

# Formation of the neuromuscular junction: molecules and mechanisms

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## Summary

The vertebrate skeletal neuromuscular junction is the site at which motor neurons communicate with their target muscle fibers. At this synapse, as at synapses throughout the nervous system, efficient and appropriate communication requires the formation and precise alignment of specializations for transmitter release in the axon terminal with those for transmitter detection in the postsynaptic cell. Classical developmental studies demonstrate that synapse formation at the neuromuscular junction is a mutually inductive event; neurons induce postsynaptic differentiation in muscle cells and myofibers induce presynaptic differentiation in motor axon terminals. More recent experiments indicate that Schwann cells, which cap axon terminals, also play an active role in the formation and maintenance of the neuromuscular junction. Here, we review recent advances in the identification of molecules mediating such inductive interactions and the mechanisms by which they produce their effects. Although our discussion concerns events at developing neuromuscular junctions, it seems likely that similar molecules and mechanisms may act at neuron–neuron synapses in the peripheral as well as the central nervous system. *BioEssays* **20**:819–829, 1998. © 1998 John Wiley & Sons, Inc.

## Introduction

In vertebrate skeletal muscle, each muscle fiber is typically innervated by a single motor axon at a site near the middle of the muscle fiber, often referred to as the motor end plate.<sup>(1)</sup>

Within the axon terminal, at such neuromuscular junctions, there are clusters of synaptic vesicles, which contain the transmitter acetylcholine. Each vesicle cluster is aligned with a patch of dense material on the presynaptic membrane, forming an active zone. At the active zone, vesicles fuse with the plasma membrane and release their content of acetylcholine. Acetylcholine diffuses across the synaptic cleft, which separates the pre- and postsynaptic cells, and binds to AChRs, which are densely packed in the postsynaptic membrane. Acetylcholine is degraded by acetylcholinesterase, which is highly concentrated in the portion of the muscle fiber's basal lamina sheath that occupies the synaptic cleft. At most neuromuscular junctions, the postsynaptic membrane has regular invaginations, called junctional folds, which are precisely aligned with the presynaptic active zones. Several "synaptic" or "end-plate" nuclei, with their associated Golgi apparatus, lie just beneath the postsynaptic membrane. The pattern of gene expression by such nuclei differs from that of typical myonuclei that occupy extrasynaptic portions of the muscle fiber. Finally, each axon terminal is covered by a Schwann cell.

Many of the molecular components comprising the pre- and postsynaptic specializations seen at the neuromuscular

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*Abbreviations:* AChR, acetylcholine receptor; MuSK, muscle-specific receptor tyrosine kinase; ARIA, acetylcholine receptor-inducing activity; CGRP, calcitonin gene-related peptide; BDNF, brain-derived neurotrophic factor; NT-3, neurotrophin-3; NT-4/5, neurotrophin 4/5; CNTF, ciliary neurotrophic factor; NDF, Neu differentiation factor; NRG, neuregulin; EGFR, epidermal growth factor receptor; PI3K, phosphatidylinositol 3-kinase; GGF, glial growth factor; MAPK, mitogen-activated protein kinase; CHO, Chinese hamster ovary; GDNF, glial cell line-derived neurotrophic factor.

junction have been identified (Table 1). Here, we describe results of recent experiments, including the creation of genetic “knockouts” by homologous recombination, that have provided new insights into how such specializations are formed and the signaling mechanisms that regulate their assembly.

#### Postsynaptic differentiation

In a pivotal series of experiments begun in the late 1970s, U.J. McMahan and colleagues demonstrated that molecules stably associated with the synaptic portion of the myofiber's basal lamina sheath trigger the formation of presynaptic and postsynaptic specializations in regenerating nerve and muscle fibers.<sup>(2)</sup> They went on to identify the basal lamina factor that induces the formation of postsynaptic specializations in muscle fibers as the protein agrin. The observation that regenerated neuromuscular junctions resemble those formed during development led to the hypothesis that agrin is released from the terminals of axons at developing synapses to trigger the formation of the postsynaptic apparatus and subsequently becomes incorporated into the synaptic basal lamina, where it helps to maintain synapses in the adult and to induce postsynaptic differentiation in regenerating muscle fibers.

#### *Agrin triggers the assembly of the postsynaptic apparatus*

The effects of agrin are most often studied in myotubes in cell culture, in which agrin induces the formation of specializations where many components of the postsynaptic apparatus accumulate.<sup>(2–4)</sup> Perhaps the most conspicuous feature of such specializations is the accumulation of AChRs. However, some features of the mature postsynaptic apparatus are not seen at agrin-induced specializations on cultured myotubes, such as junctional folds. Is this because the conditions in culture are not sufficient to support complete maturation of the postsynaptic apparatus, or is it due to the lack of additional nerve-derived differentiation signals? To address this question, extrajunctional regions of adult muscles were exposed to agrin *in vivo* by injecting expression constructs encoding for agrin into the muscle fibers themselves. At such sites, specializations form that appear to include all of the components of the mature postsynaptic apparatus.<sup>(5–8)</sup> This indicates that agrin is the only nerve-derived signal required to trigger postsynaptic differentiation.

Agrin occurs in several isoforms that arise from a single gene by alternative splicing.<sup>(3)</sup> Neurons, muscle cells, and Schwann cells all express agrin. However, only neurons express isoforms that are effective in triggering postsynaptic differentiation; the function of the isoforms expressed by muscle and Schwann cells is not known. Several functional domains in agrin have been mapped (Fig. 1). The N-terminal portion of the protein is necessary for binding of agrin to laminin.<sup>(9)</sup> Heparan sulfate glycosaminoglycan (GAG) chains,

