ELSEVIER

Developmental Brain Research 114 (1999) 171–178

DEVELOPMENTAL BRAIN RESEARCH

Research report

# Distinct phenotypes of mutant mice lacking agrin, MuSK, or rapsyn

Medha Gautam [a,*], Thomas M. DeChiara [c], David J. Glass [c], George D. Yancopoulos [c], Joshua R. Sanes [b]

[a] *Department of Molecular Biology and Pharmacology, Washington University School of Medicine, 660 S. Euclid Avenue, St. Louis, MO 63110, USA*
[b] *Department of Anatomy and Neurobiology, Washington University School of Medicine, 660 S. Euclid Avenue, St. Louis, MO 63110, USA*
[c] *Regeneron Pharmaceuticals, 777 Old Saw Mill River Road, Tarrytown, NY 10591, USA*

Accepted 12 January 1999

**Abstract**

Differentiation of the postsynaptic membrane at the neuromuscular junction requires agrin, a nerve-derived signal; MuSK, a critical component of the agrin receptor in muscle; and rapsyn, a protein that interacts with acetylcholine receptors (AChRs). We showed previously that nerve-induced AChR aggregation is dramatically impaired in knockout mice lacking agrin, MuSK, or rapsyn. However, the phenotypes of these mutants differed in several respects, suggesting that the pathway from agrin to MuSK to rapsyn is complex. Here, we compared the effects of these mutations on two aspects of synaptic differentiation: AChR clustering and transcriptional specialization of synapse-associated myonuclei. First, we show that a plant lectin, VVA-$B_4$, previously shown to act downstream of agrin, can induce AChR clusters on MuSK-deficient but not rapsyn-deficient myotubes in culture. Thus, although both MuSK and rapsyn are required for AChR clustering in vivo, only rapsyn is essential for cluster formation per se. Second, we show that neuregulin, a nerve-derived inducer of AChR gene expression, activates AChR gene expression in cultured agrin- and MuSK-deficient myotubes, even though synapse-specific transcriptional specialization is disrupted in agrin and MuSK mutants in vivo. We propose that agrin works through MuSK to determine a synaptogenic region within which synaptic differentiation occurs. 

*Keywords:* Neuromuscular synapse; Agrin; Rapsyn; MuSK; Acetylcholine receptor

## 1. Introduction

During synapse formation, pre- and postsynaptic cells provide each other with signals that coordinate the differentiation of the synaptic partners. To date, these signals, and their transduction mechanisms, have been best characterized at the vertebrate neuromuscular junction. In particular, there is now strong evidence that the motor axon uses three separate signals to trigger distinct intramuscular processes that combine to result in the selective accumulation of nicotinic acetylcholine receptors (AChRs) in the postsynaptic membrane. First, initially diffusely distributed AChRs are redistributed following nerve–muscle contact, becoming concentrated directly beneath the nerve terminal. Second, the myonuclei directly beneath the postsynaptic membrane are activated to transcribe AChR subunit genes at higher levels than the nonsynaptic nuclei in the same cytoplasm, leading to local subsynaptic synthesis of AChRs.Third, nerve-evoked electrical activity gradually represses synthesis of AChRs by nonsynaptic nuclei as the muscle matures, thereby sharpening the transcriptional distinction between junctional and extrajunctional regions (reviewed in Refs. [8,17,32]).

The nerve controls AChR clustering—the first of the processes listed above—through a pathway in which the heparan sulfate proteoglycan *agrin* is a critical nerve-derived signal, the receptor tyrosine kinase *MuSK* is a key component of the postsynaptic agrin receptor, and the cytoplasmic protein *rapsyn* is a cross-linker of AChRs. Three lines of evidence support this conclusion. The first is that agrin, MuSK and rapsyn are all concentrated at the neuromuscular junction. Agrin is synthesized by motoneurons, transported down motor axons, and secreted into the synaptic cleft, where it stably resides in the basal lamina (reviewed in Ref. [4]). MuSK is concentrated at the neuromuscular junction [34] from the earliest stages of synaptogenesis [2]. Rapsyn is a 43-kDa membrane-associated protein that is present at a 1:1 stoichiometry with AChRs, and is, like MuSK, precisely co-distributed with them from the

* Corresponding author. Department of Pharmacological and Physiological Science, St. Louis University School of Medicine, 1402 S. Grand Blvd., St. Louis, MO 63104, USA. Fax: +1-314-577-8233; E-mail: mgautam@slu.edu

0165-3806/99/$ - see front matter 
PII: S0165-3806(99)00013-9

earliest stages of synaptogenesis [21,27]. Second, agrin, MuSK, and rapsyn all exhibit activities in cultured cells consistent with their playing roles in clustering. Thus, application of agrin to cultured myotubes induces aggregation of AChRs into high-density clusters in which they are colocalized with numerous cytoskeletal and extracellular matrix components that are selectively associated with the postsynaptic apparatus in vivo [4]. MuSK is activated by agrin (although it cannot bind agrin in the absence of additional, still-unidentified components) and introduction of dominant-negative MuSK mutants into cultured myotubes inhibits the activity of agrin [15,16]. Removal of rapsyn from muscle membranes increases the mobility of AChRs [3,5] and forced expression of rapsyn in non-muscle cells induces clustering of coexpressed AChRs [11,28]. Finally, and perhaps most compelling, synaptic differentiation in general and AChR clustering is profoundly disrupted in mutant mice lacking agrin, MuSK, or rapsyn [7,12,13].

Although initial analyses of the knockout mice supported the notion that an agrin–MuSK–rapsyn pathway controls AChR clustering, several results from these studies pointed to unexpected complexities. One was that the degree of postsynaptic differentiation differed among the mutants, being least in $MuSK^{-/-}$ animals and substantial but distinguishable in the $agrin^{-/-}$ and $rapsyn^{-/-}$ mice. A second was that the transcriptional specialization in synaptic nuclei was minimal in $agrin^{-/-}$ and $MuSK^{-/-}$ mice, despite previous studies suggesting that separate signals control AChR expression and AChR clustering. In the studies reported here, we have directly compared the mutant phenotypes both in vivo and in vitro, with the aim of addressing these distinctions and thereby gaining a better understanding of the molecular mechanisms that control synaptic differentiation.

