). Here, the postsynaptic muscle membrane harbors an elaborate postsynaptic apparatus composed of many identified proteins, whose hallmark is the high density of acetylcholine receptors (AChRs), reaching up to 10,000 molecules per μm^2 [1] (fig. 1). Several extended reviews about the NMJ have recently appeared [1–7], dealing with all aspects of NMJ formation, maturation and elimination. In this review, we focus largely on one key and classic aspect of synaptogenesis at the NMJ, the clustering and maintenance of postsynaptic AChRs in high density. This topic has been intensely investigated for several decades. But we revisit AChR clustering and postsynaptic assembly here because recent studies reveal fundamentally new ways in which these processes occur, thereby changing old concepts of NMJ formation.

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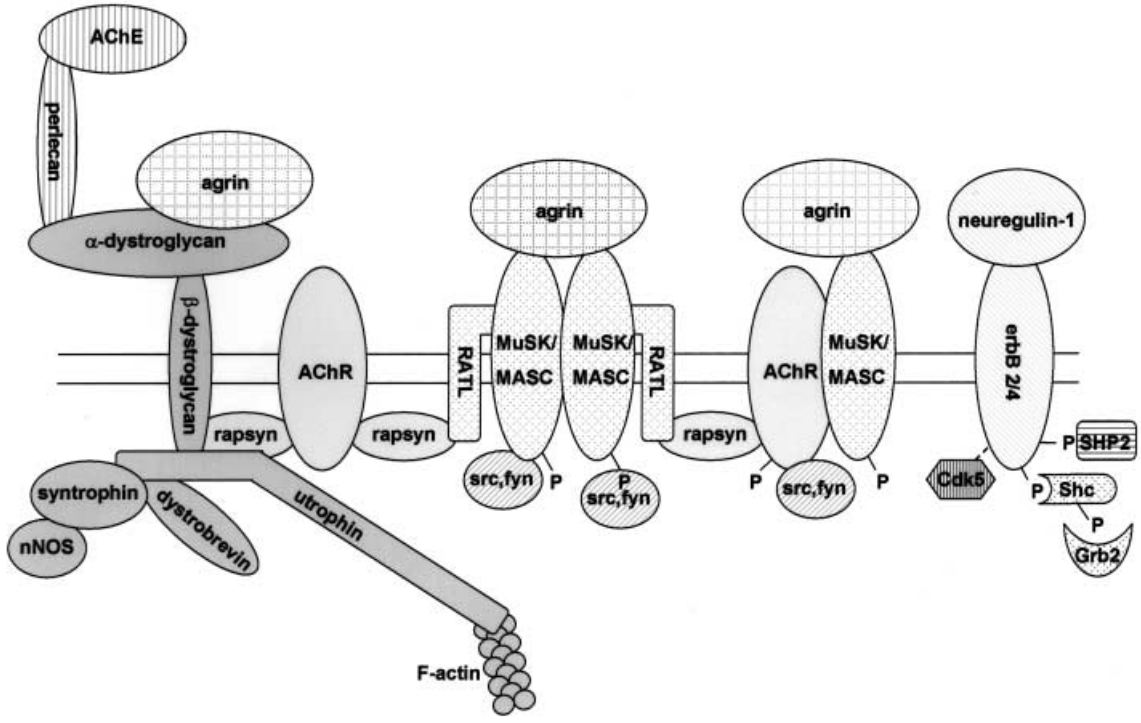

Figure 1. The postsynaptic apparatus at the neuromuscular synapse. The scheme depicts postsynaptic proteins and their interactions. For simplicity, not all isoforms of NMJ proteins are shown, and segregation of such proteins between crests and troughs of postjunctional folds is neglected. In the case of ErbB interactions, Shc and SHP2 appear to bind to tyrosine-phosphorylated ErbB receptors, while the role of tyrosine-phosphorylation for binding to Cdk5 is unknown (illustrated by a broken line). p, tyrosine phosphorylation; MuSK, muscle-specific kinase; MASC, myotube-associated specificity component; RATL, rapsyn-associated transmembrane linker; AChE, acetylcholinesterase.

Formation of AChR clusters and the postsynaptic apparatus

While AChRs at the adult NMJ are highly concentrated in the postsynaptic membrane, they are virtually absent from the extrasynaptic plasma membrane. This distribution of receptors is caused by three major signaling processes [1, 2]. First, upon contact of the growing motorneuron with the developing muscle, preexisting diffusely distributed AChRs – which are present at a density of about 1000 receptors per μm^2 – are redistributed to form clusters at sites of nerve-muscle contact, although initially some aneural AChR clustering is also observed at a distance from the nerve [8–10]. Second, underneath the nerve terminal, muscle nuclei, which will become specialized synaptic nuclei, start to transcribe genes encoding AChRs at elevated levels. Third, gene transcription of AChR subunits is arrested in extrasynaptic nuclei. Together, these three processes ensure that AChRs are maintained in high density in clusters in the postsynaptic membrane and not throughout the whole muscle sarcolemma. In this review, we largely focus on the first two of these processes, which lead to redistribution of preexisting AChRs and to enhanced new synthesis of AChRs at the synapse.

Agrin is a central player for formation of the NMJ

Recent studies show that at the time of the earliest contacts between nerve and muscle (E14.5 in the mouse), AChRs can form aneural clusters that are located at a distance from the motorneuron [9]. These clusters can, in fact, be formed in the complete absence of motorneurons, revealing a muscle program of differentiation termed prepatterning that leads to nerve-independent AChR clusters in the central endplate zone of the muscle [8, 10]. Interestingly, also myotubes cultured in vitro in the absence of motorneurons can form a limited number of spontaneous AChR clusters. The mechanism of formation of these clusters will be discussed later. However, as development proceeds, AChR clusters become concentrated in the close vicinity of the nerve and appear strictly nerve associated by E18.5 and later in the mouse. Thus, the nerve is essential for refinement of AChR clusters and can induce aggregation of AChRs in the muscle, as shown by classic studies in which neurons are cocultured with myotubes in vitro [11].

A key factor for clustering of preexisting AChRs is neural agrin, a multidomain proteoglycan released by the nerve [11] (fig. 2A). Agrin was originally isolated from the electric organ of *Torpedo californica* based on its ability to ag-

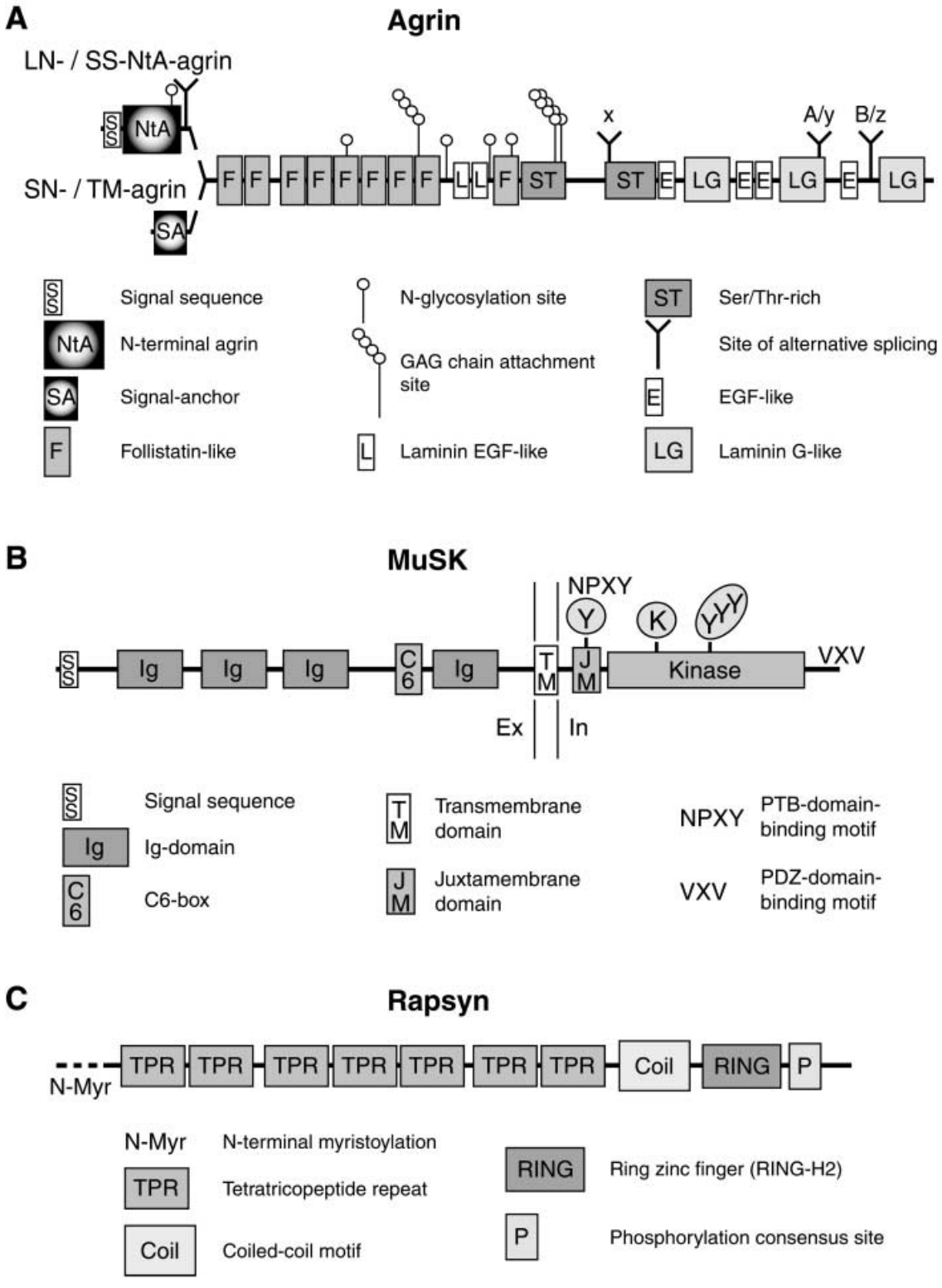

Figure 2. Schematic structures of agrin (*A*), MuSK (*B*) and rapsyn (*C*). The proteins are not drawn to scale. Protein domains are as indicated. In MuSK's cytoplasmic domain, amino acids critical for agrin-induced AChR clustering are highlighted by gray circles. GAG, glycosaminoglycan; Ig, immunoglobulin.

