Acute Severe Animal Model of Anti–Muscle-Specific Kinase Myasthenia

Combined Postsynaptic and Presynaptic Changes

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Objectives: To determine the pathogenesis of anti–muscle-specific kinase (MuSK) myasthenia, a newly described severe form of myasthenia gravis associated with MuSK antibodies characterized by focal muscle weakness and wasting and absence of acetylcholine receptor antibodies, and to determine whether antibodies to MuSK, a crucial protein in the formation of the neuromuscular junction (NMJ) during development, can induce disease in the mature NMJ.

Design, Setting, and Participants: Lewis rats were immunized with a single injection of a newly discovered splicing variant of MuSK, MuSK 60, which has been demonstrated to be expressed primarily in the mature NMJ. Animals were assessed clinically, serologically, and by repetitive stimulation of the median nerve. Muscle tissue was examined immunohistochemically and by electron microscopy.

Results: Animals immunized with 100 µg of MuSK 60 developed severe progressive weakness starting at day 16, with 100% mortality by day 27. The weakness was associated with high MuSK antibody titers, weight loss, axial muscle wasting, and decrementing compound muscle action potentials. Light and electron microscopy demonstrated fragmented NMJs with varying degrees of postsynaptic muscle end plate destruction along with abnormal nerve terminals, lack of registration between end plates and nerve terminals, local axon sprouting, and extrajunctional dispersion of cholinesterase activity.

Conclusions: These findings support the role of MuSK antibodies in the human disease, demonstrate the role of MuSK not only in the development of the NMJ but also in the maintenance of the mature synapse, and demonstrate involvement of this disease in both presynaptic and postsynaptic components of the NMJ.


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well delineated,16,17 raising the question of the mechanisms by which Ab attack on this molecule in the adult NMJ alters its function.3,6,7,18

Muscle-specific kinase is a 100-kDa transmembrane receptor tyrosine kinase with an N-terminal extracellular domain followed by a short transmembrane domain and then a C-terminal cytoplasmic domain.19-21 The extracellular domain, which appears to be required for interaction with agrin and low-density lipoprotein receptor-related protein 4, comprises 3 immunoglobulin-like domains followed by a cysteine-rich (frizzled-like) domain.13,14,20-23 It is only the extracellular domain of MuSK 60 (N–MuSK 60)24 induces all the major characteristics of the human disease with lower anti-MuSK Ab titers. Analysis of NMJ morphology in these animals suggests that Ab attack on MuSK affects both postsynaptic and presynaptic components of this synapse.

PRODUCTION, DETECTION, AND PURIFICATION OF N–MuSK 60 PROTEIN

Complementary DNA of MuSK was obtained by reverse transcription–polymerase chain reaction using total RNA from mouse adult (innervated) muscle (Invitrogen Corp). Analysis of the DNA sequence of these clones identified a variety of isoforms of mouse MuSK, including the one published sequence in GenBank (GenBank U37709). Among the other isoforms, we identified a novel MuSK splicing variant (MuSK 60) containing an additional 60 nucleotides in frame in the region between immunoglobulin-like domains 2 and 3 and expressed primarily in adult muscle.24 The N-terminal extracellular domain of MuSK 60 (N–MuSK 60) was obtained from the culture supernatants of COS7 and CHO cells transiently transfected with the expression and secretion vector pSecTag2/Hygro containing the tagged (3’ myc-epitope and polyhistidine) N–MuSK 60 complementary DNA. The secreted protein was purified using an affinity column loaded with Profinity IMAC nickel-charged resin (Bio-Rad Laboratories, Inc).

ANIMALS AND IMMUNIZATIONS

A total of 0.25 mL of purified N–MuSK 60 (the “adult” isoform; either 50 µg or 100 µg) or buffer was emulsified with an equal volume of complete Freund adjuvant. The emulsion was injected at 3 separate intradermal sites into female Lewis rats weighing 175 to 200 g, and 2.5 µg of pertussis vaccine was injected at a single separate subcutaneous site.

Ab TITRATION

Serum samples were diluted with TRIS-buffered saline containing 4% nonfat dry milk and 0.1% Tween 20. They were then subjected to immunoblot blot against 0.5 µg of affinity-purified N–MuSK 60 or 0.5 µg of bovine serum albumin (control) blotted on nitrocellulose membranes.

ELECTROPHYSIOLOGICAL STUDIES

Repetitive stimulations (3 Hz) of the median nerve, recording compound muscle action potentials from the flexor digitorum, were performed on anesthetized animals.26 Tracings were recorded using digital photography of the oscilloscope screen, and the decrement of the fifth response compared with the first response was calculated.