## 2. Materials and methods

### 2.1. Animals

Mice bearing targeted mutations in the agrin, rapsyn, and MuSK genes have been described previously [7,12,13], as have transgenic mice in which regulatory sequences from the AChR gamma subunit gene drive expression of a nuclear localized *Escherichia coli* (*E. coli*) β-galactosidase gene [AChRγ-nslacZ; Ref. [12]]. Knockout mice were maintained on a 129SV/C57BL6 hybrid background and transgenic mice were maintained on a CBA/B6 background. All lines were maintained as heterozygotes, and crossed to produce homozygous mutants. In general, homozygous mutants were identifiable by their neuromuscular phenotype at the time of sacrifice, but the genotype was always confirmed by PCR.

### 2.2. Histological analysis

Diaphragm muscles were dissected from mutants and control littermates, and fixed for 1 h in 2% paraformaldehyde in phosphate-buffered saline (PBS). Muscles were then washed with 0.1 M glycine in PBS, permeabilized with 0.5% Triton X-100 in PBS with 4% BSA, and incubated overnight with rhodamine-α-bungarotoxin (rBTX; Molecular Probes, Eugene, OR) and mounted in 90% glycerol/10% PBS, containing para-phenylenediamine to retard fading.

### 2.3. Cultures

Mononucleated cells were dissociated from hindlimbs of E18 embryos, plated on gelatin-coated glass coverslips and cultured in Dulbecco's modified Eagle Medium (DMEM) containing 10% horse serum and 5% newborn calf serum. After 2–3 days in culture, cells were switched to DMEM containing 2% horse serum, to promote fusion of myoblasts and formation of myotubes. After 1 day in fusion medium, recombinant ARIA ($rHRG\beta1_{177-244}$, Holmes et al., 1992, a generous gift of Dr. Mark Sliwkowski, Genentech) was added to some of the wells (final concentration = 3 nM). Some 3–4 days later, these cultures were fixed in 2% paraformaldehyde and stained histochemically for β-galactosidase as described in Ref. [12]. Other cultures were incubated for 2–3 days in fusion medium, then treated overnight with Vicia villosa agglutinin B4 ($VVA\text{-}B_4$; 50 μg/ml; Sigma) and/or conditioned medium containing a soluble, recombinant C-terminal fragment of *z*-agrin ($x = 12$, $y = 4$, $z = 8$ form, described in Ref. [9]). The next day, these cultures were incubated with rBTX, rinsed with PBS, fixed with 2% paraformaldehyde, and mounted for fluorescence microscopy.

## 3. Results

### 3.1. Alternative pathways for AChR clustering exhibit different requirements for agrin, MuSK and rapsyn

The distribution of AChRs in neonatal muscles is best assayed by staining whole mounts with the specific, high-affinity ligand, rBTX. This method reveals that each myotube in control muscles bears a single AChR cluster, and that most of the clusters are concentrated in a central 'endplate band' (Fig. 1A) which, as discussed below, corresponds to the position of the intramuscular nerve. We found previously that AChR distribution was perturbed in different ways in the $agrin^{-/-}$, $MuSK^{-/-}$ and $rapsyn^{-/-}$ mutants. In $agrin^{-/-}$ muscles, clusters were decreased in number and intensity relative to controls, some myotubes bore many clusters, many bore none, and a substantial fraction of the clusters were outside of the central endplate band (Fig. 1B). In MuSK mutants, AChR density was uniformly low along the entire myotube (Fig. 1D). In

