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Muscle-selective synaptic disassembly and reorganization in MuSK antibody positive MG mice

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ABSTRACT

MuSK antibody seropositive (MuSK+) Myasthenia Gravis (MG) patients present a distinct selective fatigue, and sometimes atrophy, of bulbar, facial and neck muscles. Here, we study the mechanism underlying the focal muscle involvement in mice with MuSK+ experimental autoimmune MG (EAMG). 8 week-old female wildtype C57BL6 mice and transgenic mice, which express yellow fluorescence protein (YFP) in their motor neurons, were immunized with the extracellular domain of rat MuSK and compared with control mice. The soleus, EDL, sternomastoid, omohyoid, thoracic paraspinal and masseter muscles were examined for pre- and postsynaptic changes with whole mount immunostaining and confocal microscopy. Neuromuscular junction derangement was quantified and compared between muscles and correlated with transcript levels of MuSK and other postsynaptic genes. Correlating with the EAMG disease grade, the postsynaptic acetylcholine receptor (AChR) clusters were severely fragmented with a subsequent reduction also of the presynaptic nerve terminal area. Among the muscles analyzed, the thoracic paraspinal, sternomastoid and masseter muscles were more affected than the leg muscles. The masseter muscle was the most affected, leading to denervation and atrophy and this severity correlated with the lowest levels of MuSK mRNA. On the contrary, the soleus with high MuSK mRNA levels had less postsynaptic perturbation and more terminal nerve sprouting. We propose that low muscle-intrinsic MuSK levels render some muscles, such as the masseter, more vulnerable to the postsynaptic perturbation of MuSK antibodies with subsequent denervation and atrophy. These findings augment our understanding of the sometimes severe, facio-bulbar phenotype of MuSK+ MG.

Introduction

About 40–70% of acetylcholine receptor (AChR)-antibody seronegative Myasthenia Gravis (MG) patients have antibodies against the muscle tyrosine kinase (MuSK) (Hoch et al., 2001; Sanders et al., 2003). In MuSK-antibody seropositive (MuSK+) MG patients, there is often selective involvement of bulbar-, neck- and facial muscles, as well as muscles that are usually asymptomatic in AChR-antibody seropositive (AChR+) MG, such as the paraspinal muscles (Sanders and Juel, 2008). Contrary to conventional AChR+ MG patients, the majority of MuSK+ patients does not experience symptomatic relief from acetylcholine esterase inhibitors (AChEI) (Evoli et al., 2003) but instead may respond with pronounced nicotinic adverse effects, such as muscle fasciculations and cramps (Punga et al., 2006). Pronounced atrophy of facial muscles has also been described in MuSK+ patients, although the concomitant treatment of corticosteroids in most cases has made it difficult to judge whether the MuSK antibodies or the cortisone treatment is the cause of the atrophy (Farrugia et al., 2006).

Muscle biopsy studies of the intercostal muscle and biceps brachii muscle from MuSK+ patients have shown little AChR loss (Selcen et al., 2004; Shiraishi et al., 2005), however, the neuromuscular junction (NMJ) pathophysiology in the most affected facial or bulbar muscles has not been studied. Nevertheless, MuSK antibodies have been shown to be pathogenic in animals, both after immunization with the extracellular domain of the MuSK protein itself (Jha et al., 2006; Shigemoto et al., 2008; 2006; Xu et al., 2006) and after passive transfer of sera from MuSK+ MG patients (Cole et al., 2008; ter Beek et al., 2009). MuSK is essential to the process of NMJ formation, maintenance (Wang et al., 2006) and integrity, as perturbations in MuSK protein expression cause pronounced disassembly of the entire NMJ (Hesser et al., 2006; Kong et al., 2004). Other NMJ proteins that are essential for synaptogenesis include Dok-7, a downstream adaptor protein to MuSK (Okada et al., 2006), Lrp4, the co-receptor for neural agrin (Kim et al., 2008; Zhang et al., 2008), rapsyn and the AChR subunits. The effects of MuSK antibodies in-vivo on the gene expression of those synaptic proteins in the facial or bulbar muscles have not yet been established.

Here, we hypothesized that low expression levels of MuSK may render some muscles more vulnerable to the effect of MuSK antibodies in the EAMG mouse model. We show that MuSK antibodies

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induce severe fragmentation of the postsynaptic AChR clusters in particular in the masseter and thoracic paraspinal muscles, with less fragmentation in the limb muscles. The severe postsynaptic perturbation results in subsequent denervation of muscle fibers, not previously described in EAMG or MG. We propose that one underlying mechanism for the severe involvement of the facial masseter muscle, with severely impaired NMJ architecture, atrophy and denervation, is its low intrinsic levels of MuSK. Moreover, muscles respond to the partial denervation caused by MuSK antibodies in two different ways: (1) terminal nerve sprouting in muscles with high intrinsic levels of MuSK (i.e. soleus, sternomastoid) and (2) no nerve sprouting in muscles with low intrinsic MuSK levels (i.e. masseter, omohyoid).

Methods

Production of recombinant rat MuSK

pCEP-PU vector containing the His-tagged extracellular domain of recombinant rat MuSK (aa 21-491; (Jones et al., 1999)) was transfected (Lipofectamine 2000; Invitrogen) into HEK 293 EBNA cells. The overexpressed protein was purified from the cell supernatant over a Ni-NTA superflow column (Qiagen) and was subsequently dialyzed against PBS. Protein concentration was determined at OD 280 nm and purity was ensured by SDS-PAGE.