TABLE 1. Molecular Components of the Neuromuscular Junction

#### Presynaptic terminal

Components mediating vesicle docking, priming, exocytosis

munc18<sup>(78)</sup>

SNAP-25<sup>(78)</sup>

NSF<sup>(78)</sup>

synaptobrevin<sup>(78)</sup>

$\alpha/\beta/\gamma$ -SNAP<sup>(78)</sup>

syntaxin<sup>(78)</sup>

synaptotagmin<sup>(78)</sup>

synaptophysin<sup>(78)</sup>

Hrs-2<sup>(79)</sup>

Synapsins I, II<sup>(78)</sup>

Calcium channels<sup>(78)</sup>

SV2<sup>(78)</sup>

Choline acetyltransferase<sup>(1)</sup>

Calcitonin gene-related peptide<sup>(1)</sup>

Neuregulin<sup>(45)</sup>

Integrin  $\alpha 1$ <sup>(34)</sup>

#### Synaptic basal lamina

Agrin<sup>(2)</sup>

Laminin 4 ( $\alpha 2/\beta 2/\gamma 1$ )<sup>(38)</sup>

Laminin 9 ( $\alpha 4/\beta 2/\gamma 1$ )<sup>(38)</sup>

Laminin 11 ( $\alpha 5/\beta 2/\gamma 1$ )<sup>(38)</sup>

Collagen  $\alpha 3(IV)$ ,  $\alpha 4(IV)$ ,  $\alpha 5(IV)$ <sup>(56)</sup>

Neuregulin<sup>(56)</sup>

Acetylcholinesterase<sup>(1)</sup>

Heparan sulfate proteoglycan<sup>(1)</sup>

Entactin (s-nidogen)<sup>(80)</sup>

Protease nexin I<sup>(1)</sup>

HB-GAM<sup>(81)</sup>

#### Postsynaptic membrane

Acetylcholine receptor<sup>(1)</sup>

Sodium channel (voltage-gated)<sup>(1)</sup>

N-CAM<sup>(56)</sup>

Integrins<sup>(34,35)</sup>

M-, N-cadherin<sup>(82)</sup>

MuSK<sup>(29)</sup>

erbB2/3/4<sup>(45)</sup>

$\alpha/\beta$ -dystroglycan<sup>(14)</sup>

N-Acetylgalactosaminyl transferase<sup>(1)</sup>

#### Subsynaptic cytoskeleton

Dystrophin/utrophin complex<sup>(14)</sup>

Utrophin/dystrophin

Dystrobrevin

Syntrophin  $\beta 2$

Ankyrin<sup>(1)</sup>

Rapsyn<sup>(32)</sup>

$\beta$ -Spectrin<sup>(1)</sup>

Paxillin<sup>(1)</sup>

Vinculin<sup>(1)</sup>

$\alpha$ -Actinin<sup>(1)</sup>

Filamin<sup>(1)</sup>

Talin<sup>(1)</sup>

Actin<sup>(1)</sup>

Tropomyosin 2<sup>(1)</sup>

Acetylated tubulin<sup>(1)</sup>

Lamin B<sup>(1)</sup>

Desmin<sup>(1)</sup>

SNAP, soluble NSF-attachment protein; NSF, N-ethylmaleimide sensitive factor; HB-GAM, heparin-binding growth-associated molecule; N-CAM, nerve cell adhesion molecule.

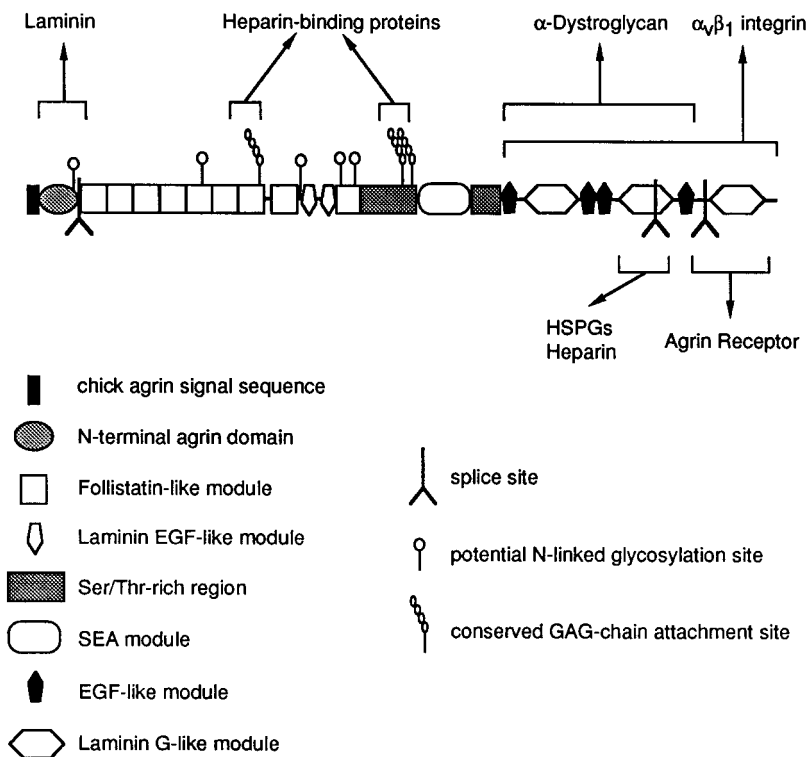


Figure 1. Identified ligand-binding domains in agrin. The structure of full-length chick agrin is illustrated.<sup>(63)</sup> Conserved sites for attachment of glycosaminoglycan side chains and N-linked glycosylation are indicated; native agrin contains heparan sulfate side chains and thereby interacts with heparin-binding proteins. Domains that mediate binding to laminin,  $\alpha$ -dystroglycan, integrin, and heparin are indicated, as is the smallest fragment that is capable of interaction with the agrin receptor that mediates acetylcholine receptor aggregation. HSPG, heparan sulfate proteoglycan; SEA, sequence found in sea urchin sperm protein, enterokinase, and agrin.

which mediate the interaction of agrin with various heparin-binding proteins, are attached to sites in the central region of agrin. Laminin G-like domains in the C-terminal half of agrin mediate binding to  $\alpha$ -dystroglycan.<sup>(10)</sup> Within this region of the protein is a domain that binds agrin to heparin.<sup>(10)</sup> The ability to induce formation of postsynaptic specializations resides in the most C-terminal domain.<sup>(10–12)</sup>

Recent dramatic confirmation of the essential role of agrin in the formation of the neuromuscular junction came as the result of “knockout” experiments in which agrin expression was prevented by homologous recombination.<sup>(13)</sup> In agrin-deficient mutant mice, myofibers appear to develop normally and axons grow into the developing muscles, but formation of neuromuscular junctions is severely perturbed. Instead, axons run unbranched for long distances and end without any apparent specializations. Most axon terminals have no associated cluster of AChRs or any other postsynaptic specialization. On the other hand, if myoblasts from mutant embryos are grown in cell culture, the myotubes respond to exogenous agrin by clustering AChRs, just as controls. Thus, in the absence of agrin, muscle cells have the potential to form

postsynaptic specializations and, indeed, do so on occasion spontaneously, but axons are unable to trigger their formation.

Why should there be a lack of presynaptic specializations in agrin-deficient mutant mice? A simple hypothesis is that agrin-induced postsynaptic differentiation generates a retrograde signal that triggers formation of presynaptic specializations. In agrin-deficient muscles, a few terminals, apparently those juxtaposed to AChR clusters, have a more normal structure. The occasional site of pre- and postsynaptic differentiation in agrin-deficient mutant muscles could mean that a signal other than agrin can induce synaptic specialization. Perhaps more likely, it could result from the chance encounter of an axon with a spontaneously occurring AChR aggregate. Such spontaneous sites of postsynaptic specialization might be expected to contain retrograde signals that trigger presynaptic differentiation.