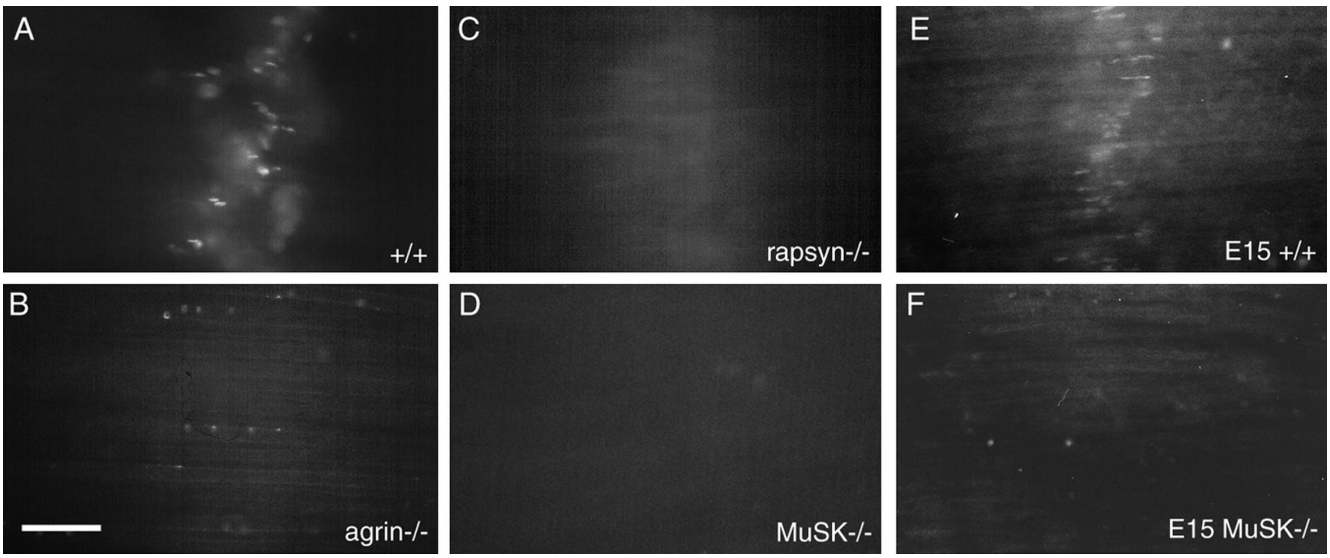


Fig. 1. Rapsyn and MuSK are essential for acetylcholine receptor cluster formation in vivo. Diaphragm muscles from E18 (A–D) or E15 (E, F) mutants and controls were fixed and stained with rhodamine-bungarotoxin and viewed as whole mounts. (A) In wild type animals, receptor clusters are present roughly in the middle of individual myofibers, forming an 'endplate band' in the central region of the muscle. In mutant animals, formation and distribution of receptor clusters is severely disrupted (B–D, F). In rapsyn$^{-/-}$ (C) and MuSK$^{-/-}$ (D, F) mutants, receptor clusters are not detectable anywhere along the fibers. In agrin$^{-/-}$ mutants (B) many regions of the muscle lacked receptor clusters; some regions, however, had small and weakly-staining clusters that were randomly distributed along the myofibers. Bar in B = 100 μm.

rapsyn mutants, no AChR clusters were present, but the density of AChRs was highest at the centers of myotubes, in the endplate band, and decreased gradually towards the tendons (Fig. 1C).

### 3.1.1. In vivo

We first needed to confirm that the differences in AChR distribution among genotypes did not reflect the fact that AChR distribution had been assessed with slightly different techniques. Accordingly, whole diaphragms from E18 agrin$^{-/-}$, MuSK$^{-/-}$, and rapsyn$^{-/-}$, and control (heterozygote and wild-type) embryos were stained in parallel with rBTX. The preparations described above (Fig. 1A–D) were generated in this way, and the patterns revealed were indistinguishable from those described previously [7,12,13]. These results confirm the existence of *z*-agrin-independent but MuSK- and rapsyn-dependent clustering stimuli. In addition, the different distributions of AChRs in MuSK$^{-/-}$ and rapsyn$^{-/-}$ muscles indicates that MuSK acts through both rapsyn-dependent and rapsyn-independent pathways, an issue discussed further below.

One possible explanation for the distinct AChR distributions among mutants is that agrin, MuSK, and rapsyn act during different periods in development. We showed previously that AChR clusters are present in agrin$^{-/-}$ muscles from the earliest stages of synaptogenesis. Moreover, AChR clusters can be induced in MuSK$^{-/-}$ myotubes in culture by exogenous stimuli other than agrin (see below, and Ref. [33]). We therefore considered the possibility that clusters formed early in development by an agrin-independent pathway might require MuSK for their stabilization rather than for their initial formation. To test this hypothesis, we examined the spatial relationship between motor axons and AChR aggregates by staining diaphragms from E15 MuSK$^{-/-}$ embryos and controls. Intramuscular nerves were present by this time in mutants (data not shown), but no AChR clusters were detectable (Fig. 1F). In muscle from controls, receptor aggregates were concentrated in the central region (Fig. 1E). Thus, MuSK is required for both agrin-dependent and agrin-independent AChR clustering throughout development.

### 3.1.2. In vitro

To test whether all AChR clustering is both MuSK- and rapsyn-dependent, we generated myotubes from homozygous mutant embryos, and challenged them with VVA-$B_4$. VVA-$B_4$ recognizes *N*-acetylgalactosamine- (GalNAc-) residues and selectively stains the neuromuscular junction. We showed previously that VVA-$B_4$ induces AChR clusters on cultured myotubes, and presented evidence that it acts on a step in agrin's signaling pathway [22]. Here, we found that VVA-$B_4$ induced AChR clustering on MuSK$^{-/-}$ and control myotubes, but had no detectable effect on rapsyn$^{-/-}$ myotubes (Fig. 2A–F). We also added a mixture of recombinant agrin and VVA-$B_4$ to sister cultures, in light of the finding that these two agents act synergistically on wild-type cells [22]. As expected, agrin induced clustering on its own in wild type myotubes, and