TISSUE PROCESSING

Muscle tissue from the diaphragm, gastrocnemius, and tibialis anterior was obtained when the animals were killed, either before or following cardiac perfusion with 4% paraformaldehyde, and prepared for immunohistochemistry, histochemistry, and electron microscopy as previously described.27,28 Frozen sections of diaphragm were labeled with Alexa Fluor 594...
conjugated α-bungarotoxin and rabbit polyclonal Abs to synapsin and neurofilament. Morphometric analyses of the electron micrographs, as previously described, used ImageJ software (National Institutes of Health).29-31

STATISTICAL ANALYSIS
The assessments involved continuous variables. Hence, they were analyzed by t test.

RESULTS

PRODUCTION AND PURIFICATION OF N–MuSK 60
For this study, we used the variant MuSK 60 that we have recently identified,24 which is expressed in high proportion in adult muscle. We purified on the order of 2 µg of N–MuSK 60/mL of culture medium of COS7 cells transiently transfected with complementary DNA encoding this domain (Figure 1).

INDUCTION OF EAMM IN LEWIS RATS
Fourteen 175- to 200-g female Lewis rats were immunized with a single injection of purified mouse N–MuSK 60 in complete Freund adjuvant, and 14 were immunized with adjuvants alone. Of the N–MuSK 60–immunized animals, 9 received 100 µg of antigen and 5 received 50 µg. Beginning at day 16 after injection, the N–MuSK 60–immunized animals developed fatigable weakness and accelerating weight loss (Figure 2). The 9 animals immunized with 100 µg of N–MuSK 60 were all moribund by day 27; the 5 animals immunized with 50 µg had less severe disease, with only 1 mortality (day 40). Aside from mild transient adjuvant arthritis in 6 animals, the adjuvant controls remained healthy for longer than 12 weeks.

All animals immunized with 100 µg of N–MuSK 60 had serum MuSK 60 Ab titers greater than 1:10⁶, whereas the 5 animals immunized with 50 µg of N–MuSK 60 had lower titers, 1:10⁵, and the 14 adjuvant controls and 4 untreated littermates had undetectable titers (Figure 3). The weakness in the N–MuSK 60–immunized animals, which began in the forelimbs, progressed rapidly to axial muscles and hind limbs. As the disease progressed, the weight loss became pronounced and the animals developed progressive axial muscle wasting, waddling gait, marked kyphosis, and ruffled, ungroomed fur (Figure 4). The ability of these animals to eat, drink, and chew was not observably abnormal until the last 2 days of life. At that time, water-soaked food pellets were placed on the

Figure 2. Clinical course. A, Rats immunized with 100 µg of the N-terminal extracellular domain of muscle-specific kinase 60 (N–MuSK 60) developed more severe weakness than those immunized with 50 µg. A clinical score of 0 indicates normal; 1, weak grip; 2, abnormal gait; 3, walking only a few steps at a time with waddle and kyphosis; 4, inability to stand; and 5, moribund. B, Mean weight of these animals, demonstrating more severe weight loss in rats immunized with 100 µg of N–MuSK 60. Error bars indicate SEM.

Figure 3. Immunodot blot of serum samples at day 27. A, Serum samples diluted from 1:10⁶ through 1:10⁸ from rats immunized with 100 µg of the N-terminal extracellular domain of muscle-specific kinase 60 (N–MuSK 60) (titer >1:10⁶) and one immunized with 50 µg (titer of 1:10⁵) were blotted against 0.5 µg of affinity-purified mouse N–MuSK 60 or 0.5 µg of bovine serum albumin (BSA) as an antigen control. B, Serum samples from 3 adjuvant control animals diluted 1:500 showed no reaction.
floor of their cages and of the control animals’ cages. Despite this maneuver, all animals immunized with 100 µg of N–MuSK 60 were moribund by day 27.

NEUROMUSCULAR TRANSMISSION

The compound muscle action potential response to 3-Hz stimulation assessed in the flexor digitorum on day 27 in the animals immunized with 100 µg of N–MuSK 60 revealed a mild decrement (mean, 9.3%), whereas the animals immunized with 50 µg, studied at a later time, days 33 to 36, demonstrated a more severe decrement (mean, 15.6%) even though they were less weak than the animals in the 100-µg group were at the time they were tested (Table 1 and eFigure 1). No abnormalities in response to repetitive stimulation were observed in the 14 adjuvant controls on either day 27 or days 33 to 66. These observations suggest that the time course of the axial muscle wasting and weakness in EAMM and of the abnormal neuromuscular transmission, measured distally, may differ.