#### *The search for the agrin receptor*

Several lines of evidence suggest that agrin induces the formation of postsynaptic specializations in myotubes by

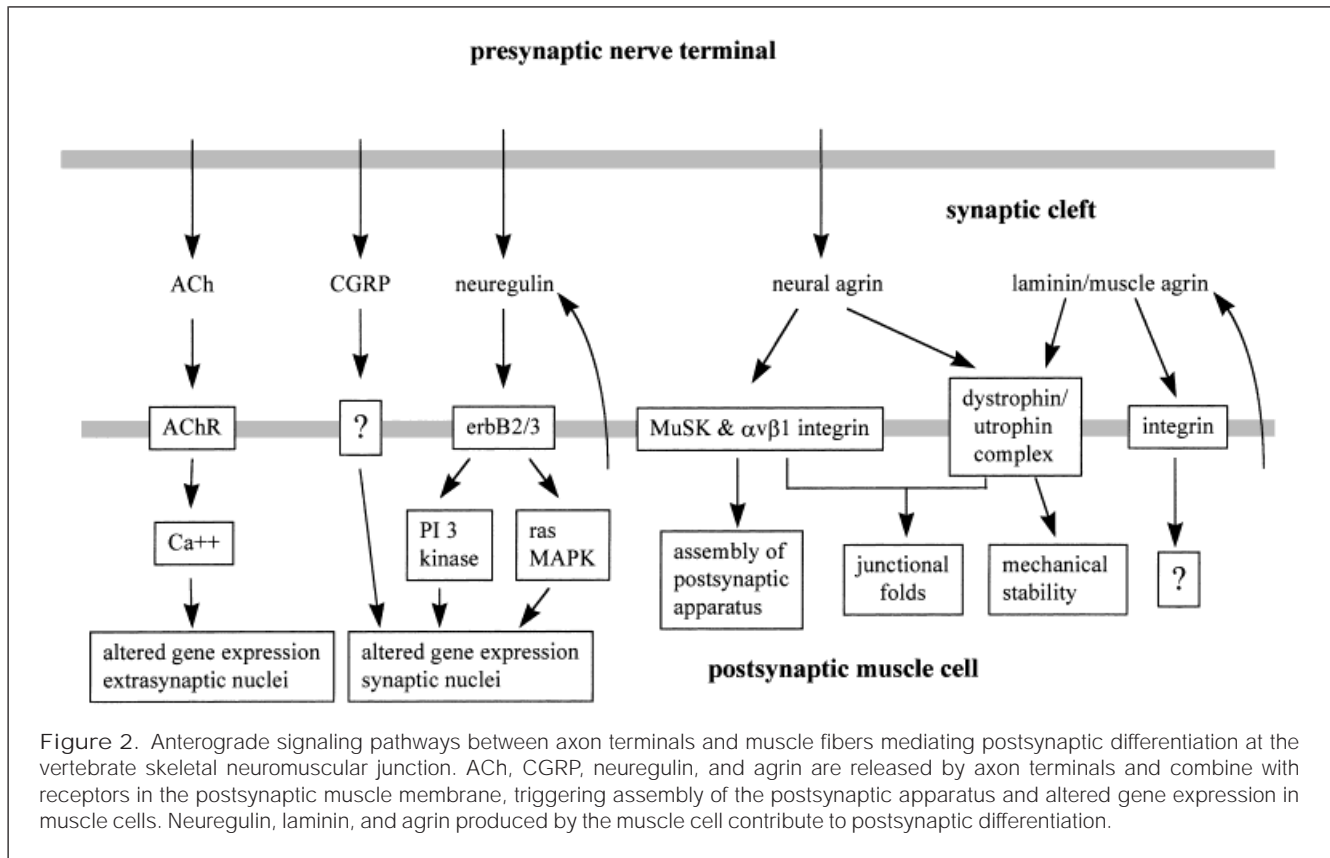


Figure 2. Anterograde signaling pathways between axon terminals and muscle fibers mediating postsynaptic differentiation at the vertebrate skeletal neuromuscular junction. ACh, CGRP, neuregulin, and agrin are released by axon terminals and combine with receptors in the postsynaptic muscle membrane, triggering assembly of the postsynaptic apparatus and altered gene expression in muscle cells. Neuregulin, laminin, and agrin produced by the muscle cell contribute to postsynaptic differentiation.

binding to a receptor on the myotube surface and thereby triggering an intracellular signaling cascade.<sup>(2)</sup> Results of recent experiments have begun to identify the critical components of this and other transduction pathways mediating synaptic differentiation (Fig. 2).

#### **The dystrophin/utrophin protein complex does not mediate agrin-induced AChR aggregation**

In muscle, the dystrophin/utrophin complex of proteins is thought to link the muscle cytoskeleton to the membrane and the surrounding extracellular matrix.<sup>(14)</sup> This complex is comprised of either dystrophin or its homologue utrophin combined with  $\alpha$ - and  $\beta$ -dystroglycan, syntrophin, dystrobrevin, and the sarcoglycans. The dystrophin/utrophin complex colocalizes with AChRs at developing and adult neuromuscular junctions and with rapsyn in heterologous coexpression systems,<sup>(15)</sup> suggesting that the formation of AChR aggregates might be mediated by interactions involving proteins in this complex. The observation that agrin binds to  $\alpha$ -dystroglycan, a surface component of the dystrophin/utrophin complex, prompted the hypothesis that  $\alpha$ -dystroglycan was, in fact, the agrin receptor.<sup>(3)</sup> However, there is no correlation between the affinity of various agrin isoforms for binding to  $\alpha$ -dystroglycan and for inducing AChR aggregation,<sup>(10,16–18)</sup>

and fragments of agrin that do not include the binding site for  $\alpha$ -dystroglycan nevertheless induce AChR aggregation.<sup>(10,12)</sup> Thus, AChR aggregation is mediated by a receptor that is distinct from  $\alpha$ -dystroglycan.

Does the dystrophin/utrophin complex have some other essential function in AChR aggregation? For example, the complex is clearly well positioned to play a structural role in anchoring AChRs and other components of the postsynaptic apparatus to the underlying cytoskeleton. Junctions in mice that are deficient in utrophin or in both utrophin and dystrophin do contain markedly reduced levels of  $\alpha$ -sarcoglycan and dystroglycan and almost no detectable dystrobrevin or  $\beta 2$ -syntrophin.<sup>(19–22)</sup> Yet AChR accumulation appears normal in such dystrophin/utrophin knockouts. Thus, neither dystrophin, utrophin, nor their associated proteins are required for agrin-induced AChR aggregation.

