Neuroscience 143 (2006) 655–659

RAPID REPORT

DELAYED SYNAPSING MUSCLES ARE MORE SEVERELY AFFECTED IN AN EXPERIMENTAL MODEL OF MuSK-INDUCED MYASTHENIA GRAVIS

K. XU, S. JHA, W. HOCH AND S. E. DRYER*

Department of Biology and Biochemistry, University of Houston, Houston, TX 77204-5001, USA

Abstract—Myasthenia gravis can be induced in mice by injecting the extracellular domain of rat muscle-specific kinase (MuSK), a transmembrane receptor tyrosine kinase involved in agrin signaling at the neuromuscular junction. About 5–10% of human myasthenia gravis patients have autoantibodies against MuSK. Here we have examined mouse neuromuscular junctions following MuSK immunization in two groups of muscles that can be distinguished on the basis of the timing of neuromuscular synaptogenesis and their response to perturbation of agrin signaling. We used confocal microscopy to characterize the distribution and expression of nicotinic acetylcoline receptors and of two presynaptic makers, neurofilament protein and synaptophysin. We observed disruption of neuromuscular junctions in all muscles examined in this model of myasthenia gravis. However delayed-synapsing muscles, including the diaphragm, sternomastoid and tibialis posterior, were significantly more severely affected than fast-synapsing muscles, including the intercostal, adductor longus and tibialis anterior. These results suggest a basis for the differential susceptibility of muscles in different classes of myasthenia gravis patients, including patients with autoantibodies against MuSK.

Key words: myasthenia gravis, agrin, muscle specific kinase, neuromusculular junction, autoimmune disorders, acetylcholine receptor.

Myasthenia gravis (MG) is an antibody-mediated autoimmune disease characterized by fluctuating muscle weakness associated with transmission block at neuromuscular junctions (NMJs). The molecular pathology of this disorder is heterogeneous. Antibodies to muscle-specific kinase (MuSK) have been reported in about 70% of so-called seronegative myasthenia gravis (SNMG) patients, a subset of MG patients who lack antibodies against muscle type nicotinic acetylcholine receptors (AChRs). MuSK is a receptor tyrosine kinase required for agrin-mediated organization and maintenance of the NMJ (Hoch 1999; Sanes and Lichtman, 2001; Hoch et al., 2001, Evoli et al., 2003). We have previously shown that injection of certain mouse strains with low amounts of recombinant rat MuSK elicits symptoms of MG comparable to those observed in animals injected with purified AChR (Jha et al., 2006). However, MuSK-injected mice exhibit a pronounced weakness in neck and respiratory muscles (Jha et al., 2006), which is a prominent feature in recent descriptions of human patients with MuSK-antibody-positive SNMG (Kurihara, 2005; Nemoto et al., 2005; Padua et al., 2006). These findings raise the possibility that muscle groups differ, at least quantitatively, in their response to MuSK immunization.

Different developing and adult muscles also show heterogeneity in their temporal patterns of synaptogenesis, and particularly in their responses to perturbation of agrin-MuSK signaling (Pun et al., 2002). One group, termed fast-synapsing (FaSyn) muscles, form synapses rapidly after initial motoneuron-muscle contact, and maintain AChR aggregates for a considerably longer period following the loss of agrin/MuSK signaling or chronic blockade of neuromuscular transmission. By contrast, delayed-synapsing (DeSyn) muscles take considerably longer to form synapses after the initial motoneuron-muscle contact, and are more sensitive to treatments that perturb agrin signaling or synaptic stability (Pun et al., 2002). We reasoned that this could provide a basis for the somewhat different MG symptom patterns caused by MuSK immunization. Therefore, in the present study we test the hypothesis that FaSyn and DeSyn muscles are differentially affected in the mouse model of SNMG. We have found that AChR clusters in DeSyn muscles in mice are more severely affected in this model.

To test this hypothesis, we selected three typical FaSyn (intercostal, adductor longus and tibialis anterior) and DeSyn (diaphragm, sternomastoid and tibialis posterior) muscles as defined by Pun et al. (2002) to investigate changes at the NMJs induced by MuSK immunization. All experiments conformed to U.S. National Institutes of Health guidelines on the ethical uses of animals, used the minimal number of animals and entailed minimal suffering. We have previously observed that mice of the C57BL/6 strain show a strong correlation between muscle weakness and anti-MuSK titers (Jha et al., 2006). Therefore, in

*Corresponding author. Tel: +1-713-743-2697; fax: +1-713-743-2636.
E-mail address: sdryer@uh.edu (P. M. Rossini); URL: http://www.bchs.uh.edu/faculty.php?155622-961-5=sdryer.

Abbreviations: AChR, nicotinic acetylcholine receptor; α-BgTX, α-bungarotoxin; ANOVA, analysis of variance; DeSyn, delayed synapsing; FaSyn, fast synapsing; HE, hemotoxylin-eosin; MG, myasthenia gravis; MuSK, muscle-specific kinase; NMJ, neuromuscular junction; PBS, phosphate buffered saline; PBST, phosphate buffered saline containing 0.5% Triton X-100; SNMG, seronegative myasthenia gravis.

