Dimerization of the Muscle-specific Kinase Induces Tyrosine Phosphorylation of Acetylcholine Receptors and Their Aggregation on the Surface of Myotubes

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During development of the neuromuscular junction, neuronal splice variants of agrin initiate the aggregation of acetylcholine receptors on the myotube surface. The muscle-specific kinase is thought to be part of an agrin receptor complex, although the recombinant protein does not bind agrin with high affinity. To specify its function, we induced phosphorylation and activation of this kinase in the absence of agrin by incubating myotubes with antibodies directed against its N-terminal sequence. Antibody-induced dimerization of the muscle-specific kinase but not treatment with Fab fragments was sufficient to trigger two key events of early postsynaptic development: acetylcholine receptors accumulated into aggregates, and their β-subunits became phosphorylated on tyrosine residues. Heparin partially inhibited receptor aggregation induced by both agrin and anti-muscle-specific kinase antibodies. In contrast, it did not affect kinase or acetylcholine receptor phosphorylation. These data indicate that agrin induces postsynaptic differentiation by dimerizing the muscle-specific kinase. They also suggest that activation of the kinase domain can account for only part of agrin’s effects. Dimerization of this molecule appears to activate an additional signal, most likely by organizing a scaffold for other postsynaptic proteins.

The basal membrane protein agrin plays a central role during the early phase of synaptic differentiation at the neuromuscular junction (1–3). Neuron-specific agrin isoforms containing an eight-amino acid insertion generated by alternative splicing (4–6) are able to induce the aggregation of AChRs1 and other synaptic proteins on the surface of myotubes (7–10). Deletion of the C2C12 mouse muscle cell line and colocalizes with AChRs at the neuromuscular junction (25, 27). Several observations suggest an important role of MuSK in the agrin pathway (28); incubation of myotubes with agrin causes the rapid tyrosine phosphorylation of MuSK (28). This reaction, a characteristic response of receptor tyrosine kinases to binding of their ligand (29, 30), is exclusively induced by biologically active fragments and isoforms of agrin.2 In addition, agrin can be cross-linked to MuSK expressed on myotubes (28). Upon transfection into the quail cell line QT-6, MuSK is concentrated in microaggregates together with rapsyn (31). Remarkably, the extracellular domain of the MuSK molecule is required for this interaction, which therefore must be indirect. It has been suggested that a hypothetical rapsyn-associated transmembrane linker (RATL) bridges these proteins (32).

Agrin does not directly bind to recombinant MuSK (28) (data not shown). Therefore, a MuSK-accessory specificity component (MASC) has been postulated, mediating its activation by agrin (28). To assess the role of MuSK in the agrin signaling pathway, it is important to activate this molecule independent of agrin. In an earlier attempt, a chimeric molecule consisting of the extracellular domain of the neurotrophin receptor TrkC and the intracellular domain of MuSK has been expressed in myotubes. The TrkC ligand neurotrophin 3 added to these myotubes induces the tyrosine phosphorylation of the chimeric receptor as well as AChRs, but not AChR aggregation (33). Here we took a different approach to bypass agrin in activating MuSK. We artificially dimerized MuSK by incubating myo-

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1 The abbreviations used are: AChR, nicotinic acetylcholine receptor; DMEM, Dulbecco’s modified Eagle’s medium; MuSK, muscle-specific kinase; MASC, MuSK-accessory specificity component; mAb, monoclonal antibody; pAb, polyclonal antibody; RATL, rapsyn-associated transmembrane linker; s-agrin, soluble agrin; PAGE, polyacrylamide gel electrophoresis; TGFβRI, transforming growth factor β receptor I.
2 C. Hopf and W. Hoch, submitted for publication.
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**EXPERIMENTAL PROCEDURES**

**Expression Constructs and Transient Transfection**—The soluble rat agrin constructs s-agrin-(4,19) and s-agrin-(0,8) have been described previously (34). The full-length MuSK construct has been reported elsewhere, and an expression construct coding for the extracellular domain of MuSK was assembled by the addition of sequence coding for a hexahistidine tag followed by a stop codon to the appropriate site of the MuSK cDNA. COS-7 cells were transiently transfected with pCMV expression vectors encoding soluble agrin (30 μg of DNA/15-cm dish) according to the method of Chen and Okayama (35). The collection of serum-free agrin-conditioned media and calibration of agrin concentrations has been described (34).