One striking phenotype of utrophin/dystrophin-deficient mutants is the paucity of junctional folds. This could be a consequence of the continual turnover of muscle fibers and neuromuscular junctions seen in dystrophic muscle. However, a paucity of junctional folds is also seen in mutants deficient only in utrophin. In such animals myofibers and junctions appear stable yet still lack folds, suggesting that utrophin plays a role in junctional fold formation.

At neuromuscular junctions, the interaction of agrin with  $\alpha$ -dystroglycan could provide structural support for the postsynaptic membrane by linking the synaptic basal lamina to the myofiber cytoskeleton via the dystrophin/utrophin complex, just as the binding of laminin to  $\alpha$ -dystroglycan is thought to reinforce the muscle plasma membrane in nonsynaptic regions.<sup>(14)</sup> The binding of agrin to  $\alpha$ -dystroglycan, as well as to components of the extracellular matrix,<sup>(9)</sup> could also serve to immobilize agrin within the synaptic cleft and increase its effective concentration in the vicinity of the receptor that triggers postsynaptic differentiation.

### ***MuSK mediates agrin-induced differentiation***

There is considerable evidence that the combination of agrin with its receptor triggers an intracellular signaling cascade that includes increased protein tyrosine phosphorylation.<sup>(23–26)</sup> To date, only two proteins have been shown to become phosphorylated on tyrosine residues as a consequence of agrin treatment, the AChR itself<sup>(23–26)</sup> and the recently cloned muscle-specific receptor tyrosine kinase MuSK.<sup>(27)</sup> Both are phosphorylated rapidly after addition of agrin to cultured myotubes, and inhibitors that prevent tyrosine phosphorylation of either MuSK or AChRs prevent AChR aggregation.<sup>(23,24,26,28)</sup> However, it has yet to be demonstrated directly that phosphorylation of MuSK or AChRs is required for AChR aggregation. MuSK is expressed predominantly in skeletal muscle and is highly localized to the postsynaptic apparatus.<sup>(29)</sup> MuSK knockouts have a phenotype similar to but more extreme than that of agrin knockouts, apparently normal nerve and muscle development, but a complete absence of any pre- or postsynaptic specializations.<sup>(30)</sup> Unlike myoblasts from agrin-deficient mice, myoblasts from MuSK-deficient mutant embryos grown in cell culture fail to respond to exogenous agrin. Thus, MuSK mediates an early and essential step in the signaling cascades triggered by the interaction of agrin with its receptor.

Is MuSK the agrin receptor? MuSK is phosphorylated soon after addition of agrin to cultured myotubes; such rapid autophosphorylation is a well-known characteristic of many receptor tyrosine kinases. In addition, antibodies against the extracellular portion of MuSK can mimic agrin's effects, triggering MuSK phosphorylation and AChR aggregation.<sup>(31)</sup> Agrin, however, does not appear to bind directly to the extracellular domain of MuSK, as might be expected if MuSK were the agrin receptor.<sup>(27)</sup> Rather, it appears that the agrin receptor may be formed by the combination of MuSK and additional, as yet unidentified, accessory proteins that are present specifically in muscle cells.<sup>(32,33)</sup>

### ***The role of integrins and laminin in AChR aggregation***

One candidate for such an agrin-binding accessory protein is integrin. Integrins are heterodimers of  $\alpha$  and  $\beta$  subunits that bind to and mediate the effects of laminin and other compo-

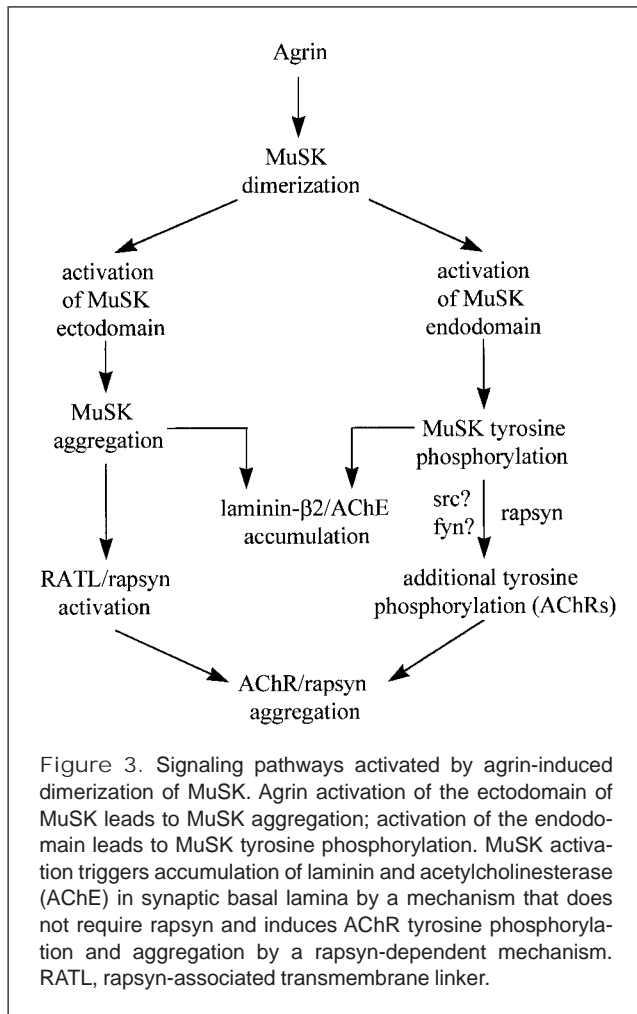
nents of the extracellular matrix. At least four integrin subunits— $\alpha$ 3,  $\alpha$ 7,  $\alpha$ v, and  $\beta$ 1—are found in the postsynaptic membrane at the adult neuromuscular junction.<sup>(34)</sup> The carboxy-terminal region of agrin contains three laminin G-like domains, which have been implicated in integrin binding.<sup>(35)</sup> Indeed,  $\alpha$ v $\beta$ 1 and  $\alpha$ v $\beta$ 3 integrins mediate adhesion of cells to immobilized agrin. Moreover, antibodies and peptides that block function of  $\beta$ 1- or  $\alpha$ v-containing integrins inhibit agrin-induced AChR aggregation, as does attenuation of  $\alpha$ v expression in myotubes with antisense oligonucleotides.<sup>(35)</sup> Thus, integrins appear to be part of the agrin-mediated signaling cascade that leads to AChR aggregation.

