Experimental Neurology 234 (2012) 506–512

Contents lists available at SciVerse ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Regular Article

Passive and active immunization models of MuSK-Ab positive myasthenia: Electrophysiological evidence for pre and postsynaptic defects

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ARTICLE INFO

Article history:
Received 30 September 2011
Revised 21 January 2012
Accepted 27 January 2012
Available online 3 February 2012

ABSTRACT

Antibodies directed against the post-synaptic neuromuscular junction protein, muscle specific kinase (MuSK) are found in a small proportion of generalized myasthenia gravis (MuSK-MG) patients. MuSK is a receptor tyrosine kinase which is essential for clustering of the acetylcholine receptors (AChRs) at the neuromuscular junction, but the mechanisms by which MuSK antibodies (MuSK-Abs) affect neuromuscular transmission are not clear. Experimental models of MuSK-MG have been described but there have been no detailed electrophysiological studies and no comparisons between the MuSK-MG and the typical form with AChR-Abs (AChR-MG). Here we studied the electrophysiology of neuromuscular transmission after immunization against MuSK compared with immunization against AChR, and also after passive transfer of IgG from MuSK-MG or AChR-MG patients. Overt clinical weakness was observed in 6/10 MuSK-immunized and 3/9 AChR-immunized mice but not in those injected with patients' IgG. Miniature endplate potentials (MEPPS) were reduced in all weak mice consistent with the reduction in postsynaptic AChRs that was found. However, whereas there was an increase in the quantal release of acetylcholine (ACh) in the weak AChR-immunized mice, no such increase was found in the weak MuSK-immunized mice. Similar trends were found after the passive transfer of purified IgG antibodies from MuSK-MG or AChR-MG patients. Preliminary results showed that MuSK expression was considerably higher at the neuromuscular junctions of the masseter (facial) than in the gastrocnemius (leg) with no reduction in MuSK immunostaining at the neuromuscular junctions. Overall, these results suggest that MuSK antibodies act in at least two ways. Firstly by indirectly affecting MuSK's ability to maintain the high density of AChRs and secondly by interfering with a compensatory presynaptic mechanism that regulates quantal release and helps to preserve neuromuscular function. These results raise questions about how MuSK is involved in retrograde signaling, and the combination of postsynaptic defects with lack of presynaptic compensation may begin to explain the more severe disease in MuSK-MG patients.

Introduction

Myasthenia Gravis (MG) is an autoimmune disorder affecting the neuromuscular junction (NMJ). It is characterized by fatigable muscle weakness resulting from failure of neuromuscular transmission. Serum antibodies directed against the AChR are found in approximately 80% of generalized MG cases (AChR-MG). These antibodies are typically of the complement fixing subclasses IgG1 and IgG3 (Vincent and Newsom-Davis, 1982) (Leite et al., 2008; Rodgaard et al., 1987). In animal models, active immunization with purified AChR (Patrick and Lindstrom, 1973) and passive transfer with AChR-MG IgG (Toyka et al., 1975) result in impaired neurotransmission, confirming that the antibodies are pathogenic. Their primary mechanism of action is through loss of the postsynaptic AChR via complement mediated destruction (Engel et al., 1977), and increased internalization and degradation of the AChR (Drachman et al., 1978). Direct pharmacological block of function can occur (Burges et al., 1990) but is probably uncommon.

A variable proportion of the remaining 20% of MG patients has antibodies to muscle specific kinase (MuSK-MG) (Hoch, 2003; Hoch et al., 2001). MuSK is also a postsynaptic protein and belongs to the receptor tyrosine kinase family. It is critical for the clustering of AChRs on the postsynaptic membrane during development (DeChiara et al., 1996) and for ongoing maintenance of the adult neuromuscular junction (Kong et al., 2004). Compared with AChR-MG, MuSK-MG more often involves bulbar, facial and respiratory muscles and can be severe and treatment-resistant (Evoli et al., 2003; Farrugia et al., 2006). MuSK-Abs are predominantly of the non-complement fixing IgG4 subclass (McConville et al., 2004), although they can still activate complement in vitro as some IgG1 is present (Leite et al., 2008).

