Neuron, Vol. 30, 399–410, May, 2001, Copyright ©2001 by Cell Press

Patterning of Muscle Acetylcholine Receptor Gene Expression in the Absence of Motor Innervation

Xia Yang,[1] Silvia Arber,[2,5] Christopher William,[2] Li Li,[3] Yasuto Tanabe,[2,6] Thomas M. Jessell,[2] Carmen Birchmeier,[3] and Steven J. Burden[1,4]

[1] Molecular Neurobiology Program
Skirball Institute
New York University Medical School
New York, New York 10011

[2] Howard Hughes Medical Institute
Department of Biochemistry and Molecular Biophysics
Columbia University
New York, New York 10032

[3] Max Delbrück Center for Molecular Medicine
13125 Berlin
Germany

Summary

The patterning of skeletal muscle is thought to depend upon signals provided by motor neurons. We show that *AChR* gene expression and AChR clusters are concentrated in the central region of embryonic skeletal muscle in the absence of innervation. Neurally derived Agrin is dispensable for this early phase of AChR expression, but MuSK, a receptor tyrosine kinase activated by Agrin, is required to establish this AChR prepattern. The zone of AChR expression in muscle lacking motor axons is wider than normal, indicating that neural signals refine this muscle-autonomous prepattern. Neuronal Neuregulin-1, however, is not involved in this refinement process, nor indeed in synapse-specific *AChR* gene expression. Our results demonstrate that AChR expression is patterned in the absence of innervation, raising the possibility that similarly prepatterned muscle-derived cues restrict axon growth and initiate synapse formation.

Introduction

During the development of the vertebrate neuromuscular junction, a precise register emerges between motor nerve terminals and the specialized transduction machinery present on the surface of the postsynaptic muscle cell, an alignment that is critical to efficient synaptic transmission (Hall and Sanes, 1993). Multinucleated skeletal muscle fibers form by fusion of precursor myoblasts and are innervated shortly after by the axons of motor neurons. Subsequent inductive interactions between nerve and muscle cells lead to the formation of a focal, and highly specialized, synaptic site on the developing myofiber (Burden, 1998; Dennis, 1981; Sanes and Lichtman, 1999). Acetylcholine receptors (AChRs) constitute the best-studied class of proteins that become localized to this small patch of the muscle fiber membrane, and their restriction to synaptic sites during development is a hallmark of the inductive events of synapse formation. The spatial patterning of AChRs on skeletal muscle cells has classically been thought to depend on focal signals provided by motor axon terminals (Burden, 1998; McMahan, 1990; Sanes and Lichtman, 1999).

Three different nerve-dependent signaling pathways have been proposed to regulate postsynaptic muscle differentiation. One signaling pathway uses Agrin, a synaptic basal lamina protein that triggers a redistribution of AChRs to synaptic sites. Agrin is critical for clustering AChRs and for promoting other aspects of postsynaptic differentiation during synapse formation (Sanes and Lichtman, 1999). Agrin-mediated signaling depends upon the activity of a receptor tyrosine kinase, MuSK, a critical component of the Agrin receptor complex (Burden, 1998; Glass and Yancopoulos, 1997). Thus, mice lacking Agrin or MuSK fail to form neuromuscular synapses, and muscle-derived proteins, including AChRs, are uniformly distributed on the surface of myofibers (DeChiara et al., 1996; Gautam et al., 1996). A second neural signaling pathway stimulates the transcription of *AChR* subunit genes in myofiber nuclei close to synaptic sites. A strong candidate for the signal that activates synapse-specific transcription is Neuregulin-1 (NRG-1), a group of polypeptides produced by alternative splicing of the *nrg-1* gene (Falls et al., 1993; Carraway and Burden, 1995; Fischbach and Rosen, 1997). A third signaling pathway, mediated by propagated electrical activity in muscle fibers, represses expression of *AChR*s and other muscle genes in myofiber nuclei (Laufer and Changeux, 1989).

The ability of motor axons to induce synaptic differentiation following brief and transient contact with muscle (Cohen et al., 1994; Ferns et al., 1993; Wallace, 1991) has made it difficult to evaluate whether the initial regional expression of AChRs occurs independently of neurally derived signals. Indeed, evidence for a possible contribution of muscle prepatterning (Braithwaite and Harris, 1979; Harris, 1981) has not been incorporated into prevailing views of neuromuscular synapse formation (Burden, 1998; Dennis, 1981; Sanes and Lichtman, 1999). In studies of mice lacking *topoisomerase 2β* (*top 2β*), we observed that motor axons fail to invade or branch within diaphragm and limb muscles, yet AChRs are clustered in the central region of these muscles (Yang et al., 2000). One interpretation of these findings is that the pattern of AChR clusters in skeletal muscles is determined, at least in part, by mechanisms that are independent of motor innervation. The proximity of motor axons and muscle in *top 2β* mutant mice, however, left open the alternative possibility that motor axons had transiently contacted muscle, or supplied diffusible signals responsible for AChR clustering.

In this study, we have analyzed several additional mouse mutants that affect motor neuron generation, motor axon projections, and motor neuron-derived sig-

[4] Correspondence: burden@saturn.med.nyu.edu
[5] Present address: Department of Cell Biology, Biozentrum, University of Basel, CH-4056, Basel, Switzerland.
[6] Present address: Mitsubishi Kasei Institute of Life Sciences, TOREST, 11, Minami-Ooya, Machida-shi, Tokyo 194-8511, Japan.

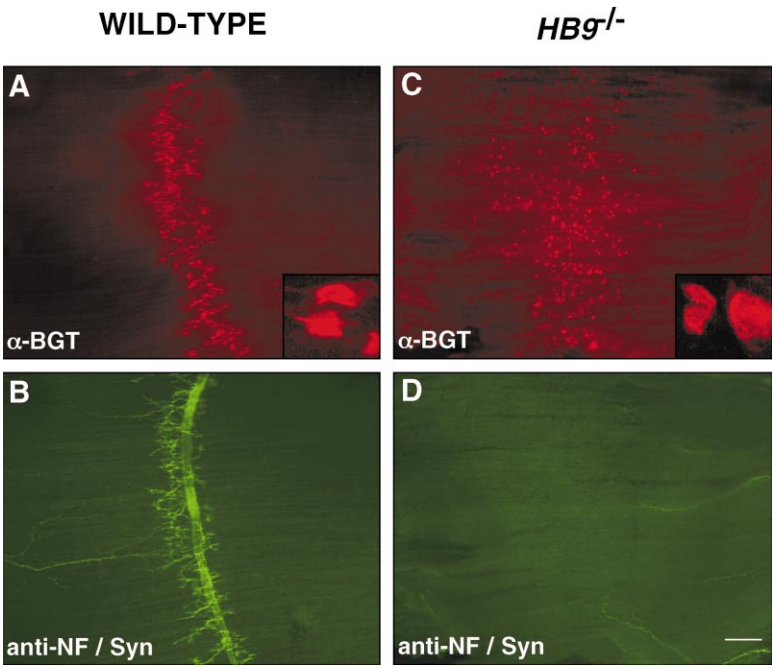

Figure 1. AChRs Are Clustered in the Central Region of Muscle Lacking Motor Axons

Whole mounts of diaphragm muscle from E18.5 wild-type (A and B) or *HB9* mutant (C and D) embryos were stained with Texas red-α-BGT and antibodies to NF and Syn. The insets in (A) and (C) show higher power views of AChR clusters in wild-type and mutant embryos. The NF-stained axons at the edge of wild-type and *HB9* mutant muscle (arrowheads) are likely to be sensory and/or autonomic axons, since these axons are absent from muscles prior to E16. The bar is 100 μm for the low magnification images and 7 μm for the insets in (A) and (C).

nals to examine more definitively whether *AChR* gene expression in muscle is spatially restricted in a manner independent of innervation. We show that *AChR* gene transcription and AChR clusters are patterned in developing skeletal muscle in the absence of motor neurons and their axons. We also find that *AChR* transcription and clustering occurs in the absence of neurally supplied Agrin but is dependent upon MuSK expression. The nerve, however, does have a role in refining the *AChR* prepattern by further restricting the domain of *AChR* gene expression. But neuronal NRG-1 is not required to promote the mature pattern of *AChR* transcription in skeletal muscle, nor to induce synapse-specific transcription. Taken together, these results demonstrate that muscle is patterned in the absence of innervation and raise the possibility that spatial cues that restrict axon growth and promote synapse formation might be provided by molecules that are also prepatterned in muscle.

Results

Skeletal Muscle Patterning Is Independent of Motor Neuron-Derived Signals

To determine whether skeletal muscle is patterned in the absence of motor axons, we first studied mice in which the homeodomain transcription factor HB9 has been inactivated by gene targeting (Arber et al., 1999; Thaler et al., 1999). In *HB9* mutant mice, motor neurons lose differentiated properties (Arber et al., 1999; Thaler et al., 1999), and the phrenic nerve, which normally innervates the diaphragm muscle, fails to form (Thaler et al., 1999). As a consequence, motor axons are not observed in the vicinity of the diaphragm muscle (Thaler et al., 1999).