Interestingly, laminin-1, another integrin ligand, induces AChR aggregates on cultured myotubes in a way that does not depend on agrin or MuSK.<sup>(36,37)</sup> The lack of AChR aggregates at neuromuscular junctions in MuSK or agrin-deficient mutants, however, suggests that the pathway activated by laminin-1 may function to supplement and/or maintain aggregates of AChRs that are initially induced by nerve-derived agrin. Moreover, since laminin-1 is not present in the synaptic basal lamina of developing or adult neuromuscular junctions,<sup>(38)</sup> such a pathway, if active, would have to be triggered by a different laminin isoform.

### ***Agrin activates multiple signaling pathways***

At least two signaling pathways appear to mediate agrin-induced synaptic specialization, one mediated by the ectodomain of MuSK, the other by the endodomain (Fig. 3). Both involve the 43 kDa AChR-associated protein rapsyn. In rapsyn-deficient mutant mice, neuromuscular junctions fail to form normally.<sup>(39)</sup> The most conspicuous deficit is the lack of aggregates comprised of AChRs, rapsyn, and the dystrophin/utrophin complex. Presynaptic differentiation is also affected; axons do not terminate as abruptly and fail to form characteristic terminal arbors. Thus, normal assembly of the synaptic apparatus occurs via pathways that require rapsyn.

On the other hand, neuromuscular deficits in rapsyn-deficient mice are not as severe as in agrin or MuSK knockouts; some limited functional synaptic transmission occurs.<sup>(39)</sup> Axonal branches tend to remain in a central band, many axons do form specialized terminals, as judged by electron microscopy, and such terminals are juxtaposed to sites of accumulation of laminin- $\beta$ 2, acetylcholinesterase, and MuSK.<sup>(32)</sup> Selective expression of AChR genes by nuclei in the synaptic region of the myofibers occurs more or less normally.<sup>(39)</sup> Compared to the complete absence of any synaptic differentiation in MuSK knockouts, the formation of such specializations in rapsyn-deficient mutant mice suggests that localized activation of MuSK by agrin released from axon terminals can trigger localized accumulation of both MuSK and components of the synaptic basal lamina, such as laminin- $\beta$ 2 and acetylcholinesterase, by a mechanism that does not require rapsyn (Fig. 3). This limited postsynaptic



differentiation, in turn, is sufficient to trigger a modest degree of presynaptic differentiation.

### **Mechanisms of rapsyn-mediated postsynaptic differentiation**

While results of knockout experiments demonstrate that rapsyn is required for many aspects of postsynaptic differentiation induced when agrin activates MuSK, the mechanism by which rapsyn contributes to the organization of the postsynaptic apparatus is not well understood. Rapsyn is relatively plentiful; throughout most of embryonic development and in the adult, rapsyn colocalizes with AChRs with a 1:1 stoichiometry. In heterologous expression systems, rapsyn forms large aggregates spontaneously. Coexpression studies demonstrate that AChRs, MuSK, and  $\alpha/\beta$ -dystroglycan are recruited to such rapsyn aggregates,<sup>(32,40)</sup> perhaps mimicking events at developing neuromuscular junctions. However, all these components are present in myotubes before the arrival of the nerve, yet their coaggregation requires agrin-induced MuSK activation and protein tyrosine

phosphorylation. Thus, the crucial question is how agrin activation of MuSK and MuSK-induced changes in phosphorylation alter the interaction among these proteins in a way that leads to aggregation.

Results of experiments on cells expressing chimeric receptors combining extracellular and intracellular domains of MuSK and related receptor tyrosine kinases<sup>(32,33)</sup> suggest that rapsyn interacts with MuSK in two ways to bring about AChR aggregation (Fig. 3). One interaction is between rapsyn and the extracellular domain of MuSK via an unidentified component (rapsyn-associated transmembrane linker, RATL)<sup>(32)</sup>. This interaction, which mediates coclustering of rapsyn and MuSK in heterologous expression systems, appears also to be required for aggregation in muscle cells.<sup>(33)</sup> Given the observation that MuSK accumulates at sites of neuromuscular contact in rapsyn-deficient mutants,<sup>(32)</sup> it seems reasonable to propose that the interaction of agrin with MuSK creates a primary scaffold to which rapsyn and AChRs are recruited.<sup>(32)</sup> In muscle cells, rapsyn and AChRs are recruited simultaneously, and such recruitment appears to require the kinase activity of the intracellular domain of MuSK.<sup>(33)</sup> Now comes the second rapsyn-dependent step, tyrosine phosphorylation of the AChR  $\beta$  subunit, an early event in agrin-induced AChR aggregation.<sup>(23,26)</sup> Although autophosphorylation of the MuSK intracellular domain occurs in the absence of rapsyn, AChR phosphorylation does not.<sup>(32,33)</sup> It is currently not clear whether MuSK phosphorylates AChRs directly or through activation of other kinases.<sup>(28)</sup> Src-like kinases, including src, fyn, and fyn, have been shown to associate with and be capable of phosphorylating AChRs, but no agrin or rapsyn dependence has been demonstrated for these events.<sup>(41–43)</sup> Thus, for agrin-induced AChR phosphorylation and aggregation to occur, it appears that rapsyn must interact, on the one hand, with the extracellular domain of MuSK and, on the other hand, with the intracellular domain of MuSK, AChRs, and perhaps one or more src-like kinases.

Rapsyn may also recruit components of the dystrophin/utrophin complex to such a MuSK/rapsyn/AChR scaffold, although, as described above, accumulation of these components is not required for AChR aggregation.

### **Regulation of postsynaptic gene expression**

In adult muscle fibers, AChR mRNA is transcribed selectively in synaptic nuclei, contributing to the 1,000-fold difference in receptor density between synaptic and extrasynaptic regions.<sup>(1)</sup> The selective expression occurs by two mechanisms: 1) nerve-induced electrical activity suppresses AChR subunit gene expression in extrasynaptic nuclei and 2) nerve-derived factors stimulate AChR subunit gene transcription in synaptic nuclei.

### **Repression of extrasynaptic AChRs**

The electrical activity of innervated muscle fibers and the resulting  $\text{Ca}^{2+}$  influx through voltage-activated  $\text{Ca}^{2+}$  channels

suppress AChR gene transcription in extrasynaptic nuclei (Fig. 2), an effect that seems to be specific for AChR subunit genes.<sup>(44)</sup> A widely accepted model proposes that increased  $\text{Ca}^{2+}$  influx activates a protein kinase C, which in turn phosphorylates and thereby inhibits the transcription factor myogenin. Phosphorylation not only inactivates preexisting myogenin but downregulates further myogenin transcription by reducing myogenin activation of its own promoter.