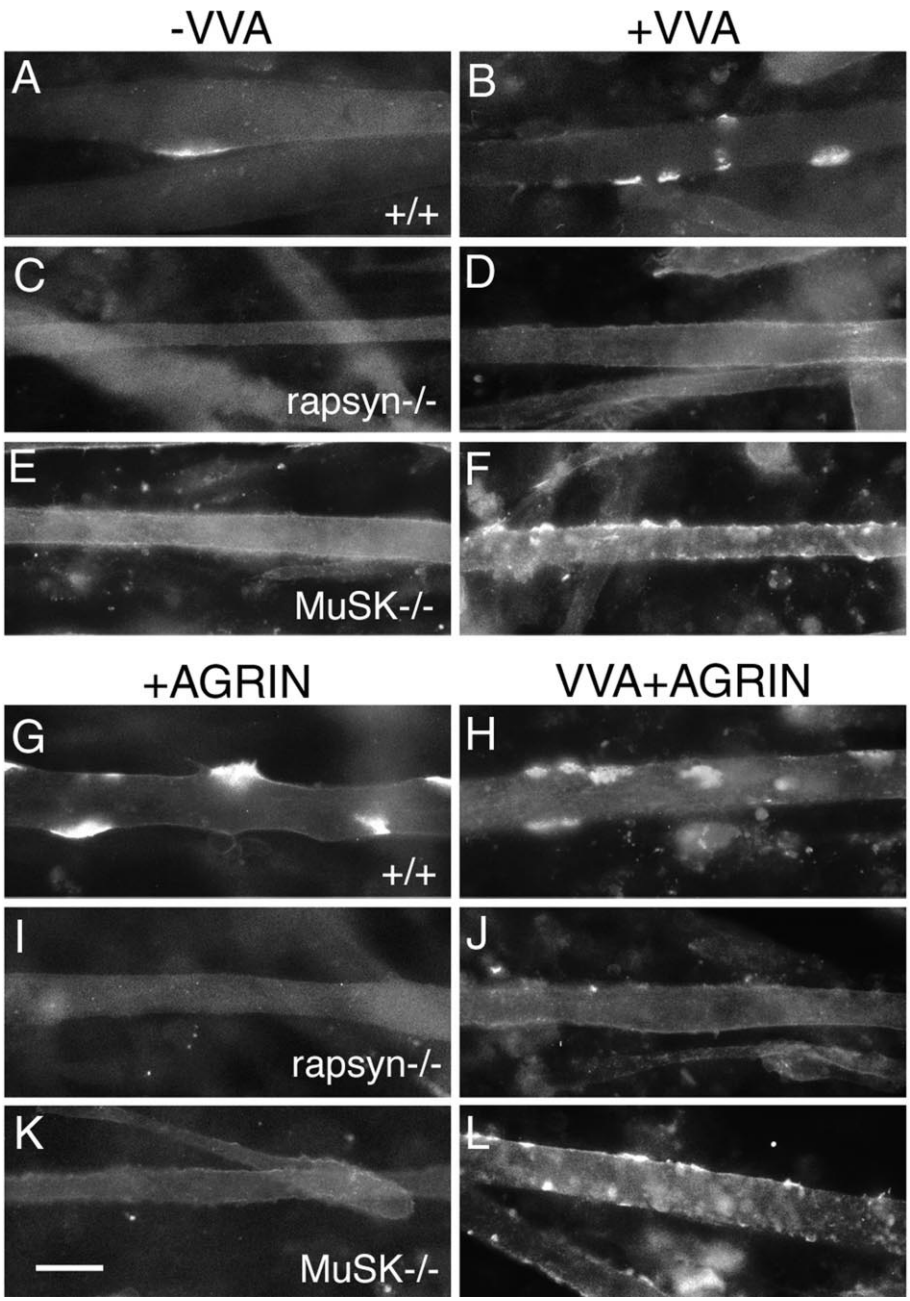


Fig. 2. Formation of acetylcholine receptor clusters in cultured myotubes in the presence and absence of exogenously added agents. Primary muscle cultures were prepared from mutants and controls and allowed to differentiate into myotubes. After 2 days in fusion medium, some cultures were left untreated (A, C, E); others were treated with the lectin VVA-$B_4$ (B, D, F), with agrin (G, I, K) or with a mixture of VVA-$B_4$ and agrin (H, J, L) for 16 h. Cultures were then stained with rh-btx and fixed with 2% paraformaldehyde to visualize AChRs. Myotubes from both rapsyn$^{-/-}$ and MuSK$^{-/-}$ cultures failed to form spontaneous (C and E, respectively), or agrin-induced receptor clusters (I and K, respectively); treatment with VVA-$B_4$, however, resulted in the formation of receptor clusters in MuSK$^{-/-}$ myotubes (F), but not in rapsyn$^{-/-}$ myotubes (D). In both rapsyn$^{-/-}$ and MuSK$^{-/-}$ cultures, distribution of AChRs in myotubes treated with VVA-$B_4$ and agrin together was similar to myotubes treated with VVA-$B_4$ alone (J and D for rapsyn, L and F for MuSK). Bar in K = 50 μm.

potentiated the effect of VVA-$B_4$ in these cultures. However, the mixture of agrin and VVA-$B_4$ had no greater effect than VVA-$B_4$ alone on MuSK$^{-/-}$ myotubes, and induced no clusters in rapsyn$^{-/-}$ myotubes (Fig. 2G–L). These results show that MuSK is essential to transduce signals that stimulate AChR clustering, but is not necessary for clustering per se. They also suggest that a GalNAc-dependent step in agrin-stimulated AChR clustering [22] is downstream of MuSK but upstream of rapsyn.

### *3.2. Interactions between signaling pathways for AChR clustering and AChR gene activation*

In normal muscles, the few myonuclei directly beneath the postsynaptic membrane express genes for several

synaptic proteins, including AChR subunits, at far higher rates than neighboring extrasynaptic nuclei in the same cytoplasm. In situ hybridization showed that synaptic transcription of AChR subunit genes was disrupted in agrin$^{-/-}$ and MuSK$^{-/-}$ mutants, but not in rapsyn$^{-/-}$ mutants [7,13,12]. Synapse-specific gene activation was also examined in agrin$^{-/-}$ and rapsyn$^{-/-}$ mutants by crossing a nuclear-localized β-galactosidase (nlacZ) reporter transgene into each of these mutant lines. Reporter expression in this transgene was driven by a promoter fragment of the gamma subunit of the AChR, providing a higher-resolution map of transcriptional activity than in situ hybridization. In fact, some tendency for increased AChR transcription in the endplate region of agrin mutants was detected with the reporter but not by in situ hybridization [13]. Accordingly, we compared reporter expression in agrin$^{-/-}$ and MuSK$^{-/-}$ mutants, to seek differences between the two.

*3.2.1. In vivo*

Mice, bearing the AChRγ-nlacZ transgene, were crossed to rapsyn$^{+/-}$, agrin$^{+/-}$, or MuSK$^{+/-}$ heterozygotes, and mice heterozygous for both the transgene and the mutant allele were detected by PCR. These mice were then crossed to mutant heterozygotes, to generate mutant homozygotes bearing a single copy of the transgene. Transgene positive littermates of these mutants served as controls.

