MuSK induces in vivo acetylcholine receptor clusters in a ligand-independent manner

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Muscle-specific receptor tyrosine kinase (MuSK) is required for the formation of the neuromuscular junction. Using direct gene transfer into single fibers, MuSK was expressed extrasynaptically in innervated rat muscle in vivo to identify its contribution to synapse formation. Spontaneous MuSK kinase activity leads, in the absence of its putative ligand neural agrin, to the appearance of ε-subunit–specific transcripts, the formation of acetylcholine receptor clusters, and acetylcholinesterase aggregates. Expression of kinase-inactive MuSK did not result in the formation of acetylcholine receptor (AChR) clusters, whereas a mutant MuSK lacking the ectodomain did induce AChR clusters. The contribution of endogenous MuSK was excluded by using genetically altered mice, where the kinase domain of the MuSK gene was flanked by loxP sequences and could be deleted upon expression of Cre recombinase. This allowed the conditional inactivation of endogenous MuSK in single muscle fibers and prevented the induction of ectopic AChR clusters. Thus, the kinase activity of MuSK initiates signals that are sufficient to induce the formation of AChR clusters. This process does not require additional determinants located in the ectodomain.

Introduction

The establishment of the neuromuscular junction involves the accumulation of acetylcholine receptor (AChR)* at the postsynaptic membrane (Burden, 1998; Sanes and Lichtman, 1999), and requires nerve terminal–secreted agrin (McMahan, 1990; Gautam et al., 1996). Recent experiments using in vivo transfection suggested that neural agrin is sufficient to induce on muscle fiber membranes changes which resemble postsynaptic specializations (Cohen et al., 1997; Jones et al., 1997; Meier et al., 1997; Rimer et al., 1997). The agrin-induced receptor accumulation requires the muscle-specific receptor tyrosine kinase (MuSK) (Valenzuela et al., 1995; DeChiara et al., 1996). According to the present view, agrin activates MuSK, which initiates an as yet unresolved complex signal transduction cascade and serves as a primary scaffolding element for the establishment of the postsynaptic membrane (Glass et al., 1996, 1997; Apel et al., 1997). Attempts to identify distinct domains of MuSK that interact with other synaptic components have highlighted the importance of the kinase activity for the induction of AChR clusters (Glass et al., 1997; Zhou et al., 1999; Herbst and Burden, 2000). The ectodomain of MuSK may be required for agrin responsiveness and for interactions with rapsyn (Zhou et al., 1999), a cytoplasmic protein that is associated with AChR (Froehner, 1991) and promotes AChR clustering at the synapse (Gautam et al., 1995).

The steps that lead to elevated levels of MuSK at synaptic sites, its interactions with agrin and rapsyn, and the signals that cause clustering of postsynaptic proteins remain elusive. Also, most experiments so far were done in cell culture systems where MuSK-dependent signaling has been assayed in the presence of the putative ligand, neural agrin. In such experiments it is not possible to exclude a direct contribution of the ligand itself to the observed effects attributed solely to the activation of MuSK. Therefore, we used ectopic expression in single muscle fibers (Jones et al., 1997; Sander et al., 2000) to analyze the function of MuSK in the formation of the postsynaptic apparatus in vivo and independent of agrin. The results show that ectopic expression of MuSK induces the formation of postsynaptic-like specializations, including the appearance of adult-type AChR ε subunit transcripts and the aggregation of synaptic proteins such as AChR and acetylcholinesterase (AChE). AChR clusters were also induced
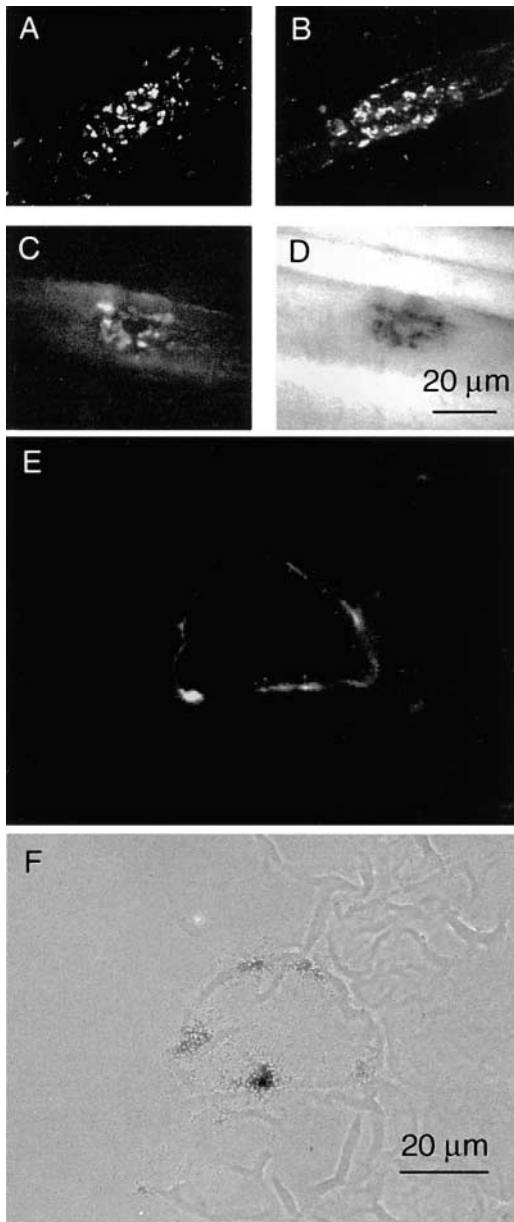
Figure 1. **MuSK induces postsynaptic-like specializations.** (A) Plasmid DNA for nGFP and MuSK was injected ectopically in single muscle fibers of innervated soleus muscles of rats. Muscles were excised 21 d after injection. Green fluorescence of nGFP in single fibers indicated expression of transgenic proteins (unpublished data). AChR clusters induced by MuSK are detected by staining with r-bgt (7 rats were used for DNA injection; a total of 96 fibers were injected and AChR clusters were detected in 61 fibers). (B) Muscle fibers were injected with plasmid DNA for nGFP, MuSK, and rapsyn and analyzed as in A. Expression of MuSK and rapsyn induces AChR clusters comparable to clusters observed in A. 10 rats were used for DNA injection; a total of 138 fibers were injected and AChR clusters were detected in 76 fibers. A and B are projections of confocal images. (C–F) Muscle fibers were injected with plasmid DNA for nGFP, MuSK, and rapsyn. Muscles were excised 28 d after injection and analyzed as in A. (C) Fluorescence microscopic view of r-bgt–labeled AChR, induced by transgenic MuSK. (D) AChE aggregates, visualized according to Koelle and Friedenwald (1949), appear colocalized with the MuSK-induced AChR clusters (3 rats were used for DNA injection and a total of 59 fibers were injected. AChR clusters were detected together with AChE aggregates on 34 fibers). In control-injected fibers, no AChE clusters were detected

by a truncated MuSK form lacking the ectodomain or a form where the ectodomain was replaced by GFP. To exclude any contribution of endogenous MuSK, we employed mutant mice (Hesser, 2000; unpublished data) where MuSK was inactivated conditionally upon expression of transgenic Cre recombinase by direct gene transfer. High resolution analysis revealed that rapsyn–green fluorescent proteins (GFPs), but not MuSK–GFP fusion proteins, were colocalized with AChR clusters. This suggests that a direct physical interaction between rapsyn and MuSK may not be a prerequisite for the formation of AChR clusters. The results show that MuSK activity initiates signals that lead to the formation of AChR clusters, a process which does not require ligand-dependent activation or ectodomain regions. Part of this work was presented in abstract form.