Experimental animals

C57BL6 mice and mice expressing yellow fluorescence protein (YFP) in their motor neurons under Thy-1 promoter (Feng et al., 2000) were originally supplied from Jackson Laboratories (Bar Harbor, Maine, US). For immunization, 8 week-old female mice were used. All mice were housed in the Animal Facility of Biozentrum, University of Basel, where they had free access to food and water in a room with controlled temperature and a 12 hour alternating light–dark cycle. All animal procedures complied with Swiss animal experimental regulations (ethical application approval no. 2352) and EC Directive 86/609/EEC.

Immunization

The immunization procedure has been described previously (Jha et al., 2006). Briefly, eleven C57BL6 and seven Thy1-YFP female mice aged 8 weeks were anesthetized (Ketamine: 111 mg/kg and Xylazine: 22 mg/kg) and immunized with 10 μg of MuSK emulsified in complete Freund's adjuvant (CFA, Difco laboratories, Detroit, Michigan, US) subcutaneously in the hind foot pads, at the base of the tail and dorsolateral on the back. At day 28 post-injection, immunization was repeated. A 3rd immunization was given to mice that did not show any myasthenic weakness after 56 days. Control mice (8 female mice) were immunized with PBS/CFA.

Clinical and neurophysiological examination

Muscle weakness was graded every week, as described (Nakayashiki et al., 2000). Briefly, mice were exercised by 20 consecutive paw grips on a grid and were then placed on an upside-down grid. The time they could hold on to the grid reflected the grade of fatigue and muscle weakness. EAMG grades were as follows: grade 0, no weakness; grade 1, mild muscle fatigue after exercise; grade 2, moderate muscle fatigue; and grade 3, severe generalized weakness. Evaluation of the response to AChEIs was performed by i.p. injection of a mix of neostigmine bromide (0.0375 mg/kg) and atropine sulfate (0.015 mg/kg) in mice with EAMG grades 2 and 3 (Berman and Patrick, 1980).

Repetitive stimulation of the sciatic nerve and recording from the gastrocnemius muscle with monopolar needle electrodes was performed under anesthesia, in mice with EAMG grades 2 (n=2) and 3 (n=2), using a Saphire 1L EMG machine (Medelec). Decrement was calculated as percent amplitude change between the 1st and 4th compound motor action potentials evoked by a train of 10 impulses where 10% was considered as pathological.

ELISA

Sera were obtained from tail vein blood on day 0 (preimmune sera) and day 35 post-immunization. ELISA plates (Nunc MaxiSorp, Fisher Thermo Scientific, Rockford, IL, US) were coated with 250 ng/ml of His-labeled rat MuSK (50 μl/well), blocked with 3% BSA/PBS and then incubated with a sera dilution row (1:3000–1:2,000,000). Preimmune sera constituted negative and rabbit-anti-MuSK antibody (Scotton et al., 2006) positive controls. After washing, plates were incubated with secondary HRPO-conjugated goat-anti-mouse (1:2000) and goat anti-rabbit antibodies (1:2000; both from Jackson Immuno Research Laboratories, Westgrove, PA, US). HRPO activation by a TMB substrate was terminated with 1 N HCl after 5 min. Absorbance was read at 450 nm.

Non-specific binding, determined by incubation of plates with preimmune serum, was subtracted. The data were displayed as "half maximum MuSK immunoreactivity", which represents the immunoreactivity at a dilution of 1:27,000, where the majority of sera obtained 50% of maximum absorbance (in the linear range of the absorption at 450 nm).

Western blot

Western blot of masseter muscles was conducted as described (Bentzinger et al., 2008). 10 μg of protein was resolved on a 4–12% NuPAGE Bis–Tris gel (Invitrogen, Eugene, OR, US), transferred to nitrocellulose membrane, probed with rat monoclonal anti-NCAM (CD56; 1:100; GeneTex) and rabbit polyclonal anti-pan-actin (1:1000; cell signaling) and then recognized with HRPO-conjugated antibodies (1:5000; Jackson Immuno Research Laboratories, Westgrove, PA, US).

Quantitative RT-PCR analysis

Mouse muscle RNA was extracted and purified as previously described (Punga et al., 2011). RT-PCR reactions (triplicates) were carried out with Power SYBR Green PCR Master Mix reagent (Applied Biosystems, Warrington, UK). β-actin was used as endogenous control (Punga et al., 2011; Murphy et al., 2003; Yuzbasioglu et al., 2010).

The following primer sets were used:

MuSK: 5′-GCCTTCAGCGGGACTGAG-3′ and 5′-GAGGCGTGGTGACAGG-3′

Lrp4: 5′-GGATGGCTGTACGCTGCCTA-3′ and 5′-TTGCCGTTGTCACAGTGGA-3′

Dok-7: 5′-CTCGGCAGTTACAGGAGGTTG-3′ and 5′-GCAATGCCACTGTCAGAGGA-3′

AChRα1: 5′-GCCATTAACCCGGAAAGTGAC-3′ and 5′-CCCCGCTCTCCATGAAGTT-3′

AChRε: 5′-CTGTGAACTTTGCTGAG-3′and 5′-GGAGATCAGGAACTTGGTTG-3′

AChR γ subunit: 5′-AACGAGACTCGGATGTGGTC-3′ and 5′-GTCGCACCACTGCATCTCTA-3′

Rapsyn: 5′-AGGTTGGCAATAAGCTGAGCC-3′ and 5′-TGCTCTCACTCAGGCAATGC-3′

MuRF-1: 5′-ACC TGC TGG TGG AAA ACA-3′ and 5′-AGG AGC AAG TAG GCA CCT CA-3′

β-actin: 5′-CAGCTTCTTTGCAGCTCCTT-3′and 5′-GCAGCGATATCGTCATCCA-3′

AChE: 5′-GGGCTCCTACTTTCTGGTTTACG-3′ and 5′-GGGCCCGGCTGATGAG-3′