In controls, nlacZ expression was restricted to synaptic regions (Fig. 3A). Reporter expression in rapsyn-deficient transgenics was indistinguishable from that in controls (Fig. 3B), In contrast, preferential expression of reporter in the endplate band was disrupted in both agrin-deficient (Fig. 3C) and MuSK-deficient embryos (Fig. 3D). In agrin$^{-/-}$ diaphragms, nuclei in the central region of muscle was distinctly more blue than outer, flanking regions. LacZ staining was less intense in diaphragms from

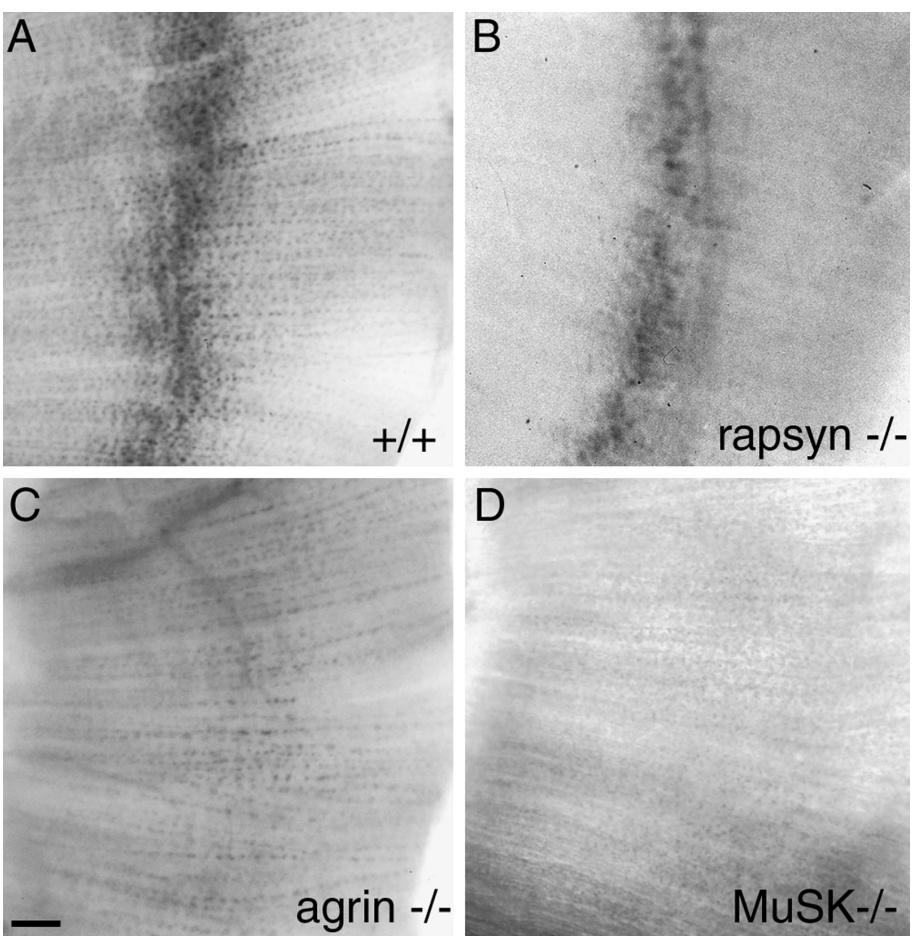


Fig. 3. Expression of beta galactosidase (LacZ) in diaphragm muscles from mutants and controls carrying the gamma-nLacZ transgene (A–D). Synapse-specific gene expression was examined in mutants and controls by crossing the gamma-nLacZ reporter transgene into individual mutant lines. Litters from such crosses were eviscerated, fixed in paraformaldehyde, and stained for LacZ. Although muscles from both rapsyn$^{-/-}$ and MuSK$^{-/-}$ mutants lack receptor clusters, synapse-specific gene expression is maintained in rapsyn mutants$^{-/-}$ (B) but is severely reduced in MuSK$^{-/-}$ mutants (D). LacZ staining was significantly reduced in agrin$^{-/-}$ mutants (C); nuclei in the central region were more blue than flanking regions of the muscle, but lacked the intensely-staining, restricted pattern seen in controls (A). Bar in C = 200 μm.

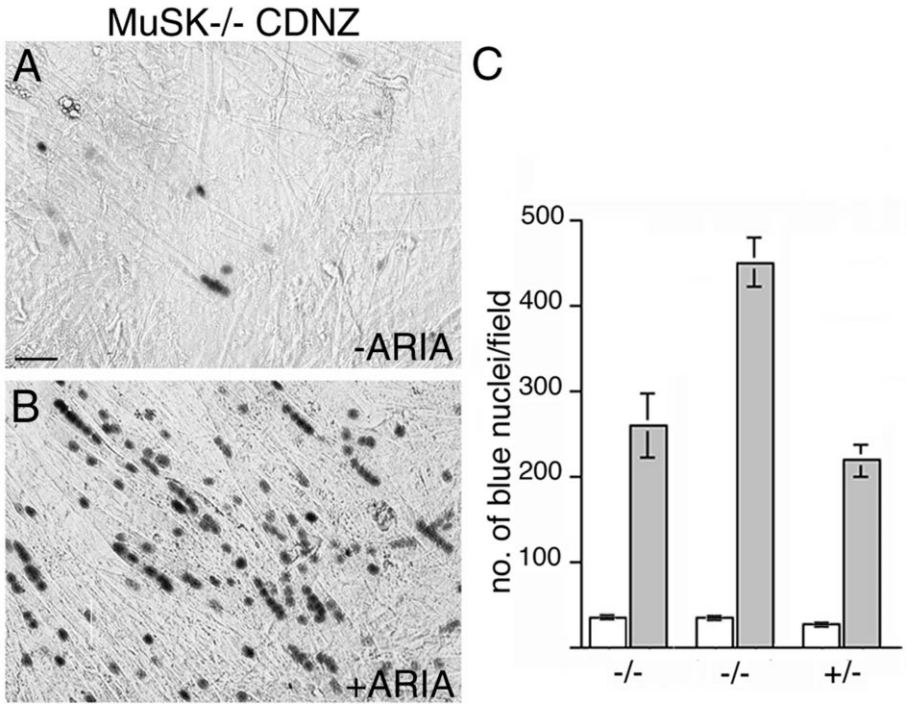


Fig. 4. ARIA/neuregulin-induced responses are normal in cultured myotubes from MuSK-deficient mice. Primary muscle cultures from MuSK$^{-/-}$/γ-nlac Z mice and control littermates treated with or without a purified recombinant fragment of neuregulin (3 nM) for 3–4 days were fixed and stained for LacZ. The number of blue nuclei in cultures treated with neuregulin (B) was about 5–10 fold greater than the number of blue nuclei in cultures grown in fusion medium alone (A) and was similar to the neuregulin-induced response in controls (C). Bar in B = 100 μm; in C, open columns = cultures in fusion medium alone, shaded columns = cultures treated with neuregulin.