Results

Transgenic MuSK, expressed in single muscle fibers, induces AChR clusters and aggregation of AChE

By direct gene transfer into individual muscle fibers it was shown that agrin induced the formation of ectopic AChR clusters (Cohen et al., 1997; Jones et al., 1997; Meier et al., 1997; Rimer et al., 1997). Using the same approach, it was found that injection of MuSK plasmid DNA alone results in the absence of neural agrin and also in the formation of AChR clusters (Fig. 1 A; Hesser et al., 1999; Jones et al., 1999). MuSK-induced AChR clusters differed, however, from clusters induced following overexpression of neural agrin. Agrin-induced clusters appeared larger in size and more spread out both on the injected fiber as well as on adjacent fibers (Jones et al., 1997; see Figs. 3 and 4). In the case of rapsyn, which is essential for the clustering of AChR at developing synapses (Gautam et al., 1995), we observed that ectopic injection of rapsyn plasmid DNA alone did not induce AChR clustering (unpublished data). Since rapsyn could be linked to MuSK and AChR (Apel et al., 1997), we asked whether MuSK-induced AChR clusters might be altered when expressed in the presence of transgenic rapsyn. Coinjection of MuSK and rapsyn DNA resulted in the formation of AChR clusters (Fig. 1 B) which were similar to the clusters induced by MuSK alone as shown in Fig. 1 A.

Transgenic MuSK mediated not only the formation of AChR clusters (Fig. 1, A–C) but also the accumulation of AChE (Fig. 1 D), which appeared colocalized with AChR clusters. No AChE clusters were detected in control fibers which had been injected with nGFP DNA only. These re-

name	cDNA construct	AChR-cluster-inducing activity
MuSK		+
MuSK$_{K608A}$		−
MuSK-GFP		+
MuSK-GFP$_{K608}$		−
Δecto-MuSK-GFP		+
Δecto-MuSK-GFP $_{K608A}$		−

Figure 2. **MuSK and MuSK mutant constructs used for direct gene transfer.** MuSK and MuSK mutant constructs are shown schematically. SP, signal peptide; TM, transmembrane domain. K608 of the wild-type kinase (o) is mutated to K608A (x) in the kinase-inactive MuSK mutants. The cDNAs cloned into pRK5 expression vectors were injected ectopically in single muscle fibers to express the transgenic proteins. Their ability to induce AChR clusters is indicated on the right.

sults demonstrate that MuSK expressed at extrasynaptic sites in innervated muscle fibers caused the formation of AChR and AChE clusters. The AChR clusters could be newly synthesized or result from aggregation of preexisting, extrasynaptic AChR. Therefore, we prepared serial cross-sections of muscle with fibers containing MuSK-induced AChR clusters to examine whether ε subunit transcript levels were elevated. Since the ε subunit transcripts of normal innervated muscle are restricted to synaptic nuclei and are not expressed in extrasynaptic fiber regions (Brenner et al., 1990), their appearance was taken as evidence of the expression of new AChR. As shown in Fig. 1 E, AChR were located in the plasma membrane of the injected muscle fiber and appeared to form aggregates of varying size. In situ hybridization of the cross-section shown in Fig. 1 E revealed ectopic expression of ε subunit transcripts in fibers containing MuSK-induced AChR clusters (Fig. 1 F). Positive hybridization signals were never observed outside the AChR cluster regions

of injected fibers nor in adjacent noninjected fibers (Fig. 1 F), suggesting that MuSK-dependent signals mediate either directly or indirectly expression of ε subunit gene transcripts and formation of AChR clusters at the sites of MuSK DNA injection.

MuSK kinase activity is required for induction of AChR clusters

Expression of the kinase-inactive mutant MuSK$_{K608A}$ (Glass et al., 1997) in single muscle fibers confirmed that no AChR clusters were induced (Fig. 2). To visualize and demonstrate directly the expression of recombinant MuSK in the injected muscle fibers, we fused GFP with MuSK (MuSK–GFP) and with the kinase-inactive mutant MuSK$_{K608A}$ (MuSK$_{K608A}$–GFP), as schematically summarized in Fig. 2. Fig. 3, A and D, demonstrate that both constructs were expressed with similar efficiency. MuSK–GFP gave rise to AChR clusters which were clearly identified with rhodamine-labeled α-bungarotoxin (r-bgt) (Fig. 3 B). The overlay of the green and red fluorescence confocal images (Fig. 3 C) shows that MuSK–GFP was not strictly colocalized with the AChR clusters. Statistical analysis revealed that in all transgene-expressing fibers, as marked by GFP, AChR clusters were formed (Fig. 3 F). No AChR clusters, however, were induced by the kinase-inactive mutant MuSK$_{K608A}$–GFP (Fig. 3, D, E, and G and Fig. 2), demonstrating that kinase activity is required for the induction of AChR clusters. Rapsyn coinjected with the MuSK mutant was unable to form AChR clusters.

Inactivation of endogenous MuSK by Cre-mediated recombination of the MuSK gene in single muscle fibers