NMJ MORPHOLOGY

Stained longitudinal frozen sections of diaphragm muscle from adjuvant control animals were normal with the characteristic pretzel-shaped appearance of the end plate membrane and with the presynaptic terminal precisely opposed to the postsynaptic AchR-stained end plates (Figure 5A). For the rats immunized with 100 µg of N–MuSK 60, the architecture and distribution of the postsynaptic components as well as the presynaptic components of the NMJs were highly abnormal. Most NMJs were disrupted, with the terminal arbors and AchR clusters being fragmented into smaller, discontinuous structures (Figure 5B). This discontinuity was accompanied by decreased alignment of the presynaptic and postsynaptic elements. Many nerve terminals appeared to be degenerating and occupied only small portions of the postsynaptic receptor clusters. In other cases, elongated globular nerve sprouts extended for short distances away from the existing NMJ (Figure 5C). In the most severe cases, only remnants of neuromuscular synapses remained; these consisted of widely dispersed, small AchR aggregates and no detectable nerve terminal (Figure 5D). No inflammatory cells were identified at any NMJs (eFigure 2). Thus, within individual muscles there were variable degrees of disruption from NMJ to NMJ. In addition, while similar changes were observed in a distal extremity muscle, the tibialis anterior, fewer end plates were involved and the severity of the changes was less.

To quantify the changes in morphology (Table 2), we analyzed the images of en face NMJs using AxioVision software (Carl Zeiss MicroImaging GmbH). Compared with control animals, postsynaptic AchR-staining segments in N–MuSK 60-immunized animals were composed of many more discontinuous regions (0.5 vs 35.6, respectively), with each region being smaller in area (44.1 vs 8.6 µm², respectively). The maximal diameter of NMJs was also increased in N–MuSK 60–immunized animals compared with control animals (56.2 µm compared with 43.7 µm, respectively). The mean area of postsynaptic AchR-stained end plates was also increased in N–MuSK 60–immunized animals compared with control animals (123.1 µm² compared with 60.5 µm², respectively). The mean area of presynaptic nerve terminal segments was also increased in N–MuSK 60–immunized animals compared with control animals (17.5 µm² compared with 9.1 µm², respectively).

![Figure 4. Clinical findings at day 25. A, Rat immunized with 100 µg of the N-terminal extracellular domain of muscle-specific kinase 60 (right) had significant weight loss, flank and neck muscle wasting, extremity weakness, kyphotic posture, and ruffled, ungroomed fur, whereas the adjuvant control (left) was healthy. B, Lateral view of the same immunized rat.](http://archneur.jamanetwork.com/)

**Table 1. Compound Muscle Action Potential Responses to 3-Hz Repetitive Median Nerve Stimulation**

<table>
<thead>
<tr>
<th>Immunization</th>
<th>Amplitude of First Response, mV (SEM)</th>
<th>Amplitude of Fifth Response, mV (SEM)</th>
<th>Mean (SEM)</th>
<th>P Value of Decrement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adjuvant control (n=14)</td>
<td>68.6 (5.7)</td>
<td>69.1 (5.5)</td>
<td>69.1 (5.5)</td>
<td>-0.9 (0.4)</td>
</tr>
<tr>
<td>N–MuSK 60 100 µg (n=9)</td>
<td>64.0 (3.5)</td>
<td>57.9 (3.4)</td>
<td>64.0 (3.5)</td>
<td>9.3 (1.7)</td>
</tr>
<tr>
<td>N–MuSK 60 50 µg (n=5)</td>
<td>65.0 (5.2)</td>
<td>54.6 (4.0)</td>
<td>65.0 (5.2)</td>
<td>15.6 (2.3)</td>
</tr>
</tbody>
</table>

Abbreviation: N–MuSK 60, N-terminal extracellular domain of muscle-specific kinase 60.
vs 34.2 µm, respectively). These results indicate a dramatic fragmentation of postsynaptic AChR segments in the N–MuSK 60–immunized animals, accompanied by a dispersal of the postjunctional fragments.

We also assessed cholinesterase-stained end plates in teased gastrocnemius bundles (to identify synaptic regions for electron microscopic study). This unexpectedly revealed variable degrees of patchy granular cholinesterase activity in extrajunctional regions of all myofibers (Figure 6A), many quite distant from the innervation band, a finding not seen in experimental autoimmune MG (EAMG) or our controls. Some of these extrajunctional stained structures had the size and appearance of end plates. At higher magnification of end plate regions (Figure 6B), punctate cholinesterase staining was observed perijunctionally.