### **Induction of synaptic gene expression by neuregulin**

In adult muscle, AChR expression is restricted to synaptic nuclei and the embryonic pattern of  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$  subunit expression is changed to the adult pattern of  $\alpha$ ,  $\beta$ ,  $\epsilon$ , and  $\delta$ .<sup>(44)</sup> Regulation of AChR  $\epsilon$ -subunit gene expression is controlled by factors of motoneuronal origin thought to be associated with the synaptic portion of the basal lamina. The first identified candidate for such a factor is ARIA, originally isolated from chicken brain.<sup>(44,45)</sup> ARIA is synthesized and secreted by motor neurons and is stably incorporated into the synaptic basal lamina of the neuromuscular junction.<sup>(44,45)</sup> When tested in vitro, ARIA induces AChR  $\alpha$ -,  $\gamma$ -,  $\epsilon$ , and  $\delta$ -subunit mRNA expression.<sup>(44–46)</sup>

ARIA is an  $\sim 45$  kDa protein cleaved from a larger membrane-associated proARIA precursor.<sup>(45)</sup> Rat and human homologues of the same *nrg-1* gene encode NDF and HRG, respectively, as well as GGF. The family of mosaic proteins derived from this gene by alternative splicing are collectively named the neuregulins (NRGs).<sup>(45)</sup> All members of the NRG-1 family are characterized by a conserved EGF-like domain and are subdivided into types I, II, and III according to additional functional domains.<sup>(47)</sup> ARIA belongs to the type I group of NRGs.

Receptors for NRG are members of the type I EGFR-related family of receptor tyrosine kinases, comprising the EGFR (*erbB1*) as well as the closely related *erbB2/neu*, *erbB3*, and *erbB4* receptors.<sup>(45)</sup> *ErbB2*, *erbB3*, and *erbB4* receptors accumulate at the neuromuscular junction, where the heterodimer consisting of *erbB2*, with its functional tyrosine kinase domain, and the ligand-binding *erbB3* or *erbB4* likely comprise the functional NRG receptor.<sup>(45)</sup> NRG-induced stimulation of *erbB* receptors appears to activate both Ras/MAPK and PI3K pathways, leading to enhanced AChR gene expression.<sup>(48–50)</sup> Thus, NRG type I might be the factor that activates synapse-specific gene expression in subsynaptic nuclei (Fig. 2).

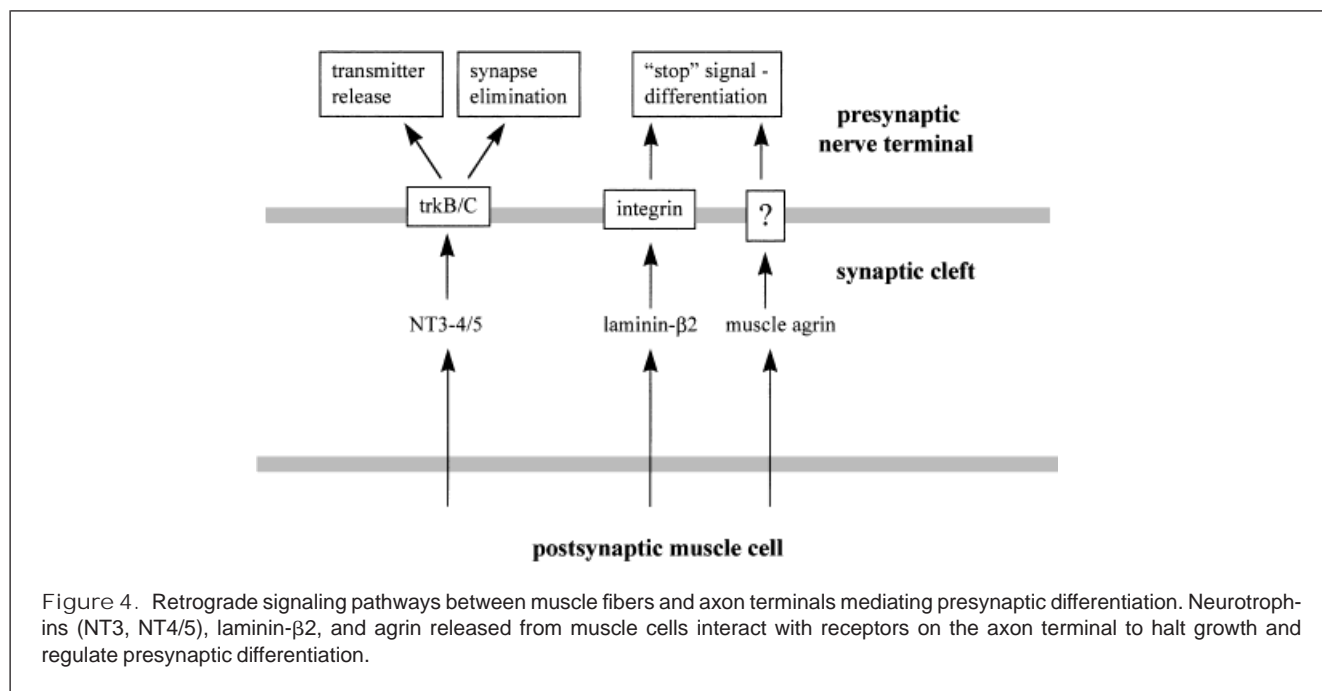
If NRG were responsible for synapse-specific AChR gene expression, then the regional distribution of AChR gene expression would be determined by the localization of either NRG or the *erbB* receptors. In intercostal muscles from MuSK- and agrin-deficient mutant mice, AChR gene expression is not confined to a central band, as usual, but is widespread.<sup>(13,30)</sup> The uniform level of expression could result from the absence of gene repression due to the lack of nerve-evoked electrical activity in such mutant mice. In addition, the

interaction of NRG, released from axons that fail to terminate within the central portion of such mutant muscles, with *erbB* receptors, which also appear to be scattered over the myofiber surface, could promote widespread AChR gene expression. In rapsyn-deficient mice, on the other hand, AChR gene expression is localized to a central band of myonuclei.<sup>(39)</sup> In such rapsyn-deficient mutant mice, nerve terminals tend to be restricted to more central regions of the myofibers and there appears to be some functional neuromuscular transmission. Thus, localized expression could be due to the normal pattern of repression of AChR gene expression in extrasynaptic regions by electrical activity and NRG-dependent induction of expression in nuclei near axon terminals. It should be noted that these experiments monitor  $\alpha$ - and  $\gamma$ -subunit expression. Agrin-, MuSK-, and rapsyn-deficient mice die shortly after birth, before expression of the  $\epsilon$  subunit normally begins, so whether or not synapse-specific expression of the  $\epsilon$  subunit would occur in such mice cannot be determined.