The results so far suggest that expression of transgenic MuSK initiates signals which lead to the formation of AChR clusters. However, overexpression of transgenic MuSK could activate the expression of endogenous MuSK, as suggested by Jones et al. (1999). In this case, it would be difficult to ana-
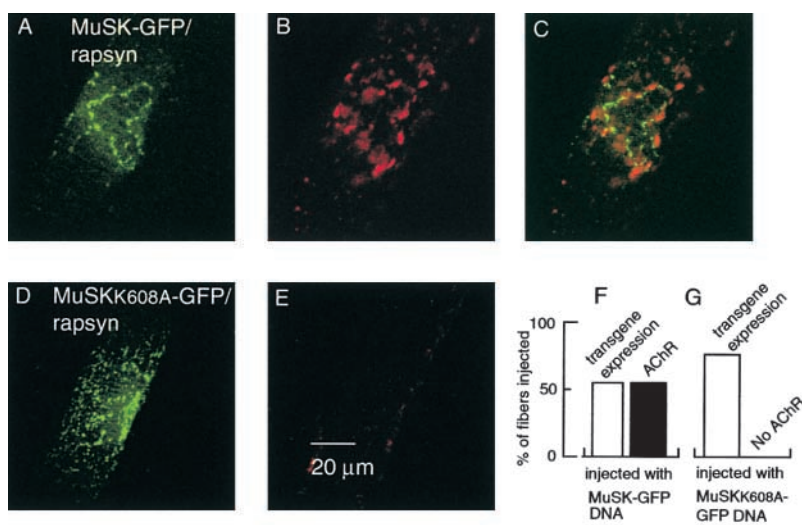
Figure 3. **Induction of ectopic AChR clusters requires MuSK kinase activity.** Rat muscle fibers were injected with expression vectors encoding MuSK–GFP or MuSK$_{K608A}$–GFP and rapsyn. After 21 d, the muscles were excised. Maximum projections of confocal image series show expression of GFP fusion protein (green) and the formation of AChR clusters. AChR clusters are visualized by r-bgt (red). (A) Expression of MuSK–GFP and rapsyn, as indicated. (B) AChR clusters induced by MuSK–GFP. (C) overlay of A and B indicates that MuSK–GFP and AChR clusters are not colocalized. (2 rats were used for DNA injection; a total of 26 fibers were injected and AChR clusters were detected in 14 fibers.) (D) Expression of kinase-inactive MuSK$_{K608A}$–GFP and rapsyn, as indicated. (E) MuSK$_{K608A}$–GFP does not induce AChR clusters and coinjected rapsyn is unable to form AChR/rapsyn aggregates. 4 rats were used for DNA injection; a total of 50 fibers were injected and 38 GFP-positive fibers were detected. (F) Graph illustrates that AChR clusters are detected on all fibers expressing transgenic MuSK. (G) Fibers expressing MuSK$_{K608A}$–GFP display no ectopic AChR clusters.

lyze the functional role of transgenic MuSK expressed in single muscle fibers. To exclude any contribution of endogenous MuSK in live muscle fibers, endogenous MuSK should be inactivated. Since mice lacking MuSK die at birth (DeChiara et al., 1996), we made use of genetically modified mice where the exon encoding the kinase domain of *MuSK* was flanked by *loxP* sites (unpublished data). In these mice it is possible to express Cre recombinase upon DNA injection. Transgenic Cre should mediate the recombination of the loxP-containing MuSK gene and thus lead to the inactivation of endogenous MuSK. Biochemical analysis of single muscle fibers or immunochemical detection of endogenous MuSK in whole mount muscle preparations using commercially available antibodies lack the sensitivity required to unequivocally establish the presence or absence of endogenous MuSK. Therefore, we developed a bioassay based on the AChR cluster–inducing activity of agrin, which revealed that endogenous MuSK was indeed inactivated locally in fibers expressing Cre: injection of agrin DNA into the extrasynaptic regions of muscle fibers led to the appearance of AChR clusters on the injected and adjacent fibers (Fig. 4 A). In such a view, however, it was not possible to decide on the exact location of the AChR clusters. Cross-sections of single confocal images revealed that the r-bgt–labeled AChR were located in the plasma membrane of the injected and noninjected muscle fibers (unpublished data). Fig. 4 B represents a reconstructed xz projection of the complete confocal image series over the range indicated by white arrows shown in Fig. 4 A. It demonstrated that the injected fiber contained AChR clusters in the plasma membrane "surrounding" the nGFP-expressing fiber.

Since agrin-mediated AChR clustering requires MuSK (Glass et al., 1996; Zhou et al., 1999), AChR clusters should not appear in fibers lacking endogenous MuSK. Therefore, muscle fibers were injected with Cre DNA together with agrin and nGFP. The maximum projection of a confocal image series in Fig. 4 C demonstrates successful gene transfer by the appearance of agrin-induced AChR clusters. Cross-sections of single confocal images (unpublished data) as well as the reconstructed xz projection (Fig. 4 D) of the confocal image series (Fig. 4 C) demonstrated clearly that the Cre-injected fibers, identified by nGFP, were not surrounded by r-bgt fluorescence and thus had not formed AChR clusters. However, AChR clusters were located on fibers adjacent to or in the vicinity of the injected fibers. Thus, the locally restricted gene knock-out of MuSK in the Cre-injected muscle fibers inhibited agrin-mediated AChR cluster formation.

To strengthen the observation that AChR cluster formation was completely prevented in muscle fibers expressing Cre, we developed an additional bioassay using rapsyn–GFP. In cell culture experiments it has been shown previously that rapsyn–GFP forms fluorescently labeled complexes with AChR (Ramarao and Cohen, 1998). If such complexes were also formed in rapsyn–GFP-injected muscle fibers, one could directly and reliably monitor the appearance of AChR clusters. In fact, coinjecting rapsyn–GFP with agrin DNA resulted in the appearance of AChR clusters that were exactly colocalized with rapsyn–GFP (Fig. 5 A) and demonstrated that induction and aggregation of AChR can be readily detected and assigned to the injected muscle fiber. Transgenically expressed rapsyn–GFP is therefore a specific and sensitive marker to monitor
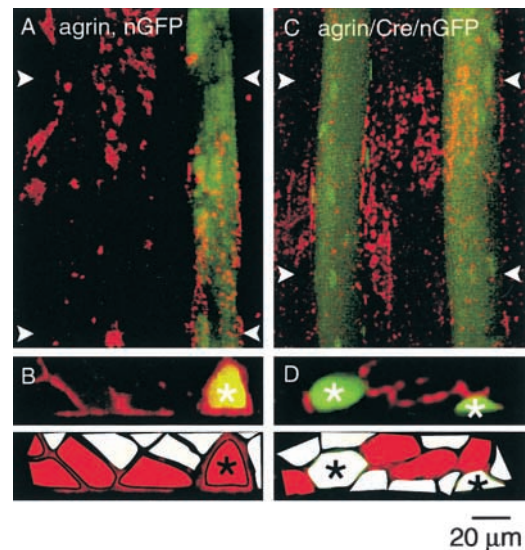

20 μm

Figure 4. **Cre-mediated inactivation of the *MuSK* gene in single muscle fibers.** Single muscle fibers of *MuSK*^loxP/− mice were injected with DNA and expression of transgenic products was analyzed 21 d after injection. Excised muscles were incubated with r-bgt to identify AChR clusters. Overlays of green and red fluorescence images are shown. (A) Maximum projection of a confocal image series shows ectopic AChR clusters (red) induced by agrin. The nGFP-expressing fiber (green) on the right has been injected with agrin and nGFP DNA as indicated. Expression of nGFP and the appearance of AChR clusters shows successful expression of the transgenic proteins. The clusters are apparently located on the injected as well as on noninjected fibers. (B) Cross-sections of the confocal images allow to assign AChR clusters to individual muscle fibers. Reconstructed xz projection of the confocal image series (A) over the range (y) marked by white arrowheads. AChR clusters, located in the plasma membrane, surround the nGFP, transgene-expressing muscle fiber (marked by asterisk). AChR clusters are also located on neighboring noninjected fibers. (Bottom) Location of muscle fibers is indicated schematically and AChR cluster–expressing fibers are labeled red. Transgene-expressing fiber is marked by asterisk. (C) Maximum projection of a confocal image series. Two nGFP-expressing fibers (green) had been injected with agrin and nGFP DNA plus Cre DNA to conditionally inactivate endogenous MuSK. Agrin-induced AChR clusters (red) are apparently distributed as in A. Expression of nGFP and AChR clusters shows successful expression of the transgenic proteins. (D) Reconstructed xz projection of the confocal image series (C) over the range (y) marked by white arrowheads. AChR clusters are not found on the nGFP, Cre-expressing muscle fibers (marked by asterisks), but only on neighboring noninjected fibers. Cre-mediated inactivation of endogenous MuSK prevents formation of AChR clusters. (Bottom) Location of muscle fibers is indicated schematically. AChR cluster-expressing fibers are labeled red. Transgene-expressing fibers are marked by asterisks. 7 mice were used for DNA injection; a total of 39 GFP-expressing fibers were analyzed but contained no ectopic AChR clusters.