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doi:10.1016/j.expneurol.2012.01.025

There have been in vitro studies of the effects of MuSK antibodies on AChR clustering on mouse myotubes (Hoch et al., 2001; Jha et al., 2006; Shigemoto et al., 2006) as well as reports of effects of active immunization against purified MuSK (Jha et al., 2006; Shigemoto et al., 2006) and passive transfer of MuSK-MG IgG (Cole et al., 2008; Cole et al., 2010) to experimental animals. These studies emphasized the clinical and morphological effects of the antibodies but did not investigate in detail the underlying neurophysiology or explain why MuSK-MG is more severe than AChR-MG. Nor is it clear whether active immunization against MuSK induces the same changes as passive transfer of patient's IgG. Here we compared serological and electrophysiological studies on mice with actively-induced MuSK-MG and AChR-MG, and compared with the effects of injection of patients' MuSK or AChR antibodies.

Methods

Purification of recombinant MuSK and Torpedo AChR

The DNA for the extracellular domain of human MuSK (residues 21–465) was amplified by PCR using the primers 5′-AGTCAGATCTGAGAAACTTCCAAAAGCT CCTGTC and 5′-GCATACCGGTATGTGGCAGTCTGGCACAGGCCGT. This was sub-cloned into the vector pMT/BiP/V5-HisA. Drosophilia S2 cells were maintained in Schneider's Drosophila-S2 media (Invitrogen, USA) supplemented with 2 mM glutamine, penicillin/streptomycin/amphotericin B (100 units/ml) and 10% fetal bovine serum at 28 °C. A stable line expressing MuSK (E22-H452) was generated by cotransfection of the cloned extracellular MuSK in pMT/BIP-MuSK and pCoblast using standard calcium phosphate transfection. These transfected S2 cells were grown in selective growth media containing Blastocidin (25 μg/ml). Protein expression was induced with 500 μM copper sulfate for 5 days. The presence of a histidine tag on the recombinant MuSK allowed purification on a 7 ml Ni-NTA column. 10 mM Imidazole was also added to the supernatant before loading onto the column. This column was washed with equilibration buffer (50 mM Tris, 500 mM NaCl, 10 mM Imidazole, pH 7.0). Following further washes with equilibration buffer, the recombinant MuSK was eluted with 250 mM Imidazole. This was dialyzed against phosphate buffered saline (PBS) overnight before determining the concentration by spectrometry. The purity of MuSK was checked by SDS/PAGE and western blotting with MuSK-Ab positive MG sera and commercial anti-MuSK antibodies (R&D, UK). Torpedo AChR was purified by affinity chromatography as previously described (Jermy et al., 1993).

IgG purification

IgG was purified from plasma of MuSK-MG, AChR-MG patients and healthy individuals on a Protein G-Sepharose fast flow (Sigma, UK) column. The plasma was first centrifuged for five minutes, diluted 1:1 with PBS solution and passed through the Protein-G column. The IgG was eluted with 0.1 M glycine solution (pH 2.3) and neutralized immediately with 1 M Tris pH 8. The protein content of the eluted fractions was measured using a Coomassie Plus assay kit (Pierce, USA), and the peak fractions pooled and dialyzed against Hartmann's physiological solution for thirty six hours with three changes. The IgG was concentrated using polyethelene glycol 6000 (Sigma, UK) and the concentration determined by spectrometry.

Immunizations

Active immunizations were undertaken on groups of ten eight week-old female C57BL/6 mice and carried out in accordance with UK Home Office guidelines. Recombinant human MuSK (30 μg), Torpedo AChR (20 μg) and PBS were emulsified 1:1 with complete Freund's adjuvant (Sigma, UK). A total of 200 μl of emulsion was injected into the base of the tail at day 0. Boosts in Freund's incomplete adjuvant were given at days 28 and 56. The mice were bled at days 40 and 80, and serum prepared and stored at −20 °C. The mice were observed daily and weighed twice weekly.

For passive transfer 12 female C57BL/6 mice, aged 8 weeks, were injected intraperitoneally with 50 mg of each purified IgG daily for five days. They were weighed daily and assessed for motor functions (see below). At termination of all immunizations, blood was taken for serology and muscles were removed for electrophysiology and other studies.