0306-4522/06$30.00+0.00
doi:10.1016/j.neuroscience.2006.09.022

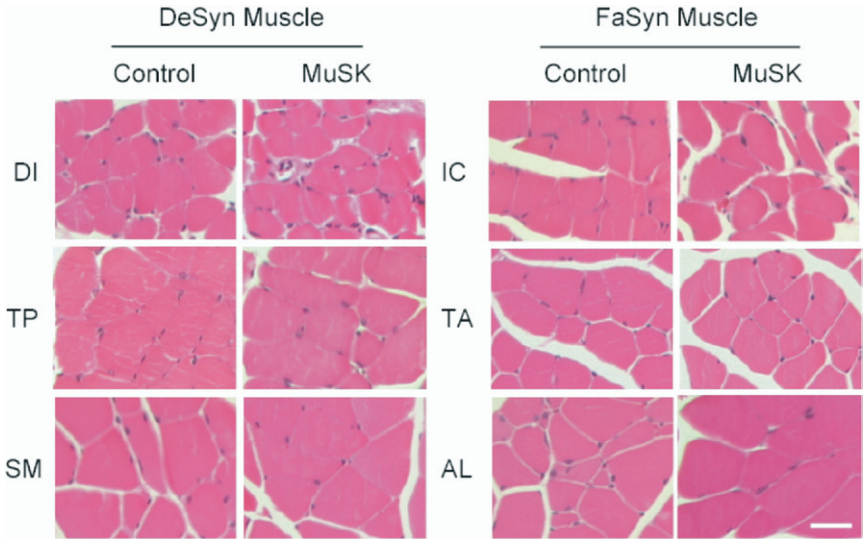

Fig. 1. MuSK-immunized mice at stage 2 of MG do not exhibit muscular atrophy. HE staining of three DeSyn and FaSyn muscles in control and MuSK-immunized mice. There is no evidence of muscle pathology in any of the muscles examined. Scale bar=50 μm. Abbreviations used in the figures: AL, adductor longus; DI, diaphragm; IC, intercostal; SM, sternomastoid; TA, tibialis anterior; TP, tibialis posterior.

the present study female C57BL/6 mice obtained from Jackson Laboratory (Bar Harbor, ME, USA) were used at 8 weeks of age. Immunization and clinical evaluation of mice have been detailed previously (Jha et al., 2006). All MuSK-injected mice were at stage 2 of MG at the time of killing, which typically occurs about 2 weeks after the last immunization. Muscles were dissected and fixed in 1% paraformaldehyde for 60 min at room temperature, rinsed, and incubated with 0.1 M glycine in phosphate buffered saline (PBS). After removal of overlying connective tissue, muscles were permeabilized in 0.5% Triton X-100 in PBS (PBST), and incubated overnight at 4 °C with rabbit polyclonal antibodies against the presynaptic markers neurofilament protein (1:500, Chemicon, Temecula, CA, USA) or synaptophysin (1:50, Zymed, San Francisco, CA, USA) in PBST containing 4% goat serum and 2% bovine serum albumin. The muscles were washed repeatedly in PBST, and then incubated overnight at 4 °C with Cy2-conjugated goat anti-rabbit IgG (1:200, Jackson Immunoresearch) and rhodamine-conjugated α-bungarotoxin (α-BgTX; 1:2500, Molecular Probes, Eugene, OR, USA), which labels endplate AChRs. After repeated rinsing, muscles were flat mounted and images of endplates were acquired using an Olympus FV1000 confocal microscope. Simple-PCI™ software (Compix Inc., Sewickley, PA, USA) was used to compute the areas stained by the markers. Data obtained with different markers were analyzed using a two-way analysis of variance (ANOVA) and Scheffe's post-hoc test (with α=0.05) using Statistica™ software (Statsoft Inc., Tulsa, OK, USA). In this experimental design, the two treatments under consideration were immunization (MuSK injection versus control), and muscle type (DeSyn versus FaSyn), and the dependent variable was the computed area of the fluorescent signal from each endplate. The individual muscle type was the covariable. Data points from each mouse used in the statistical analysis are the means of measurements of at least 13 endplates in each muscle from each mouse. Muscles from control and MuSK-injected mice were also stained with hemotoxylin-eosin (HE) using standard methods. Muscles were processed in parallel to avoid interassay variations.

MuSK-injected mice showed muscle weakness in exercise tests. They also exhibited gasping after the exercise tests, as noted previously (Jha et al., 2006), suggesting effects on respiratory muscle. However, HE staining showed no evidence of significant muscle atrophy in any of the muscle types examined in control or MuSK-injected mice (Fig. 1). This observation is consistent with previous observations of rats immunized with AChRs (Lennon et al., 1978). It is possible that muscle atrophy would eventually occur in more severely affected mice, however for ethical reasons we did not allow for such long survival times.

We observed that MuSK immunization severely disrupted endplate organization, as evidenced by a marked reduction in the area of AChR clusters measured with rhodamine-conjugated α-BgTx. The overall effect of MuSK immunization (pooled over all muscle types) was significant ($F_{1,55}$=237.7986, P<0.00001). Although all of the muscles that we examined were affected, the key observation of this study is that the reduction in AChR cluster area was significantly more severe in DeSyn muscles than in FaSyn muscles. This conclusion is based on the significant interaction effect between muscle type and MuSK immunization ($F_{1,55}$=5.881, P<0.02). It bears noting that there was no difference in mean AChR cluster area between the DeSyn and FaSyn muscles in the control mice (P>0.90 by Scheffe's test). Therefore, the observed significant difference in AChR areas between DeSyn and FaSyn muscles in the overall two-way ANOVA ($F_{1,55}$=34.8580, P<0.0001) can be attributed to the more severe effect of MuSK immunization in the DeSyn muscles. In all three DeSyn muscles examined, the AChR area in MuSK-injected mice was reduced to about 40% of that seen in controls. In FaSyn muscles,