the appearance of newly formed AChR clusters in vivo. We never observed rapsyn–GFP–AChR clusters in Cre-expressing fibers (Fig. 5 B), demonstrating that local, Cre-mediated DNA recombination in single muscle fibers efficiently inactivated endogenous MuSK and thus prevented agrin-induced activation of AChR expression.

Finally, if MuSK signaling activates AChR expression it should be possible to rescue the Cre-mediated MuSK knock-out by expressing MuSK transgenically. MuSK DNA was therefore coinjected together with Cre and agrin DNA.
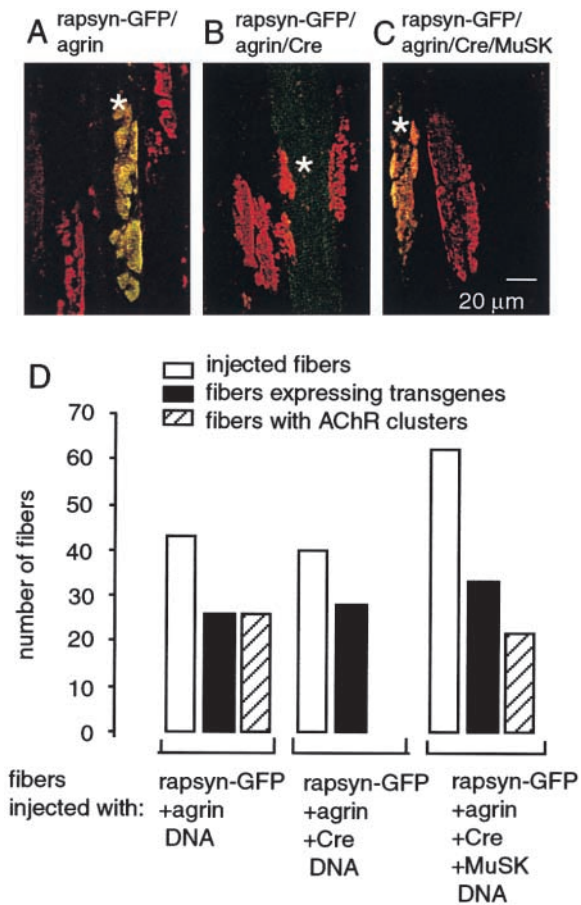
Figure 5. **Rapsyn–GFP identifies ectopically induced AChR clusters.** Single muscle fibers of *MuSK^{loxP/−}* mice were injected with DNA, as indicated by asterisks, and expression of transgenic products was analyzed 21 d after injection. The excised muscles were incubated with r-bgt to identify AChR clusters. Overlays of green and red fluorescence images of a confocal image series are shown as maximum projections. (A) Expression of agrin and rapsyn–GFP as indicated. Agrin induces ectopic AChR clusters. AChR in the injected muscle fiber form complexes with rapsyn–GFP and AChR clusters appear yellow in the overlay. The r-bgt labeled AChR clusters on noninjected fibers are red. (2 *MuSK^{loxP/−}* mice were used for injection; a total of 43 fibers were injected and 26 fibers expressed agrin and rapsyn–GFP. In all injected transgene-expressing fibers AChRs were colocalized with rapsyn–GFP and appeared yellow). (B) Expression of agrin, Cre, and rapsyn–GFP as indicated. Transgenic Cre inactivates endogenous MuSK. The absence of AChR/rapsyn–GFP clusters demonstrates that the formation of AChR clusters was prevented in injected muscle fibers. Agrin-induced AChR clusters are found only on neighboring noninjected fibers. 2 *MuSK^{loxP/−}* mice were used for DNA injection; a total of 40 fibers were injected; 28 fibers expressed agrin and rapsyn–GFP; no AChR clusters were detected on the injected fibers. (C) Expression of agrin, Cre, rapsyn–GFP, and MuSK as indicated. AChR induced in the injected fiber form complexes with rapsyn–GFP and AChR clusters appear yellow in the overlay. The Cre-mediated inactivation of the *MuSK* gene was rescued by expression of transgenic MuSK (3 *MuSK^{loxP/−}* mice were used for DNA injection; a total of 62 fibers were injected and 33 fibers expressed agrin and rapsyn–GFP. Endogenous MuSK inactivation was rescued in 22 fibers, which contained AChR/rapsyn–GFP complexes.) (D) Graphic summary of experiments described in A–C shows that Cre-mediated inactivation of endogenous MuSK prevents the formation of AChR clusters. The conditional MuSK gene knock out is rescued with high efficiency by transgenic MuSK: AChR clusters are found in 73% of transgene-expressing fibers.

Fig. 5 C reveals that AChR/rapsyn–GFP complexes were clustered in the injected muscle fiber and successful expression of transgenic proteins was again confirmed by the agrin-induced AChR clusters on the neighboring fibers. These results, summarized in Fig. 5 D, demonstrate that endogenous MuSK can be inactivated conditionally in single muscle fibers and transgenic MuSK mediates the appearance of AChR clusters in the absence of endogenous MuSK.

Ectodomain of transgenic MuSK is not required for induction of AChR clustering

There is evidence that MuSK signaling requires the ectodomain of MuSK (Apel et al., 1997; Glass et al., 1997). To determine whether MuSK-induced AChR clustering requires the ectodomain, we constructed a mutant lacking the complete extracellular region (ΔectoMuSK; see Fig. 2). Fig. 6 A shows several representative AChR cluster patterns induced by transgenic MuSK. The number and size of the AChR clusters vary to some extent and is probably due to the variable amounts of MuSK expressed upon injection. The expression of ΔectoMuSK led to the formation of AChR clusters (Fig. 6 B), which were comparable to the clusters induced by full length MuSK. Again, to prove successful expression of the transgene in the injected muscle fibers, the ΔectoMuSK construct was fused with GFP where GFP replaced the ectodomain (ΔectoMuSK–GFP; see Fig. 2). Injection of this DNA led to efficient expression of Δecto-MuSK–GFP, which induced AChR clusters (Fig. 6 C). The kinase-inactive mutant ΔectoMuSK$_{K608A}$–GFP (Fig. 2) was also expressed efficiently, but was unable to induce AChR clusters (Fig. 6 D). Thus, deletion of the ectodomain does not interfere with MuSK's ability to induce aggregation of AChR.