MuSK$^{-/-}$ muscles when compared with agrin$^{-/-}$ muscles, and increased staining in the central region was less visible. Thus, defects in synapse-specific gene expression were more severe in MuSK$^{-/-}$ mutants than in agrin$^{-/-}$ mutants.

*3.2.2. In vitro*

One possible explanation for the lack of localized transcription in MuSK$^{-/-}$ mutants is that activation of MuSK is required for mediating postsynaptic responses to an inducing factor. The best candidate inducing factor is ARIA/neuregulin [10], which is localized at synaptic sites, interacts with erbB receptors in the postsynaptic membrane, and stimulates AChR gene expression [1,6, 18,26,30,31,35]. Accordingly, we tested the idea, that MuSK is required for neuregulin to exert its effects. To this end, we adapted the assay system of Chu et al. [6] and Missias et al. [24]. Myoblasts were dissociated from MuSK$^{-/-}$ mutants bearing the γ-nLacZ transgene, and cultured under conditions that promoted fusion into myotubes. Following fusion, a subset of the cultures were treated with recombinant neuregulin. Three or four days later, all cultures were fixed and stained for lacZ and labeled nuclei were counted. In both control and MuSK$^{-/-}$ cultures, the number of nlacZ-positive nuclei was increased 5–10 fold by treatment with neuregulin (Fig. 4). Therefore, in MuSK$^{-/-}$ mutants the postsynaptic gene expression defects probably arise from a lack of neuregulin or its localization rather than a direct involvement of MuSK in synaptic gene activation. Likewise, agrin$^{-/-}$/γ-nLacZ cultures also showed similar increases in neuregulin-induced activation of the lacZ reporter (data not shown).

## 4. Discussion

We have reported previously that the absence of agrin, MuSK and rapsyn causes profound defects in neuromuscular development. Here, we have used these lines of mutant mice to further characterize the pathways affecting the formation of nerve–muscle synapses in vivo and in vitro.

### *4.1. Neural induction of AChR receptor clustering*

Our results are consistent with the notion that postsynaptic differentiation in vivo requires agrin as a nerve-derived signal, MuSK as a component of agrin's signal transduction pathway, and rapsyn as a crucial effector of AChR clustering [32]. The direct comparison of phenotypes also revealed three new features of the signaling pathway. First, the finding of a significant number of nerve-associated AChR clusters in agrin$^{-/-}$ mutants indicates that nerves can induce postsynaptic differentiation by a *z*-agrin-independent mechanism. On the other hand, the absence of any postsynaptic differentiation in the MuSK$^{-/-}$ mutants indicates that the second signal acts through MuSK. One explanation for these findings is that

receptor clusters in agrin mutants are induced by ligand-independent activation of MuSK; in favor of this hypothesis is the finding by Gillespie et al. [14] that rapsyn-dependent autophosphorylation of MuSK occurs in transfected QT6 fibroblasts. Another possibility is that the second signal is an alternatively spliced form of agrin: in the allele we have studied, the '*z*' exons, which are essential for bioactivity in vitro, have been deleted, but low levels of *z*-minus agrin are produced [13]. However, the only agrin isoforms capable of activating MuSK in cultured cells are those that contain the *z* exons [15,16]. Moreover, since neural and aneural receptor clusters are also present in muscles from agrin-null mutants (R. Burgess and J.R. Sanes, in preparation), it is unlikely that muscle agrin is the second signal. A third possibility is that there is a second nerve-associated ligand capable of activating MuSK. Therefore, activation of MuSK, either by multiple ligands or by autophosphorylation, appears to be essential for cluster formation in vivo.

Second, the result that the GalNAc-specific lectin, VVA-B4, causes AChR clustering in MuSK$^{-/-}$ myotubes indicates that MuSK is not an essential structural component of clusters. A similar conclusion was reached by Montanaro et al. [25] and Sugiyama et al. [33] who recently reported that laminin induces AChR clustering in both wild-type and MuSK$^{-/-}$ myotubes. We had previously argued that GalNAc-terminated glycoconjugates are components of agrin's signal transduction pathway [22], whereas Montanaro et al. [25] and Sugiyama et al. [33] proposed that laminin acts by an independent, parallel pathway. Unfortunately, our present results do not provide any means of distinguishing between these possibilities. However, the inability of VVA-$B_4$ (and laminin; J. Sugiyama, E. Apel, Z. Hall and J.R.S., unpublished) to cluster AChRs in rapsyn$^{-/-}$ myotubes supports the idea that rapsyn is necessary for all clustering to occur.

Finally, evidence for a second nerve-derived signal, along with the MuSK-independent clustering abilities of VVA-$B_4$ and laminin, raised the possibility that cluster formation is initiated by an agrin- and MuSK-independent pathway, with MuSK being required for maintenance rather than initiation of clustering. We tested this idea by examining MuSK$^{-/-}$ muscles at early developmental stages, but found no evidence for transient clustering. In addition, the observation that no AChR clustering occurs in MuSK$^{-/-}$ muscle in vivo suggests that the second signal is neither laminin nor a GalNAc-terminated glycoconjugate, even though both are present in embryonic muscle.