Motor axons and nerve terminals in diaphragm muscles were visualized by expression of neurofilament (NF) and synaptophysin (Syn), respectively, and AChRs were visualized by α-bungarotoxin (α-BGT) labeling. In wild-type mice, the main intramuscular nerve is located in the central region of the muscle and is oriented perpendicular to the long axis of the muscle fibers. Motor axons branch and terminate adjacent to the main intramuscular nerve, resulting in a narrow, distinct endplate zone in the middle of the muscle, marked by presynaptic nerve terminals and a high density of AChR clusters (Figure 1). In *HB9* mutant embryos, no motor axons were detected in or near the diaphragm muscle (E12.5–E18.5) (Figure 1; data not shown), yet AChRs were still clustered in the central region of the muscle (Figure 1).

We also examined muscle AChR distribution in mice lacking motor neurons themselves, from early developmental stages. To eliminate motor neurons early in development, we crossed an $HB9^{cre}$ mouse line, which expresses Cre recombinase selectively in motor neurons, with an $Isl2^{DTA}$ mouse line in which a diphtheria toxin (DTA) gene, preceded by an IRES-loxP-stop-loxP sequence (Lee et al., 2000), had been introduced into the 3′ UTR of the *Isl2* gene (Brown et al., 2000). In mice carrying both transgenes, the translational stop sequence is deleted selectively in motor neurons, leading to expression of DTA at the time that motor neurons exit the cell cycle (E9.5–E11) (S.A. et al., unpublished data). Examination of these mice from E12 to E18.5 revealed a selective and virtually complete absence of motor neurons, as assessed by the lack of $Isl1^{+}/HB9^{+}$ neurons in the ventral spinal cord (Figure 2; data not shown). Despite the absence of motor neurons, AChRs were still clustered in the central region of the diaphragm muscle (Figure 2). Taken together, these results provide strong evidence that AChR expression in skeletal muscle is patterned in the absence of motor neurons and their axons.

We were concerned, however, by the fact that sensory

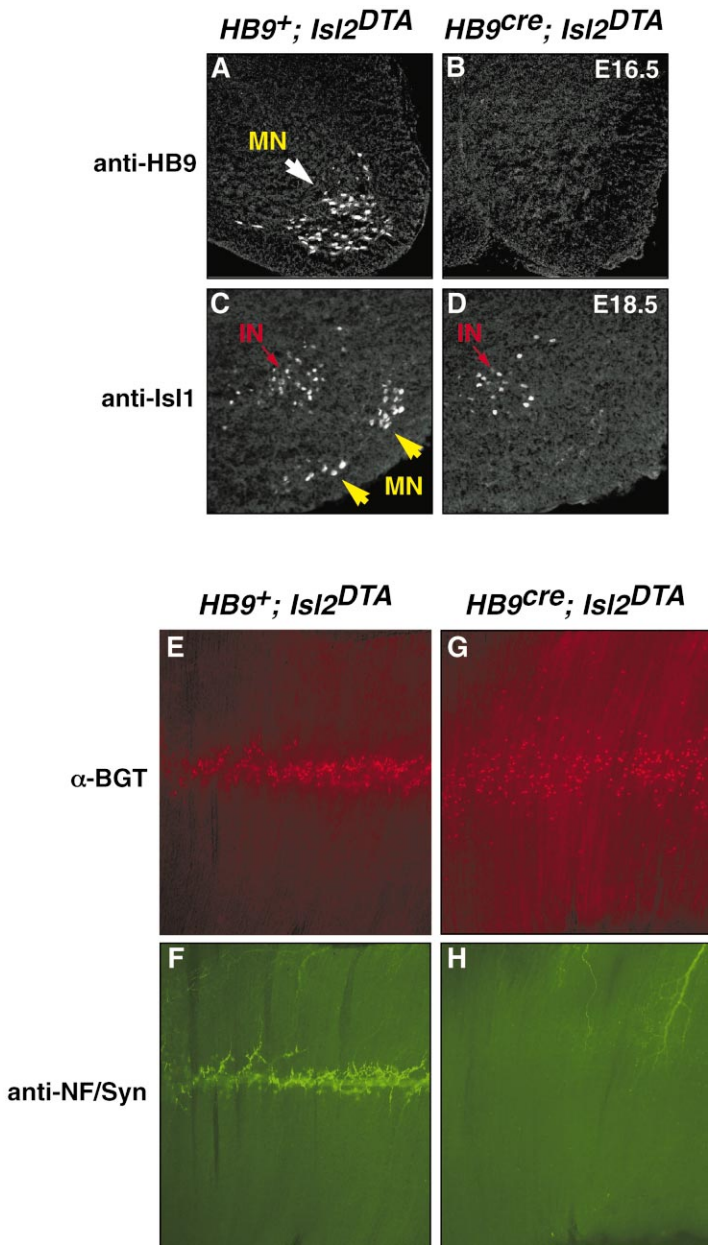

Figure 2. AChRs Are Clustered in the Central Region of Muscle Lacking Motor Neurons

Transverse sections of the spinal cord from *HB9*$^{+}$; *Isl2*^{DTA} (A and C) or *HB9*^{cre}; *Isl2*^{DTA} (B and D) embryos were stained with antibodies to HB9 (E16.5) or Isl1 (E18.5). *HB9*^{cre}; *Isl2*^{DTA} mice lack motor neurons (MN) but have normal numbers of Isl1-expressing dorsal interneurons (IN). Whole mounts of diaphragm muscle from E18.5 *HB9*$^{+}$; *Isl2*^{DTA} (E and F) or *HB9*^{cre}; *Isl2*^{DTA} (G and H) embryos were stained with Texas red-α-BGT and antibodies to NF and Syn.

and/or autonomic axons that are found at the periphery of diaphragm muscle by E16.5 (Yang et al., 2000), and synthesize neural isoforms of Agrin (Ma et al., 1995), might provide signals that pattern muscle AChR expression at late embryonic stages. We therefore analyzed the diaphragm muscle of *HB9* and *top 2*β mutant embryos at E15, prior to the arrival of sensory and/or autonomic axons, and found that AChRs were still clustered in the central region of the muscle (Figures 3A and 3B). Thus, muscle exhibits patterned AChR expression in the absence of all peripheral innervation.

The existence of a prepattern of AChRs in noninnervated muscle in turn raised the issue of the distribution of AChRs during the early stages of muscle innervation in wild-type embryos. In embryos examined at E15, AChRs were clustered both at synaptic sites and at sites in the central region of muscle lacking nerve terminals (Figures 3C and 3D). The position and width of the band of nonsynaptic AChR clusters in innervated muscle was similar to that found in muscle of *HB9* mutant mice lacking motor axons (Figures 1 and 2, and see below). In contrast, in innervated muscle of wild-type mice examined at E18.5, AChRs were clustered only at synaptic sites (Figures 1 and 2). These results provide evidence that motor innervation ultimately extinguishes nonsynaptic AChR clusters present in the central region of muscle at an earlier stage of synapse formation.

Prepatterning of AChR Gene Expression

The mechanisms that control *AChR* subunit gene transcription are thought to be distinct from those that control AChR clustering (Burden, 1998). We therefore exam-

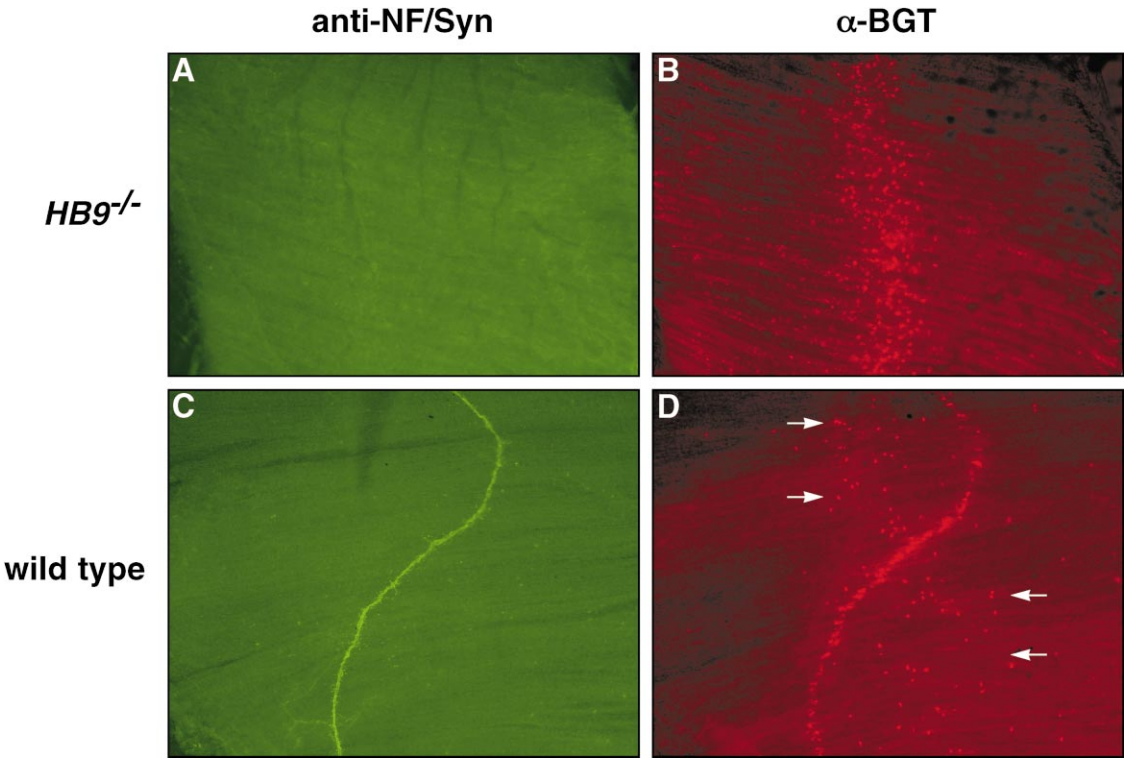

Figure 3. Patterning of AChRs in the Central Region of Muscle at E15

In *HB9* mutant embryos at E15, motor, sensory, and autonomic axons are absent from muscle, but AChR clusters are concentrated in the central region of muscle (A and B). In normal muscle at E15, AChRs are clustered both at synaptic sites and at nonsynaptic sites in the central region of muscle (C and D). Whole mounts of diaphragm muscle from E15 *HB9* mutant (A and B) or wild-type (C and D) embryos were stained with Texas red-α-BGT and antibodies to NF and Syn.