Motor function

Mice were tested before each immunization and two weeks after the last injection by a blinded observer. Prolonged strength was tested using an inverted screen (Cossins et al., 2004) with a maximum time limit of 600 s. If the mice fell off before 600 s, they were retested. The average time they were able to maintain their grip is shown. To assess their bulbar function, the mice were partially starved overnight, and then given condensed milk diluted 1:1 with water (total 2 ml placed in a container on the floor of the test cage). The total volume consumed in the 90 s after drinking commenced was recorded.

Antibody assays

For the radio-immunoprecipitation assays, 2.5 μl mouse or human serum was made up to 25 μl with PTX buffer (0.02 M phosphate, 0.1% Triton X-100 pH 7.2). These were serially diluted fivefold, added to 25 μl ^{125}I recombinant human MuSK (RSR, UK) and left overnight at 4 °C. 25 μl anti-human IgG (RSR, UK) was added with appropriate amounts of normal human or mouse serum to ensure comparable IgG levels and left for 90 min. A further 0.5 ml PTX buffer was then added, samples microfuged at 13,000 rpm, washed and counted on an automated gamma counter (Packard, Meriden). Cell-based assays looking for IgG and C3b binding were performed and analyzed as previously described (Leite et al., 2008) with the exception that mouse serum was diluted 1:100 for initial assays, or by serial titrations for accurate levels.

Ex-vivo electrophysiology

Phrenic nerve/hemidiaphragm preparations were placed in Krebs' solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4 \cdot 7H_2O$, 1.2 mM KH_2PO_4, 24.9 mM $NaHCO_3$, 11.1 mM D-glucose and 2.5 mM $CaCl_2$ pH7.4), before determining miniature endplate potentials (MEPPs), endplate potentials (EPPs) and quantal contents (QC) as previously described (Cossins et al., 2004).

Recordings, from at least ten endplates from one hemidiaphragm of each animal, were accumulated over a 90 minute period at room temperature. Analysis was performed using pClampfit 9 software (Axon laboratory, CA, USA) and for each endplate, a minimum of 20 MEPPs and EPPs were required to generate a mean value. All MEPPs and EPPs were standardized to a resting membrane potential of −80 mV. Mean quantal content (m) was calculated by the direct method using the formula described elsewhere (Wood and Slater, 2001).

AChR quantification

Quantification of endplate AChR was undertaken as previously described (Cossins et al., 2004; Jermy et al., 1993). The unused hemidiaphragm was incubated for 2 h in Krebs' solution containing an excess of ^{125}I-α-bungarotoxin. It was then washed and left overnight in Krebs' solution. The following day it was fixed in 2.5% glutaraldehyde in 80% Krebs' solution for two hours at room temperature before being washed in distilled water. The diaphragms were stained for

acetylcholinesterase and the region containing endplates excised, weighed, measured and the cpm bound to the endplate regions counted on a gamma counter. The cpm of a similar weight of non-endplate containing muscle was subtracted and specific endplate binding calculated as cpm/mg/cm.

MuSK staining and quantification

Masseter and gastrocnemius muscles were dissected and placed in Cryotek molds containing 15% gelatin and 25% Tissue Tek/OCT (Sakura Finetek, Japan) and allowed to set at 4 °C, frozen in isopentane cooled in liquid nitrogen, and stored at −80 °C. Muscle sections (10–15 μm) were cut using a Leica CM1900 cryostat at −18 °C and mounted on to polytetrafluoroethylene coated slides. The muscle sections were rinsed with PBS, and blocked with 1% BSA and 10% goat serum in PBS for 30 min. Rabbit anti-MuSK antibody (83033), a polyclonal antibody directed against an extracellular epitope (kindly donated by Dr Steve Burden, New York), was diluted 1:6000 in the blocking solution and applied to the sections overnight at 4 °C. After washing, anti-rabbit IgG Alexa fluor 488 (Invitrogen, Paisley) diluted 1:500 in blocking solution was added for 60 min at room temperature. After further PBS washes, α-bungarotoxin Alexa Fluor 568 diluted 1:5000 in PBS with 1% BSA was applied for 30 min at room temperature before the final washes. In order to prevent bias, individual endplates were identified by means of their AChR staining before the corresponding MuSK images were acquired on an Axion 200 inverted Zeiss fluorescence microscope using Open Lab software with all photographs acquired under the same conditions. Overall at least six sections were obtained for each mouse muscle with a minimum of four photographs per muscle section, each typically containing several endplates. The integrated intensity of individual endplates was then measured using Metamorph software (Universal Imaging Corporation, USA).