**Antibodies and Fab Fragments**—Polyclonal antibody (pAb) Cyt-MuSK against a bacterial fusion protein comprising the first half of the cytoplasmic domain of MuSK was affinity-purified by absorption to the antigen immobilized on Affi-Gel (Bio-Rad). pAb N-MuSK was purified from a crude antisera against a peptide (N-peptide) corresponding to residues 14–34 of the MuSK N-terminal cytoplasmic domain of MuSK by affinity chromatography on an immobilized N-terminal bacterial fusion protein. Both pAbs specifically recognize MuSK in immunoprecipitation and Western blot experiments. For the antibody specificity analysis shown in Fig. 1, membrane proteins were extracted from COS cells, whereas a plasma membrane fraction of C2C12 cells differentiated for 5 days in fusion medium was prepared as described (36).

The phosphotyrosine antibody mAb 5E2 was a kind gift from Dr. A. Ullrich (Max-Planck-Institute for Biochemistry). The phosphotyrosine antibodies mAb PY20 and mAb 4G10 were purchased from Transduction Laboratories and Upstate Biotechnology Inc., respectively. mAb 124 (rat monoclonal) directed against the β-subunit of the AChR was a kind gift from Dr. J. Lindseth (University of Pennsylvania). Purified polyclonal antibodies against extracellular epitopes of the TGFβR I and M-cadherin were purchased from Santa Cruz Biotechnology. Iodinated and horseradish peroxidase-conjugated secondary antibodies were obtained from Amersham Corp. and Jackson/Dianova.

Fab fragments of pAb N-MuSK were generated by digestion with papain conjugated to agarose beads (Sigma) for 10 h at 37 °C. FC fragments and undigested antibodies were removed by absorption to protein A-agarose (Calbiochem). Binding of the Fab fragments and pAb N-MuSK to N-peptide as well as the recombinant extracellular domain of MuSK was compared by enzyme-linked immunosorbent assay. Briefly, microtiter plates were coated with N-peptide, purified recombinantly expressed extracellular domain of MuSK, or bovine serum albumin. Thereafter, the plates were incubated with several concentrations of pAb N-MuSK or Fab fragments of these antibodies for 2 h at room temperature. Unbound antibodies were removed by extensive washing, and bound antibodies were reacted with biotin-conjugated anti-rabbit-Fab-antibodies (Jackson/Dianova) followed by streptavidin-conjugated horseradish peroxidase (Amersham). As the “active concentration” of Fab fragments, the concentration of pAb N-MuSK was defined according to the same reactivity against the N-peptide as in this enzyme-linked immunosorbent assay. At this concentration, Fab fragments also showed a similar reactivity toward the extracellular domain of MuSK as the intact antibody. For staining of MuSK- or mock-transfected COS cells under native conditions, COS cells were grown on polylysine-coated slides. Medium was replaced by C2C12 fusion medium containing antibody or Fab fragments (“active concentration” 30 mM) and incubated for the same time as in the enzyme-linked immunosorbent assay described above. After incubation for 1 h at 37 °C, cells were washed, fixed in 2% paraformaldehyde, and incubated with biotin-conjugated anti-rabbit-Fab-antibodies followed by Cy3 conjugated to streptavidin.

Analysis of MuSK and AChR Tyrosine Phosphorylation—C2C12 myoblasts were propagated as described previously (7). Unless indicated otherwise, cells were allowed to immunoblot 2.5% horse serum, 2 mM glutamine in DMEM (fusion medium) for 4–5 days. They were switched to 0.25% horse serum in DMEM for 3–12 h prior to stimulation with agrin or antibodies in various concentrations.

Immunoprecipitation of MuSK with Cyt-MuSK antisera and enrichment of AChRs by binding to bixin-α-bungarotoxin (Molecular Probes) followed by incubation with streptavidin-agarose beads has been described previously. Precipitated proteins were resolved by SDS-PAGE on 10% gels and transferred to Fluorotrans membrane (Pall Filtron). Immunoreactive bands were visualized with horseradish peroxidase-conjugated secondary antibodies and chemiluminescent SuperSignal substrate (Pierce). Antibodies were stripped from the membranes with 0.2 M glycine, pH 2.5, 150 mM NaCl, 0.1% Nonidet P-40; blocked again; and reprobed with suitable antibodies. Some experiments were performed using 125I-conjugated secondary antibodies. Bound radioactivity was quantitated by PhosphorImager analysis (Molecular Dynamics) after 5–30 days of exposure.