ined whether *AChR* gene transcription is also patterned in the absence of motor innervation. We used in situ hybridization to assess the pattern of *AChR* subunit gene expression in whole-mount preparations of muscle from wild-type, *HB9* mutant, and *HB9*^{cre}; *Isl2*^{DTA} mice. In diaphragm muscle of wild-type embryos examined at E18.5, *AChR* α and δ subunit mRNAs were restricted to a narrow band, corresponding to the central zone of innervation (Figures 4A and 4B; data not shown). In diaphragm muscles from *top 2*β and *HB9* mutant mice, as well as in *HB9*^{cre}; *Isl2*^{DTA} mice, we found that *AChR* α and δ subunit mRNAs were also enriched in the central region of muscle (Figures 4C–4E; data not shown). In addition, the level of *AChR* mRNA expression throughout the muscle was higher in *top 2*β and *HB9* mutant, as well as in *HB9*^{cre}; *Isl2*^{DTA} mice, than in wild-type embryos (Figure 4) (see below). This latter finding is consistent with the elevated levels of *AChR* gene expression observed in denervated, electrically inactive, adult muscle (Goldman and Staple, 1989). These results therefore provide evidence for a spatially restricted pattern of muscle *AChR* gene transcription in the absence of motor innervation.

Distinct Roles of Agrin and MuSK in the Early Control of AChR Expression

Previous studies in newborn mice have provided genetic evidence that neurally derived Agrin has a critical role in clustering AChRs at synapses (Gautam et al., 1996). How can these findings be reconciled with our observations that AChR clusters are spatially patterned in muscle that has never received motor innervation? One potential explanation invokes the idea that the arrival of motor axons is associated with two activities: one that directs the extinction of the AChR prepattern, and a second, dominant and Agrin-associated activity, that ensures the persistence of clustered AChRs at nascent synaptic sites. One prediction of this model is that the elimination of neural Agrin, when coupled with the absence of motor axons, should permit the maintenance of the AChR prepattern. To test this idea, we examined the pattern of AChR expression in neural *agrin*; *HB9* double mutant embryos at E18.5. AChR clusters were indeed patterned in the central region of muscle from neural *agrin*; *HB9* double-mutant mice, in a manner similar to that observed in *HB9* single-mutant mice (Figure 5). These results, taken together with the phenotype of neural *agrin* mutant mice, support the idea that the major role of Agrin at developing neuromuscular synapses is to maintain a patterned distribution of AChRs in the face of an independent neural signal that would otherwise extinguish the preexisting AChR prepattern.

The dispensability of neural Agrin raised the issue of whether MuSK is involved in patterning AChRs in the central region of developing, uninnervated muscle. To examine this issue, we analyzed the pattern of AChR clustering in *top 2*β; *MuSK* and *HB9*; *MuSK* double-

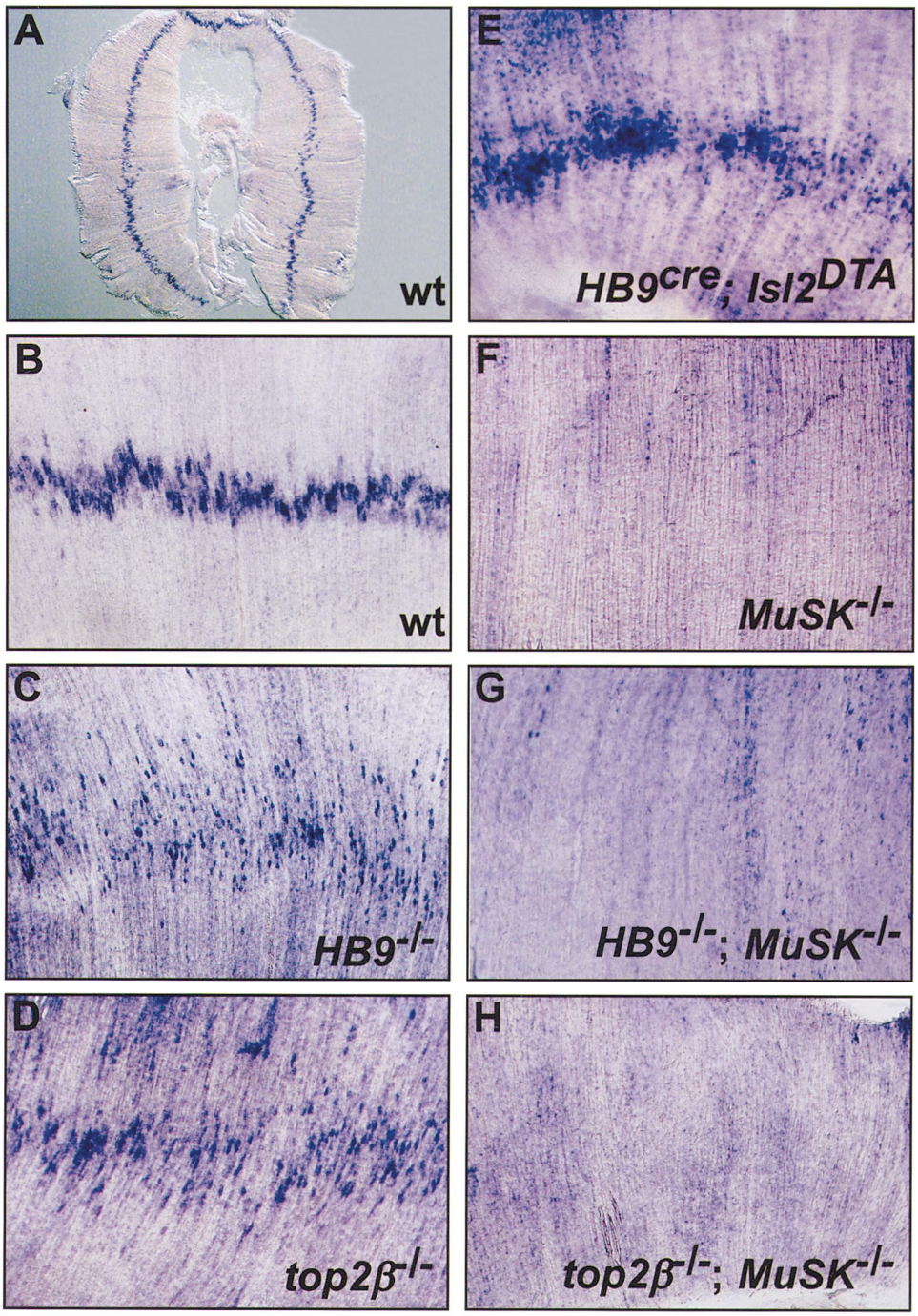

Figure 4. Muscle Is Transcriptionally Patterned in the Absence of Motor Axons

Whole mounts of diaphragm muscle from E18.5 wild-type (A and B), *HB9* mutant (C), *top 2*β mutant (D), *HB9*^{cre}; *Isl2*^{DTA} (E), *MuSK* mutant (F), *HB9*; *MuSK* double-mutant (G), and *top 2*β; *MuSK* double-mutant (H) embryos were processed for in situ hybridization using a probe from the *AChR* α subunit gene. *AChR* transcription is patterned in the central region of wild-type, *HB9* mutant, *top 2*β mutant, and *HB9*^{cre}; *Isl2*^{DTA} muscle but not in *MuSK* mutant, *HB9*; *MuSK* double-mutant, or *top 2*β; *MuSK* double-mutant embryos. The zone of enhanced transcription is wider in *HB9* mutant, *top 2*β mutant, and *HB9*^{cre}; *Isl2*^{DTA} muscle than in wild-type embryos, and the level of transcription outside this zone is greater in these mutants than in wild-type embryos. We obtained similar results using a probe from the *AChR* δ subunit gene (data not shown). A low magnification view of the diaphragm muscle is shown in (A); the other panels show higher magnification views.

mutant embryos at E18.5. In both double-mutant backgrounds, motor axons fail to grow or enter the diaphragm muscle, and although AChR expression was detected, no evidence of AChR clustering was observed (Figure 6). Thus, MuSK is required to pattern AChRs in the absence of motor innervation. In addition, *AChR*