To exclude any contribution of endogenous MuSK, we performed the same experiments in *MuSK^{loxP/−}* mice where endogenous MuSK was inactivated locally in the injected muscle fiber by transgenic Cre. The results demonstrate that ΔectoMuSK–GFP was expressed efficiently (Fig. 7 A) and induced the appearance of AChR clusters (Fig. 7 B). Since endogenous MuSK had been inactivated by Cre, this observation confirms that the ectodomain of MuSK is not an integral component of the mechanisms underlying MuSK-induced AChR expression. The overlay of green and red fluorescence images (Fig. 7 C) suggests that ΔectoMuSK–GFP is not colocalized with r-bgt–labeled AChR.

Distribution of AChR clusters, MuSK–GFP, and rapsyn–GFP

When comparing the distribution of r-bgt–labeled AChR clusters and MuSK–GFP (Fig. 3 C) or ΔectoMuSK–GFP (Fig. 7 C), it appeared that the two proteins were not strictly colocalized on the surface of the muscle fibers and MuSK–GFP fusion proteins were observed outside of AChR clusters and vice versa. This was surprising in view of the current model, in which the ectodomain of MuSK has to be structurally linked to AChR. Therefore, one would expect that a significant portion of the AChR clusters overlaps with the GFP-labeled MuSK molecules. Rapsyn is thought to interconnect AChR and MuSK via the hypothetical linker RATL (Apel et al., 1997). Therefore, we analyzed at higher resolution whether rapsyn was distributed like MuSK or was associated more strictly with AChR (as
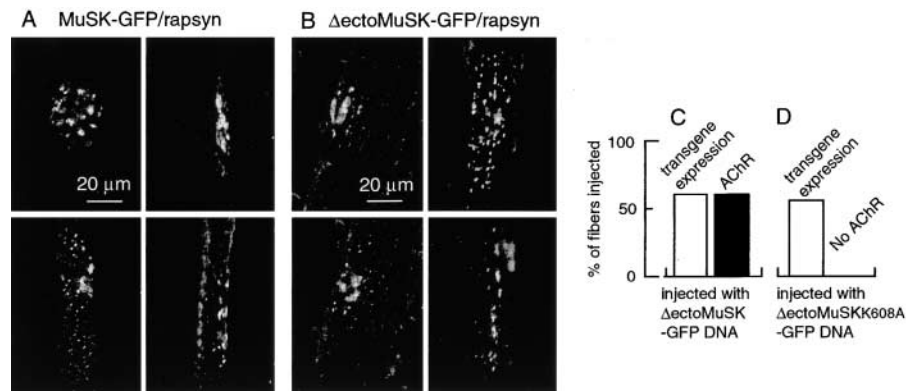
Figure 6. MuSK lacking the ectodomain induces AChR clusters. Rat muscle fibers were injected with DNA of rapsyn and MuSK mutants. After 21 d, muscles were excised. MuSK-induced AChR clusters were visualized by r-bgt. (A) Expression of MuSK–GFP and rapsyn, as indicated, induces AChR clusters. Several different AChR cluster patterns are presented and demonstrate that size and shape of MuSK-induced clusters may vary, probably depending on the concentration of transgenic MuSK expressed upon direct gene transfer. (B) Expression of ΔectoMuSK–GFP and rapsyn, as indicated, induces AChR clusters similar to that shown in A (4 rats were used for DNA injection; a total of 47 fibers were injected and AChR clusters were detected on 28 fibers). (C) Diagram: ΔectoMuSK–GFP induces AChR clusters as observed in A and B. In all transgene-expressing fibers, AChR clusters are formed (3 rats were used for DNA injection; a total of 48 fibers were injected; 28 fibers expressed ΔectoMuSK–GFP and AChR clusters were detected on all 28 fibers). (D) Diagram: kinase-inactive $ΔectoMuSK_{K608A}$–GFP fails to induce AChR clusters (2 rats were used for DNA injection; a total of 25 fibers were injected and 14 $ΔectoMuSK_{K608A}$–GFP-expressing fibers were detected; none of the fibers had ectopic AChR clusters).

indicated already in Fig. 5). MuSK and rapsyn–GFP were injected ectopically to induce AChR clusters and the distribution of rapsyn–GFP relative to AChR clusters was analyzed by confocal microscopy. Single sections revealed that rapsyn–GFP was strictly colocalized with AChR stained by r-bgt (Fig. 8, A–C). In contrast, when MuSK–GFP and rapsyn were injected, MuSK–GFP displayed a different distribution and was spatially distinct from AChR clusters (Fig. 8, E–G).

This was confirmed by a colocalization analysis using all sections of the confocal image series (see Materials and methods). Fig. 8, D and H, show two-dimensional graphs from colocalization histograms of the MuSK/rapsyn–GFP- and MuSK–GFP/rapsyn-injected fibers, respectively. The intensity of the GFP fluorescence is plotted on the x-axis and r-bgt fluorescence on the y-axis; the intensity contrast of the two channels was scaled similarly. Data points located

near the diagonal line (running through the origin, slope of 1) thus indicate colocalization. From Fig. 8 D it can be seen that all data points were located proximal to the diagonal and are therefore in accordance with the view that rapsyn–GFP and AChR form stoichiometric complexes. In Fig. 8 H, the data points displayed no high degree of codistribution, indicating that MuSK–GFP and AChR were not colocalized. This suggests that a physical link between MuSK–GFP and rapsyn is not a prerequisite for the formation of AChR clusters, which are induced by MuSK kinase activity.

Discussion

Using the gene transfer method, in which plasmid DNA is directly injected into individual muscle fibers of adult rats (Jones et al., 1997; Sander et al., 2000), we tested whether
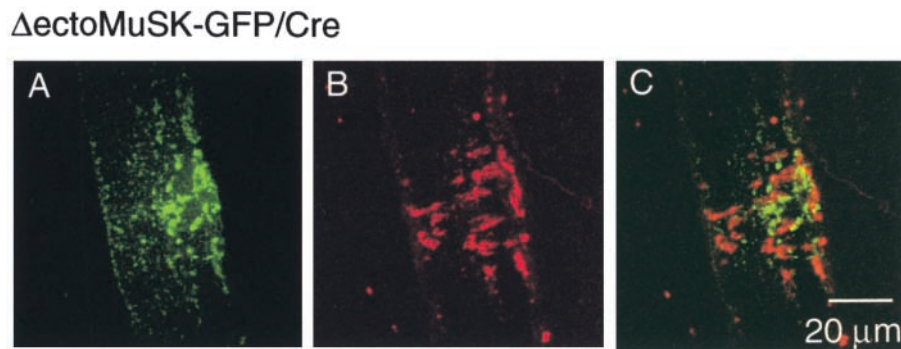
Figure 7. AChR clusters are induced by transgenic MuSK lacking the ectodomain. Single muscle fibers of $MuSK^{loxP/-}$ mice were injected with DNA of ΔectoMuSK–GFP and Cre. Expression of transgenes was analyzed 21 d after injection. The excised muscles were incubated with r-bgt to identify AChR clusters. Maximum projections of confocal image series are shown. (A) Expression of ΔectoMuSK–GFP (green fluorescence indicates successful expression of transgenes) and Cre. (B) AChR clusters are formed in absence of endogenous MuSK which has been inactivated by transgenic Cre (a $MuSK^{loxP/-}$ mouse was used for injection; a total of 22 fibers were injected; 11 fibers expressed ΔectoMuSK–GFP and 10 of these fibers had ectopic AChR clusters). (C) Overlay of green and red fluorescence images: ΔectoMuSK–GFP is not colocalized with AChR clusters.