To assess the ability of the N-peptide or heparin to neutralize pAb N-MuSK effects, myotubes were pretreated with a 10,000-fold molar excess of N-peptide (approximately 500 μg/ml) or suitable concentrations of heparin in DMEM for 45 min prior to stimulation. Antibody was preincubated with the same excess of N-peptide for 2 h at 4 °C.

Quantitation of Antibody-induced AChR Aggregation—C2C12 myotubes were cultured on chamber slides (Nunc). After 2% days in fusion medium, they were stimulated with antibody preparations or agrin for 10–16 h. AChRs were visualized with rhodamine-α-bungarotoxin, and the number of AChR aggregates in at least 12 microscopic fields was quantitated as described previously (34). Many small AChR clusters were observed when formation of aggregates was induced with pAb N-MuSK. These were not included in our quantitation, since only clusters >5 μm in length were counted. In experiments with heparin or N-peptide, the cells were pretreated with these agents as outlined above. All experiments were performed 2–6 times. The number of AChR aggregates was displayed as the mean of 3–5 determinations ± S.E. Statistical significance of the observed differences was verified by t test analysis (p < 0.05).

**RESULTS**

**MuSK Antibodies Induce Tyrosine Phosphorylation of the Kinase**—Ligand-induced dimerization is an essential step for activation of receptor tyrosine kinases and in many cases is sufficient to activate these kinases (29, 30, 39). We therefore attempted to artificially dimerize and activate MuSK in the absence of agrin using a peptide antisera directed against the N terminus of the MuSK protein. Polyclonal antibodies (pAb N-MuSK) affinity-purified from this serum recognized a single band in detergent extracts from COS cells transfected with a MuSK expression construct (Fig. 1A). A band of corresponding size was recognized by these antibodies in a plasma membrane preparation of the muscle cell line C2C12 (Fig. 1B). At least a subset of these antibodies was able to react with undenatured MuSK protein, since intact antibodies as well as Fab fragments bound to MuSK-transfected unfixed COS cells but not to mock-transfected controls (Fig. 1C). MuSK was concentrated in small patches on the surface of transfected COS-cells. Similar immunoreactive patches were observed when cells were fixed by incubation with paraformaldehyde prior to exposure to antibodies or Fab fragments (data not shown). This suggests a tendency for MuSK to self-aggregate.

In a first set of experiments, we assessed the ability of these antibodies to induce tyrosine phosphorylation of MuSK by incubating differentiated C2C12 myotubes for 1 h. After this stimulation, MuSK was immunoprecipitated from detergent extracts of the cells using a fusion protein antiseraum directed against its intracellular domain. Phosphorylation of the MuSK molecule on tyrosine residues was detected by probing of Western blots with phosphotyrosine-specific antibodies. Incubation of myotubes with two concentrations of pAb N-MuSK indeed significantly induced tyrosine phosphorylation of the kinase (Fig. 2A). Several control experiments indicated that antibody-induced phosphorylation was specific: 1) phosphorylation was greatly reduced in the presence of a 10,000-fold molar excess of the N-peptide, against which the antisera had been raised; and 2) high concentrations of control antibodies directed against an intracellular region of MuSK (pAb Cyt-
MuSK did not cause phosphorylation of MuSK; 3) Fab fragments derived from N-MuSK antibodies had no effect, although they bound similar amounts of antigen as compared with pAb N-MuSK (Fig. 1C and data not shown). This observation indicated that bivalency of the antibodies was necessary to induce phosphorylation of MuSK, suggesting that pAb N-MuSK was able to dimerize the kinase. Reprobing the blot with MuSK-specific antibodies demonstrated that variations in the amounts of MuSK protein that had been precipitated did not account for the differences in tyrosine phosphorylation.

**Antibodies against MuSK TriggerTyrosine Phosphorylation of the β-Subunit of the AChR**—Agrin induces the tyrosine phosphorylation of the β-subunit of the AChR in chick and C2C12 myotubes cultures (18, 40). We therefore investigated whether antibody-induced dimerization of MuSK had similar effects. We isolated AChRs from detergent extracts of myotubes treated with pAb N-MuSK or from control preparations. Antibody-induced dimerization of MuSK caused a significant and dose-dependent increase in tyrosine phosphorylation of the AChR β-subunit (Fig. 2B). We conclude that dimerization of MuSK induced not only kinase autophosphorylation but also the phosphorylation of a downstream target. Only bivalent N-MuSK-antibodies were able to induce AChR phosphorylation; Fab fragments or control antibodies had no effect. Reprobing of the blot with a monoclonal antibody directed against the β-subunit showed that comparable amounts of AChR were precipitated from the detergent extracts in all samples.