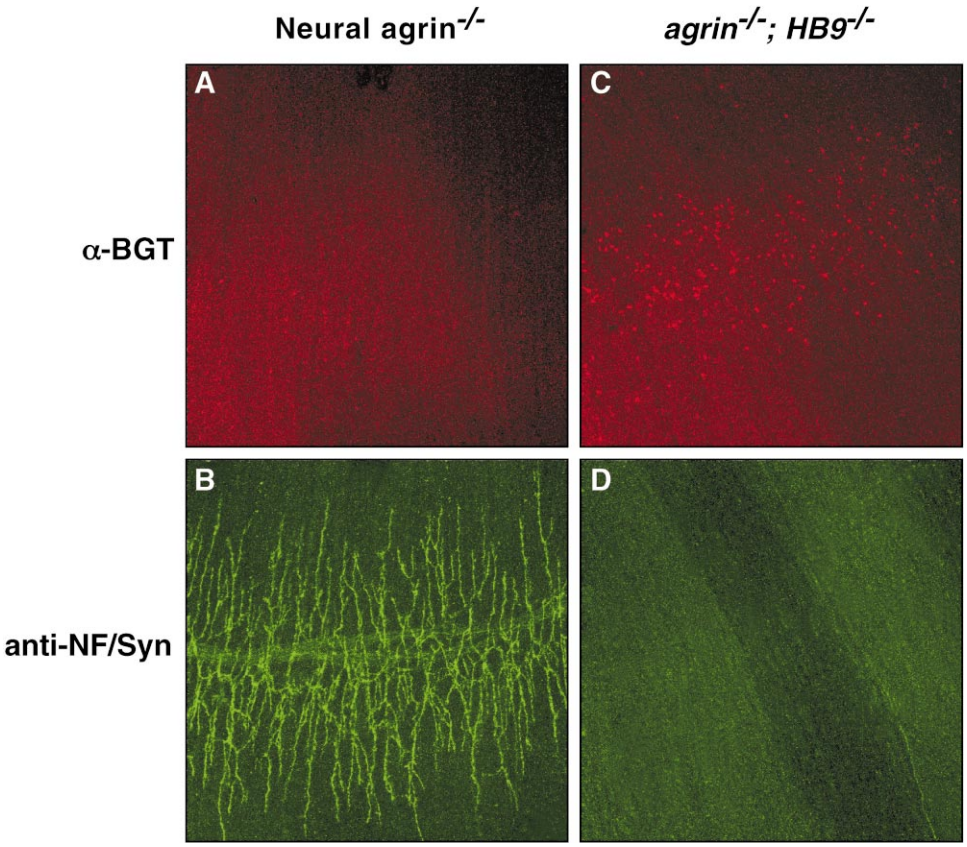

Figure 5. AChRs Are Clustered in the Central Region of E18.5 Muscle Lacking both Neural Agrin and Motor Axons

Whole mounts of diaphragm muscle from E18.5 neural *agrin* mutant (A and B) or neural *agrin*; *HB9* double-mutant (C and D) embryos were stained with Texas red-α-BGT and antibodies to NF and Syn.

transcription was uniform in muscle from *top 2*β; *MuSK* and *HB9*; *MuSK* double-mutant mice (Figure 4), indicating that MuSK is required, directly or indirectly, to regulate the pattern of *AChR* transcription in muscle lacking motor axons. Thus, even in the absence of motor innervation, MuSK has a critical role in patterning *AChR* transcription. Our results also indicate that the uniform pattern of *AChR* expression observed in *MuSK* mutant mice is unlikely to be caused by the broad and uniform release of acetylcholine (or other neural signals) from the widely distributed ectopic motor axons evident in these mutants.

A NRG-1-Independent Neural Signal Refines the Muscle-Autonomous AChR Pattern

Even though the results described above provide evidence for a prepattern of *AChR* gene transcription and AChR clusters, the spatial features of AChR expression were distinct from those observed normally at developing synapses. In wild-type, innervated embryos examined at E18.5, the band of AChR clusters was 7% ± 4% of muscle length, and the band of *AChR* mRNA was 8% ± 3% of muscle length (Figures 1 and 4). In contrast, in *HB9* mutant mice, the band of AChR clusters and *AChR* transcription was markedly wider (AChR clusters were 17% ± 5% of muscle length and *AChR* mRNA was 16% ± 5% of muscle length) (Figures 1 and 4). These results raise the possibility that signals from motor neurons normally restrict and refine the muscle-autonomous pattern of *AChR* mRNA and AChR protein to a narrower region of the muscle. Moreover, because the zones of *AChR* mRNA expression and AChR clustering are similar in width, we infer that the domain of *AChR* transcription is likely to define the pattern of AChR clusters found in the absence of innervation.

How might the pattern of *AChR* transcription be refined? One possibility is that nerve-induced electrical activity in muscle reduces *AChR* transcription throughout the myofiber, and this repressive effective is counteracted by the ability of neuronal NRG-1 to stimulate *AChR* transcription locally in prospective synaptic nuclei. We therefore examined whether neuronal NRG-1 is required to promote the mature localized pattern of *AChR* transcription in skeletal muscle. Mice lacking NRG-1 die from defects in cardiac development at E10.5, several days prior to neuromuscular synapse formation (Meyer and Birchmeier, 1995). To circumvent this early lethality, we inactivated NRG-1 selectively in motor and sensory neurons, leaving muscle NRG-1 expression intact. To achieve this, we crossed *Isl1*^{cre} mice, which express Cre recombinase selectively in motor and sensory neurons (Srinivas et al., 2001) (Supplementary Figure S1 [http://www.neuron.org/cgi/content/full/30/2/399/DC1]), with mice containing floxed and null alleles of *nrg-1*.

Isl1$^{cre/+}$; *nrg-1*$^{flox/-}$ embryos were recovered at ex-

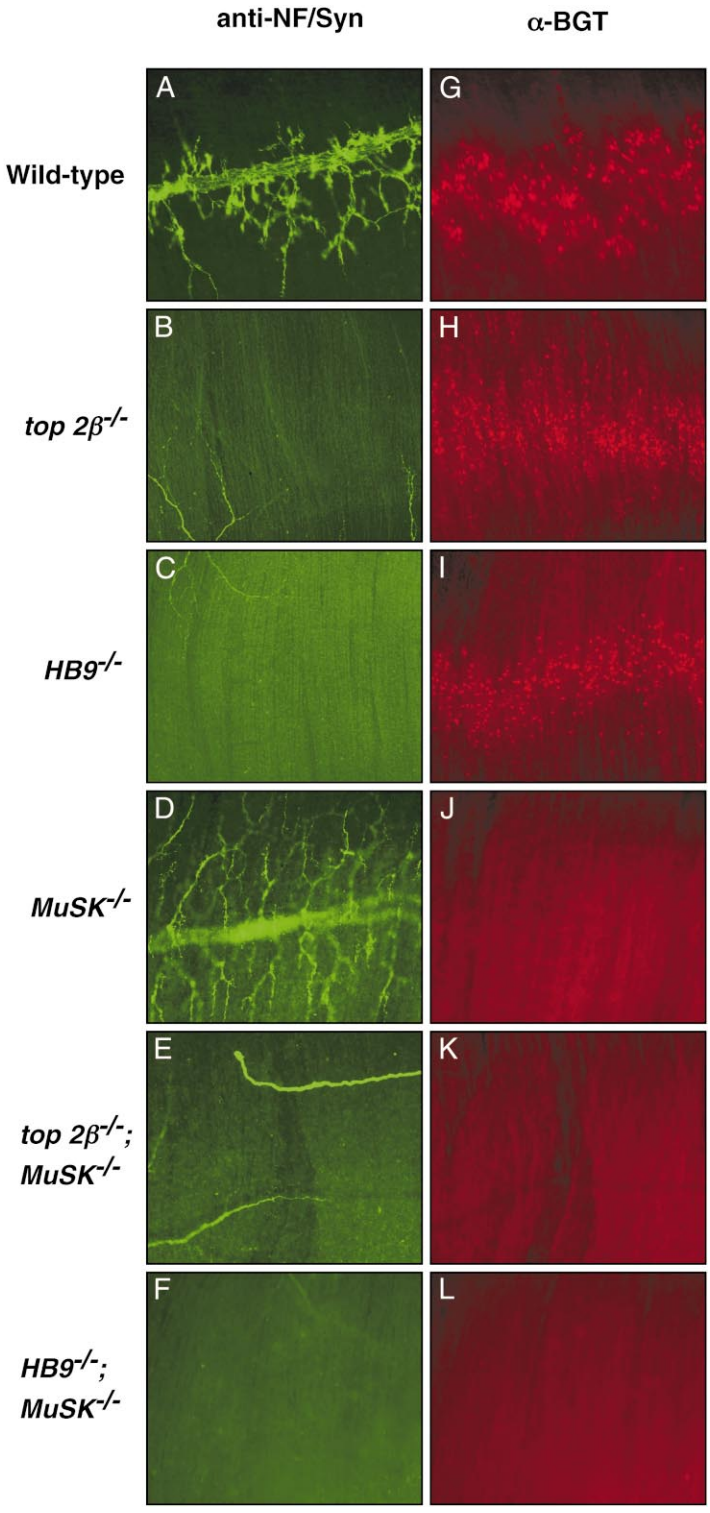

Figure 6. MuSK Is Required to Cluster AChRs in Muscle Lacking Motor Axons

Whole mounts of diaphragm muscle from E18.5 wild-type, *top* 2β mutant, *HB9* mutant, *MuSK* mutant, *HB9*; *MuSK* double-mutant, and *top* 2β; *MuSK* double-mutant embryos were stained with Texas red-α-BGT and antibodies to NF and Syn. AChRs are clustered at synaptic sites in muscle from wild-type embryos and in the central region of muscle from *HB9* and *top* 2β mutant embryos. AChRs are not clustered in muscle from *MuSK* mutant, *HB9*; *MuSK* double-mutant or *top* 2β; *MuSK* double-mutant embryos.