### *4.2. Activation of receptor clustering and receptor gene expression*

The best characterized effect of agrin is the stimulation AChR clustering; another signaling molecule, neuregulin, has been implicated as an inducer of AChR gene expression in synaptic nuclei [10]. The finding that synapse-specific transcription is markedly defective in agrin$^{-/-}$ and MuSK$^{-/-}$ mice provided the first evidence that pathways for clustering and transcription interact. One possible explanation that we considered earlier was that agrin and MuSK are necessary to recruit neuregulin receptors, the erbB2-4 kinases, to the postsynaptic membrane. However, synapse-specific transcription is normal in rapsyn$^{-/-}$ mutants, even though erbB kinases are not synaptically concentrated in those mutants [26]. Likewise, the defect is unlikely to be an indirect consequence of the sprouting of motor axons beyond the bounds of the normal endplate band, because considerable sprouting is observed in the rapsyn$^{-/-}$ mice. Here, we examined a third possibility, that agrin and MuSK are required for the transduction of a neuregulin/erbB kinase-initiated signal to the nucleus. In fact, exogenous neuregulin stimulated AChR gene expression to similar extents in myotubes from control, agrin$^{-/-}$ and MuSK$^{-/-}$ mutants. Thus, neuregulin signaling itself is not completely MuSK-dependent.

One possibility raised by this result is that agrin rather than neuregulin is the physiological inducer of synaptic transcription. Indeed, recent reports have shown that agrin is capable of inducing AChR gene expression to a modest extent [19,20]. Inconsistent with this idea, however, is the finding of Sandrock et al. [31] that AChR expression is reduced in neuregulin$^{+/-}$ mice (neuregulin$^{-/-}$ mice die before muscles form, so are not informative in this regard). Alternatively, nerve terminals or muscle fibers may be unable to release sufficient neuregulin in the absence of an agrin- and MuSK-dependent retrograde signal. In either case, our results indicate that synapse-specific gene expression is initiated by agrin–MuSK interactions by a pathway that is not dependent on rapsyn, or cluster formation; subsequently, neuregulin may be necessary to maintain high levels of synaptic gene transcription by a MuSK-independent mechanism. The results that agrin can induce assembly of a complete postsynaptic apparatus in the absence of the nerve [20,29] and that muscle fibers express neuregulin [23,26] have raised the possibility that agrin stimulates AChR transcription indirectly, by means of an effect on the release or accumulation of muscle-derived neuregulin. Our results are consistent with, but do not prove this mechanism.

## Acknowledgements

We thank M. Nichol, M. Elam, and B. Klocke for technical assistance. This work was supported by grants from the NIH.

## References

[1] N. Altiok, J.L. Bessereau, J.P. Changeux, ErbB3 and ErbB2/neu mediate the effect of heregulin on acetylcholine receptor gene expression in muscle: differential expression at the endplate, EMBO J. 14 (1995) 4258–4266.