Interestingly, NRGs are synthesized and secreted not only by motor neurons but also by muscle cells.<sup>(51)</sup> What prevents muscle-derived NRGs from constitutively inducing widespread AChR gene expression? One explanation could be that agrin is necessary for activation of gene expression by muscle-derived NRG, perhaps because muscle-derived NRG is present at a lower concentration than that derived from axon terminals. For example, agrin, by virtue of its heparan sulfate GAG chains (Fig. 1), or other heparan sulfate proteoglycans induced to accumulate by agrin might increase the local concentration of muscle-derived NRG by localizing it to the synaptic basal lamina.<sup>(52)</sup> Additionally, agrin might influence synaptic AChR subunit gene expression by aggregating *erbB* receptors in the myofiber membrane. Tissue-specific prevention of NRG expression would allow the contributions of nerve- and muscle-derived NRGs to be distinguished.

### **Does agrin alter gene expression?**

Soluble forms of agrin do not alter AChR gene expression in cultured myotubes.<sup>(53)</sup> On the other hand, there is evidence suggesting that agrin might regulate synaptic gene expression under certain conditions. For example, rat myotubes cultured on a basal lamina substrate impregnated with recombinant agrin show focal expression of AChR  $\epsilon$ -subunit mRNA, colocalized with sites of AChR aggregation.<sup>(53)</sup> In addition, extrajunctional regions of muscle fibers injected with agrin expression plasmids in vivo develop postsynaptic specializations resembling those at normal adult synapses, including expression of AChR  $\epsilon$  subunits, despite the absence of a nerve terminal.<sup>(5,8)</sup> In both situations, however, agrin would induce a focal accumulation of muscle-derived NRGs and *erbB* receptors. Thus, the simplest interpretation of available data is that agrin has no direct effect on gene expression but acts indirectly to promote gene expression via muscle-derived NRGs and *erbBs*.



#### ***NRG- and erbB-deficient mutant mice***

The issue of whether agrin directly affects gene expression or influences it indirectly through NRG could, in principle, be settled by knockout experiments. However, mice deficient in functional NRG, erbB2, or erbB4 genes die in utero, too early to study the effects of such mutations on the formation of neuromuscular junctions.<sup>(45)</sup> Normal induction of AChR subunit gene expression, as well as nerve-induced aggregation of AChRs, is observed in erbB3-deficient mice, but in such mice NRG-dependent AChR gene induction might depend entirely on the activation of erbB2/erbB4 heterodimers.<sup>(54)</sup> Support for NRG as a major regulator of AChR gene expression comes from studies of NRG-deficient heterozygotes in which NRG mRNA levels are decreased by 50% compared to controls. In such mutants, the density of AChRs in the postsynaptic membrane is also 50% of normal.<sup>(55)</sup> A more definitive answer could come from muscle-specific or late-onset knockouts of NRGs and erbB receptors.

#### ***Regulation of gene expression by calcitonin gene-related peptide (CGRP)***

One other factor known to stimulate AChR expression in cultured myotubes is CGRP.<sup>(44)</sup> CGRP is present during development in many motor nerve terminals. However, in  $\alpha$ CGRP-deficient mutant mice, the appearance of neuromuscular junctions and the density and distribution of AChRs and AChR mRNA are normal.<sup>(56)</sup> Thus, CGRP appears not to be required for the normal pattern of synaptic gene expression.

#### **Presynaptic differentiation**

As mentioned above, experiments by McMahan<sup>(2)</sup> demonstrated that molecules stably associated with the synaptic basal lamina could trigger the formation of presynaptic specializations in regenerating axons in adult muscle. However, compared to the wealth of information on differentiation of the postsynaptic apparatus, little is known about differentiation of the presynaptic motor axon terminal (Fig. 4).

#### ***Laminin- $\beta$ 2***

Evidence for the identity of one retrograde, basal lamina-associated signal mediating presynaptic differentiation comes from mice deficient in laminin- $\beta$ 2 (originally called s-laminin), a subunit of the ubiquitous heterotrimeric basal lamina protein laminin.<sup>(57)</sup> Laminin- $\beta$ 2 is synthesized by muscle and accumulates selectively in the synaptic basal lamina<sup>(38)</sup> and at sites of ectopic agrin-induced AChR aggregates,<sup>(6,7)</sup> as a component of laminin 4 ( $\alpha_2\beta_2\gamma_1$ ), laminin 9 ( $\alpha_4\beta_2\gamma_1$ ), and laminin 11 ( $\alpha_5\beta_2\gamma_1$ ).<sup>(38)</sup> In vitro, recombinant fragments of laminin- $\beta$ 2 are selectively adhesive for motor neurons, inhibit neurite outgrowth promoted by other matrix molecules, and appear to act as “stop” signals for growing neurites.<sup>(58)</sup> Whether or not the laminin- $\beta$ 2 chain inhibits neurite growth in the context of a native laminin molecule depends on the identity of the other subunits; for example, laminin 11 displays such activity, laminin 4 does not.<sup>(38,59)</sup> Indeed, neurites grow robustly on surfaces coated with laminin 4 but tend not to cross from laminin 4 onto other growth-promoting surfaces, an effect that might serve to hold axon terminals at synaptic sites. In

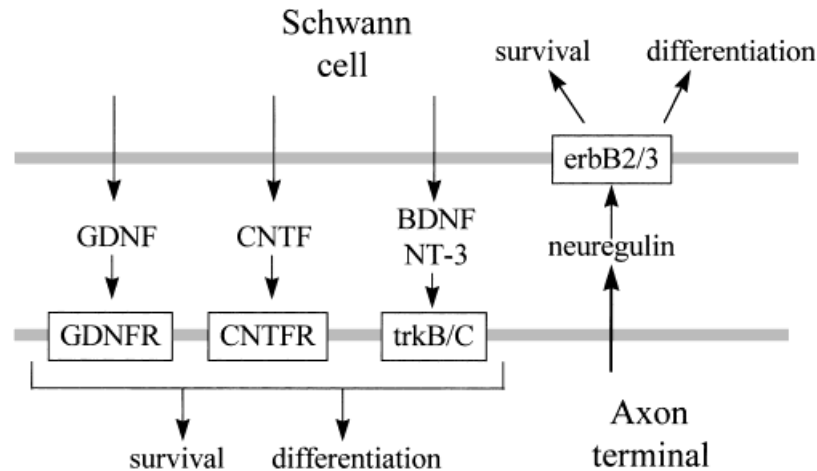


Figure 5. Signaling pathways between Schwann cells and axon terminals. GDNF, CNTF, BDNF, and NT-3 released from Schwann cells interact with receptors on axon terminals to regulate motoneuron survival and presynaptic differentiation. Release of neuregulin from axon terminals influences the survival and differentiation of Schwann cells.

laminin- $\beta$ 2 knockout mice, however, axons stop growing when they contact developing muscle fibers, just as in controls, indicating that laminins containing the  $\beta$ 2 subunit are not the only signals inhibiting further growth.<sup>(57)</sup> On the other hand, presynaptic differentiation is clearly abnormal in such mutant mice; complex terminal arbors fail to develop, and no active zones are formed. Thus, although laminin- $\beta$ 2 may not be required to stop the advance of motor axons as they contact myofibers in developing muscle, it is required for normal presynaptic differentiation.