pected frequencies at E18.5, indicating that inactivation of *nrg-1* selectively in motor and sensory neurons circumvents the early embryonic lethality of constitutive *nrg-1* mutant mice. Nonetheless, most (~90%) neuronal *nrg-1* mutant mice died at birth with a phenotype identical to that observed in mice lacking NRG-1 signaling in Schwann cells (Morris et al., 1999; Riethmacher et al., 1997; Woldeyesus et al., 1999) (Figure 7) (Experimental Procedures). This result provides strong evidence that NRG-1 expression is markedly reduced or absent from motor and sensory neurons.

Strikingly, we found that the pattern of *AChR* α and δ subunit transcription was normal in neuronal *nrg-1* mutant embryos (Figure 7C). Importantly, the band of *AChR* mRNA was as narrow in neuronal *nrg-1* mutant embryos as in wild-type embryos (Figure 7C). These

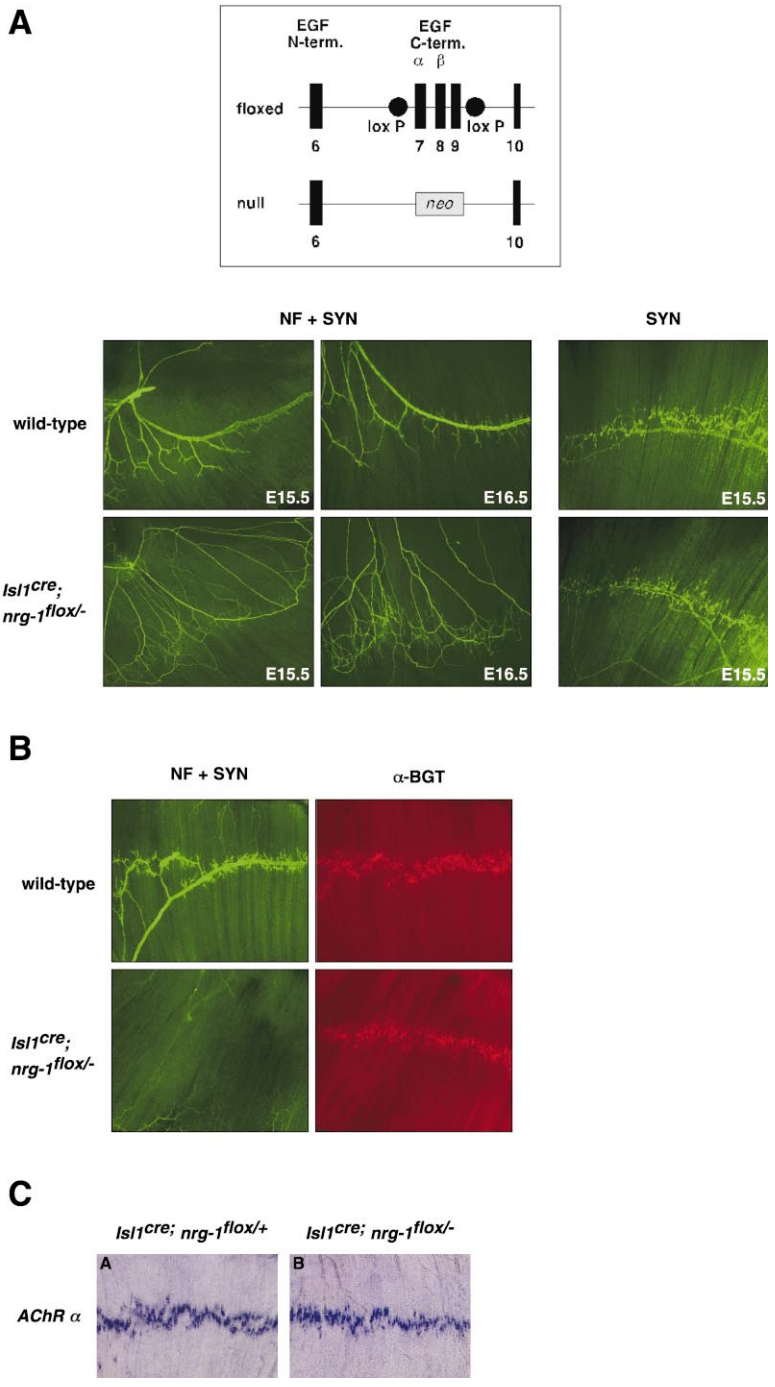

Figure 7. Neuronal NRG-1 Is Not Required for Synapse-Specific Transcription

We inactivated the *nrg-1* gene selectively in motor and sensory neurons by crossing *Isl1*$^{cre/+}$ mice with mice containing floxed and null alleles of *nrg-1* (A). The floxed allele of the *nrg-1* gene contains one loxP site in intron 6 and a second loxP site in intron 9; the null allele lacks exons 7, 8, and 9.

(A) In E15.5–E16.5 neuronal *nrg-1* mutant embryos, motor axons, stained with antibodies to neurofilaments (NF), are highly defasciculated but form terminal arbors, stained with antibodies to SYN, that are correctly positioned in the middle of the muscle.

(B) By E18.5, motor axons and terminals are absent from neuronal *nrg-1* mutant embryos, but AChRs remain clustered in the middle of the muscle.

(C) AChR transcription is patterned normally in muscle from *Isl1*^{cre}; *nrg-1*$^{flox/-}$ muscle. Whole mounts of diaphragm muscle from *Isl1*^{cre}; *nrg-1*$^{flox/+}$ control embryos and *Isl1*^{cre}; *nrg-1*$^{flox/-}$ embryos were processed for in situ hybridization using a probe from the *AChR* α subunit gene.

findings suggest that neuronal NRG-1 is not required to refine the domain of *AChR* subunit gene expression during development. They also imply that neuronal NRG-1 is not required for synapse-specific *AChR* gene transcription.

Discussion

AChR genes are expressed and AChRs are clustered selectively at synaptic sites in skeletal muscle. Prevailing views suggest that AChR expression, and other features of muscle patterning, are established by signals derived from motor neurons as they contact developing muscle. Our results provide several lines of evidence that the pattern of *AChR* transcription and AChR protein is restricted to the central region of developing muscle in the absence of motor neurons and their axons. Thus, certain features of the early patterning of skeletal muscle are established in a manner independent of neurally derived signals.

Taken together, our findings provide insight into the sequential steps involved in establishing the pattern of AChR expression on developing skeletal muscle fibers (Figure 8). An initial spatially restricted pattern of *AChR* gene expression and AChR clusters is clearly generated in muscle prior to motor innervation and is achieved

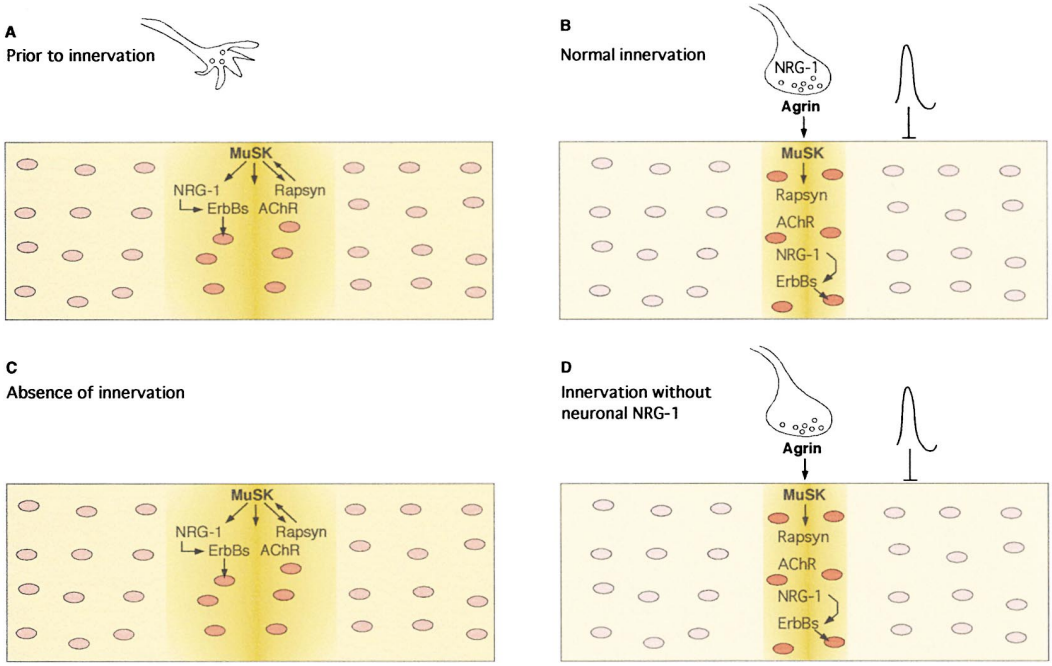

Figure 8. Model for Prepatterning and Refining AChR Expression in Skeletal Muscle

(A) MuSK is required for patterning AChR expression, suggesting that MuSK is clustered and activated, possibly by Rapsyn, in the central region of the muscle. We speculate that activated MuSK clusters ErbBs and muscle-derived NRG-1, thereby establishing an autocrine signaling pathway that stimulates *AChR* transcription.