gregate AChRs in myotubes in culture [11] and was shown in subsequent studies to be the main organizer of the NMJ. Due to differential splicing, agrin exists in several isoforms that differ in their ability to induce AChR clusters [12, 13] (fig. 2A). Isoforms containing inserts at the B/z site are synthesized by motorneurons, collectively termed neural agrin, and are highly active in AChR clustering assays. In contrast, muscle and Schwann cells express forms that lack B/z inserts and are inactive in clustering. With respect to the N-terminus, agrin exists in two main forms (fig. 2A). An isoform with a longer N-terminus contains a unique region termed NtA domain in chick [14]. In mouse, this agrin form is designated LN-agrin and in chick SS-NtA-agrin [15, 16]. It is expressed in both neural and nonneural tissue, appears to account for most of the agrin in motorneurons and muscle, and is efficiently secreted and incorporated into the basal lamina [14, 15]. SN-agrin in mouse (called TM-agrin in chick), in contrast, contains a shorter N-terminus, was the first agrin to be cloned (from rat [17]), is mainly expressed in the brain [15, 16] and associates with the plasma membrane without being secreted. In fact, SN-agrin is a type II transmembrane protein with a cytoplasmic N-terminus, because its N-terminal region serves as a uncleaved signal-anchor domain [16]. When expressed in a cell-bound form in transfected cells, SN-agrin is also able to induce AChR clusters in myotube cultures [16].

While the biological roles of SN-agrin in vivo have yet to be elucidated, two main approaches have demonstrated that LN-agrin containing the NtA domain and B/z inserts is required to induce AChR clustering and assembly of the postsynaptic apparatus at the NMJ. In the first approach, the agrin gene was inactivated by homologous recombination. The resulting agrin-deficient knockout mice lack differentiated NMJs, and clusters of AChRs and other postsynaptic marker proteins are no longer observed in association with the nerve at birth, by which time the animals die due to failure to breathe [18]. Mice in which agrin isoforms containing B/z inserts were selectively abolished [19], and mice that selectively lack LN agrin isoforms [15], show the same deleterious phenotype as the full agrin knockout (that lacks all agrin proteins), confirming the importance of these specific isoforms in NMJ formation.

In the second approach, agrin complementary DNA (cDNA) was microinjected ectopically into myofibers in vivo. In the case of neural but not muscle agrin isoforms, the injection causes the formation of ectopic postsynaptic specializations that contain clustered AChRs in association with many other postsynaptic proteins, deep membrane folds and adult-type AChRs with ε subunits [20–24]. This gain-of-function approach demonstrates that neural agrin is sufficient to induce postsynaptic specialization in muscle. In fact, apparently through an indirect mechanism (see below), ectopically injected agrin also causes local gene expression of postsynaptic proteins such as AChRs, MuSK and ErbB receptor tyrosine kinases, whose messenger RNAs (mRNAs) are likewise normally accumulated at NMJs [25]. Thus, agrin not only directs clustering of preexisting postsynaptic proteins, but through interaction with other signaling pathways, also leads to local gene expression of at least some of these postsynaptic components [25].

In addition, presynaptic differentiation may also be induced by agrin, as illustrated in agrin-deficient mice, where motorneurons grow extensively over muscle fibers of the diaphragm without being restricted to the central endplate zone [18]. In line with this are tissue-culture experiments that show that agrin expressed in heterologous cells induces growth inhibition and differentiation of neurites from cocultured ciliary ganglion neurons [26].

The receptor tyrosine kinase MuSK is a component of the agrin receptor

Many steps in clustering of AChRs induced by agrin remain unclear, although agrin signaling and postsynaptic assembly have been the topic of intense research for many years. Clearly, however, a receptor complex in the muscle membrane is activated by agrin released from the nerve. This receptor initiates a signaling cascade that culminates in clustering and anchoring of AChRs with the help of intracellular and AChR-associated signaling and anchoring proteins [2, 6, 11]. A key component of the agrin receptor is the muscle-specific kinase MuSK, a receptor tyrosine kinase (RTK) originally identified in *Torpedo* electric organ [27] and highly expressed in myotubes and muscle fibers [28] (fig. 2B). Gene targeting has been applied to examine the role of MuSK in NMJ development. MuSK-deficient mice lack differentiated NMJs, clusters of AChRs and other postsynaptic marker proteins, and thus show similarities in their phenotype to animals lacking agrin [29]. Furthermore, whereas wild-type myotubes respond to agrin treatment by a rapid tyrosine phosphorylation of MuSK followed by AChR clustering, cultured MuSK –/– myotubes fail to cluster the AChR in response to agrin [30].

In a gain-of-function approach, a constitutively active recombinant form of MuSK was injected and expressed at ectopic sites in innervated muscle in vivo. Interestingly, at these sites, a full complement of postsynaptic proteins accumulated including adult-type AChRs, and local gene expression of endogenous MuSK, AChR ε and ErbB receptors was induced [25, 31]. Together, results from these two approaches demonstrate that MuSK is part of the functional receptor for agrin and that it is both necessary and, upon activation, sufficient to drive postsynaptic assembly and at least some aspects of synaptic gene transcription.

MuSK, however, is itself unable to directly bind to agrin, which represents a parallel to certain other RTKs that require coreceptors to bind to their extracellular ligands [30]. Based on this observation, it has been proposed that MuSK needs to associate with a myotube-associated specificity component (termed MASC; fig. 1), in order to constitute a fully functional receptor that both binds and responds to agrin [30]. Mutational analysis of MuSK suggests that the first, most N-terminal immunoglobulin-like domain in MuSK's exodomain (fig. 2B) interacts with MASC [32], but the nature of MASC has remained largely elusive so far. Since α-dystroglycan, a component of the dystrophin/utrophin glycoprotein complex (D/UGC), efficiently binds to agrin, this protein could in principle be a candidate for MASC. However, agrin fragments that do not bind to α-dystroglycan still cause AChR clustering and activate MuSK [33–35], and agrin-induced MuSK activation is normal in antisense-treated myotubes that express low levels of α-dystroglycan [36]. These observations strongly suggest that α-dystroglycan is not the MASC coreceptor for MuSK.

MASC, however, may in part originate from posttranslational modification of one or several synaptic proteins, in particular from glycosylation. This idea stems from the observation that certain galactose-based oligosaccharides, including the so-called CT carbohydrate, are concentrated at the NMJ [37, 38]. Agrin itself contains the CT carbohydrate, which potentiates agrin's clustering ability in myotubes [39]. Also, when heavily glycosylated agrin forms are purified by peanut agglutinin, they do trigger MuSK autophosphorylation in vitro. This activation can be inhibited by adding components of the CT carbohydrate, and such components bind to agrin and MuSK in vitro [38]. Thus, the CT carbohydrate, at least in vitro, can serve a similar role to that proposed for MASC in that it can bind to agrin and MuSK and stimulate the MuSK kinase activity.

Another important question is how, following stimulation by agrin, MuSK transmits a signal into myotubes. In response to agrin, MuSK undergoes autophosphorylation, probably on several cytoplasmic tyrosine residues [40]. Such phosphorylated tyrosines could then act as docking sites for binding of signal-transducing molecules, in analogy to many other RTKs. Several of MuSK's cytoplasmic tyrosine residues are indeed essential for AChR clustering in response to agrin in myotubes, as shown by mutational analysis of MuSK and transfection into MuSK –/– myotubes (fig. 2B). Three of these critical tyrosines reside closely together in the kinase activation loop, which illustrates the requirement for MuSK kinase activity in agrin signaling [41]. In agreement with this, inactivating MuSK's kinase activity by mutation of a lysine residue in the ATP-binding site also abolishes the AChR clustering activity of MuSK [32]. Another critical tyrosine is located close to the membrane and can, along with the sequence surrounding it, actually confer agrin responsiveness when expressed in otherwise inactive MuSK-TrkA chimeras [32, 41]. Interestingly, the sequence context of this juxtamembrane tyrosine (NPXY; fig. 2B) corresponds to the consensus binding site for PTB (phosphotyrosine binding) domains of Shc and related adaptor proteins.

Thus, MuSK signaling depends on MuSK kinase activity and a juxtamembrane domain, which is very likely to bind to an important Shc-like protein as downstream mediator. In contrast, the extreme C-terminus of MuSK, which contains a consensus motif for interaction with PDZ domains, is dispensable for clustering of AChRs at least in transfected myotubes, suggesting that binding of PDZ-domain proteins to MuSK is not important for this process [32]. Interestingly, cross-linking and mass spectrometry recently identified a PDZ-domain-containing protein, MAGI-1c, a member of the membrane-associated guanylate kinase protein family, as a protein that interacts with MuSK [42]. Whether MAGI-1c binds to the C-terminus of MuSK and what role it plays in NMJ formation is currently unknown; the synaptic location of this protein at the NMJ, however, suggests a role in later steps of synapse formation, following AChR clustering.