Statistics

Repeated ANOVA was used to analyze differences in weight between the different groups of mice over time, and the titrations of mouse MuSK antibody levels. One-way ANOVA was used to analyze differences in bulbar function and prolonged strength on the inverted screen. The electrophysiological parameters, AChR and MuSK quantification data were all analyzed by means of one-way ANOVA (Kruskal–Wallis test with Dunn's multiple comparison test). The correlations between MEPP amplitude and AChR numbers were assessed by linear correlation. Graph Pad Prism was used for all graphs and analyzes.

Results

Preliminary studies of immunization against MuSK

We first immunized mice with 30 μg of MuSK or PBS, boosting at 28 and 56 days and terminating at day 70. The mouse sera were positive for IgG antibodies binding to MuSK expressed on HEK cells, indicating that they recognized extracellular epitopes (Supplementary Fig. 1a). These antibodies were equally IgG1, IgG2b and IgG2c subtypes, with little IgG3 or IgM (data not shown). At termination, there was a significant reduction in MEPP and EPP amplitudes (Supplementary Fig. 1b) in MuSK-immunized mice compared with PBS-immunized mice. MuSK-immunized mice showed loss of AChR staining at the endplates in both masseter (facial) and gastrocnemius (limb) muscles but surprisingly no loss of MuSK expression in these muscles (Supplementary Fig. 1c). Moreover, MuSK expression was considerably higher in masseter than in gastrocnemius (Supplementary Fig. 1c).

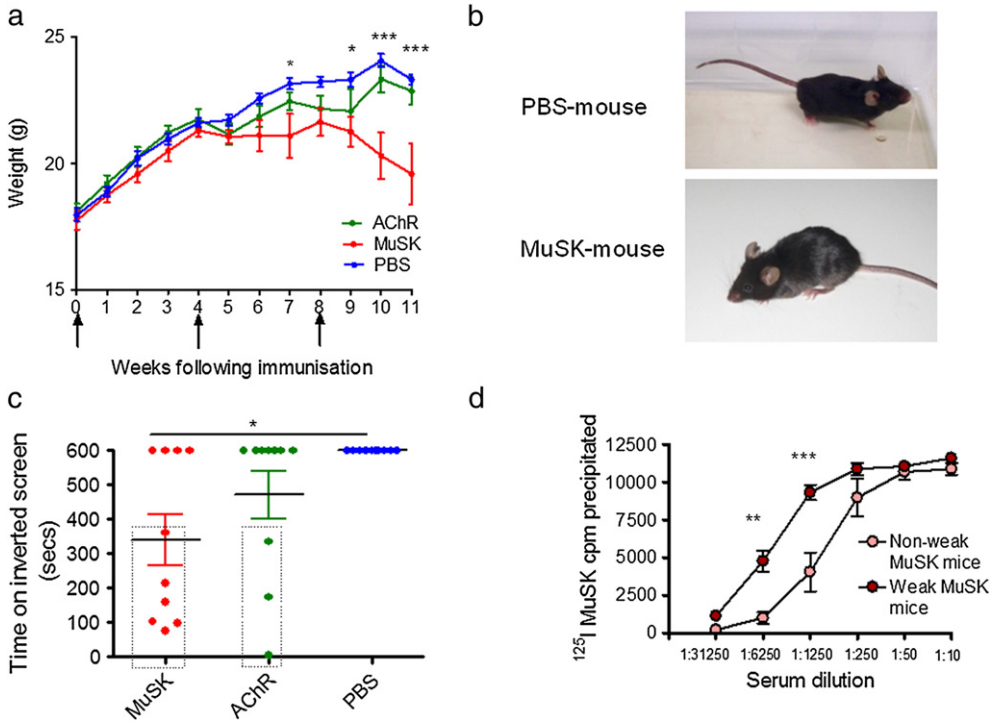

Fig. 1. Results of active immunization.(a) Body weight (mean + SEM) of AChR, MuSK and PBS immunized mice following immunization. Arrows show initial immunization and boosts. Repeated ANOVA and Bonferroni post test *p<0.05; ***p<0.001. (b) