INTRODUCTION

Neuromuscular synapses form following a series of complex interactions between motor neurons, muscle fibers, and Schwann cells that culminate in the formation of a highly specialized postsynaptic membrane and a highly differentiated nerve terminal (Son and Thompson, 1995; Burden, 1998; Sanes and Lichtman, 2001; Schaeffer et al., 2001). In adult muscle, motor axon terminals are situated in shallow depressions of the muscle cell membrane, which is invaginated further into deep and regular folds, termed postjunctional folds. Acetylcholine receptors (AChRs) and additional proteins are concentrated at the crests of these postjunctional folds, while other proteins, including sodium channels, are enriched in the troughs of the postjunctional folds. The genes encoding many of these muscle-derived synaptic proteins are transcribed preferentially in the myofiber nuclei that are situated near the synaptic site, resulting in an accumulation of the encoded mRNAs near synaptic nuclei. The nerve terminal is likewise spatially organized, and its substructural organization reflects that of the postsynaptic membrane. Synaptic vesicles are clustered adjacent to poorly characterized specializations of the postsynaptic membrane, termed active zones, which are the sites of synaptic vesicle fusion. Active zones are organized at regular intervals and are aligned precisely with the mouths of the postjunctional folds. This precise registration of active zones and postjunctional folds insures that acetylcholine encounters a high concentration of AChRs within microseconds after release, thereby facilitating synaptic transmission. This precise organization of molecules in presynaptic and postsynaptic membranes belies the concept that the neuromuscular synapse is a simple synapse. Rather, the substructure of presynaptic and postsynaptic membranes suggests that complex mechanisms, requiring spatially restricted signaling between presynaptic and postsynaptic cells, are required to assemble the synapse and to coordinate presynaptic and postsynaptic differentiation.

Myogenesis and the First Contacts between Motor Axons and Muscle

Multinucleated skeletal muscle fibers form during development (beginning at ~E11 in the mouse) by fusion of precursor myoblasts. Shortly after myotubes begin to form, motor axons grow into the developing muscle (at E12–E13 in the mouse) and form a main intramuscular nerve, which in mammals extends through the central region of the muscle, perpendicular to the long axis of the myotubes. Individual motor axons branch and terminate adjacent to the main intramuscular nerve, resulting in a narrow, distinct endplate zone, marked by presynaptic nerve terminals, clusters of AChRs, and elevated levels of AChR gene expression, in the middle of the muscle. This spatial patterning of skeletal muscle cells has
been thought to arise from the focal release of motor neuron-derived signals, including Agrin, that activate receptor tyrosine kinases in the myotube.

**Agrin**

Agrin is a 200-kDa protein that is synthesized by motor neurons, transported in motor axons, and released at synaptic sites, where it organizes postsynaptic differentiation (McMahan, 1990; Ruegg and Bixby, 1998; Sanes and Lichtman, 2001). Agrin is necessary for synapse formation, as mice lacking Agrin fail to form neuromuscular synapses (Gautam et al., 1996). Moreover, Agrin is sufficient to induce certain aspects of postsynaptic-like differentiation in cultured muscle cells and at ectopic sites in adult muscle (Wallace, 1989; Meier et al., 1997, 1998; Rimer et al., 1998). Agrin stimulates postsynaptic differentiation by activating MuSK, a receptor tyrosine kinase that is expressed selectively in skeletal muscle and concentrated at synaptic sites (Jennings et al., 1993; Valenzuela et al., 1995; Glass and Yancopoulos, 1997).

Agrin contains a laminin-binding domain, nine follistatin-like repeats, one laminin type III domain, three laminin G domains and four epidermal growth factor (EGF)-like signaling domains (Ruegg and Bixby, 1998) [Fig. 1(A)]. The four EGF-like domains and the three laminin G domains are contained in the carboxyl-terminal region, which is sufficient for activating MuSK and inducing the clustering of MuSK, AChRs, Rapsyn, and Acetylcholinesterase (AChE) in cultured myotubes. The laminin-binding site at the amino-terminus is thought to be responsible for the association of Agrin with the extracellular matrix.

The *agrin* gene is expressed in a variety of cell types. Alternative splicing generates multiple Agrin isoforms that differ in their AChR clustering efficiency. The isoform that is most effective in clustering synaptic proteins is expressed in neurons, including motor neurons, whereas other Agrin isoforms are expressed in additional cell types, including skeletal muscle cells. The active, neuronal-specific isoforms of Agrin contain 8, 11, or 19 amino acids at a splice site, referred to as the Z site in rat Agrin and the B site in chick Agrin (Ruegg and Bixby, 1998; Sanes and Lichtman, 2001).

**MuSK**

MuSK is a critical component of an Agrin receptor complex. MuSK is expressed in *Torpedo* electric organ and in skeletal muscle, where it is concentrated in the postsynaptic membrane. The extracellular region of mammalian MuSK contains four immunoglobulin-like domains and a cysteine-rich domain; in avians, fish, and amphibians, the extracellular region also contains a kringle domain (Jennings et al., 1993; Valenzuela et al., 1995; Fu et al., 1999; Ip et al., 2000) [Fig. 1(A)]. The intracellular region of MuSK contains a ~50 amino acid juxtamembrane domain, a kinase domain and a short, eight amino acid carboxy-terminal tail. Mice deficient in *MuSK* (DeChiara et al., 1996), like *agrin* mutant mice, lack normal neuromuscular junctions.

![Figure 1](image-url)
cular synapses. The similar phenotype of *agrin* and *MuSK* mutant mice, at least at birth, is consistent with the idea that MuSK is a component of an Agrin receptor complex. Both *agrin* and *MuSK* mutant mice are immobile, cannot breathe and die at birth. Muscle differentiation is normal in *agrin* and *MuSK* mutant mice, but muscle fibers in *MuSK* mutant mice lack all known features of postsynaptic differentiation at all stages of development. Muscle-derived proteins, including AChRs, AChE, ErbB3, and ErbB4, receptors for Neuregulin-1 (Nrg-1), which are concentrated at synapses in normal mice, are uniformly distributed in *MuSK* mutant myofibers. In addition, AChR genes, which are normally transcribed selectively in synaptic nuclei of normal muscle fibers, are transcribed at similar rates in synaptic and nonsynaptic nuclei of muscle fibers from *MuSK* mutant mice.

Synaptic differentiation is similarly defective in *agrin* and *MuSK* mutant mice, at least at birth. Earlier in development (E14), however, AChR clusters are evident and concentrated in the central region of muscles from *agrin* mutant mice (Lin et al., 2001), whereas AChR clusters are absent from *MuSK* mutant mice at all stages of development. These results indicate that Agrin is neither required to cluster AChRs *in vivo* nor to position these AChR clusters in the central region of the muscle. Therefore, Agrin appears to be necessary to maintain, rather than to induce AChR clusters *in vivo* (see below) (Lin et al., 2001; Yang et al., 2001). In contrast, in cultured myotubes, Agrin induces the formation of AChR clusters. These differing roles for Agrin may be a consequence of the neural-independent mechanisms that can pattern AChR expression in developing muscle *in vivo* (see below) and the disruption of these muscle-autonomous patterning mechanisms in cultured myotubes, which are derived from myoblasts that are dissociated from embryonic muscle.

Presynaptic differentiation is also aberrant in *agrin* and *MuSK* mutant mice, as motor axons fail to stop or differentiate, and instead wander throughout the muscle. Taken together with experiments showing that MuSK is activated by Agrin, these results indicate that Agrin stimulation of MuSK leads to the clustering of critical muscle-derived proteins, including MuSK, AChRs, and ErbBs, activation of synapse-specific gene expression, and the induction of a retrograde signal for presynaptic differentiation (DeChiara et al., 1996; Gautam et al., 1996). Although clustering of postsynaptic proteins is thought to be a direct consequence of MuSK signaling [Fig. 1(B)], defects in presynaptic differentiation and synapse-specific transcription could be attributed to a direct or indirect role for Agrin/MuSK in these aspects of synaptic differentiation. For example, although clustered MuSK could itself function as a “stop/differentiation” signal for presynaptic differentiation, MuSK signaling may regulate presynaptic differentiation by controlling the synthesis or organization of other proteins that serve as retrograde signals. Likewise, although synapse-specific transcription may be regulated directly by a transcriptional pathway stimulated by MuSK, activated MuSK may indirectly control synapse-specific transcription by clustering other ligands/receptors, notably Nrg-1 and ErbBs, that stimulate synaptic gene expression [Fig. 1(B), see below].

How does MuSK activation lead to postsynaptic differentiation? Agrin stimulates the rapid phosphorylation of MuSK, and the kinase activity of MuSK is essential for Agrin to stimulate clustering and tyrosine phosphorylation of AChRs. Well-characterized signaling pathways (e.g., MAP kinase, PI3-kinase, PLC-gamma), however, are neither activated by Agrin/MuSK signaling nor are required for Agrin to stimulate AChR clustering (Wallace, 1988; Herbst and Burden, 2000). Signaling downstream from MuSK depends upon phosphorylation of a tyrosine residue (Y553) in the juxtamembrane region of MuSK (Zhou et al., 1999; Herbst and Burden, 2000). Phosphorylation of this tyrosine is required to fully activate MuSK kinase activity and to recruit a downstream signaling component(s) required for all aspects of MuSK signaling, including tyrosine phosphorylation and clustering of AChRs, synapse-specific transcription and presynaptic differentiation (Herbst and Burden, 2000; Herbst et al., in press). At present, however, little is known about the protein(s) that is recruited to the juxtamembrane tyrosine in MuSK, which presumably recruits additional proteins that mediate the signaling pathways leading to presynaptic and postsynaptic differentiation. There is evidence, however, that Rac and Cdc 42, small GTP-binding proteins that regulate actin organization, are required for Agrin to stimulate AChR clustering (Weston et al., 2000). Moreover, at least one kinase acts downstream from MuSK and is recruited and/or activated by Agrin-activated MuSK, because staurosporine, a protein kinase inhibitor, inhibits Agrin-induced AChR clustering without blocking tyrosine phosphorylation of MuSK (Wallace, 1994; Ferns et al., 1996).

Agrin stimulates the rapid tyrosine phosphorylation of MuSK expressed in myotubes (Glass et al., 1996). Agrin, however, does not stimulate tyrosine phosphorylation of MuSK that is force-expressed in fibroblasts or myoblasts (Glass et al., 1996). These experiments suggest that Agrin does not bind directly to MuSK and that other components expressed selectively by skeletal muscle, termed MASC, are required...
for Agrin to activate MuSK. Despite substantial effort and encouraging reports (Glass and Yancopoulos, 1997), MASC has yet to be identified. It remains possible that a coligand or posttranslational modification of Agrin or MuSK, rather than a coreceptor, is required for Agrin to stimulate MuSK.

Activated MuSK clusters MuSK (Jones et al., 1999; Moore et al., 2001), and this positive-feedback mechanism may be important to achieve an adequate level of MuSK expression at the synapse, sufficient to cluster more than 10 million AChR molecules per synapse. Nonetheless, an unrestrained positive-feedback mechanism could lead to MuSK activation and postsynaptic differentiation beyond the synaptic site. Thus, it may be important to impose restraints on MuSK activation at synaptic sites. There may be instances when an absence of such restraints, leading to Agrin-independent MuSK activity, however, is exploited during development. For example, clustering of AChRs in the central region of muscle early in development, which is independent of Agrin but dependent upon MuSK (Lin et al., 2001; Yang et al., 2001), may be initiated by adventitious MuSK activation and facilitated by the absence of an inhibitory protein that otherwise restrains MuSK activity.

Rapsyn, a 43-kDa peripheral membrane protein that is associated with AChRs at 1:1 stoichiometry, is a required intermediate on the pathway that couples MuSK activation to AChR clustering (Gautam et al., 1995) [Fig. 1(A)]. The tetratricopeptide repeats in Rapsyn mediate Rapsyn self-association, likely to be important for clustering Rapsyn at synapses, the coiled-coil domain in Rapsyn directs association between Rapsyn and the main intracellular loop of AChR subunits, and the RING-H2 domain in Rapsyn interacts with beta-dystroglycan (Ramarao and Cohen, 1998; Bartoli et al., 2001; Ramaaro et al., 2001). The mechanisms by which MuSK activation leads to clustering of Rapsyn are poorly understood, but clustering of Rapsyn is essential for clustering AChRs. Although Rapsyn is central to building a synapse, certain aspects of synaptic differentiation do not require Rapsyn. In the absence of Rapsyn, MuSK remains concentrated at synaptic sites and AChR genes are selectively transcribed by synaptic nuclei, leading to an enrichment of AChR protein in the central region of the muscle (Gautam et al., 1995; Apel et al., 1997). The mechanisms that regulate the clustering of MuSK, independent of Rapsyn, at synaptic sites are not understood. The carboxy-terminus of MuSK can bind PDZ domain-containing proteins (Strochlic et al., 2001), but this binding site is dispensable for MuSK function in cultured muscle cells and in vivo (Zhou et al., 1999; Herbst et al., in press). It is possible that engagement by Agrin captures MuSK or that Agrin activation of MuSK stimulates MuSK clustering at synapses independent of Rapsyn and PDZ domain-containing proteins.

Differing results have been reported for the role of the ectodomain of MuSK in the formation of AChR clusters. Like Agrin-induced AChR clusters, the formation of spontaneous AChR clusters in cultured myotubes depends upon Rapsyn and MuSK (Gautam et al., 1995; Zhou et al., 1999; Herbst and Burden, 2000). Because the formation of spontaneous AChR clusters is not dependent upon the ectodomain of MuSK (Zhou et al., 1999), sequences in the MuSK cytoplasmic domain are sufficient to mediate the Rapsyn-dependent formation of spontaneous AChR clusters. Consistent with this idea, MuSK mutants, lacking the ectodomain can stimulate the formation of AChR clusters at ectopic sites in adult muscle (Sander et al., 2001). These findings would suggest that the cytoplasmic domain of MuSK is sufficient to activate a pathway leading to AChR clustering. In contrast to this idea, NT3-stimulated tyrosine phosphorylation of a TrkC/MuSK chimera, expressed in cultured myotubes fails to stimulate AChR clustering (Glass et al., 1997). Moreover, Rapsyn-induced clustering of MuSK in QT6 cells requires the MuSK ectodomain, indicating that the ectodomain of MuSK interacts with Rapsyn indirectly, via a putative transmembrane protein termed RALT, to mediate Rapsyn/MuSK co-clustering in QT6 cells (Apel et al., 1997).

AChR Tyrosine Phosphorylation

Agrin stimulates tyrosine phosphorylation of the AChR β and δ subunits (Wallace et al., 1991; Qu and Huganir, 1994; Mittaud et al., 2001). The function of AChR tyrosine phosphorylation is not understood, but it is insufficient to cluster AChRs, as AChR tyrosine phosphorylation but not AChR clustering is stimulated in muscle cells that are transfected with a TrkC/MuSK chimera and treated with NT3 (Glass et al., 1997). In myotubes expressing both wild-type and mutant AChR pentamers, containing an epitope-tagged, mutant β subunit (Y390F), mutant pentamers are clustered by Agrin, although less (twofold) efficiently than wild-type pentamers (Meyer and Wallace, 1998; Borges and Ferns, 2001). Although these results indicate that tyrosine phosphorylation of the AChR β subunit contributes to AChR clustering induced by Agrin, it remains possible that AChR tyrosine phosphorylation is essential for AChR clustering. First, because the transfected myotubes express both mutant and wild-type AChR pentamers, wild-
Tyrosine phosphorylation of AChRs could regulate AChR clustering by initiating a link between AChRs and the cytoskeleton (Wallace, 1992). Consistent with this idea, AChR pentamers containing a mutant β subunit (Y390F) are extracted more readily by non-ionic detergent from cultured myotubes treated with Agrin (Borges and Ferns, 2001). In addition, a phosphotyrosine (Y393)-containing sequence in the β subunit serves as a docking site for Grb2 (Colledge and Froehner, 1997), raising the possibility that Grb2 serves as an adaptor to link tyrosine phosphorylated δ subunits to additional proteins, possibly a cytoskeletal complex.