(B) Innervation refines the prepattern by restricting AChR clusters and *AChR* transcription to synaptic sites. This refinement may depend on neural Agrin, which enhances *AChR* transcription and clustering at synaptic sites, and ACh-induced electrical activity, which represses *AChR* transcription and clustering in nonsynaptic regions.

(C) In the absence of innervation (e.g., in *top 2*β, *HB9*, or *HB9*^{cre}; *Isl2*^{DTA} mutant mice), the pattern of *AChR* transcription and clustering persists in a zone in the central region of the muscle.

(D) Our experiments indicate that the refinement and restriction of *AChR* transcription to synaptic nuclei does not require neuronal NRG-1.

in a manner independent of neurally derived Agrin, or indeed of any other neural signal. Nevertheless, the steps involved in the emergence of this muscle AChR prepattern appear to co-opt at least some of the same molecules used during normal synaptogenesis, since the AChR prepattern is dependent on MuSK expression. Arrival of the nerve does, however, have a critical role in converting the AChR prepattern into the more refined pattern of *AChR* transcription and AChR clustering characteristic of mature synapses. The arrival of the nerve triggers two separable molecular programs of muscle differentiation. One program appears to utilize neurally derived Agrin to counteract a latent nerve-evoked dissolution of the AChR prepattern and thus ensures the stable expression of AChR clusters at nascent synapses. A second program involves a neural signal that refines the pattern of *AChR* gene transcription, ensuring focal expression of *AChR* genes in subsynaptic nuclei. The neural signal responsible for this refinement program has not yet been defined but appears not to depend on neuronal NRG-1 activity.

Below, we discuss some of the implications of this developmental scenario: first, the background to the idea that muscle can be patterned independent of motor innervation; second, the relative contributions of Agrin, MuSK, and NRG-1 signaling to muscle patterning; third, the possible role of muscle prepatterning in regulating the pattern of innervation; and fourth, how muscle prepatterning might be achieved.

Muscle AChR Patterning Is Independent of Innervation

Although muscle prepatterning has not been incorporated into conventional views of synapse formation, the possibility that muscle AChRs are patterned in the absence of motor innervation has been examined previously by Harris and colleagues, who attempted to study muscle development in the absence of innervation, through elimination of motor axons with the neurotoxin β-bungarotoxin, (Braithwaite and Harris, 1979). In β-bungarotoxin-treated rat embryos, AChRs were clustered in the central region of muscle, despite the absence of motor axons. Although these experiments suggested that muscle might be patterned in the absence of innervation (Harris, 1981), this interpretation was complicated by the difficulty in establishing that β-bungarotoxin exposure completely eliminated motor axons before they had contacted developing muscle fibers. The present studies, together with previous findings in *top 2*β mutant mice (Yang et al., 2000), strongly reinforce the idea that *AChR* gene expression and AChR clusters are patterned in developing skeletal muscle in the complete absence of motor axons.

The Contribution of Agrin and MuSK Signaling to AChR Patterning in Muscle

Are the mechanisms used to establish the muscle prepattern similar to those involved in promoting synaptic differentiation? Our results show that MuSK is required

to pattern AChRs and *AChR* transcription in muscle lacking motor axons. Thus, this aspect of the pathway involved in patterning AChR expression in the absence of motor axons appears to be similar to that used for synaptic differentiation. This role for MuSK, however, is not dependent on neural Agrin, since AChR expression is patterned in mutant embryos in which motor axons never invade the muscle, and in mutant embryos lacking both neural Agrin and motor axons.

Non-neural Agrin, when presented on the cell surface, can stimulate clustering of AChRs in cultured muscle cells (Ferns et al., 1992, 1993), and thus it remains possible that non-neural Agrin, supplied by the muscle, is involved in patterning AChRs in the absence of innervation. However, levels of muscle Agrin are markedly reduced in the *agrin* mutants we studied (Gautam et al., 1996), and thus we favor the idea that MuSK is activated in developing muscle in a manner independent of Agrin. Consistent with this view, MuSK can be activated independently of Agrin by its overexpression or by coexpression with Rapsyn, an intracellular peripheral membrane protein that clusters MuSK (Apel et al., 1997; Gillespie et al., 1996; Watty et al., 2000). Thus, a high level of MuSK and/or Rapsyn expression in the central region of developing muscle could, in principle, activate MuSK, independent of Agrin, and thus pattern *AChR* transcription and AChRs (Figure 8).

Neural Refinement of AChR Prepattern

The neural signals that regulate synapse-specific transcription in muscle remain elusive (Morris et al., 1999; Riethmacher et al., 1997; Woldeyesus et al., 1999; Wolpowitz et al., 2000). Here, we show that *AChR* genes are expressed preferentially in the central region of muscle in the absence of motor axons and its attendant signals. These results show that motor neuron-derived signals are not required to pattern *AChR* transcription in developing muscle. But because the zone of enhanced transcription is narrower in muscle from wild-type mice than in muscle lacking motor axons, neural signals appear to be required to refine the initial muscle-autonomous pattern of *AChR* transcription (Figure 8). NRG-1 has been considered a strong candidate for the neuronal signal that induces synapse-specific transcription (Falls et al., 1993; Carraway and Burden, 1995; Fischbach and Rosen, 1997). In principle, two possible mechanisms, each involving NRG-1 signaling, might refine the pattern of *AChR* expression. First, neuronal NRG-1 might stimulate *AChR* transcription locally in synaptic nuclei, while nerve-induced electrical activity reduces *AChR* transcription throughout the myofiber. Second, NRG-1 might activate *AChR* transcription in the synaptic region and induced synaptic nuclei may signal laterally to perisynaptic nuclei and repress *AChR* expression. Each model predicts that *AChR* transcription would not be appropriately patterned in the absence of neuronal NRG-1.

However, the pattern of *AChR* transcription in neuronal *nrg-1* mutants is indistinguishable from that of wild-type mice, indicating that neuronal NRG-1 is not required for the refinement of the AChR prepattern, or for synapse-specific transcription. We have not excluded that a very low level of neuronal NRG-1 expression, insufficient to support survival of Schwann cells, but sufficient to activate synapse-specific transcription, is initiated prior to Cre-mediated inactivation of *nrg-1*. Our results, however, are most easily explained by the idea that neural signals other than NRG-1 refine the pattern of *AChR* transcription in skeletal muscle (Figure 8D). Our experiments also leave open the possibility that muscle-derived NRG-1 may be required for synapse-specific transcription. We note that neural Agrin can cluster muscle-derived NRG-1 (Jones et al., 1997; Meier et al., 1998; Rimer et al., 1998), and thus, it is possible that neural Agrin is responsible for refining the muscle-autonomous pattern of *AChR* transcription, as well as AChR clustering, by defining the limits of NRG-1 and NRG-1 receptor expression and thus restricting an autocrine NRG-1 signaling pathway to nascent synaptic sites in muscle (Burden and Yarden, 1997) (Figures 8B and 8D).

In addition, we emphasize that we have not analyzed expression of the *AChR* ϵ subunit gene, which is activated later than other *AChR* subunit genes and is induced to a greater extent by NRG-1 in cultured muscle cells (Martinou et al., 1991). It remains possible therefore that neuronal NRG-1 is involved in activation of the *AChR* ϵ subunit gene, or in maintaining *AChR* gene expression in synaptic nuclei. Such an action of NRG-1 might provide a basis for the finding that adult mice heterozygous for the Ig isoform of NRG-1 express fewer AChRs at neuromuscular synapses (Sandrock et al., 1997).

Functional Relevance of a Muscle Prepattern

What is the significance of the AChR prepattern in muscle? AChRs can cluster "spontaneously" in noninnervated myotubes in cell culture, indicating that aspects of postsynaptic differentiation can occur in the absence of presynaptic input (Fischbach and Cohen, 1973; Sytkowski et al., 1973). These preexisting AChR clusters are distributed randomly on cultured myotubes and do not preconfigure presumptive synaptic sites (Anderson and Cohen, 1977; Frank and Fischbach, 1979), leading to the idea that motor axons do not form synapses with muscle cells at predetermined sites. In vivo, motor axons typically terminate and create a synaptic zone in the central region of the muscle. This arrangement of synapses has been proposed to arise from random contact between motor axons and developing myotubes, followed by extensive growth of muscle (Bennett and Pettigrew, 1974; Burden, 1998; Dennis, 1981; Hall and Sanes, 1993). This view, however, does not readily explain the observation that in neuronal *nrg-1* mutant embryos, which exhibit exuberant growth of motor axons, synaptic terminals are still located normally in the central region of the muscle (Figure 7A).