MuSK/rapsyn-GFP
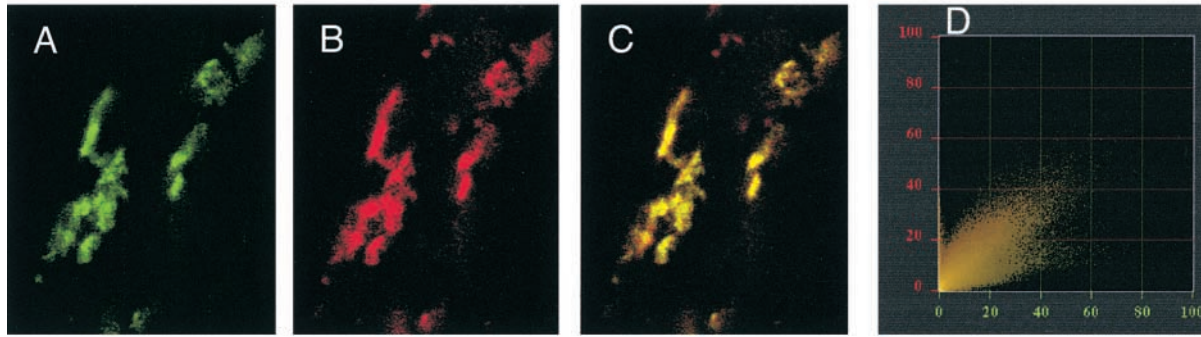
MuSK-GFP/rapsyn

Figure 8. **Rapsyn–GFP/AChR and MuSK–GFP/AChR distribution at ectopic MuSK-induced AChR clusters.** Rat muscle fibers were injected with plasmid DNA. After 21 d, the muscles were excised. AChR were stained with r-bgt. Confocal image series were recorded to analyze at high resolution whether rapsyn and MuSK are colocalized with AChR clusters. (A) A single section of a confocal imag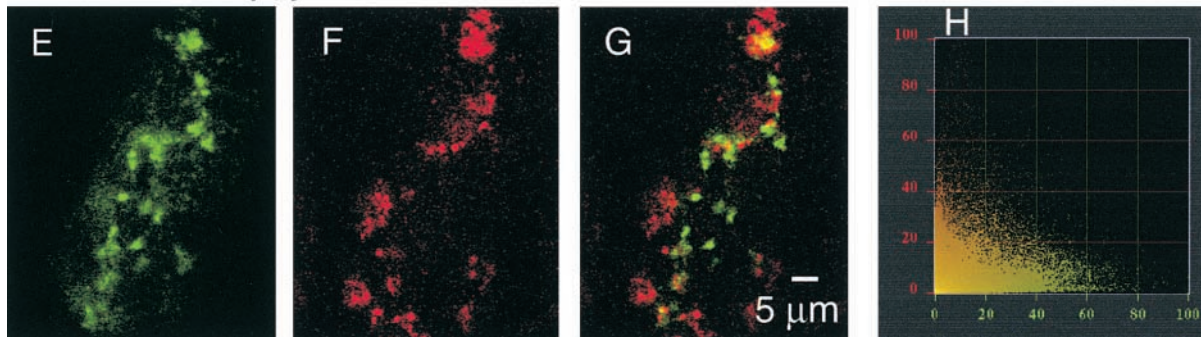e series shows expression of MuSK and rapsyn–GFP (green), as indicated. (B) MuSK-induced AChR clusters (red). (C) AChR clusters and rapsyn–GFP are colocalized as shown by overlay of green and red fluorescence images. (E) A single section of a confocal image series shows expression of MuSK–GFP (green) and rapsyn, as indicated. (F) MuSK-induced AChR clusters (red). (G) AChR clusters and MuSK–GFP are not colocalized as shown by overlay of green and red fluorescence images. (D) Statistical colocalization analysis of all sections of a confocal image series of a transfected fiber was performed (see Materials and methods). The relative fluorescence intensities of rapsyn–GFP (x-axis) are plotted against the relative fluorescence intensities of r-bgt–labeled AChR clusters (y-axis). Data points are located near the diagonal, showing that rapsyn–GFP is colocalized with AChR. (H) Statistical colocalization analysis, as in D, for MuSK–GFP. Data points are scattered, showing that MuSK–GFP is not colocalized with AChR.

MuSK, as a component of the putative agrin receptor complex, could induce postsynaptic specializations at ectopic sites in a ligand-independent manner. Overexpression of receptor tyrosine kinases has been shown to lead to their ligand-independent activation, presumably by increased formation of dimers (Schlessinger and Ullrich, 1992; Hubbard et al., 1998). A similar mechanism may activate MuSK when it is expressed ectopically in muscle fibers. Our results show that the expression of MuSK, but not $MuSK_{K608A}$, leads to the appearance of AChR clusters and ϵ subunit gene transcripts at the site of gene transfer. Furthermore, AChE aggregates were colocalized with the AChR clusters. So far, no kinase-signaling pathways have been detected that respond to agrin/MuSK activation. However, the fact that overexpression of MuSK in the absence of neural agrin leads to the ectopic appearance of ϵ subunit mRNA indicates that MuSK-mediated signals could influence gene transcription of the AChR subunit genes and possibly other synaptically expressed genes. Whether this gene induction is directly dependent on MuSK kinase activity or on proteins that bind to activated MuSK (Zhou et al., 1999; Herbst and Burden, 2000) remains to be investigated. There is also the possibil-

ity that signaling was indirectly mediated by erbB receptors, which appear to become concentrated at the MuSK scaffold (Burden, 1998; Meier et al., 1998). Recent findings showed that erbB3-deficient mice (Riethmacher et al., 1997), as well as erbB2-deficient mice with a heart-specific erbB2 rescue (Morris et al., 1999), display only minor deficits in early postsynaptic development, suggesting that erbB receptor kinase signaling may not be linked to MuSK to activate AChR subunit gene expression.