Rapsyn is a downstream mediator in AChR clustering

The agrin signaling pathway downstream of MuSK involves rapsyn as a central player. Originally identified as a 43-kDa AChR-associated protein in *Torpedo* synaptic membranes [43–45], rapsyn turned out to be necessary for clustering of AChRs and many aspects of postsynaptic differentiation in mammalian muscle. Thus, at mutant endplates of rapsyn –/– mice, clusters of AChRs and other postsynaptic proteins such as dystroglycan, utrophin or ErbB receptors are missing, and the animals die at birth due to failure to breathe [46]. Likewise, myotubes cultured from these mutant mice do not form AChR clusters spontaneously nor in response to agrin. MuSK is the only postsynaptic protein to still be localized synaptically at mutant NMJs in rapsyn-deficient mice, and MuSK is activated by agrin in cultured rapsyn –/– myotubes, showing that rapsyn acts downstream of MuSK [47].

In agreement with its role in protein aggregation in vivo, rapsyn is able to cluster certain postsynaptic markers upon expression in transfected or microinjected heterologous cells in vitro. When expressed heterologously, rapsyn organizes itself in small clusters, illustrating an intrinsic capability of rapsyn to form aggregates. Upon coexpression in heterologous cells, rapsyn causes coclustering of itself with AChRs, dystroglycan and MuSK [48–51]. Such heterologous expression of rapsyn and AChRs was used to determine which domains in both proteins mediate steps in rapsyn-induced clustering of the receptor. The protein structure of rapsyn includes, from the N-terminus, a

myristoylation site, several tetratricopeptide repeats (TPRs), a coiled-coil motif, a ring zinc finger (RING-H2) domain and a serine phosphorylation site (reviewed in [52]; fig. 2C). Whereas myristoylation mediates plasma membrane targeting of rapsyn, at least two of the TPRs are necessary and sufficient for rapsyn self-aggregation [53–55]. The coiled-coil motif, in turn, is required for co-clustering of AChRs in rapsyn aggregates [54, 55]. With respect to the AChR, rapsyn is able to cluster individually expressed single subunits of the receptor, a process which requires the intracellular portion of the subunits, as shown for the AChR α subunit [56]. Thus, in heterologous cells, rapsyn appears to interact with all AChR subunits; furthermore, self-aggregation and AChR clustering are mediated by different domains of the rapsyn molecule.

Besides the AChR, MuSK and dystroglycan are two postsynaptic proteins that are clustered by rapsyn in heterologous cells. Rapsyn directly binds to the cytoplasmic portion of β-dystroglycan, and this binding is mediated by the RING-H2 domain of rapsyn as shown by biochemical assays, interactions in yeast two-hybrid systems and co-clustering in transfected cells [57, 58]. In contrast, the association of rapsyn with MuSK does not require the cytoplasmic tail of MuSK, but involves the fourth (membrane-proximal) immunoglobulin-like domain in MuSK's exodomain [32, 47] (fig. 2B, C). Therefore, the extracellular domain of MuSK may be linked to rapsyn by a rapsyn-associated transmembrane linker protein (RATL) of as yet unknown identity [47] (fig. 1), whose function may be to recruit rapsyn and its associated proteins to MuSK. Taken together, these studies assign different functions to the different modules in rapsyn: while TPR domains mediate rapsyn self-aggregation, the coiled-coil region causes AChR clustering and the RING-H2 domain clustering of dystroglycan.

It is worth mentioning that these results are largely based on heterologous protein expression. In myotubes, clustering appears to be regulated by additional, myotube-specific mechanisms. This is illustrated by the requirement for agrin and MuSK to efficiently cluster AChRs in muscle and by the larger size of clusters in myotubes. Furthermore, the expression level of rapsyn is an important parameter that determines its ability to mediate agrin-induced AChR clustering in myotubes. In muscle, as well as *Torpedo* electric organ, endogenous rapsyn occurs in roughly a 1:1 stochiometry with the AChR [59, 60]. When rapsyn is overexpressed in myotubes, fewer spontaneous and agrin-induced AChR clusters are seen than in myotubes expressing physiological levels of rapsyn [61–63]. This implies that in muscle, rapsyn aggregation may be regulated by further muscle proteins.

Moreover, rapsyn may be involved in trafficking of AChRs to the plasma membrane, which is supported by the colocalization of rapsyn with AChRs in post-Golgi transport vesicles of *Torpedo* electric organ [64, 65]. The possible role of rapsyn in AChR surface transport is also illustrated by myotubes overexpressing rapsyn, in which AChRs accumulate intracellularly in aggregates that colocalize with rapsyn [63]. Furthermore, in transfected fibroblasts expressing both rapsyn and AChRs, levels of surface AChRs are much lower than in fibroblasts expressing only AChRs [63]. These data suggest, in fact, that rapsyn may be a negative regulator of AChR surface transport and may thus control expression levels of the receptor at the plasma membrane.

Finally, unlike in heterologous cells, rapsyn cannot form clusters independent of AChRs in myotubes, but requires some sort of association with the receptor to form aggregates. This is illustrated by ectopic injection of rapsyn into myofibers in vivo, where this rapsyn does not form aggregates, and by downregulation of surface AChRs by anti-receptor antibodies in myotubes; in such cells, residual rapsyn does not form agrin-induced clusters [66].

Src-family kinases and phosphorylation of the AChR β subunit are involved in efficient AChR clustering induced by agrin

Well-known signaling steps downstream of MuSK are tyrosine-phosphorylation of the AChR β subunit and a link of the receptor to the cytoskeleton. Recently it has been shown that AChR phosphorylation and Src-family kinases are involved in efficient agrin-induced cytoskeletal linkage and clustering of the AChR [67–70].

Agrin-induced tyrosine-phosphorylation of the AChR occurs on its β and δ subunits in cultured myotubes and is paralleled by a link of the receptor to the cytoskeleton [68, 71, 72]. Similarly, AChRs at developing NMJs are progressively stabilized, most likely through a cytoskeletal link, and these junctions contain phosphotyrosine proteins that codistribute with the receptors [73, 74]. Several experimental approaches were used to determine whether agrin-induced clustering and cytoskeletal linkage of the AChR require its tyrosine-phosphorylation. For example, herbimycin and staurosporine, two inhibitors of tyrosine kinases, block both agrin-induced AChR β and δ phosphorylation and clustering [68, 72, 75]. Conversely, pervanadate, an inhibitor of tyrosine phosphatases, causes increased AChR phosphorylation and an increased cytoskeletal link of AChRs in myotubes [76]. Most important, upon transfection into myotubes, mutant AChRs lacking cytoplasmic tyrosine residues in their β subunits, although still able to form agrin-induced clusters [77], show a clearly reduced extent of clustering and cytoskeletal linkage [67]. This demonstrates that tyrosine phosphorylation of the AChR β subunit induced by agrin regulates the link of the AChR to the cytoskeleton and contributes to efficient clustering of the receptors [67].

Other strategies have been used to analyze which kinase(s) phosphorylates the AChR following treatment of myotubes with agrin and what role such a kinase plays in receptor clustering. AChR phosphorylation is unlikely to be the direct result of agrin-activated MuSK, because staurosporine blocks phosphorylation (and clustering) of the receptor without interfering with MuSK activation [68, 78]. These observations strongly imply the existence of some other kinase(s) downstream of MuSK. Accumulating evidence suggests that this kinase(s) is a member of the Src family. Thus, two members of this family, Src and Fyn, interact with AChRs in myotubes [79], whereas Fyn together with Fyk (another Src-like kinase) associate with AChRs in the electric organ of *Torpedo* [80]. In agrin-treated myotubes, the Src-like kinases that are bound to the AChR are activated and tyrosine phosphorylated; this activation is abolished by staurosporine and not observed in rapsyn-deficient myotubes and thus correlates highly with AChR clustering [68]. A fraction of the Src and Fyn pool in myotubes also forms a complex with MuSK, and these kinases can phosphorylate MuSK and increasingly interact with MuSK following agrin treatment [69]. This suggests, interestingly, that the rapid phosphorylation of MuSK in response to agrin, widely thought to reflect autophosphorylation, may actually originate from Src-like kinases that are constitutively bound to MuSK. Furthermore, PP1, a specific inhibitor of the Src family, blocks the agrin-induced cytoskeletal linkage of the AChR, while expression of dominant-negative, kinase-inactive Src in myotubes interferes with clustering induced by agrin, leading to AChR aggregates that are very small [69].

A genetic approach, using doubly-deficient mice lacking both Src and Fyn, tested the role of Src and Fyn in postsynaptic differentiation, AChR clustering and phosphorylation more directly [70]. These animals die around birth and move and breathe only weakly, consistent with neuromuscular failure. However, all aspects of synaptic development examined such as innervation, AChR gene expression and clustering of postsynaptic proteins are normal [70]. Likewise, agrin induces normal AChR clustering and AChR β phosphorylation in myotubes derived from the doubly-deficient mice. However, in these myotubes, the Src-family kinase Yes is upregulated and may thus compensate. Most interesting, after induction of clustering and withdrawal of agrin from these myotubes, clusters of AChRs are dramatically less stable than in wild-type cells [70].

Taken together, these recent data show that kinases of the Src family play a role in agrin signaling: a subpopulation of the total pool of these kinases interacts with and phosphorylates MuSK in response to agrin, while those Src-like kinases that constitutively bind to the AChR are activated by agrin. Furthermore, cytoskeletal linkage of the AChR and growth of AChR clusters involves Src-like kinase functioning. However, Src and Fyn themselves are dispensable for AChR clustering and phosphorylation, although in their absence, Yes may compensate. Nonetheless, Src and Fyn are clearly required to stabilize agrin-induced AChR aggregates, showing that the mechanisms for formation versus stabilization of these clusters are different.

Postsynaptic preassembly: an active role for AChRs, by association with rapsyn, in postsynaptic organization

As detailed above, both MuSK and the AChR interact with Src-family kinases in myotubes. Some interaction occurs constitutively, i.e. independent of agrin in both cases, thus revealing the existence of preassembled protein complexes. Since these Src-related kinases react differently in response to agrin, however, interactions with MuSK and AChR appear to originate from two distinct subpopulations of Src-family kinases: while agrin causes Src-like kinases to increasingly bind to MuSK [69], agrin activates Src-like kinases constitutively associated with the AChR without affecting their interaction with the receptor [68]. Further differences between the subpopulations of Src-like kinases bound to MuSK and the AChR are illustrated by rapsyn. Rapsyn is not necessary for agrin-induced MuSK phosphorylation [47], implying that activation of MuSK-bound Src-like kinases and Src-mediated phosphorylation of MuSK [69] occurs independent of rapsyn. In contrast, rapsyn is required for agrin-induced activation of AChR-bound Src-family kinases [68]. These data provide increasing evidence for the existence of two independent preassembled signaling complexes in myotubes, a MuSK-bound complex and an AChR-bound complex (fig. 3).

Accumulating evidence suggests that these complexes both contribute to postsynaptic assembly, thereby providing a step-by-step mechanism for postsynaptic organization. One first key aspect of this emerging mechanism is that the MuSK-bound complex acts as a primary synaptic scaffold and is clustered first due to agrin; this scaffold then recruits preassembled AChR complexes via rapsyn (fig. 3). This concept is based on several observations. First, in the absence of rapsyn, MuSK is the only identified postsynaptic protein that is still clustered postsynaptically, leading to the idea that MuSK is a primary synaptic scaffold [47]. Second, in agreement with this, a recombinant constitutively active form of MuSK is able, upon ectopic injection in myofibers in vivo, to organize postsynaptic specializations containing AChRs and most other postsynaptic components [31]. Third, AChRs, dystroglycan and MuSK are coclustered with rapsyn upon expression in heterologous cells [48–51], suggesting that rapsyn recruits other proteins to the MuSK scaffold.

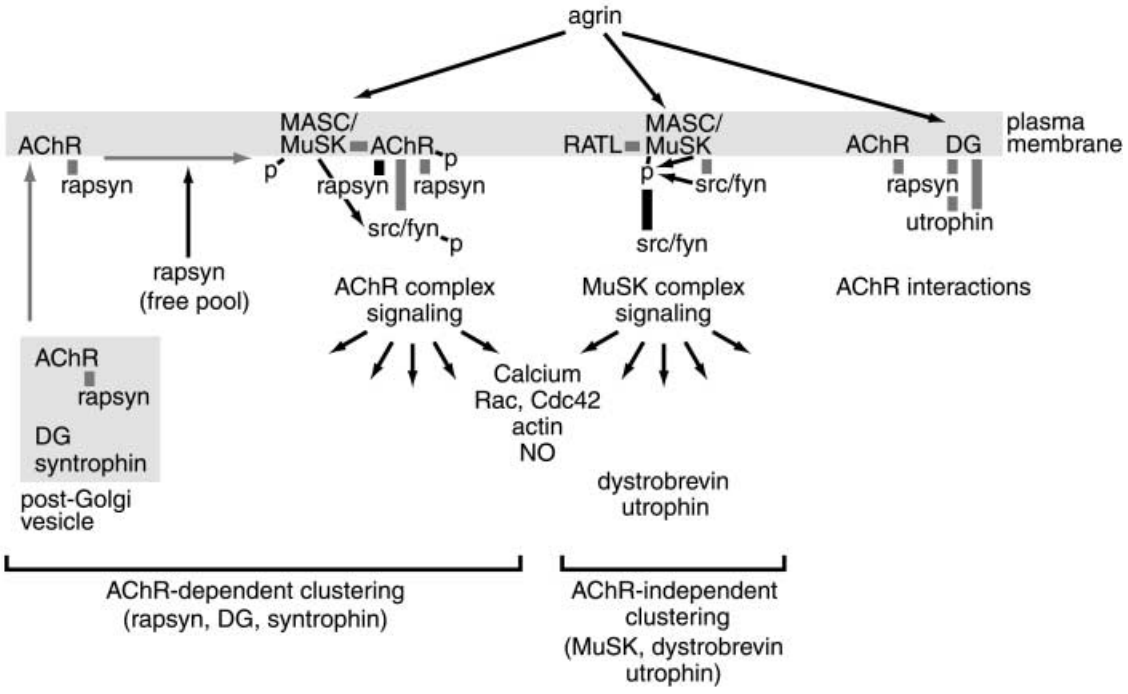

Figure 3. Model for agrin-induced clustering of postsynaptic proteins in myotubes. Gray arrows and bars indicate constitutive steps and protein interactions that occur in the absence of neural agrin. Black arrows and bars refer to neural agrin-induced events and protein interactions. In the absence of neural agrin, at least two protein complexes exist, an AChR-based complex and a MuSK-based complex; the latter may act as a primary synaptic scaffold. Assembly of these complexes may in part occur intracellularly within the secretory pathway (post-Golgi vesicles and Golgi apparatus). Within the AChR complex, agrin activates MuSK, which, in a rapsyn-dependent way, causes phosphorylation of AChR-bound Src-like kinases and AChR β and δ subunits. Agrin also recruits more rapsyn to bind to AChRs before clustering. Within the MuSK complex, following agrin-induced MuSK activation, Src and Fyn can phosphorylate MuSK and increasingly bind to MuSK. Both the AChR and MuSK complexes are likely to initiate multiple downstream signaling events (indicated by the multiple arrows). Further elements in agrin signaling are calcium, Rac and Cdc42, actin polymerization and NO. Cluster formation occurs at least in part by rapsyn-dependent linkage of preassembled AChR-rapsyn complexes to the MuSK complex. DG, dystroglycan; p, tyrosine phosphorylation.

A second key aspect of this step-by-step mechanism of postsynaptic assembly is that AChRs are not passively inserted into a postsynaptic lattice formed by other proteins, but instead play an active role themselves in this postsynaptic organization [66, 81, 82]. In particular, AChRs are necessary for clustering of rapsyn (fig. 3). Thus, in zebrafish mutants lacking functional AChRs, NMJs are formed and appear ultrastructurally normal [83], but lack synaptic rapsyn [82], showing that AChRs are required for synaptic accumulation of rapsyn. Similarly, upon ectopic injection into myofibers in vivo, rapsyn cannot form clusters in the absence of AChRs [66]. In cultured myotubes, AChRs interact with rapsyn (and some other postsynaptic proteins) independent of agrin [84], illustrating the presence of preassembled rapsyn-AChR complexes. Some of these complexes are observed intracellularly, but most of them reside in a diffusely distributed form in the myotube plasma membrane [66] [M. Moransard and C. Fuhrer, unpublished data]. Rapsyn can also interact with unclustered AChRs in extrasynaptic areas of denervated diaphragm in vivo [M. Moransard and C. Fuhrer, unpublished data]. Most important, rapsyn-AChR complexes at the surface are essential for clustering because upon downregulation of surface receptors by AChR-antibodies in myotubes, residual rapsyn cannot form agrin-induced clusters but redistributes along with the AChR [66]. This antibody treatment affects the cellular level of rapsyn roughly half as much as the level of AChRs, suggesting that about 50% of the total rapsyn is complexed with the AChR while the rest represents free rapsyn [66].

These data change the traditional view that rapsyn forms clusters first and then recruits AChRs into such clusters. Instead, rapsyn is not able to aggregate independent of the AChR in muscle but requires association with the AChR in preassembled complexes to form agrin-induced clusters in myotubes. Rapsyn may act in clustering by increasingly binding to AChRs and linking such receptors to the cytoskeleton, because agrin triggers increased interaction of rapsyn with unclustered surface AChRs in myotubes. Such an increase correlates highly with AChR clustering, and when analyzed by a differential extraction assay [67] those receptors that increasingly interact with rapsyn are increasingly bound to the cytoskeleton [M. Moransard, M. J. Ferns and C. Fuhrer, unpublished data].