**Antibodies against MuSK Induce Aggregation of AChRs**—Next, we asked whether activation of MuSK alone is sufficient to induce not only phosphorylation but also clustering of AChRs. We incubated C2C12 myotubes with pAb N-MuSK or with soluble nerve agrin (s-agrin (4, 19)) for 12 h, visualized AChRs with rhodamine-conjugated α-bungarotoxin, and ana-
MuSK Antibodies Induce AChR Aggregation

Fig. 3. The polyclonal antibodies N-MuSK induce the aggregation of AChRs on the surface of C2C12 myotubes. C2C12 myotubes were incubated with s-agrin (4, 19) (20 pm) (A); pAb N-MuSK (15 nM) (B); DMEM (control) (C), or with pAb M-cadherin (30 nM) (D) for 10 h. AChRs were visualized with rhodamine-α-bungarotoxin. Bar, 20 μm.

Agrin caused the redistribution of AChRs into large aggregates on the surface of myotubes (Fig. 3A). Strikingly, N-MuSK antibodies were able to trigger a similar aggregation in the absence of agrin (Fig. 3B), whereas untreated myotubes rarely bore AChR clusters (Fig. 3C). The effect of anti-MuSK antibodies was specific; antibodies against an extracellular region of M-cadherin (pAb M-cadherin), which stains the neuromuscular junction in adult mouse skeletal muscle (41), did not induce a significant number of AChR clusters (Fig. 3D). Antibodies directed against TGFβR I and a cytoplasmic region of MuSK also had no effect (data not shown). Typically, upon extended incubation, agrin induced long AChR patches (>5 μm in length) on C2C12 myotubes (Fig. 3A). In addition to these aggregates, pAb N-MuSK treatment often induced small AChR-rich patches that were not included in our quantitative analysis of antibody-induced AChR clustering.

The aggregating activity of pAb N-MuSK was specific (Fig. 4A) and dose-dependent (Fig. 4B). Even higher concentrations of antibodies directed against extracellular domains of M-cadherin and TGFβR I, an unrelated receptor protein of the muscle surface, had no effect on the distribution of AChRs. An excess of N-peptide abolished the AChR-aggregating effects of pAb N-MuSK. As in our MuSK and AChR phosphorylation experiments, dimerization of the MuSK molecule was required for AChR clustering; Fab fragments of pAb N-MuSK did not induce AChR aggregation (Fig. 4A).

**Fig. 4.** Antibody-induced AChR clustering is specific and dose-dependent. AChR aggregates induced by pAb N-MuSK and control antibodies were quantified. A, C2C12 myotubes were treated with pAb N-MuSK (30 nM) alone or in the presence of a 10,000-fold molar excess of N-peptide, with pAbs against M-cadherin and against TGFβR I (both 60 nM), with Fab fragments (active concentration 30 nM) derived from pAb N-MuSK, or with DMEM (control) for 16 h. After staining with rhodamine-α-bungarotoxin, AChR clusters in at least 12 microscopic fields were counted. One representative experiment performed in triplicate is shown (mean ± S.E.). B, concentration dependence of antibody-induced aggregation of AChR. C2C12 myotubes were treated with different concentrations of pAb N-MuSK. Mean numbers of aggregates ± S.E. from five culture chambers are shown. The number of AChR aggregates induced by all concentrations of MuSK antibodies used in these experiments was significantly different from the number of spontaneous clusters observed in the absence of effector (t test; p < 0.01).