Neuromuscular junctions of gastrocnemius (Figure 7) from 2 of the animals immunized with 100 µg of N–MuSK 60 and 2 of the adjuvant control animals were examined with the electron microscope. The NMJs from the N–MuSK 60–immunized animals compared with the adjuvant control animals demonstrated hypersegmentation of some junctions (as manifested by increased numbers of junctional segments per unit of fiber length; 0.25/µm vs 0.15/µm, respectively) and increased total nerve terminal area (7.74 vs 0.064 µm², respectively) (Table 3). In addition, there was marked simplification of postsynaptic membranes, resulting in reduced end plate index (ratio of the length of the postsynaptic membrane to the length of the apposed presynaptic membrane)²⁷ (1.67 vs 5.90, respectively) and reduced numbers of secondary end plate folds per length of the primary cleft²⁷,³⁰ (0.37/µm vs 2.08/µm, respectively). No inflammatory cells were observed in any of these electron micrographs.

Together, these morphologic observations demonstrate that anti-MuSK attack produces severe disruption
of both components of the NMJ and even complete loss of these structures.

**COMMENT**

Experimental studies have supported the hypothesis that AMM is the result of the autoimmune response directed against MuSK, by observing weakness and NMJ changes in animals actively or passively immunized with MuSK. In rabbits and mice repeatedly immunized with MuSK, mild weakness has been observed and there is mild electrophysiological evidence of disordered neuromuscular transmission. In addition, in mice passively injected with human AMM IgG repeatedly over 14 days (total of 0.68 g), mild to moderate weakness occurred in conjunction with reduced MuSK and AChR staining and reduced registration between nerve terminals and end plates at NMJs.

In contrast, the form of EAMM induced in Lewis rats by a single immunization with 100 µg of xenogeneic N-MuSK 60 is extremely severe, with 100% mortality by 27 days and very high anti-MuSK antibody titers (>1:10⁶). The animals exhibit marked weight loss and axial muscle wasting, the latter not described in the other models. As the disease progresses, the axial weakness and wasting lead to a striking kyphotic posture and eventually the inability of the forelimbs to lift the chest from the floor of the cage. It is of note that similar posture and gait abnormalities have been observed in adult mice in which MuSK expression was turned off using Cre recombinase-mediated MuSK gene deletion. The reproduction of all the characteristics of AMM in the current form of EAMM supports the hypothesis that the autoimmune response to MuSK in AMM is pathogenically important in this disease rather than representing an epiphenomenon. Moreover, these observations highlight the potential usefulness of this model for studying the pathogenesis and future treatments of AMM.

It is unclear why the disease in our study is so much more severe than that induced in the 3 previous studies.
involved in active immunization. Possible factors include differences in species susceptibility to autoimmunity, or species differences in sensitivity to the anti-MuSK attack, perhaps related to differences in the safety factor of neuromuscular transmission. A third possibility relates to the differences in the antigens used to induce the disease and, hence, the epitope targets of the possibility relates to the differences in the antigens used to induce the disease and, hence, the epitope targets of the disease-inducing Abs. The MuSK 60 isoform used as the immunogen in this study appears to be an adult form of the protein, which may be an important target of the auto-Abs in EAMM and AMM. For the rabbit and mouse forms of EAMM, the immunogens have been either the fetal isoform or another splicing variant of the protein that is missing not only the 20-amino acid extra domain of MuSK 60 but also the entire third immunoglobulin-like domain.

Immunization with a lower dose of N–MuSK 60 resulted in lower titers of anti-N–MuSK 60 Abs (1:10³) and less severe disease with a much lower mortality. The correlation between anti-N–MuSK 60 Ab titer and disease severity also supports the hypothesis that EAMM and AMM are the result of the action of the MuSK Abs. The observation that the low-dose animals exhibit greater diminution in neuromuscular transmission (at least as measured in a distal extremity muscle) (Table 1), albeit at later times following the immunization, may simply relate to the differential effect observed on axial vs distal muscles in EAMM. However, the observation also raises the possibility that the weakness, weight loss, and muscle wasting associated with the rapidly fatal high-dose disease may not solely be the consequence of reduced neuromuscular transmission per se but rather that other physiological activities at the NMJ may also play a role.