AChRs are also involved in clustering of other postsynaptic proteins in myotubes (fig. 3). In variants of C2 lacking AChRs, and in C2 treated with anti-AChR antibodies to downregulate surface receptors, rapsyn, dystroglycan, syntrophin and acetylcholinesterase cannot form agrin-induced clusters, while clustering of MuSK, dystrobrevin and utrophin is unchanged [66, 85]. The absence of clusters of acetylcholinesterase under these circumstances [85] fits well with its binding to perlecan, which in turn interacts with dystroglycan (fig. 1; [86, 87]). Strikingly, many of the proteins that require AChRs to form agrin-induced clusters in cultured myotubes (i.e. rapsyn, dystroglycan and syntrophin) are cotransported with AChRs to the postsynaptic membrane in post-Golgi transport vesicles in *Torpedo* electric organ [65]. Dystrophin and dystrobrevin are not found on these vesicles and thus reach the postsynaptic membrane independent of AChRs and the secretory pathway in *Torpedo* [65], in analogy with the AChR-independent clustering of utrophin and dystrobrevin in cultured myotubes [66]. These data strongly suggest that cotransport of rapsyn, dystroglycan and syntrophin with the AChR through parts of the secretory pathway is a requirement for subsequent agrin-driven clustering at the surface. This role of AChRs in clustering of other proteins is paralleled by biochemical demonstrations of preassembly of the AChR with other postsynaptic proteins including MuSK, Src-family kinases, dystroglycan and utrophin independent of agrin [84]. Such complexes appear to be linked to MuSK – presumably the MuSK primary synaptic scaffold – in response to agrin, leading to a rapsyn-dependent increase in the AChR-MuSK interaction, whereas other AChR-protein interactions are not affected by agrin (fig. 3) [84].

In addition, once NMJs are formed, AChRs are required to maintain the synaptic density of several postsynaptic proteins [88]. In AChR ε subunit-deficient mice at 2 months of age, AChRs and rapsyn are strongly reduced in parallel at NMJs, whereas agrin, dystrobrevin and utrophin are still efficiently clustered [89]. Thus, the synaptic densities of dystrobrevin and utrophin, but not rapsyn, can be regulated independent of AChRs at agrin-containing NMJs, similarly to clustering of these proteins in agrin-treated myotubes lacking aggregated AChRs [66].

The emerging picture is that AChRs play an active and early role in postsynaptic assembly, at least in part by forming preassembled complexes with other postsynaptic components. Moreover, AChRs are required later in development in order to maintain the integrity of the postsynaptic apparatus.

An important consequence of these studies is that formation of a postsynaptic apparatus can occur by preassembly of neurotransmitter receptors with scaffolding proteins. This concept of postsynaptic preassembly may well apply also to synapses in the central nervous system. At excitatory synapses, preassembly, although not shown on a biochemical level, has been proposed based on hippocampal neurons where clusters of PSD-95 first appear in dendritic shafts or dynamic spine precursors before moving to their final position in the mature spine head [90]. Furthermore, at inhibitory synapses, much like AChRs and rapsyn at the NMJ, gephyrin and GABAA-receptors are mutually important for clustering [91], most likely by forming interdependent components of a protein complex. Postsynaptic preassembly is an interesting parallel to the more established observations that presynaptic components of the nerve terminal are transported together in packets along axons, reflecting preassembled active zone precursors [92, 93]. Thus, both pre- and postsynaptic specializations of synapses may be constructed from preassembled complexes of their components; such preassembly may ensure that pre- and postsynaptic membranes can be formed and modified rapidly according to physiological needs.

Calcium, GTPases and nNOS are further signaling intermediates in agrin-induced AChR clustering

Given the complexity of the postsynaptic apparatus and its plasticity during embryonic and early postnatal development, the mechanism of agrin-induced postsynaptic assembly (fig. 3) is likely to be subject for regulation by further signaling intermediates. Inhibition of agrin-induced AChR clustering by a fast chelator, BAPTA-AM, has indeed shown that calcium fluxes are required for receptor aggregation in cultured myotubes [94, 95].

AChR clustering in myotubes also depends on the small GTPases, Rac and Cdc42, because dominant-negative forms of these signaling molecules inhibit aggregation of AChRs in transfected myotubes [96]. A role of these GTPases in agrin signaling is also indicated by the activation of one of their downstream kinases, c-Jun NH_2-terminal kinase (JNK), by agrin [96]. In other signaling systems, JNK, via phosphorylation of c-Jun, is known to induce expression of target genes, while Rac and Cdc42 can control actin polymerization, thereby causing focal reorganization of the actin cytoskeleton in response to extracellular cues. F-actin assembly is indeed required for AChR aggregation, because latrunculin A inhibits agrin-induced AChR clustering in cultured *Xenopus* muscle cells [97]. Together, these data show that Rac and Cdc42 activation is required for receptor clustering, most likely via controlled reorganization of the actin cytoskeleton. It remains unclear, however, how F-actin acts in AChR clustering. Although utrophin is an actin-binding protein, utrophin itself is dispensable for receptor aggregation [98], implying that actin acts through other binding proteins.

Another signaling intermediate recently found to be important in AChR clustering is nitric oxide, NO [99]. The enzyme producing NO, neuronal nitric oxide synthetase

(nNOS), was known for several years to be concentrated at NMJs, through binding to isoforms of syntrophin, components of the D/UGC (fig. 1) [1]. Recent reports indicate that nNOS is coaggregated with AChRs in cultured myotubes treated with agrin [100]; furthermore, NO is a messenger and is required in agrin-induced AChR clustering and AChR β phosphorylation as shown by NOS inhibitors and NO donors [99]. Although the downstream targets of NO are not known at this point, they could in principle involve Src-family kinases because these are activated by NO in fibroblasts [101]. The idea of NO-mediated Src-family activation in muscle is particularly attractive because of the activation of Src-family kinases by agrin in myotubes and their role in stabilization of AChR clusters [68, 70].

Neuregulin-1 is a key factor for AChR gene expression

Apart from redistribution of existing proteins by agrin, the high synaptic density of NMJ components, particularly of the AChR, is also achieved through a specific upregulation in the transcription of their genes in subsynaptic nuclei. This activity originates at least in part from neuregulin-1, a protein expressed by motorneurons and skeletal muscle which is concentrated at the NMJ [3, 102]. Neuregulin-1 is a member of the neuregulin gene family and was first isolated from chick brain as AChR-inducing activity (ARIA) because of its ability to induce transcription of AChR subunits in myotube cell cultures (reviewed in [102]). In heterozygous mice lacking one copy of the neuregulin-1 gene, the AChR density at the endplate is reduced and the mice are myasthenic [103], showing that neuregulin-1 is indeed required to maintain high receptor density in vivo.

The pathway leading from neuregulin-1 to gene expression is now clarified to some extent [104, 105]. Neuregulin-1 binds and activates ErbB receptor tyrosine kinases (ErbB2–4) which are expressed by muscle and concentrated at the NMJ [106, 107]. Since ErbB3 is detected in presynaptic Schwann cells while ErbB2 and ErbB4 are found in the postsynaptic muscle membrane, the functional receptor for neuregulin-1 to signal postsynaptic gene transcription is likely to be a combination of ErbB2 and ErbB4 [108]. Like MuSK, ErbB receptors belong to the class of receptor tyrosine kinases, characterized by autophosphorylation on intracellular tyrosine residues upon ligand binding. ErbB receptors trigger a signaling cascade that involves recruitment of the adapter proteins Grb2 and Shc and activation of Ras, Raf and ERK, a subgroup of the MAP kinase family [109–112]. Still controversial is the role of phosphatidylinositol-3 (PI3) kinase in this pathway, since interaction of this kinase with ErbB receptors does occur in muscle cells, but it is not clear whether its activity is required for AChR subunit expression [110, 112]. ERK ultimately regulates the transcription of c-Jun and c-Fos transcription factors that represent the major components of the transcription factor AP-1 [113]. JNK, another member of the MAP kinase family that regulates the function of c-Jun, is also activated by neuregulin-1 in muscle cell cultures and required for neuregulin-1–induced AChR transcription [113]. Together, these studies indicate that neuregulin-1 activates multiple signaling cascades that converge to regulate the transcription of AChR subunits (fig. 4). Since c-Jun functioning is essential in this process but possible targets such as the AChR ε subunit gene lack the consensus sequence for AP-1 binding [113], the transcription of AChR subunits seems to ultimately be driven by transcription factors other than AP-1. Indeed, members of the Ets family of transcription factors such as GABP α and GABP β bind to N-box motifs in the regulatory region of the AChR subunit genes, and this mechanism drives synaptic AChR transcription [114–118]. The evidence for GABP being a critical transcription factor for synaptic AChR expression fits well with the observations that Ets-family transcription factors are classical targets for MAP kinases and that such kinases (for example ERK) are activated by neuregulin-1 [105].

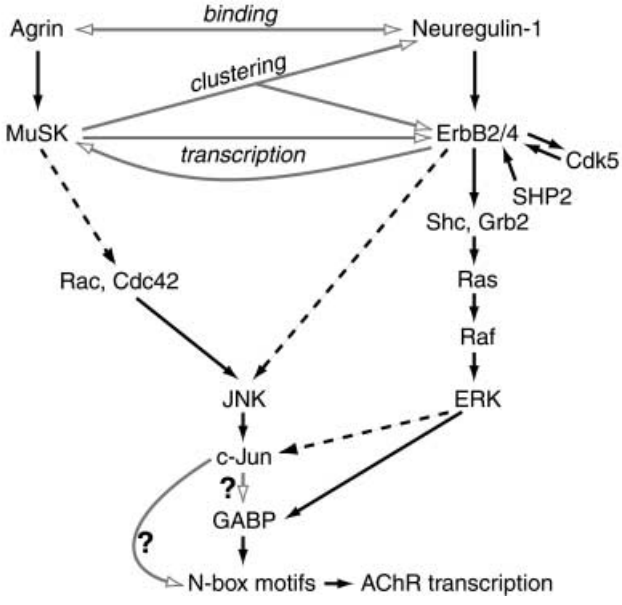

Figure 4. Cross-talk between the agrin and neuregulin-1 pathways. Agrin appears to be able to organize the neuregulin-1 pathway by causing, through MuSK, clustering of neuregulin-1 and ErbBs. Neuregulin-1, on the other hand, causes increased expression of MuSK. Both pathways lead to activation of JNK. Solid and dashed black arrows indicate direct and indirect signaling steps, respectively, and grey arrows with question marks refer to still hypothetical steps. Grey arrows (*binding, clustering, transcription*) indicate general principles of interactions between the agrin and neuregulin-1 pathways. Some of these interactions, in particular clustering and transcription, occur through intracellular signaling pathways, possibly the JNK or ERK pathway.