Staurosporine blocks Agrin-stimulated tyrosine phosphorylation of AChRs without inhibiting tyrosine phosphorylation of MuSK, indicating that tyrosine kinases other than MuSK catalyze tyrosine phosphorylation of AChRs (Ferns et al., 1996; Fuhrer et al., 1997). Src-family kinases were considered good candidates to catalyze AChR tyrosine phosphorylation (Swope and Huganir, 1993; Ferns et al., 1996; Fuhrer and Hall, 1996; Fuhrer et al., 1999), but Agrin stimulates tyrosine phosphorylation and clustering of AChRs in mutant muscle cells lacking either Src and Fyn or Src and Yes and in muscle cells treated with inhibitors of Src-family kinases (Smith et al., 2001; but see Mohamed et al., 2001). Thus, kinases other than Src-family kinases appear to catalyze AChR tyrosine phosphorylation and clustering.

Src-like kinases, however, regulate the stability of AChR clusters (Smith et al., 2001). Following withdrawal of Agrin, AChR clusters disassemble more rapidly in myotubes lacking Src and Fyn than in wild-type myotubes. Nonetheless, the role of Src and Fyn in stabilizing AChR clusters does not require their kinase activities, because inhibitors of Src-family kinase activity do not decrease the stability of AChR clusters. These results suggest that the adapter activities, rather than the kinase activities of Src/Fyn are essential to stabilize and anchor AChRs and that Src/Fyn recruit additional kinases that modulate interactions between the postsynaptic membrane and the cytoskeleton.

These ideas are supported by two observations. First, upon removal of Agrin, herbimycin and staurosporine, unlike inhibitors of Src-family kinases, disperse pre-formed AChR clusters (Ferns et al., 1996). As MuSK phosphorylation is not inhibited by staurosporine (Fuhrer et al., 1997), these findings suggest that the activity of a kinase, other than MuSK or a Src-family member, is necessary to maintain AChR clusters. Second, the stability of Agrin-induced AChR clusters is also reduced in myotubes lacking α-Dystrobrevin (Grady et al., 2000). Dystrobrevin is a substrate for tyrosine kinases and is strongly tyrosine phosphorylated in Torpedo electric organ (Wagner et al., 1993). Although Dystrobrevin is not tyrosine-phosphorylated by Agrin stimulation nor required for early steps in Agrin-induced AChR clustering (Nawrotzki et al., 1998), its phosphorylation may play a role in stabilization of AChR clusters. Taken together, these studies suggest that tyrosine phosphorylation, catalyzed by kinases other than Src family members, may have a role in anchoring AChRs to cytoskeletal components, including Dystrobrevin and its associated Utrophin glycoprotein complex.

**Certain Genes Are Expressed Selectively in Synaptic Nuclei of Myofibers**

The mRNAs encoding the five AChR subunits are concentrated at synaptic sites (Burden, 1998; Sanes and Lichtman, 2001; Schaeffer et al., 2001). Studies with transgenic mice carrying gene fusions between regulatory regions of AChR subunit genes and reporter genes have demonstrated that AChR genes are transcribed selectively in myofiber nuclei near the synaptic site. Thus, localized transcription of AChR genes in synaptic nuclei is responsible, at least in part, for the accumulation of AChR mRNA at synaptic sites. This pathway is important for ensuring that AChRs are expressed at the required density in the postsynaptic membrane, as defects in synapse-specific gene expression of the AChr ε subunit gene is the cause of a congenital myaesthenia (Nichols et al., 1999; Ohno et al., 1999).

Like AChR subunit genes, the utrophin gene is transcribed selectively in synaptic nuclei, resulting in accumulation of utrophin mRNA and protein at synaptic sites (Gramolini et al., 1999). mRNAs encoding Rapsyn, N-CAM, MuSK, sodium channels, and the catalytic subunit of AChE are also concentrated in the synaptic region of skeletal myofibers, consistent with the idea that these genes are likewise transcribed preferentially in synaptic nuclei. Thus, synapse-specific transcription is likely to be a common and important mechanism for concentrating gene products at the neuromuscular synapse.
Neuregulin-1 Is a Candidate for the Signal That Activates Gene Expression in Synaptic Nuclei

A signal for synapse-specific transcription is contained in the synaptic basal lamina. Nonetheless, because soluble forms of Agrin do not increase AChR expression in cultured muscle, Agrin has not been considered a favored candidate for the transcriptional signal in the synaptic basal lamina. Instead, the favored candidate is Nrg-1 (Falls et al., 1993; Carraway and Burden, 1995; Rosen et al., 1996; Fischbach and Rosen, 1997; Schaeffer et al., 2001) [Fig. 2(A)]. The nrg-1 gene encodes more than a dozen alternatively spliced products that have multiple activities. Although originally purified as a ligand that stimulates tyrosine phosphorylation of the neu oncogene, Nrg-1 was independently purified as an activity in the CNS, termed AChR inducing activity (ARIA), that induces AChR synthesis in cultured myotubes, and as an activity in the pituitary, termed glial growth factor (GGF), that stimulates proliferation of Schwann cells.

Nrg-1 is concentrated at neuromuscular synapses, and can activate AChR gene expression in muscle cells grown in cell culture (Chu et al., 1995; Jo et al., 1995) [Fig. 2(B)]. Nrg-1 contains a single EGF-like domain, which is necessary and sufficient for cell signaling. Motor neurons synthesize Nrg-1, and Nrg-1 protein is detectable in motor axons, indicating that some of the Nrg-1 protein at synaptic sites is synthesized by motor neurons (Sandrock et al., 1995). Skeletal myofibers, however, also synthesize Nrg-1 (Moscoso et al., 1995; Rimer et al., 1998), and some of the Nrg-1 at synaptic sites is synthesized by myofibers (Rimer et al., 1998). These findings raise the possibility that Nrg-1 could act as an autocrine and/or paracrine signal at neuromuscular synapses (Fig. 3).

ErbB3 and ErbB4, members of the EGF receptor family, are receptors for Nrg-1, and each receptor is concentrated in the postsynaptic membrane at neuromuscular synapses (Altiok et al., 1995; Moscoso et al., 1995; Zhu et al., 1995) [Fig. 2(B)]. The co-clustering of Nrg-1, ErbB3, and ErbB4 at synapses supports the idea that Nrg-1 is a signal that regulates synaptic differentiation. Nonetheless, because mice mutant for nrg-1, erbB2, or erbB4 die, owing to a failure of heart development, at E10.5, several days prior to neuromuscular synapse formation (Gassmann et al., 1995; Lee et al., 1995; Meyer and Birchmeier, 1995), these mutant mice are not suitable for analyzing whether Nrg-1–mediated signaling is required for synapse-specific transcription.

Adult mice that are heterozygous for the Ig-isoform of Nrg-1 express fewer AChRs at neuromuscular synapses, supporting the idea that Nrg-1, supplied by motor neurons and/or muscle, has a role in regulating AChR expression at adult synapses (Sandrock et al., 1997). Neuronal Nrg-1, however, is not required for synapse-specific transcription at developing synapses.
as the pattern of $AChR$ transcription is normal in newborn mice lacking Nrg-1 specifically in motor and sensory neurons (Yang et al., 2001). These experiments leave open the possibility that muscle-derived Nrg-1 may be required for synapse-specific transcription or that neuronal- and muscle-derived Nrg-1 have redundant roles in regulating synapse-specific transcription.