### Agrin

A second candidate for the signal that stops the growth of motor axon terminals is, somewhat surprisingly, agrin. In neuron-CHO cell cocultures, neurites fail to grow on CHO cells expressing recombinant agrin on the surface,<sup>(60,61)</sup> and axon terminals that abut agrin-expressing CHO cells label with antibodies to synaptotagmin, suggesting an accumulation of synaptic vesicles.<sup>(60,62)</sup> These observations led to the suggestion that agrin is a differentiation-inducing stop signal for motor axon terminals. However, the results described above for recombinant fragments of laminin together with the observation that neurites in culture can be induced to differentiate when they come in contact with a variety of stimuli, such as polystyrene beads coated with polycations or with basic fibroblast growth factor,<sup>(63)</sup> suggest that results of such experiments with cultured neurons must be interpreted cautiously. The lack of presynaptic differentiation seen in agrin knockouts could also be interpreted as support for such a hypothesis. However, the fact that a similar phenotype is seen in MuSK knockouts suggests that the lack of presynaptic differentiation in agrin knockouts is not owing directly to

lack of agrin but to lack of a retrograde signal released in response to the activation of MuSK by agrin.

### Neurotrophins and the *trk* receptor family

BDNF, NT-3, and NT-4/5 support motoneuron survival during development and protect motoneurons from degeneration after nerve lesions in the adult.<sup>(64)</sup> Such effects are thought to be mediated by the release of the neurotrophin from the target tissue and activation of the corresponding member of the *trk* family of receptor tyrosine kinases on the axon terminal, leading ultimately to changes in gene expression in the cell soma. NT-3 and NT-4/5 are expressed in embryonic and adult muscle fibers, and *trkB* and *trkC* neurotrophin receptors are expressed by motoneurons.<sup>(64,65)</sup> Interaction of NT-3 and NT-4/5 with *trkB/C* receptors not only affects motoneuron survival, however, but also stimulates synapse formation in nerve-muscle cocultures,<sup>(66)</sup> potentiates transmission at synapses developing in culture by enhancing transmitter release,<sup>(67)</sup> and delays the elimination of polyneuronal innervation that normally occurs in neonatal muscles.<sup>(68)</sup> Thus, muscle-derived trophic factors can influence synapse formation in developing muscle.

### Signaling between axon terminals and Schwann cells

Although much less thoroughly studied than interactions between nerve and muscle, interactions between Schwann cells and axon terminals are important to the formation of the neuromuscular junction (Fig. 5). Some interactions mediate cell survival, a topic that is beyond the scope of this review. For example, if neonatal rat junctions are denervated, Schwann cells undergo rapid apoptotic death.<sup>(69)</sup> This can be

prevented by injection of GGF II, a secreted form of NRG. Likewise, mutant mice deficient in erbB3 receptors lack Schwann cells.<sup>(54)</sup> Since motor neurons are known to synthesize NRGs and Schwann cells are known to express erbB NRG receptors,<sup>(70)</sup> it seems reasonable to conclude that at developing neuromuscular junctions nerve-derived NRG sustains terminal Schwann cells. Conversely, Schwann cells appear to sustain motor neurons through the release of neurotrophic factors. Schwann cells synthesize CNTF, GDNF, BDNF, and NT-3<sup>(71,72)</sup> and axon terminals express trkB, trkC, GDNF, and CNTF receptors.<sup>(64,72,73)</sup> BDNF, GDNF, and CNTF promote the survival of motoneurons in vitro and after axotomy in vivo. Motor neuron survival is not affected in BDNF knockout mice,<sup>(74)</sup> but a null mutation in CNTF results in motor neuron atrophy and death.<sup>(75)</sup>

More pertinent to the subject of this review, the NRG-erbB, neurotrophin-*trk*, GDNF, and CNTF signaling pathways also influence synaptic differentiation. For example, BDNF, GDNF, and CNTF potentiate transmission at synapses developing in nerve-muscle cocultures,<sup>(73,76)</sup> and BDNF can delay the elimination of polyneuronal innervation in neonatal muscles.<sup>(68)</sup> If exogenous GGF II is applied to intact developing muscles, Schwann cells extend processes and migrate from the synapse and axon terminals retract from postsynaptic sites and grow in association with the migrating Schwann cells to the ends of the muscle.<sup>(77)</sup> Such results suggest that Schwann cell-axon terminal interactions play an active role in regulating synapse formation and maturation at developing neuromuscular junctions.

### Conclusions

Historically, the study of the structure and function of the vertebrate skeletal neuromuscular junction, with its seductive simplicity, has provided valuable insights and probes for investigating the apparently more complicated synapses that occur between neurons. Here, we have focused on proteins and signaling pathways that are involved in the formation of the neuromuscular junction. Many of the same proteins, or their homologues, are likely to mediate synaptic differentiation throughout the nervous system. In addition, three generalizations that emerge from the developmental studies reviewed here are also likely to be generally applicable. First, synaptic differentiation is regulated by a plethora of signaling molecules and receptors whose functions are often interdependent and overlapping. Second, the pathways for synaptic differentiation are hierarchically arranged. Accordingly, the interaction of a single signaling molecule with its receptor, such as the activation of MuSK by agrin, can trigger a cascade of events that involves a variety of structural proteins as well as proteins involved in additional intra- and intercellular signaling pathways. Third, many of the proteins required for synapse formation and function are present within the pre- and postsynaptic cells before synaptogenesis begins. Thus, at least the initial stages of synapse formation rely solely on

the recruitment and reorganization of preexisting components, rather than on a change in gene expression and synthesis of new proteins. This allows functional synapses to be formed rapidly and, as in the case of axon terminals, at a great distance from the cell soma. Finally, the results described here are of more than developmental interest. Many of the processes involved in synapse formation are likely to remain active at adult synapses, where they could mediate changes in synaptic efficacy that underlie neuronal plasticity.

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