ErbB signaling is under more complex regulation than implied from the studies summarized above. First, besides kinases, the protein tyrosine phosphatase SHP2 is also involved in the regulation of the neuregulin-1 pathway. An initial indication for this was the increased expression of AChR ε subunits in myotubes treated with the phosphatase inhibitor orthovanadate [119]. Later, SHP2 was found to interact with ErbB receptors through its SH2 domain and to act as a negative regulator in the pathway by keeping low the number of phosphorylated tyrosines on ErbB receptors [120]. This implicates a possible role for SHP2 in limiting the number of ErbB-interaction partners, thereby selecting the outcome of the signaling cascade.
Second, the cycline-kinase Cdk5, a serine kinase, is activated by neuregulin-1 in muscle cells [121]. On the other hand, Cdk5-activity is necessary for neuregulin-1-induced activation and autophosphorylation of ErbB receptors. One explanation for this observation is that a basal level of Cdk5 activity is always present that keeps ErbBs in a 'primed' state and that binding of neuregulin-1 increases ErbB kinase activity [121]. This, in turn, further activates Cdk5, thereby leading to a positive feedback loop onto ErbBs themselves (fig. 4).
Besides AChRs, mRNAs encoding other synaptic proteins are concentrated at the NMJ, including acetylcholinesterase, MuSK, rapsyn, laminin-β2 and N-CAM [28, 122–124]. For some of these, for example acetylcholinesterase, utrophin or MuSK, N-box motifs have been reported in the regulatory regions of their genes [25, 125, 126]. In the case of acetylcholinesterase, N-box motifs are indeed required for its synaptic expression in vivo [126]. Utrophin, in turn, is increasingly expressed in response to neuregulin-1 through the GABP/N-box pathway [125]. This suggests that this pathway regulates the synaptic expression of several proteins, not only AChRs. Since not all synaptically expressed genes have been reported to contain N-boxes, however, other pathways are likely to exist and to regulate synaptic gene expression at the NMJ.

Interaction between the neuregulin and agrin signaling pathways

Interestingly, a growing body of evidence suggests that the agrin and neuregulin-1 signaling pathways, which both contribute to the high synaptic density of AChRs at the NMJ, are linked and that agrin acts upstream of or in parallel with neuregulin-1, by using a cross-talk with the neuregulin-1 pathway that ultimately allows agrin to increase the expression of AChRs. Of crucial importance for this was the initial observation that agrin, when presented to cultured myotubes in a substrate-bound form, is not only able to cluster preexisting AChRs but also to induce new expression of AChR ε subunits [127]. Upon ectopic injection of agrin cDNA into adult muscle, agrin was then found to cause local transcription of AChR ε subunits in vivo [22, 24]. Furthermore, such injected agrin causes clustering of neuregulin-1 and ErbB receptors (along with AChRs and many other postsynaptic proteins) [21, 23] and also induces local transcription of MuSK and ErbBs [25]. Following ectopic injection of wild-type or constitutively active recombinant MuSK, AChR ε transcripts are likewise produced around the injection site [31, 128].
Interestingly, agrin-induced AChR ε transcription in both cultured myotubes and ectopically injected myofibers is dependent on signaling through ErbB2, as shown using dominant-negative ErbB2 expression and conditional homologous recombination, respectively [25, 129]. Conversely, neuregulin-1 induces the expression of MuSK mRNA in cultured myotubes [130]. This strongly implies a feedback loop between ErbBs and MuSK, in that the agrin/MuSK pathway stimulates ErbB transcription, while the neuregulin-1/ErbB pathway triggers enhanced MuSK transcription (fig. 4).
Another level of cross-talk between the two pathways comes from the observations that in the agrin –/– mice, ErbBs are no longer clustered at the mutant synapses of these animals [18] and that ectopically expressed agrin causes clustering of muscle-derived neuregulin-1 and ErbBs, as mentioned above. This implies that agrin organizes the neuregulin-1 pathway by causing clustering of its major components (fig. 4). This may also involve direct binding of agrin to neuregulin-1 as shown in vitro [129].
The neuregulin-1 involved in AChR cluster formation may be entirely muscle-derived, because nerve-derived neuregulin-1 is not required for AChR gene transcription in vivo (see below, [10]) and because extracts of muscle cells show neuregulin-1-like biological activity as demonstrated by tyrosine phosphorylation of ErbB2 in cultured myotubes [129].
Finally, interaction between the agrin and neuregulin-1 pathways is also found on the level of JNK, because agrin requires Rac and Cdc42 for AChR clustering and this pathway leads to activation of JNK [96], a kinase which is also essential for neuregulin-1 signaling.
The emerging picture, thus, is that agrin acts as a master organizer of the NMJ by activating its own MuSK-based signaling pathway and by organizing, through aggregation, the neuregulin-1 pathway which can be entirely derived from the muscle (fig. 4). Through multiple levels of cross-talk, however, both the agrin and neuregulin-1 signals appear important to lead to full differentiation of the NMJ, including synaptic transcription of many of its components.

AChR clustering in the absence of motorneurons

Much evidence over the last decade pointed out a key role of nerve-derived agrin in clustering of AChRs on the mus-

cle surface. As detailed above, crucial observations for this were that in agrin –/– mice no nerve-bound AChR clusters are present in myofibers at E18.5 and that ectopic injection of agrin into myofibers in vivo induces formation of a postsynapse-like apparatus.

However, this agrin-centered view of synapse formation ignores some early experiments that showed AChR clusters aligned in the central portion of embryonic mouse muscle fibers that were rendered aneural by treatment with β-bungarotoxin [131, 132]. In this model, it was difficult to establish whether the neurotoxin treatment had actually eliminated motorneurons and their contacts with the muscle completely. The results, though, have to be reconsidered in view of several recent studies that show that clustering of AChRs can occur in the complete absence of motorneurons in the central portion of myofibers in vivo [8–10].

A strong indication for nerve-independent AChR clustering first came from the analysis of mice lacking DNA topoisomerase II β, in which motorneurons do not invade or branch on the diaphragm or limb muscles, while AChRs are still clustered in the central portion of these myofibers at E18.5 [8]. Yet, in these mice nerves grow close to muscles, leaving open the possibility that a short contact may take place or that nerve-derived agrin may diffuse into the close vicinity of muscle fibers. Therefore, mice lacking the transcription factor HB9 were used that fail to differentiate many motorneurons so that the phrenic nerve is completely absent and the diaphragm never innervated. Strikingly, also in these HB9 –/– animals, AChRs along with acetylcholinesterase are clustered in the central portion of muscle fibers, although in a wider band than in the wild type; these aneural clusters of AChRs are seen at E14.5 and are maintained through E18.5 [9, 10]. Furthermore, in the agrin –/– mice, abundant clusters of AChRs and acetylcholinesterase are observed in the central portion of diaphragm muscle at E14.5 [9, 10]. By E18.5, these clusters are much reduced in number and size, confirming earlier observations in these agrin –/– animals [18, 19]. This represents a contrast to HB9 –/– mice, where AChR clusters are present in abundance at E18.5 [9, 10]. Finally, in wild-type diaphragm at E14.5, AChR clusters appear in the central endplate zone of muscle fibers but many of these clusters are at a distance from the nerve and not opposite to a nerve ending, while at E18.5, AChR clusters are strictly nerve bound [9].

Interestingly, in aneurally cultured myotubes, spontaneous AChR clusters are formed and several postsynaptic proteins interact with the AChR independently of neural agrin as described above [84]. This shows that myotubes have the intrinsic ability to preassemble AChR protein complexes in the absence of motorneurons, a striking parallel to the capability of fetal muscle to cluster AChRs and other proteins independent of neural input (see above).

Together, these studies show that, first, motorneurons and their secreted factors are not required for clustering of synaptic proteins in the central endplate zone of muscle fibers at E14.5, the time of the earliest contact between nerve and muscle in normal development. Second, cooperation must exist between a cluster-maintaining system driven by neural agrin and a cluster-disrupting system driven by other nerve-released factors, because AChR clusters disappear at E18.5 if the nerve is present but neural agrin is missing (in agrin –/– mice), and the clusters are maintained if the nerve is also absent (in HB9 –/– mice). The nature of the nerve-released cluster-disrupting factor remains elusive. However, a good candidate is acetylcholine and evoked electrical activity in the muscle because these factors are well-known to downregulate AChR synthesis in extrasynaptic nuclei (reviewed in [1]). The emerging picture is that muscle fibers undergo a program of prepatterning even in the absence of motorneurons, so that AChR are clustered independent of neurons and agrin in a central end-plate zone of the muscle [133, 134]. This prepatterning is refined by nerve contact in that neural agrin stabilizes nerve-bound clusters and induces new clusters, while another signal, perhaps acetylcholine leading to electrical muscle activity, disperses clusters that are farther away. Together, these mechanisms cause refinement and compacting of the central endplate zone bearing AChR clusters, leading to AChR clusters that are precisely located underneath the nerve terminal as seen at E18.5 and thereafter.