MuSK Antibodies Induced MuSK Phosphorylation with Higher Efficiency than AChR Phosphorylation and Aggregation—The experiments described above demonstrated that MuSK activation alone mimics effects normally triggered by agrin. However, they could not exclude the possibility that interactions of agrin with components of the myotube surface not connected to MuSK (e.g. α-dystroglycan) play a synergistic role in initiating AChR aggregation. To set a limit for the potential effects of such MuSK-independent effects of agrin, it was important to compare the ability of agrin and anti-MuSK antibodies to induce different effects more quantitatively. C2C12 myotubes were stimulated with various concentrations of pAb N-MuSK and s-agrin (4, 19) or with DMEM (control). From one aliquot of the cell lysates, MuSK was immunoprecipitated; from another, AChRs were affinity-purified. In both preparations, tyrosine phosphorylation evoked by the two effectors was measured by quantitative Western blot analysis. In the concentration range used in this experiment, pAb N-MuSK induced a higher degree of MuSK phosphorylation than agrin, whereas AChR phosphorylation was triggered with reversed efficiencies (Fig. 5). For example, 40 pm agrin induced a comparable level of MuSK phosphorylation as 24 nM pAb N-MuSK, but 3-fold higher antibody concentrations were required to match the ability of 40 pm agrin to cause AChR phosphorylation. Similarly, 3-fold higher concentrations of antibody were required to induce AChR aggregation in comparison with MuSK phosphorylation (data not shown). Thus, a potential activation of MuSK-independent receptors by agrin can at best play a small synergistic role in the agrin signaling pathway.

**Antibody-induced AChR Aggregation, but Not Tyrosine Phosphorylation of MuSK or AChRs Is Inhibited by Heparin.—**The possibility of activating AChR aggregation in the absence of agrin allowed us to further delineate a target for the action of heparin, a well known inhibitor of nerve- as well as agrin-induced clustering of AChRs (42, 43). This inhibitor could prove useful in studies aiming at an understanding of the mechanisms by which MuSK activation triggers AChR aggregation. While heparin directly binds to a subset of agrin isoforms (21, 22, 44), this direct binding to agrin only accounts for part of its...
inhibitory effects. Recently, we showed that heparin acts as an inhibitor at an additional step in the agrin pathway (34), which has not been identified.

To narrow down this second target of heparin, we investigated whether heparin differentially affects AChR aggregation induced by agrin or anti-MuSK antibodies. High concentrations of heparin reduce the amount of AChR aggregates induced by a non-heparin-binding agrin isoform by 55–75% (34). Upon inducing AChR aggregation by incubation of myotubes with anti-MuSK antibodies, we observed an ~80% reduction in the number of AChR clusters (Fig. 6A), demonstrating that the target mediating this heparin inhibition is not localized upstream of MuSK.

Next, we assessed whether heparin blocks phosphorylation of MuSK and AChRs to a similar extent as it inhibits AChR aggregation. We incubated C2C12 myotubes with a non-heparin-binding agrin isoform or MuSK antibodies in the presence of different concentrations of heparin. Then we precipitated MuSK by incubation with our fusion protein antiserum and AChRs by absorption to biotin-a-bungarotoxin and analyzed their phosphotyrosine content (Fig. 6B). Surprisingly, neither MuSK nor AChR phosphorylation was significantly affected by the presence of heparin. Similar results were obtained with lower concentrations of agrin and pAb N-MuSK (data not shown).

These experiments provided additional evidence that heparin acts downstream from MuSK. In addition, they identify heparin as the first agent that selectively affects AChR aggregation but not its phosphorylation.

**DISCUSSION**

The goal of our study was to specify the role of MuSK in the assembly of the postsynaptic apparatus induced by agrin. We have shown that incubation of myotubes with antibodies against MuSK triggers the tyrosine phosphorylation of MuSK. More importantly, we have demonstrated that this antibody-induced cross-linking is sufficient to induce similar effects as treatment of myotubes with agrin; AChRs started to aggregate, and their β-subunits became phosphorylated on tyrosine residues.

Fab fragments of pAb N-MuSK did not trigger similar effects, although they bound to MuSK to a similar extent as bivalent antibodies in several control experiments. We found no evidence for binding of our antibodies to other cell surface proteins besides MuSK. Furthermore, the small size of the N-peptide (14 amino acids) against which N-MuSK antibodies are directed makes it very unlikely that more than one antibody at a time bound to MuSK’s N terminus. Based on these considerations, we conclude that the observed AChR aggregation is caused by a selective dimerization of MuSK and cannot be attributed to extensive cross-linking of this molecule.