The MuSK kinase signal is thought not to be sufficient to elicit MuSK action (Glass et al., 1997). Additional domains located in the extracellular region of MuSK appear to be required for the clustering of AChR. The ectodomain mediates agrin-dependent activation via the putative receptor MASC and may interact physically with a hypothetical linker, RATL, to allow MuSK to recruit rapsyn–AChR complexes to a primary scaffold in the postsynaptic membrane (Apel et al., 1997). However, in myotubes cultured from $MuSK^{-/-}$ mutant mice it was observed that AChR clusters are also induced in a ligand-independent manner by MuSK, even without being colocalized with AChR clusters (Zhou et al., 1999). Using cultured $MuSK^{-/-}$ myotubes, the authors

demonstrated that formation of agrin-induced as well as spontaneous AChR clusters requires MuSK.

Gene transfer experiments in single muscle fibers demonstrate now that AChR clusters can be induced by spontaneous MuSK kinase activity in vivo. Since the AChR-inducing activites of MuSK and ΔectoMuSK are similar, the clustering process appears not to require additional signals mediated by the ectodomain of MuSK. Jones et al. (1999) induced AChR clusters in muscle fibers by expressing transgenic MuSK chimeras. They found endogenous MuSK associated with the ectopic AChR clusters and concluded that endogenous MuSK, recruited by the transgenic kinase activity, induced AChR clustering via its ectodomain. If the ectodomain was actually required for the formation of AChR clusters one would assume that expression of transgenic full length MuSK, which would not need to recruit endogenous MuSK, was more potent in the induction of AChR clusters than the truncated ΔectoMuSK mutant. However, this was not the case, indicating that kinase activity is sufficient to induce AChR clusters.

Nevertheless, endogenous MuSK has to be inactivated to exclude any contribution to the observed changes induced by transgenic MuSK and MuSK mutants. Conditional MuSK gene knock out has been achieved in $MuSK^{loxP/-}$ mice by expressing transgenic Cre. Using the agrin-mediated formation of ectopic AChR clusters as bioassay we showed that endogenous MuSK is inactivated, which prevents the formation of ectopic AChR clusters. The fact that the local MuSK gene knock out is rescued by transgenic MuSK supports the view that agrin requires MuSK to induce AChR clusters. The inactivation of endogenous MuSK, combined with the expression of MuSK mutants lacking the entire ectodomain, demonstrates in addition that the ectodomain of MuSK can be deleted or replaced by GFP without obvious effects on the ability to induce AChR clusters. The apparently ligand-independent activation of MuSK is thus sufficient to initiate signals that lead to the formation of AChR clusters and postsynaptic-like specializations. As there are only very few preexisting AChR in extrasynaptic regions of innervated muscle fibers, it is assumed that transgenic MuSK leads to the production of new AChR which are then organized into clusters.

The AChR clusters induced upon expression of MuSK or of MuSK and rapsyn together do not differ significantly in size and shape, indicating that the endogenous pool of rapsyn is sufficient for the formation of ectopic AChR clusters. In heterologous expression systems transgenic rapsyn self-aggregates and promotes clustering of AChR when both proteins are coexpressed (Froehner et al., 1990; Phillips et al., 1991). However, expression of rapsyn in ectopic muscle fiber regions does not result in the appearance of AChR clusters, demonstrating that AChR are not simply recruited and clustered from preexisting AChR by transgenic rapsyn, as may be the case in cultured myotubes.

AChR clusters are also induced by agrin in $MuSK^{-/-}$ cell cultures transfected with MuSK mutants lacking the putative RATL binding domain, indicating that MuSK–rapsyn/RATL interactions are dispensable for at least some aspects of MuSK function (Zhou et al., 1999). The finding that the GFP-labeled kinase and AChR clusters can be spatially distinct in muscle fibers lends support to the view that the
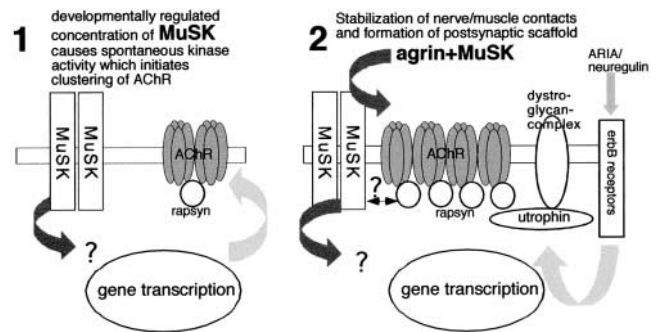

Figure 9. **Model of MuSK-initiated synapse formation.** (1) MuSK accumulates to reach a critical threshold in central regions of developing muscle fibers. Spontaneous kinase activity induces small accumulations of AChR, similar to those we observe upon transgenic expression of MuSK. (2) Agrin released by incoming motor axons supports the formation of denser accumulations by locally activating MuSK, possibly by mechanisms suggested in previous models (Zhou et al., 1999). The local action of MuSK and the local secretion of agrin reinforce each other to generate the extreme density of postsynaptic proteins encountered at the neuromuscular junction. See Discussion for further information.

rapsyn/AChR clustering process does not require a structural linker protein between MuSK and AChR. MuSK signals act locally restricted, since transgenic MuSK–GFP is not transported over a wide distance but is expressed within a spatially limited range at the DNA injection site, where it determines formation of AChR clusters.

These results lead to the hypothesis that MuSK signals mediate an increased expression of AChR subunits which are assembled to form complexes with endogenous rapsyn. Postsynaptic specializations develop only at sites where MuSK is concentrated, under natural conditions at contact sites of nerve and muscle. The clustering of AChR that we observe upon transgenic expression of MuSK may thus reflect a physiological process occurring before synapse formation. It was reported that in topoisomerase-deficient mice, motor axons are not developed. Yet, there are AChR concentrated in the central region of the diaphragm muscle (Yang et al., 2000). This suggests that the accumulation of AChR occurs in the absence of neuronal factors. However, AChR are not accumulated in muscle fibers lacking MuSK, although motor axons are present in these muscles (DeChiara et al., 1996), demonstrating that MuSK is required for the accumulation of AChR. While this paper was under review, two papers were published where AChR expression was investigated in embryonic mutant mice lacking agrin, MuSK, rapsyn, and/or motor nerves (Lin et al., 2001; Yang et al., 2001). The results show that MuSK and rapsyn are required for early postsynaptic differentiation which occurs in absence of agrin or motor axons. In this scenario (Fig. 9) MuSK accumulates in the central, developmentally oldest region of muscle fibers. Reaching a critical threshold, spontaneous kinase activity is strong enough to induce accumulations of AChR, similar to those we observe upon transgenic expression of MuSK. In a second step, agrin (and possibly other factors) released by incoming motor axons might favor the formation of denser accumulations by locally activating MuSK. The local action of MuSK and the local secretion of

agrin might reinforce each other to generate the extreme density of postsynaptic proteins encountered at the neuromuscular junction.

Materials and methods

Expression plasmids

nGFP plasmid DNA. The pRK5/pRK7 expression vectors (Schall et al., 1990) contain the cytomegalovirus early promoter/enhancer that mediates transcription of downstream DNA inserts fused to SV-40 termination and polyadenylation signals. The modified version of GFP with enhanced fluorescence and "humanized codon usage" in pTR-UF5 (Zolotukhin et al., 1996) was amplified by PCR and ligated using the XbaI-HindIII restriction sites into a pRK5 vector containing the SV-40 nuclear transport signal (Lanford et al., 1988) and named pRK5-nGFP.

MuSK. MuSK-cDNA was isolated by RT-PCR and cloned with EcoRI (5′) and XbaI (3′) in pRK5 according to Hesser et al. (1999).

MuSK–GFP. A XhoI cloning site was introduced into the MuSK cDNA at nucleotides 100–105 and an EcoRV site at nucleotides 106–111. Both mutations were introduced by site-directed mutagenesis in two steps using PCR (QuickChange Site-directed Mutagenesis Kit; Stratagene). GFP was amplified by PCR using the corresponding primers to ligate the product into the mutated MuSK cDNA. The following primer pair was used for PCR amplification of GFP: the 5′ sense primer was AACTCGAGAGCAAGGGC-GAGGAACTGTTC, adding an XhoI site and at the same time replacing the start codon; the 3′ antisense primer was TTGATATCGTACAGCTCGTC-CATGCCATG, adding an EcoRV site and replacing the stop codon of GFP. The ligation of the GFP with XhoI (5′) and EcoRV (3′) in the mutated MuSK (XhoI, EcoRV) resulted in MuSK–GFP.

ΔectoMuSK. XhoI cloning sites were introduced into the MuSK cDNA at nucleotides 100–105 and at nucleotides 1,432–1,437. These mutations were also introduced by site-directed mutagenesis. Restriction with XhoI and religation resulted in a MuSK mutant construct where the entire ectodomain but not the signal peptide was deleted.

ΔectoMuSK–GFP. The PCR-amplified GFP sequence, flanked by XhoI sites, was inserted at the XhoI site of ΔectoMuSK as described above to yield ΔectoMuSK–GFP, where the ectodomain excluding the signal peptide was replaced by GFP. The following primer pair was used for PCR amplification of GFP: the 5′ sense primer was AACTCGAGAGCAAGGGC-GAGGAACTGTTC, adding an XhoI site and replacing the start codon; the 3′ antisense primer was TTCTCGAGGTACAGCTCGTCCATGCCATG, also adding an XhoI and replacing the stop codon of GFP.

MuSK$_{K608A}$. MuSK cDNA in the pRK5 vector was mutated by replacing K608 to A608 using the site-directed mutagenesis kit from Stratagene. The kinase activity–deficient mutant MuSK$_{K608A}$ was described by Glass et al. (1997).

ΔectoMuSK$_{K608A}$–GFP. The K608 residue of the fusion construct, ΔectoMuSK–GFP, was mutated as described for MuSK$_{K608A}$. The resulting gene product has its ectodomain replaced by GFP and lacks kinase activity.

Rapsyn. The 1A15 cDNA clone encoding mouse muscle 43K protein, rapsyn (Froehner, 1989), was obtained from Dr. S. Froehner (University of North Carolina, Chapel Hill, NC) and was subcloned in pRK7.

Rapsyn–GFP. The rapsyn–GFP expression vector was obtained from Dr. J. Cohen (Harvard Medical School, Boston, MA) (Ramarao and Cohen, 1998).

Agrin. The vector containing neural agrin, cAgrin$_{7A4B8}$ (Denzer et al., 1995) was provided by Dr. M. A. Ruegg (University of Basel, Basel, Switzerland).

Cre recombinase. The Cre cDNA has a nuclear localization signal n and was excised by EcoRI and XbaI from pMC-Cre (Gu et al., 1993) and ligated into pRK5 to yield pRK5-nCre.

Transgenic mice

Homozygous MuSK$^{loxP/loxP}$ and heterozygous MuSK$^{loxP/-}$ mutant mice were generated by Dr. B.A. Hesser (Hesser, 2000; unpublished data). A genomic fragment of 6.3 kb that contained the MuSK kinase exon domain was cloned into pSP72 (Promega) and used for the construction of the targeting vector. In the introns flanking the kinase domain at the 5′ and 3′ end loxP sites were introduced. The targeting vector was introduced into embryonic stem cells and the resulting cell clones were injected into blastocysts derived from C57Bl/6J mice (at BRL Füllinsdorf, Switzerland).

Muscle fiber injection

Muscle fibers were injected as described previously (Jones et al., 1997; Sander et al., 2000). Sprague Dawley rats (*rattus norvegicus*) of ∼150 g

body weight (4–5-wk-old) were anesthetized with Nembutal (Abbott Laboratories) at a dosage of 50 mg/kg. The soleus muscle of the left hind leg was surgically exposed. Micropipettes were filled with injection solution that consisted of 90 mM KCl, 15 mg/ml Fast Green FCF (Sigma-Aldrich) and each plasmid DNA at a concentration of 100 ng/μl. Air pressure pulses of 1 psi for 0.2–0.3 s were used to inject the DNA into individual muscle fibers. A reference electrode was used to measure the membrane potential of the injected fiber and injection was visualized directly by Fast Green FCF.

Histological analysis

3 or 4 wk after injection, animals were killed by CO_2 to excise soleus muscles, which were kept in Ringer's solution (135 mM NaCl, 5.4 mM KCl, 1 mM MgCl$_2$, 1.8 mM CaCl$_2$, 5 mM Hepes) for further analysis. The muscles were pinned to Sylgard-lined plastic dishes and then incubated for 1 h at RT in 2 μg/ml r-bgt (Molecular Probes) in Ringer's solution. Labeled AChR and GFP fluorescence were viewed with a ZEISS Axioplan microscope.

AChE activity was visualized according to Koelle and Friedenwald (1949). The number of fibers expressing AChR clusters, nGFP, or GFP-tagged proteins (as applicable) was determined and compared with the total number of fibers initially injected.

For confocal microscopy, fluorescently labeled muscles were fixed for 3 min in PBS containing 4% paraformaldehyde. A thin layer of muscle fibers containing the regions of interest was prepared and mounted in CITI-FLUOR onto high quality glass coverslips. Confocal image series were recorded on a Leica confocal laser scanning unit, TCS NT, which was coupled to a Leica DM IRB microscope. The image series were processed using the TCS NT software (Leica). For colocalization analysis, the original three-dimensional data were first deconvolved with the HUYGENS2 software (SVI; Hilversum) and then further processed with the CO-LOCALISATION module of the IMARIS 3D visualization program (Bitplane). Intensity values of corresponding individual voxels (volume pixels) of the green and the red fluorescence channel of a three-dimensional dataset were plotted as a two-dimensional graph (displaying occurrence of individual combinations of green fluorescence intensities, x-axis and red fluorescence intensities, y-axis). In situ hybridization was performed as detailed in Kues et al. (1995).

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