Besides lacking neural agrin, aneural muscle also lacks nerve-derived neuregulin-1. Surprisingly, mRNAs encoding the AChR α and δ subunits are concentrated in the central endplate zone of muscle fibers in HB9-deficient mice although on a wider band than in the wild type, similarly to AChR clusters [9, 10]. Furthermore, in mice where neuregulin-1 is selectively inactivated in sensory and motor neurons to circumvent the lethality at E10.5 of neuregulin-1–null mice, AChR α and δ mRNAs are concentrated in the middle of muscle fibers as in the wild type [10]. Thus, nerve-derived neuregulin-1 is not required for synaptic AChR transcription at E14.5 and E18.5, nor for refining the zone of AChR expression.

These results strengthen the previous suggestion (see above) that muscle-derived neuregulin-1 may elicit to some degree the expression of synaptic proteins such as the AChR. Thus neural agrin seems sufficient to induce AChR transcription by initiating a muscle-intrinsic neuregulin-1/ErbB receptor pathway. It cannot be excluded, however, that another signal, unrelated to neuregulin-1, regulates AChR transcription in the absence of neural neuregulin-1.

MuSK is required for nerve-independent AChR clustering

Nerve-independent clustering of AChRs in the central endplate zone of the muscle raises the questions of how such clusters are assembled and how their formation, in the process of muscle prepatterning, is limited to the middle of myofibers, although to a significantly wider area than in the presence of the nerve.

Cluster assembly at early (E14.5) and later (E18.5) stages was shown to depend on MuSK and rapsyn, because in both MuSK –/– as well as rapsyn –/– mice no AChR clusters are present at these stages [9, 10, 46]. Furthermore, in HB9-MuSK double mutants, no AChR clusters or AChR α subunit-mRNAs are seen in the central endplate zone of the muscle [9, 10]. Thus, MuSK is required for nerve-independent synaptic gene transcription, and MuSK and rapsyn are necessary for AChR clustering in the absence of motorneurons. The signaling machinery that drives early aneural AChR clustering is therefore similar if not identical to that used by neural agrin at later developmental stages. This raises the issue of how MuSK signaling is initiated at the earliest synapses in the absence of neural agrin. Mice lacking all forms of agrin, including the muscle isoforms, show the same NMJ phenotype as mice specifically lacking only neural agrin [9], implying that it is not muscle-derived agrin that activates MuSK early in NMJ formation. The possibility thus remains that at this stage MuSK activation is achieved through other ligands or due to spontaneous self-activation. Agrin-independent MuSK activation has indeed been shown upon overexpression of MuSK or coexpression of MuSK with rapsyn in heterologous cells [40, 47, 49]. Most telling, upon ectopic injection into myofibers in vivo, MuSK constructs lacking the ligand-binding extracellular domain are able to elicit AChR transcription and clustering even in a MuSK-conditional-knockout background, showing that no ligand binding is required for MuSK's synaptogenic activity in this paradigm [128].

Regarding the question of how AChR clusters are limited to the central endplate zone of the muscle, one interesting possible explanation takes into consideration the differences in the developmental state along muscle fibers [133–135]. Myotubes grow from the middle longitudinally by fusion of myoblasts at their ends. Therefore, nuclei and basal lamina in the central part of fibers could be ahead in their differentiation state with respect to the more distal parts. As MuSK and rapsyn are necessary for central AChR clustering, such clustering may be initiated at sites where these and other proteins are expressed a higher levels, that is, in the center of myofibers.

Muscle prepatterning could in principle also involve components of the extracellular matrix, in particular laminins. In cultured myotubes, AChR clustering can be induced by laminin-1 and laminin 2/4 (merosin) [136–138], forms of laminin that are all expressed in the muscle at the earliest stages of NMJ formation [139]. Interestingly, laminin-1 activates a signaling pathway that requires tyrosine kinases downstream of MuSK as well as rapsyn to cluster AChRs in myotubes; this signaling mechanism thus overlaps with that of agrin strongly [139a]. Laminins may thus preactivate myofibers by activating tyrosine kinases, and such signaling could be involved in agrin-independent AChR clustering and prepatterning at E14.5. This mechanism could in principle involve MuSK activation through phosphorylation via the laminin-activated downstream kinases. Three observations are consistent with such a model. First, MuSK can indeed be phosphorylated by other kinases such as Src and Fyn in cultured myotubes [69]. Second, laminin-induced AChR clustering occurs at a lower rate in cultured MuSK –/– myotubes than in wild-type cells [136]. Third, in ectopic injection experiments in myofibers in vivo, the MuSK kinase domain but not extracellular domain is required to induce AChR transcription and clustering [128].

Stabilization of AChR clusters and postsynaptic maturation

Neuromuscular synaptogenesis not only involves initial formation of the NMJ during late embryogenesis but also synaptic maturation, particularly in the first weeks and months after birth. At birth, muscle fibers are multiply innervated, and several nerve terminals are intermingled over the postsynaptic apparatus in which clustered AChRs are homogeneously arranged in a flat postsynaptic membrane [1, 6]. After birth, activity-dependent mechanisms lead to the process of synaptic elimination, in which all but one nerve terminal disappear from the synaptic contact [140]. At the same time, the postsynaptic membrane dramatically changes its morphological appearance, in that postsynaptic folds are formed causing segregation of some proteins to become concentrated either at the crests or in the troughs of these folds [6, 141]. Sodium channels, for example, reside in the troughs while AChRs are found at the crests where they closely colocalize with the nerve endings. As a result, AChR clusters and nerve terminals adopt a pretzel-like morphology at adult NMJs [6, 141].

Recent studies indicate that synaptic components of the D/UGC, while largely dispensable for initial AChR clustering, are required for this process of postjunctional fold formation as well as for consolidation and stabilization of AChR clusters. Furthermore, several tyrosine kinases are as well necessary for maintenance of AChRs in clusters.

The D/UGC stabilizes AChR clusters and is required for postnatal maturation of the postsynaptic membrane

Since myofibers undergo contractions, their plasma membrane must be stabilized to withstand mechanical stress, and this is the major role of the D/UGC, which links the muscle cytoskeleton to the extracellular matrix [142]. Accordingly, when components of this complex are damaged or missing, a phenotype often observed is muscular dystrophy [143]. Roles of the D/UGC at the synapse were for a long time less obvious than this general role of muscle membrane stabilization. However, molecular cloning in combination with detailed immunocytochemical analysis revealed that while some components of the D/UGC are found along the muscle fibers, others are highly concentrated at the NMJ, thus giving raise to synaptic and extrasynaptic variants of the D/UGC. For example, utrophin, β2-syntrophin and α-dystrobrevin-1 are highly concentrated at NMJs and virtually absent from extrasynaptic areas, whereas α-syntrophin, α-dystrobrevin-2, α- and β-dystroglycan and dystrophin are found both at NMJs and extrasynaptic areas [144–146] (fig. 1). This shows that some components of the D/UGC are strongly enriched at the NMJ, and recent progress has now revealed, largely using gene targeting, that several synaptic components of the D/UGC play a role in stabilization of AChRs in clusters and formation of postjunctional folds.

Utrophin and dystrophin, two homologous proteins, are the largest D/UGC components and together necessary for muscle fiber integrity but largely dispensable for NMJ formation. Thus, mice lacking utrophin appear surprisingly healthy and have functional NMJs at which AChR densities and postjunctional folding are slightly reduced [98, 147]. Similarly, NMJs of *mdx* mice that lack functional dystrophin are largely normal apart from a reduction in postsynaptic folds. This reduced folding may be the consequence of muscle fiber necrosis and cycles of regeneration rather than originating from the absence of functional dystrophin protein per se [148, 149]. These results raise the possibility of compensation between utrophin and dystrophin in the maintenance of myofiber integrity, and indeed, mice lacking both dystrophin and utrophin develop a severe muscular dystrophy closely resembling human Duchenne muscular dystrophy [150, 151]. Because NMJs are remarkably normal even in these doubly-deficient mice and only show reduced densities of AChRs and postsynaptic folding, the D/UGC as such appears dispensable for initial formation of the NMJ and AChR clusters, but critical for their maturation, in particular formation of postsynaptic folds.

A more critical role in consolidation and maintenance of AChR clusters and the NMJ is played by dystroglycan. To circumvent the early lethality of dystroglycan –/– mice, chimaeric mice were generated that lack dystroglycan in selected striated muscle fibers [152]. Such dystroglycan-deficient myofibers develop severe muscular dystrophy in the first months after birth, and in the adult NMJs are fragmented and lack the typical high density of AChRs and acetylcholinesterase [152]. The fibers also show a wider distribution of AChR clusters, with a corresponding increase in the size of the nerve terminal at the mutant NMJs [153]. In cultured dystroglycan –/– myotubes, agrin induces pronounced clustering of AChRs, but many other D/UGC components such as utrophin, α-dystrobrevin, laminin chains, perlecan and acetylcholinesterase are not found at these clusters in contrast to the wild type [153, 154]. In the cultured dystroglycan –/– myotubes, similarly to the aberrant NMJs of the chimaeric mice in vivo, the AChR clusters induced by agrin occupy a larger area, appear fragmented and actually consist of many microclusters [153, 154]. Most dramatically, these clusters disperse very rapidly after withdrawal of agrin from the culture medium [153]. Formation of normal AChR clusters is also compromised in antisense derivatives of C2 myotubes expressing low amounts of dystroglycan, while agrin-induced phosphorylation of MuSK and AChR β subunits is normal in these derivatives [36]. Taken together, these data indicate that dystroglycan is required for the integrity of the D/UGC and serves as a membrane anchor to recruit components of the complex to the plasma membrane. Although dystroglycan is not a coreceptor for MuSK, it is necessary for consolidation of AChR microclusters into aggregates of normal size as well as for stabilization of preformed AChR clusters and of the NMJ.