Neural Agrin, or constitutively active MuSK, can cluster muscle-derived Nrg-1 and ErbBs, and stimulate transcription of $AChR$ genes (Meier et al., 1997, 1998; Rimer et al., 1998) (Fig. 3). Ectopic induction of $AChR$ transcription is inhibited by a dominant negative form of ErbB2, demonstrating that Agrin/MuSK stimulate $AChR$ transcription in an ErbB-dependent manner, at least at ectopic sites (Meier et al., 1998; Moore et al., 2001). Moreover, a dominant negative form of GABP, a transcription factor implicated in Nrg-1-mediated induction of $AChR$ transcription in cultured myotubes, likewise inhibits induction of $AChR$ transcription at ectopic Agrin sites (Meier et al., 1998; Moore et al., 2001). Taken together, these experiments suggest that neural Agrin regulates synapse-specific transcription by defining the limits of Nrg-1 and ErbB expression, thus restricting an autocrine Nrg-1 signaling pathway to synaptic sites in muscle [Figs. 1(B) and 3]. Moreover, because Nrg-1 can stimulate MuSK expression (Ip et al., 2000), Agrin/MuSK and Nrg-1/ErbB signaling pathways mutually reinforce one another (Fig. 4), thereby sharpening the boundaries of transcription and protein localization at synaptic sites.

Nrg-1 can activate $AChR$ gene expression in muscle cells grown in cell culture, and the Nrg-1 response element in the $AChR$ δ subunit gene is a binding site for Ets-domain transcription factors (Burden, 1998; Schaeffer et al., 2001). In the $AChR$ ε subunit gene, both an Ets-site as well as a binding site for Sp1 are thought to mediate Nrg-1 responsiveness (Alroy et al., 1999). Mutations in the Ets-binding site in the human $AChR$ ε subunit gene result in reduced $AChR$ expression and a myopathy, termed congenital myasthenic syndrome (Nichols et al., 1999; Ohno et al., 1999); these findings are currently the best evidence that synapse-specific transcription has a critical role at neuromuscular synapses. Nrg-1 stimulation of ErbBs leads to activation of MAP kinase signaling pathways, and both Erk and Jnk kinases are required for Nrg-1 to induce $AChR$ genes in cultured myotubes (Si et al., 1998; Schaeffer et al., 2001). The mechanisms that couple MAP kinase signaling to activation of GABP are poorly understood.

### Muscle-Autonomous Patterning of AChR Expression

Analysis of $agrin$ mutant mice supported the idea that neural signals are required to pattern $AChR$ gene expression independent of innervation and for refinement of this patterning by innervation. In the absence of innervation, and possibly prior to innervation, weak MuSK activation in the central region of the muscle leads to clustering (black arrows) of $AChRs$, muscle-derived Nrg-1 and ErbBs in this central region (light blue zone). Muscle-derived Nrg-1 activates ErbBs (blue arrow), leading to an increase in $AChR$ and MuSK transcription (red arrow). Motor innervation modifies the prepattern to the more refined pattern of $AChR$ transcription and $AChR$ clustering characteristic of mature synapses. This conversion appears to depend upon two distinguishable nerve-dependent programs: one program utilizes motor neuron-derived Agrin to maintain $AChR$ expression at nascent synaptic sites and a second program, possibly triggered by electrical activity, extinguishes $AChR$ expression throughout the muscle. Together, the two programs ensure the stable expression of $AChR$ clusters selectively at nascent synapses (blue zone).
expression and AChR clusters in skeletal muscle cells (Gautam et al., 1996). Small AChR clusters are present in muscle from agrin mutant newborn mice, but they were found to be scattered throughout the muscle (Gautam et al., 1996). These findings indicated that Agrin is required to restrict AChR gene expression and AChR clusters to the central region of the muscle, consistent with the notion that neuronal signals are required to pattern muscle.