The demonstration of regional differences in AChR expression within muscle thus raises the possibility of other prepatterned aspects of muscle cell differentiation that may have a role in defining sites of motor axon termination and synapse formation (Figure 8A). In this context, it is noteworthy that prospective synaptic sites on *Drosophila* muscle fibers are thought to be marked in a manner independent of innervation and to direct the site of axon termination and synapse formation (Broadie and Bate, 1993).

Finally, it remains unclear how mammalian muscle is patterned in the absence of innervation. In *Drosophila*,

muscle patterning is dependent on a population of specialized founder cells that have an early role in muscle development, and are thought to regulate the targeting of motor axons (Dohrmann et al., 1990; Knirr et al., 1999; Landgraf et al., 1999; Williams et al., 1991). One intriguing possibility is that mammalian muscle fibers also contain founder cells that establish a pattern of AChRs and other aspects of muscle differentiation in an innervation-independent manner, thus providing preexisting spatial cues that could be recognized by incoming motor axons.

Experimental Procedures

Immunohistochemistry

Diaphragm muscles were dissected from embryos, fixed in 1% formaldehyde in 0.1 M sodium phosphate, pH 7.3 at 4°C for several hours, rinsed briefly in PBS, washed twice for 10 min in PBS, washed for 15 min in 0.1 M glycine in PBS, washed for 5 min in PBS, permeabilized for 5 min with 0.5% Triton X-100 in PBS (PBT), and incubated overnight at 4°C with rabbit antibodies to the 150 kDa subunit of Neurofilaments (Chemicon) and Synaptophysin (Zymed) in PBT with 2% BSA. The muscles were subsequently rinsed in PBT, washed three times for 1 hr in PBT, incubated with fluorescein-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch) and Texas red-conjugated α-bungarotoxin (Molecular Probes) in PBT overnight at 4°C, washed three times for 1 hr with PBT, washed once for 5 min in PBS, post-fixed for 10 min in 1% formaldehyde, further fixed in 100% methanol at −20°C, mounted in Vectashield (Vector Labs), and viewed with filters selective for either Texas red or fluorescein. Images were captured on a CCD camera (Princeton Instruments).

In Situ Hybridization

Diaphragm muscles were fixed in 4% formaldehyde, dehydrated in methanol, digested with Proteinase K, probed with a digoxigenin-labeled riboprobe transcribed from either an *AChR* α (DeChiara et al., 1996) or δ subunit cDNA (Simon et al., 1992), and processed as described elsewhere (Wilkinson, 1992).

Mutant Mice

A floxed allele of the *nrg-1* gene, containing loxP sites flanking exons 7, 8, and 9, was generated by homologous recombination in ES cells. *nrg-1* mutant mice, lacking exons 7, 8, and 9, have been described previously (Meyer and Birchmeier, 1995). *HB9* mutant mice were generated by introducing an IRES-Cre cassette into the *HB9* locus using homologous recombination in ES cells with an analogous strategy to the one described previously (Arber et al., 1999). The phenotype of $HB9^{cre}$ mutant mice is indistinguishable from that of *HB9* mutant mice (Arber et al., 1999). The construction of $Isl1^{cre}$ mice have been described elsewhere (Srinivas et al., 2001); the pattern of Cre expression was assessed by crossing these mice with lacZ reporter mice (Supplementary Figure S1 [http://www.neuron.org/cgi/content/full/30/2/399/DC1]). $Isl2^{DTA}$ mice were generated by inserting an *IRES-loxP-neo-loxP-DTA* cassette (Lee et al., 2000) into the 3′ UTR of the *Isl2* locus (Brown et al., 2000) (S.A. et al., unpublished data). Mice lacking neural Agrin (Gautam et al., 1996) and mice lacking MuSK (DeChiara et al., 1996) were kindly supplied by Fabio Rupp (Johns Hopkins Medical School) and George Yancopoulos (Regeneron Pharmaceuticals), respectively. Mice were maintained on a hybrid C57BL/6J and 129S7/SvEvBrd background.

Neuronal *nrg-1* Mutant Mice

Mice lacking neuronal *nrg-1* would be expected to phenocopy mice lacking ErbB receptors in Schwann cells (Garratt et al., 2000; Morris et al., 1999; Riethmacher et al., 1997; Woldeyesus et al., 1999). Such mutant embryos lack peripheral Schwann cells, leading to defasiculation and ultimately withdrawal of intramuscular axons (Lin et al., 2000; Morris et al., 1999; Riethmacher et al., 1997; Woldeyesus et al., 1999). Consequently, these mutant mice die at birth.

Several lines of evidence indicate that in $Isl1^{cre/+}$; $nrg\text{-}1^{flox/-}$ embryos lack neuronal NRG-1. First, motor and sensory neurons in neuronal *nrg-1* mutant embryos, unlike in wild-type embryos, are not stained by antibodies to NRG-1 (data not shown). Second, like *erbB* mutant embryos, Schwann cells are absent from intramuscular motor axons in neuronal *nrg-1* mutant embryos (E13.5–E16.5), and as a consequence, motor axons are defasiculated and disorganized, resulting in exuberant axon growth throughout the muscle (Figure 7A). Third, as in *erbB* mutant embryos, despite the ectopic location of motor axons in developing muscle, Syn-stained nerve terminals (Figure 7A) and clusters of AChRs (Figure 7B) are located normally in the central region of the muscle. Fourth, like *erbB* mutant embryos, these motor axons largely retract by birth (Figure 7B). Thus, the motor nerve phenotype of neuronal *nrg-1* mutant mice mimics that of mice lacking ErbB signaling in Schwann cells, indicating that NRG-1 is entirely, or largely, lacking from motor and sensory neurons in $Isl1^{cre/+}$; $nrg\text{-}1^{flox/-}$ mice.

Acknowledgments

We thank Dr. Xiang-qing Li, Ekaterina Avetisova, Barbara Han, and Monica Mendelsohn for expert technical assistance, and Gerry Fischbach and Ruth Lehmann for their thoughtful comments on the manuscript. This work was supported by a postdoctoral fellowship from the NIH to X.Y., by grants from the NIH to S.J.B. (NS27963 and NS36193), and grants to T.M.J. from NINDS and Project ALS. T.M.J. is an Investigator of the Howard Hughes Medical Institute. S.A. is supported by a grant from the Swiss National Science Foundation.

Received March 15, 2001; revised April 23, 2001.

References

Anderson, M.J., and Cohen, M.W. (1977). Nerve-induced and spontaneous redistribution of acetylcholine receptors on cultured muscle cells. J. Physiol. Lond. *268*, 757–773.

Apel, E.D., Glass, D.J., Moscoso, L.M., Yancopoulos, G.D., and Sanes, J.R. (1997). Rapsyn is required for MuSK signaling and recruits synaptic components to a MuSK-containing scaffold. Neuron *18*, 623–635.

Arber, S., Han, B., Mendelsohn, M., Smith, M., Jessell, T.M., and Sockanathan, S. (1999). Requirement for the homeobox gene Hb9 in the consolidation of motor neuron identity. Neuron *23*, 659–674.

Bennett, M.R., and Pettigrew, A.G. (1974). The formation of synapses in striated muscle during development. J. Physiol. Lond. *241*, 515–545.

Braithwaite, A.W., and Harris, A.J. (1979). Neural influence on acetylcholine receptor clusters in embryonic development of skeletal muscles. Nature *279*, 549–551.

Broadie, K., and Bate, M. (1993). Innervation directs receptor synthesis and localization in Drosophila embryo synaptogenesis. Nature *361*, 350–353.

Brown, A., Yates, P.A., Burrola, P., Ortuno, D., Vaidya, A., Jessell, T.M., Pfaff, S.L., O'Leary, D.D., and Lemke, G. (2000). Topographic mapping from the retina to the midbrain is controlled by relative but not absolute levels of EphA receptor signaling. Cell *102*, 77–88.

Burden, S.J. (1998). The formation of neuromuscular synapses. Genes Dev. *12*, 133–148.

Burden, S., and Yarden, Y. (1997). Neuregulins and their receptors: a versatile signaling module in organogenesis and oncogenesis. Neuron *18*, 847–855.

Carraway, K.L., III, and Burden, S.J. (1995). Neuregulins and their receptors. Curr. Opin. Neurobiol. *5*, 606–612.

Cohen, M.W., Moody-Corbett, F., and Godfrey, E.W. (1994). Neuritic deposition of agrin on culture substrate: implications for nerve-muscle synaptogenesis. J. Neurosci. *14*, 3293–3303.

DeChiara, T.M., Bowen, D.C., Valenzuela, D.M., Simmons, M.V., Poueymirou, W.T., Thomas, S., Kinetz, E., Compton, D.L., Rojas, E., Park, J.S., et al. (1996). The receptor tyrosine kinase MuSK is required for neuromuscular junction formation in vivo. Cell *85*, 501–512.

Dennis, M.J. (1981). Development of the neuromuscular junction: inductive interactions between cells. Annu. Rev. Neurosci. *4*, 43–68.