α-Dystrobrevin plays a similar role as dystroglycan in that it is also necessary to stabilize AChR clusters. In mice lacking a functional gene for α-dystrobrevin, muscular dystrophy is observed which may originate in part from the absence of D/UGC-linked signaling by nNOS, which normally interacts with dystrobrevin indirectly [155] (fig. 1). Initial NMJ formation proceeds relatively normally in these animals, but after birth, maturation of the NMJ is strongly affected, as the number of postsynaptic folds is reduced and AChRs are found at crests as well as in troughs of these folds, and because AChR clusters become fragmented in older α-dystrobrevin –/– animals [154]. Synaptic localization of α- and β2–syntrophin and nNOS is selectively reduced at the mutant endplates. In mutant myotubes in tissue culture, AChRs form clusters after addition of agrin, but these clusters disappear rapidly upon removal of agrin [154]. Thus, dystrobrevin is required for maintenance of clusters of the AChR both in vivo and in vitro, and plays a role in postsynaptic folding and segregation of AChRs between crests and troughs.

Structurally abnormal NMJs are also observed in the absence of α-syntrophin [156]. In mice with an inactivated α-syntrophin gene, muscle fibers appear healthy histologically, and the mice are mobile and fertile [157]. At the

NMJs of these animals, utrophin and nNOS are absent, densities of AChRs and acetylcholinesterase are reduced and abnormal postsynaptic folds with fewer openings to the synaptic cleft are observed [156]. Thus, α-syntrophin causes synaptic localization of utrophin and nNOS, which may explain some of the similarities in the NMJ phenotype between α-syntrophin –/– and utrophin –/– mice.

A role for tyrosine kinases in stabilization of AChR clusters induced by agrin

Several studies have recently shown that besides the D/UGC, stabilization of agrin-induced AChR clusters in myotubes requires tyrosine kinases. First, the kinase inhibitors herbimycin and staurosporine disperse preformed AChR clusters after removal of agrin from myotubes [72], showing that tyrosine kinase activity is necessary to maintain AChR clusters. Since staurosporine does not affect the phosphorylation status of MuSK [78], the critical kinase activity for cluster stabilization seems to originate from a kinase other than MuSK.

Second, although dispensable for AChR clustering, Src together with Fyn are necessary for stabilization of AChR clusters, as shown in myotubes derived from Src-Fyn doubly-deficient mice: in such cultures, agrin-induced AChR clusters disappear rapidly after removal of agrin from the culture medium [70]. Although performed in cell culture, these studies seem relevant also for NMJ development in vivo because results from dystroglycan –/– and α-dystrobrevin –/– myotubes in culture (see above) are strictly in agreement with the observed alterations at the NMJs in vivo in these mice: in both experimental setups (cultured myotubes vs. NMJs in vivo), the stability of clustered AChRs is compromised. Interestingly, the stability of AChR clusters in myotubes is not affected by the Src-family inhibitors PP1 and PP2, suggesting that the kinase activities of Src and Fyn are not primarily required for cluster maintenance [70]. These observations reveal a parallel to focal adhesion sites, where Src, independent of its kinase activity, can recruit and activate other proteins such as focal adhesion kinase (FAK) [158, 159]. At the NMJ, thus, Src and Fyn may act at least in part as adapters, by recruiting other proteins including kinases in order to stabilize AChR clusters.

Third, the receptor tyrosine kinase TrkB is also involved in cluster stabilization. Overexpression of a dominant-negative TrkB form leads to disruption of TrkB-initiated signaling and to a decrease in the stability of AChR clusters both in vivo and in agrin-treated myotubes in culture [160]. Since TrkB is concentrated in the postsynaptic membrane at the NMJ and can associate with Fyn in cortical neurons [160, 161], TrkB may interact with Src-family kinases also in muscle and act through these kinases, offering an explanation why AChR clusters are unstable when Src and Fyn are absent or when TrkB signaling is affected. The ligand for TrkB that triggers cluster stabilization is not known at this point. Although BDNF is a prominent ligand of TrkB, BDNF actually inhibits agrin-induced AChR clustering in cultured myotubes, similar to the effects of NT-4 [162]. This raises the possibility that TrkB may exert two different effects at the neuromuscular synapse, depending on the stage of NMJ differentiation. While early in postsynaptic assembly it may negatively regulate AChR clustering (triggered by BDNF), it may later stabilize the postsynaptic membrane.

Taken together, several tyrosine kinases (Src, Fyn and TrkB) are necessary for stabilization of clusters of the AChR. It remains to be investigated how these kinases act in this process. One attractive possibility is that they ultimately direct a link of the AChR to the D/UGC, because this protein complex is – like Src, Fyn and TrkB – essential in cluster maintenance (see above). Such a link may involve dystrobrevin, because this protein is a substrate for tyrosine kinases and heavily phosphorylated in *Torpedo* electric organ [163]. Although α-dystrobrevin is not phosphorylated by short agrin treatments of myotubes in culture [145], its phosphorylation may play a role in stabilization of AChR clusters at the developing NMJ in vivo.

AChRs themselves are required for postsynaptic stabilization and postnatal maturation

Besides synaptic elimination and formation of postjunctional folds, the development of the NMJ after birth includes a change in the composition of the AChR itself, as the embryonic γ subunit is replaced by the adult ε subunit. The role of AChRs in this phase of NMJ development was studied by deleting the ε gene in mice [88, 164]. In AChR ε –/– mice, neuromuscular synaptogenesis proceeds normally until the first weeks after birth, when normally the γ subunit is replaced by ε. Since the mice downregulate γ but cannot synthesize ε, they fail to produce functional AChRs in high enough amounts to keep the NMJ healthy. Instead, the high postsynaptic density of AChR seen at birth is gradually reduced in the first weeks after birth, leading to gradual muscle weakness and atrophy and premature death in the mutants [88, 164]. Interestingly, postjunctional folds disappear along with the AChR, such that the postsynaptic membrane, although rich in folds at 20 days, is flattened out by 60 days of age [89]. Furthermore, other postsynaptic proteins disappear also as the AChR density decreases [88]: rapsyn disappears strictly in parallel with the AChR, while utrophin, α-dystrobrevin and also agrin lag behind and can still be efficiently clustered at mutant synapses that have already lost most of their AChRs [89]. These data show that ε-containing AChRs are re-

quired for synaptic localization of rapsyn and maintenance of the postsynaptic apparatus at the postnatal NMJ, including postjunctional folds. Together with very recent results showing an active role of the AChR in clustering of other postsynaptic proteins (see above), these studies on the ε AChR subunit lead to the conclusion that AChRs themselves are actively involved both in formation as well as maintenance of the postsynaptic apparatus at the NMJ.

Conclusion

Formation and maturation of the NMJ occur in several well-defined stages, and the nerve and its derived factors were previously thought to be the key inducers of the postsynaptic apparatus, in particular clusters of the AChR. Although recent progress has revealed a program of muscle prepatterning that can lead to aneural AChR clustering, the importance of the nerve, particularly of neural agrin, remains largely undisputed: without motorneurons, clusters of AChRs are not precisely aligned in a narrow band in the center of the muscle, and without agrin (in the presence of the nerve), no functional synapses are present at birth. Neural agrin not only redistributes preexisting AChRs but, through its interaction with the neuregulin-1-pathway, also causes new synthesis of AChRs. Moreover, agrin stabilizes AChR clusters against their nerve-induced elimination.

While classic studies on rapsyn-induced clustering of AChRs in heterologous cells led to the conclusion that rapsyn is the driving force and is necessary for aggregation of the AChR, recent progress has shown that likewise AChRs are required for agrin-induced clustering and synaptic localization of rapsyn. In muscle cells in culture, rapsyn and AChRs interact even in the absence of neurons or neural agrin. Thus, both the AChR and its anchoring protein rapsyn are mutually important for aggregation, by forming a preassembled protein complex. In cultured myotubes, also other postsynaptic proteins, for example MuSK, preassemble in complexes independent of neural agrin, suggesting that preassembly may represent the mechanism of formation of spontaneous protein clusters in muscle cells. This nerve-independent preassembly is a striking parallel to the ability of embryonic myofibers to form aneural AChR clusters in the process of muscle prepatterning and suggests that formation of preassembled protein complexes may also play a role in this process.

Finally, AChRs are also actively involved in agrin-induced clustering of other postsynaptic proteins (besides rapsyn) and in maintaining the synaptic accumulation of some proteins, revealing that the AChR itself is an active player both in the formation as well as the maintenance of the postsynaptic apparatus.

Recent studies have also shown that the protein and signaling requirements for formation of AChR clusters are different from those for stabilization of such clusters. While components of the D/UGC, Src and Fyn are largely dispensable for initial AChR clustering, they are essential, along with TrkB, for maintenance of previously induced clusters. It remains to be investigated how the signaling mechanisms for formation versus maintenance of AChRs clusters are fine tuned, in particular, what sets of proteins closely interact with the AChR at each step in the formation, elimination and maturation of the postsynaptic apparatus.

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