Studies of mice lacking Topoisomerase IIβ (Top IIβ), however, led to a reconsideration of the possibility that muscle may be patterned independent of motor innervation (Yang et al., 2000). Although motor axons in top 2β mutant embryos fail to grow into or branch within diaphragm and limb muscles, AChRs are clustered in the central region of muscle, in a zone that is only twofold wider than in normal mice (Yang et al., 2000). These findings indicated that the pattern of AChR clusters in skeletal muscle might be determined, at least in part, by mechanisms that are independent of motor innervation. Subsequent analysis of additional mouse mutants that affect motor neuron generation, motor axon projections, and motor neuron-derived signals confirmed that AChR gene transcription and AChR clustering are patterned in developing skeletal muscle in the absence of motor neurons and motor axons (Lin et al., 2001; Yang et al., 2001). Moreover, these studies indicated that neural signals have a role in maintaining and refining, rather than initiating this pattern of AChR expression (Yang et al., 2000, 2001; Lin et al., 2001).

These findings suggest that an initial, spatially restricted pattern of AChR expression is generated in muscle-independent of neurally derived Agrin (Fig. 5). Arrival of the nerve, and its attendant signals, modifies the AChR prepattern into the more refined pattern of AChR transcription and AChR clustering characteristic of mature synapses. This alteration appears to depend upon two distinguishable nerve-dependent programs: one program utilizes neurally derived Agrin to maintain AChR expression at nascent synaptic sites and a second program, possibly triggered by electrical activity (Goldman et al., 1988), extinguishes AChR expression throughout the muscle. Together, the two programs ensure the stable expression of AChR clusters selectively at nascent synapses (Fig. 5).

Prior studies of developing mammalian muscle provided hints of such nerve-independent, muscle-patterning mechanisms (Braithwaite and Harris, 1979; Harris, 1981). In the earlier studies, however, it remained unclear whether developing muscle was indeed deprived of innervation or whether some motor axons had established transient contacts with muscle prior to their ablation. The lack of a well-defined synaptic zone in chick muscles may explain the discrepant reports on AChR expression in developing chick muscles deprived of innervation (Sohal, 1988; Dahm and Landmesser, 1991).

Muscle patterning does not appear to be required for synapse formation, as motor neurons can form synapses with muscle cells in cell culture, which do not display an AChR prepattern (Anderson and Cohen, 1977; Frank and Fischbach, 1979). Likewise, regenerating motor axons can form synapses at non-synaptic regions of denervated adult muscle (Frank et al., 1974). Nevertheless, muscle patterning may provide a preferred region for motor innervation during development in vivo. Physiologic reasons may dictate why the central region of a muscle is commonly the preferred site for innervation, as the contraction mechanism is optimally synchronized by initiating an action potential from a synapse near the middle of a muscle fiber.

Although the muscle AChR prepattern does not require Agrin, this prepattern is not observed in mice lacking MuSK (Lin et al., 2001; Yang et al., 2001). These results indicate that MuSK can be activated independent of Agrin, and suggest that the mechanisms that control MuSK activation have an important role in establishing the AChR prepattern. Because activated MuSK recruits and clusters MuSK (Jones et al., 1999), an early and modest bias in MuSK activity in the central region of the muscle may be converted, via this positive feedback loop, into a significant increase in MuSK activity in the central region of the muscle, leading to patterned expression of additional muscle genes. Although such mechanisms could explain how MuSK regulates muscle patterning, this model does not address how MuSK might initially be expressed preferentially in the prospective synaptic region of developing muscle.

Developing myotubes grow, in large part, by the fusion of myoblasts symmetrically to either side of the neuromuscular synapse. Because a large number of genes expressed in skeletal muscle, including MuSK, are activated concomitant with fusion, gene activation will proceed temporally from the central to the peripheral regions of the muscle. Because activated MuSK clusters and recruits MuSK, an early bias in MuSK expression would function to stabilize and enhance this disposition. Thus, a low level of ligand-independent MuSK activation, initiated when MuSK is first expressed, may be sufficient to establish a muscle pre-pattern in the absence of innervation.
REFERENCES


Gramolini AO, Angus LM, Schaeffer L, Burton EA, Tinsley

Neuromuscular Synapse 509


Sandrock AW, Jr., Goodeal AD, Yin QW, Chang D,