Dohrmann, C., Azpiazu, N., and Frasch, M. (1990). A new Drosophila homeo box gene is expressed in mesodermal precursor cells of distinct muscles during embryogenesis. Genes Dev. *4*, 2098–2111.

Falls, D.L., Rosen, K.M., Corfas, G., Lane, W.S., and Fischbach, G.D. (1993). ARIA, a protein that stimulates acetylcholine receptor synthesis, is a member of the neu ligand family. Cell *72*, 801–815.

Ferns, M., Hoch, W., Campanelli, J.T., Rupp, F., Hall, Z.W., and Scheller, R.H. (1992). RNA splicing regulates agrin-mediated acetylcholine receptor clustering activity on cultured myotubes. Neuron *8*, 1079–1086.

Ferns, M.J., Campanelli, J.T., Hoch, W., Scheller, R.H., and Hall, Z. (1993). The ability of agrin to cluster AChRs depends on alternative splicing and on cell surface proteoglycans. Neuron *11*, 491–502.

Fischbach, G.D., and Cohen, S.A. (1973). The distribution of acetylcholine sensitivity over uninnervated and innervated muscle fibers grown in cell culture. Dev. Biol. *31*, 147–162.

Fischbach, G.D., and Rosen, K.M. (1997). ARIA: a neuromuscular junction neuregulin. Annu. Rev. Neurosci. *20*, 429–458.

Frank, E., and Fischbach, G.D. (1979). Early events in neuromuscular junction formation in vitro: induction of acetylcholine receptor clusters in the postsynaptic membrane and morphology of newly formed synapses. J. Cell Biol. *83*, 143–158.

Garratt, A.N., Voiculescu, O., Topilko, P., Charnay, P., and Birchmeier, C. (2000). A dual role of erbB2 in myelination and in expansion of the schwann cell precursor pool. J. Cell Biol. *148*, 1035–1046.

Gautam, M., Noakes, P.G., Moscoso, L., Rupp, F., Scheller, R.H., Merlie, J.P., and Sanes, J.R. (1996). Defective neuromuscular synaptogenesis in agrin-deficient mutant mice. Cell *85*, 525–535.

Gillespie, S.K., Balasubramanian, S., Fung, E.T., and Huganir, R.L. (1996). Rapsyn clusters and activates the synapse-specific receptor tyrosine kinase MuSK. Neuron *16*, 953–962.

Glass, D.J., and Yancopoulos, G.D. (1997). Sequential roles of agrin, MuSK and rapsyn during neuromuscular junction formation. Curr. Opin. Neurobiol. *7*, 379–384.

Goldman, D., and Staple, J. (1989). Spatial and temporal expression of acetylcholine receptor RNAs in innervated and denervated rat soleus muscle. Neuron *3*, 219–228.

Hall, Z.W., and Sanes, J.R. (1993). Synaptic structure and development: the neuromuscular junction. Cell Suppl. *72*, 99–121.

Harris, A.J. (1981). Embryonic growth and innervation of rat skeletal muscles. III. Neural regulation of junctional and extra-junctional acetylcholine receptor clusters. Phil. Trans. R. Soc. Lond. B Biol. Sci. *293*, 287–314.

Jones, G., Meier, T., Lichtsteiner, M., Witzemann, V., Sakmann, B., and Brenner, H.R. (1997). Induction by agrin of ectopic and functional postsynaptic-like membrane in innervated muscle. Proc. Natl. Acad. Sci. USA *94*, 2654–2659.

Knirr, S., Azpiazu, N., and Frasch, M. (1999). The role of the NK-homeobox gene slouch (S59) in somatic muscle patterning. Development *126*, 4525–4535.

Landgraf, M., Baylies, M., and Bate, M. (1999). Muscle founder cells regulate defasciculation and targeting of motor axons in the Drosophila embryo. Curr. Biol. *9*, 589–592.

Laufer, R., and Changeux, J.P. (1989). Activity-dependent regulation of gene expression in muscle and neuronal cells. Mol. Neurobiol. *3*, 1–53.

Lee, K.J., Dietrich, P., and Jessell, T.M. (2000). Genetic ablation reveals that the roof plate is essential for dorsal interneuron specification. Nature *403*, 734–740.

Lin, W., Sanchez, H.B., Deerinck, T., Morris, J.K., Ellisman, M., and Lee, K.F. (2000). Aberrant development of motor axons and neuromuscular synapses in erbB2- deficient mice. Proc. Natl. Acad. Sci. USA *97*, 1299–1304.

Ma, E., Morgan, R., and Godfrey, E.W. (1995). Agrin mRNA variants are differentially regulated in developing chick embryo spinal cord and sensory ganglia. J. Neurobiol. *26*, 585–597.

Martinou, J.C., Falls, D.L., Fischbach, G.D., and Merlie, J.P. (1991). Acetylcholine receptor-inducing activity stimulates expression of the epsilon-subunit gene of the muscle acetylcholine receptor. Proc. Natl. Acad. Sci. USA *88*, 7667–7673.

McMahan, U.J. (1990). The agrin hypothesis. Cold Spring Harb. Symp. Quant. Biol. *55*, 407–418.

Meier, T., Masciulli, F., Moore, C., Schoumacher, F., Eppenberger, U., Denzer, A.J., Jones, G., and Brenner, H.R. (1998). Agrin can mediate acetylcholine receptor gene expression in muscle by aggregation of muscle-derived neuregulins. J. Cell Biol. *141*, 715–726.

Meyer, D., and Birchmeier, C. (1995). Multiple essential functions of neuregulin in development. Nature *378*, 386–390.

Morris, J.K., Lin, W., Hauser, C., Marchuk, Y., Getman, D., and Lee, K.F. (1999). Rescue of the cardiac defect in ErbB2 mutant mice reveals essential roles of ErbB2 in peripheral nervous system development. Neuron *23*, 273–283.

Riethmacher, D., Sonnenberg-Riethmacher, E., Brinkmann, V., Yamaai, T., Lewin, G.R., and Birchmeier, C. (1997). Severe neuropathies in mice with targeted mutations in the ErbB3 receptor. Nature *389*, 725–730.

Rimer, M., Cohen, I., Lomo, T., Burden, S.J., and McMahan, U.J. (1998). Neuregulins and erbB receptors at neuromuscular junctions and at agrin- induced postsynaptic-like apparatus in skeletal muscle. Mol. Cell. Neurosci. *12*, 1–15.

Sandrock, A.W., Jr., Dryer, S.E., Rosen, K.M., Gozani, S.N., Kramer, R., Theill, L.E., and Fischbach, G.D. (1997). Maintenance of acetylcholine receptor number by neuregulins at the neuromuscular junction in vivo. Science *276*, 599–603.

Sanes, J.R., and Lichtman, J.W. (1999). Development of the vertebrate neuromuscular junction. Annu. Rev. Neurosci. *22*, 389–442.

Simon, A.M., Hoppe, P., and Burden, S.J. (1992). Spatial restriction of AChR gene expression to subsynaptic nuclei. Development *114*, 545–553.

Srinivas, S., Watanabe, T., Lin, C.S., William, C.M., Tanabe, Y., Jessell, T.M., and Costantini, F. (2001). Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. BMC Dev. Biol. *1*, 4.

Sytkowski, A.J., Vogel, Z., and Nirenberg, M.W. (1973). Development of acetylcholine receptor clusters on cultured muscle cells. Proc. Natl. Acad. Sci. USA *70*, 270–274.

Thaler, J., Harrison, K., Sharma, K., Lettieri, K., Kehrl, J., and Pfaff, S.L. (1999). Active suppression of interneuron programs within developing motor neurons revealed by analysis of homeodomain factor HB9. Neuron *23*, 675–687.

Wallace, B.G. (1991). The mechanism of agrin-induced acetylcholine receptor aggregation. Phil. Trans. R. Soc. Lond. B Biol. Sci. *331*, 273–280.

Watty, A., Neubauer, G., Dreger, M., Zimmer, M., Wilm, M., and Burden, S.J. (2000). The in vitro and in vivo phosphotyrosine map of activated MuSK. Proc. Natl. Acad. Sci. USA *97*, 4585–4590.

Wilkinson, D.G. (1992). In Situ Hybridization: A Practical Approach (Oxford, UK: Oxford University Press. IRL Press).

Williams, J.A., Bell, J.B., and Carroll, S.B. (1991). Control of Drosophila wing and haltere development by the nuclear vestigial gene product. Genes Dev. *5*, 2481–2495.

Woldeyesus, M.T., Britsch, S., Riethmacher, D., Xu, L., Sonnenberg-Riethmacher, E., Abou-Rebyeh, F., Harvey, R., Caroni, P., and Birchmeier, C. (1999). Peripheral nervous system defects in erbB2 mutants following genetic rescue of heart development. Genes Dev. *13*, 2538–2548.

Wolpowitz, D., Mason, T.B., Dietrich, P., Mendelsohn, M., Talmage, D.A., and Role, L.W. (2000). Cysteine-rich domain isoforms of the neuregulin-1 gene are required for maintenance of peripheral synapses. Neuron *25*, 79–91.

Yang, X., Li, W., Prescott, E.D., Burden, S.J., and Wang, J.C. (2000). DNA topoisomerase IIbeta and neural development. Science *287*, 131–134.