Within individual EAMM muscles, NMJs exhibit varying degrees of disruption. The architecture of some end plates is altered at the ultrastructural level by hypersegmentation consisting of multiple small axon terminals with marked simplification of postsynaptic membranes (Figure 7 and Table 3). The nerve terminals at other NMJs are more severely affected, with complete or partial loss of these structures (Figure 5 and Table 2). In addition, some axons have an abnormal globular appearance and others exhibit local extension beyond the NMJ, or even frank terminal axon sprouting (Figure 5). In some NMJs, there is misalignment between the presynaptic and postsynaptic portions of these synapses. The latter findings, some of which were also observed in the study of passive transfer of human AMM serum into mice and in 1 of the 3 studies of AMM, suggest that there is abnormal signaling between the nerve terminal and muscle end plate in both directions, resulting in failure of maintenance of the mature synapse in these animals.

In addition, cholinesterase staining of teased muscle bundles from N–MuSK 60–immunized animals reveals segmented and dispersed cholinesterase-stained patches away from the compact synaptic region (motor point) (Figure 6) reminiscent of newly formed synapses, which raises the possibility that the Ab attack leads first to frank denervation at some NMJs with subsequent and possibly ongoing attempts at reinnervation. It is of special note that severe damage to the end plate membrane, as is seen in acute forms of EAMG and MG and was shown in MG by Fambrough et al, produces local cholinesterase spreading but minimal nerve terminal abnormalities and/or denervation and distant reinnervation. Hence, the Ab attack on MuSK appears to have wider-ranging effects on the NMJ than are seen with the highly destructive Ab attack on the more abundant end plate AChRs in EAMG and MG.

The mechanism of the prominent axial muscle wasting in EAMM rats, also not seen in EAMG and MG, remains unclear. Both histological and electrophysiological studies in human AMM suggest that the muscle wasting is not the result of denervation but rather is the consequence of a myopathic process. Such observations support a role for MuSK in mediating trophic effects on muscle, perhaps through complex 2-way communication between nerve and muscle at the NMJ. On the other hand, focal denervation with accompanying reinnervation, as suggested by some of the morphologic studies we have described, might also lead to muscle wasting.

Finally, the observations presented here demonstrate that an immune attack on MuSK can result in weakness, muscle wasting, and severe disruption of the architecture of both the postsynaptic and presynaptic portions of the mature NMJ. They support the hypothesis that, in addition to its role in the developing NMJ, MuSK plays a role in the maintenance and function of the mature structure.

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Table 3. Ultrastructural Analysis of Neuromuscular Junctions

<table>
<thead>
<tr>
<th>Immunization</th>
<th>Junctional Segments/µm of Muscle Fiber, No.</th>
<th>End Plate Indexa</th>
<th>Secondary Clefts/µm of Primary Cleft, No.</th>
<th>Nerve Terminal Area, µm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adjuvant control</td>
<td>0.15 (0.013)</td>
<td>5.80 (0.49)</td>
<td>2.08 (0.18)</td>
<td>0.064 (0.011)</td>
</tr>
<tr>
<td>Muscle fibers/NMJs, No.</td>
<td>18</td>
<td>23</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>N–MuSK 60</td>
<td>0.25 (0.002)</td>
<td>1.67 (0.11)</td>
<td>0.37 (0.001)</td>
<td>7.74 (0.52)</td>
</tr>
<tr>
<td>Muscle fibers/NMJs, No.</td>
<td>15</td>
<td>43</td>
<td>43</td>
<td>43</td>
</tr>
</tbody>
</table>

Abbreviation: NMJs, neuromuscular junctions; N–MuSK 60, N-terminal extracellular domain of muscle-specific kinase 60.

aLength of postsynaptic membrane divided by length of apposed presynaptic membrane.
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Author Contributions: Study concept and design: Richman and Agius. Acquisition of data: Richman, Nishi, Morell, Chang, Ferns, Wollmann, Maselli, and Schnier. Analysis and interpretation of data: Richman, Nishi, Morell, Chang, Ferns, Maselli, Schnier, and Agius. Drafting of the manuscript: Richman, Nishi, Chang, and Ferns. Critical revision of the manuscript for important intellectual content: Richman, Nishi, Morell, Chang, Ferns, Wollmann, Maselli, Schnier, and Agius. Statistical analysis: Richman, Chang, and Ferns. Obtained funding: Richman, Ferns, Maselli, and Agius. Administrative, technical, and material support: Richman and Wollmann. Study supervision: Richman, Ferns, Maselli, and Agius.

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