In comparison with agrin, anti-MuSK antibodies displayed slightly different efficiencies of MuSK phosphorylation versus
AChR phosphorylation and aggregation. These differences could indicate the synergistic participation of a hypothetical MuSK-independent signal, which is triggered by agrin but not by the antibodies. However, in a detailed study of the ligand specificity of agrin-induced effects, no evidence was found for the existence of such a signal. Alternatively, the reduced level of AChR phosphorylation and aggregation in our experiments was due to a slightly altered conformation of the agrin receptor complex in response to antibody-induced but not agrin-induced MuSK dimerization and/or an incomplete activation of the kinase domain of MuSK (Fig. 7). While the mode of activation of MuSK has not been analyzed so far, a stepwise autophosphorylation and activation process has previously been described for other receptor tyrosine kinases, e.g., the insulin receptor (45).

While this manuscript was in preparation, another report described the activation of MuSK by a single chain antibody (46). In agreement with our results, this activation was sufficient to trigger the phosphorylation and aggregation of AChRs, although these effects were not studied quantitatively. In contrast to our antibodies directed against the N terminus of MuSK, several monovalent antibodies directed against unknown regions of the extracellular domain of MuSK caused the activation of the kinase. Surprisingly, bivalency of these antibodies was not required, suggesting a different mode of activation (47).

The effects of antibody-induced activation of MuSK described here demonstrate that anti-MuSK antibodies are a useful tool for elucidating MuSK’s role in the agrin pathway. The comparison of our results with those of other attempts to activate MuSK agrin-independently (33) highlights a major difference between MuSK and other receptor tyrosine kinases: the crucial role of the extracellular domain of MuSK. Glass et al. (33) stimulated a chimeric receptor consisting of the extracellular domain of TrkC and the intracellular domain of MuSK with neurotrophin 3 and thereby efficiently induced phosphorylation of the chimera and the AChR. However, activation of TrkC/MuSK did not lead to the aggregation of AChR on the surface of C2C12 myotubes (Fig. 7). Clearly, stimulating the kinase activity of MuSK alone cannot account for AChR aggregation. In addition to the MuSK/TrkC chimera, antibody-induced dimerization of the full-length MuSK molecule and concomitant redistribution of putative MuSK-associated proteins was able to induce not only the phosphorylation of AChRs but also their aggregation.

This functional difference directly points at an essential role of the extracellular domain of MuSK. An inherent organizing function has previously been suggested by cotransfection experiments in a quail fibroblast cell line (31, 32). In this system, a kinase-defective mutant of the Torpedo MuSK ortholog (31) and a MuSK fragment in which most of the cytoplasmic domain had been deleted (32) were aggregated by cotransfected rapsyn. In muscle cells, a kinase-defective mutant of rat MuSK suppressed AChR clustering (33), demonstrating the requirement of tyrosine kinase activity for this process.

Our data complement the suggestion that two signals are necessary to induce the aggregation of AChRs in myotubes (Fig. 7) (32, 48); the first signal is the kinase activity of MuSK, and the second signal appears to originate from the physical association of other proteins with MuSK. It is neither known in which way this scaffolding depends on MuSK activation nor which proteins associate with MuSK. The most likely candidate appears to be RATL, which might directly tether rapsyn and the stoichiometrically complexed AChRs to MuSK (32). Alternatively, the passive redistribution of MASC induced by MuSK dimerization could be important for AChR aggregation. This second possibility appears less likely, since our data showed that a direct activation of MASC by the binding of agrin is not essential for this pathway. Any signal that might be triggered by the binding of agrin to MASC can be bypassed by the dimerization of MuSK.

Two types of inhibitors of the agrin pathway have been characterized so far. Staurosporin, an inhibitor of tyrosine kinases, blocks both phosphorylation and aggregation of AChRs (17) and apparently inhibits the first signal in the pathway (Fig. 7); our data suggest that heparin represents a second type of inhibitor, which interferes with the second signal. Heparin treatment caused a MuSK/TrkC-like “phenotype”; it inhibited AChR aggregation induced by a non-heparin-binding agrin isoform (34) and by anti-MuSK antibodies by more than 80%. Strikingly, it did not affect the phosphorylation of either MuSK or AChRs. This selective interference with receptor aggrega-
tion would be expected from a reagent interfering exclusively with the second signal in the agrin pathway. The extracellular domain of MuSK, which is involved in this step, is accessible to heparin and other polyanions added into the medium. The protein directly interacting with heparin has not been identified so far, but RATL is an interesting candidate.

The availability of specific activators and inhibitors of the agrin signaling pathway should be useful in the future to identify the missing players and understand how they interact with the already identified components.

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