[2] E.D. Apel, D.J. Glass, L.M. Moscoso, G.D. Yancopoulos, J.R. Sanes, Rapsyn is required for MuSK signalling and recruits synaptic components to a MuSK-containing scaffold, Neuron 18 (1997) 623–635.
[3] F.J. Barrantes, D.-Ch. Neugebauer, H.P. Zingsheim, Peptide extraction by alkaline treatment is accompanied by rearrangement of the membrane-bound acetylcholine receptor from *Torpedo marmorata*, FEBS Lett. 112 (1980) 73–78.
[4] M.A. Bowe, J.R. Fallon, The role of agrin in synapse formation, Annu. Rev. Neurosci. 18 (1995) 443–462.
[5] J. Cartaud, A. Sobel, A. Rousselet, P.F. Devaux, J.P. Changeux, Consequences of alkaline treatment for the ultrastructure of the acetylcholine receptor-rich membranes from *Torpedo marmorata* electric organ, J. Cell Biol. 90 (1981) 418–426.
[6] G.C. Chu, L.M. Moscoso, M.X. Sliwkowski, J.P. Merlie, Regulation of the acetylcholine receptor epsilon subunit gene by recombinant ARIA: an in vitro model for transynaptic gene regulation, Neuron 14 (1995) 329–339.
[7] T. DeChiara, D.C. Bowen, D.M. Valenzuela, M.V. Simmons, W.T. Poueymirou, S. Thomas, E. Kinetz, C. Smith, D.L. Compton, J.S. Park, P.S. DiStefano, D.J. Glass, S.J. Burden, G.D. Yancopoulos, The receptor tyrosine kinase MuSK is required for neuromuscular junction formation in vivo, Cell 85 (1996) 501–512.
[8] A. Duclert, J.-P. Changeux, Acetylcholine receptor gene expression at the developing neuromuscular junction, Physiol. Rev. 75 (1995) 339–368.
[9] M. Ferns, J.T. Campanelli, W. Hoch, R.H. Scheller, Z.M. Hall, The ability of agrin to cluster AChRs depends on alternative splicing and on cell surface proteoglycans, Neuron 11 (1993) 491–502.
[10] G.D. Fischbach, K.M. Rosen, ARIA: a neuromuscular junction neuregulin, Annu. Rev. Neurosci. 20 (1997) 429–458.
[11] S.C. Froehner, C.W. Luetje, P.B. Scotland, J. Patrick, The postsynaptic 43 k protein clusters muscle nicotinic acetylcholine receptors in Xenopus oocytes, Neuron 5 (1990) 403–410.
[12] M. Gautam, P.G. Noakes, J. Mudd, M. Nichol, G.C. Chu, J.R. Sanes, J.P. Merlie, Failure of postsynaptic specialization to develop at neuromuscular junctions of rapsyn-deficient mice, Nature 377 (1995) 232–236.
[13] M. Gautam, P.G. Noakes, L.M. Moscoso, F. Rupp, R.H. Scheller, J.P. Merlie, J.R. Sanes, Defective neuromuscular synaptogenesis in agrin-deficient mutant mice, Cell 85 (1996) 525–535.
[14] S.K. Gillespie, S. Balasubramanian, E.T. Fung, R.L. Huganir, Rapsyn clusters and activates the synapse-specific receptor tyrosine kinase MuSK, Neuron 16 (1996) 953–962.
[15] D.J. Glass, D.C. Bowen, T.N. Stitt, C. Radziejewski, J. Bruno, T.E. Ryan, D.R. Gies, S. Shah, K. Mattsson, S.J. Burden, P.S. DiStefano, D.M. Vlaenzuela, T.M. De Chiara, G.D. Tancopoulos, Agrin acts via a MuSK receptor complex, Cell 85 (1996) 513–523.
[16] D.J. Glass, E.D. Apel, S. Shah, D.C. Bowen, T.M. DeChiara, T.N. Stitt, J.R. Sanes, G.D. Yancopoulos, Kinase domain of the muscle-specific receptor tyrosine kinase (MuSK) is sufficient for phosphorylation but not clustering of acetylcholine receptors: required role for the MuSK ectodomain?, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 8848–8853.
[17] Z.W. Hall, J.R. Sanes, Synaptic structure and development: the neuromuscular junction. Cell 71/Neuron 10 (Suppl.), 1993, pp. 99–121.
[18] S.A. Jo, X. Zhu, M.A. Marchionni, S.J. Burden, Neuregulins are concentrated at nerve–muscle synapses and activate AChR-gene expression, Nature 373 (1995) 158–161.
[19] G. Jones, A. Herczeg, M.A. Ruegg, M. Lichtsteiner, S. Kroger, H.R. Brenner, Substrate-bound agrin induces expression of acetylcholine receptor epsilon-subunit gene in cultured mammalian muscle cells, Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 5985–5990.
[20] G. Jones, T. Meier, M. Lichtsteiner, V. Witzemann, B. Sakmann, H.R. Brenner, Induction by agrin of ectopic and functional postsynaptic-like membrane in innervated muscle, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 2654–2659.
[21] W.J. La Rochelle, S.C. Froehner, Determination of the tissue distributions and relative concentrations of the postsynaptic 43 kDa protein and the acetylcholine receptor in *Torpedo*, J. Biol. Chem. 261 (1986) 5270–5274.
[22] P.T. Martin, J.R. Sanes, Role for a synapse-specific carbohydrate in agrin-induced clustering of acetylcholine receptors, Neuron 14 (1995) 743–754.
[23] T. Meier, F. Masciulli, C. Moore, F. Schoumacher, U. Eppenberger, A.J. Denzer, G. Jones, H.R. Brenner, Agrin can mediate acetylcholine receptor gene expression in muscle by aggregation of muscle-derived neuregulins, J. Cell Biol. 141 (1998) 715–726.
[24] A.C. Missias, G.C. Chu, B.J. Klocke, J.R. Sanes, J.P. Merlie, Maturation of the acetylcholine receptor in skeletal muscle: regulation of the AChR γ-to-ε switch, Dev. Biol. 179 (1996) 138–223.
[25] F. Montanaro, S.H. Gee, C. Jacobson, M.H. Lindenbaum, S.C. Froehner, S. Carbonetto, Laminin and alpha-dystroglycan mediate acetylcholine receptor aggregation via a MuSK-independent pathway, J. Neurosci. 18 (1998) 1250–1260.
[26] L.M. Moscoso, G.C. Chu, M. Gautam, P.G. Noakes, J.P. Merlie, J.R. Sanes, Synapse-associated expression of an acetylcholine receptor-inducing protein, ARIA/heregulin, and its putative receptors, ErbB2 and ErbB3, in developing mammalian muscle, Dev. Biol. 172 (1995) 158–169.
[27] P.G. Noakes, W.D. Phillips, T.A. Hanley, J.R. Sanes, J.P. Merlie, 43 k protein and acetylcholine receptors colocalize during the initial stages of neuromuscular synapse formation in vivo, Dev. Biol. 155 (1993) 275–280.
[28] W.D. Phillips, C. Kopta, P. Blount, P.D. Gardner, J.H. Steinbach, J.P. Merlie, ACh receptor-rich membrane domains organized in fibroblasts by recombinant 43-kilodalton protein, Science 251 (1991) 568–570.
[29] M. Rimer, I. Mathiesen, T. Lomo, U.J. McMahan, Gamma-AChR/epsilon-AChR switch at agrin-induced postsynaptic-like apparatus in skeletal muscle, Mol. Cell. Neurosci. 9 (1997) 254–263.
[30] A.W. Sandrock Jr., A.D. Goodearl, Q.W. Yin, D. Chang, G.D. Fischbach, ARIA is concentrated in nerve terminals at neuromuscular junctions and at other synapses, J. Neurosci. 15 (1995) 6124–6136.
[31] A.W. Sandrock Jr., S.E. Dryer, K.M. Rosen, S.N. Gozani, R. Kramer, L.E. Theill, G.D. Fischbach, Maintenance of acetylcholine receptor number by neuregulins at the neuromuscular junction in vivo, Science 276 (1997) 599–603.
[32] J.R. Sanes, Genetic analysis of postsynaptic differentiation at the vertebrate neuromuscular junction, Curr. Opin. Neurobiol. 7 (1997) 93–100.
[33] J. Sugiyama, D.J. Glass, G.D. Yancopoulos, Z.W. Hall, Laminin-induced acetylcholine receptor clustering: an alternative pathway, J. Cell Biol. 139 (1997) 181–191.
[34] D.M. Valenzuela, T.N. Stitt, P.S. DiStefano, E. Rojas, K. Mattsson, D.L. Compton, L. Nunez, J.S. Park, J.L. Stark, D.R. Gies, S. Thomas, M.M. Le Beau, A.A. Fernald, N.G. Copeland, N.A. Jenkins, S.J. Burden, D.J. Glass, G.D. Yancopoulos, Receptor tyrosine kinase specific for the skeletal muscle lineage: expression in embryonic muscle, at the neuromuscular junction, and after injury, Neuron 15 (1995) 573–584.
[35] X. Zhu, C. Lai, S. Thomas, S.J. Burden, Neuregulin receptors, erbB3 and erbB4, are localized at neuromuscular synapses, EMBO J. 14 (1995) 5842–5848.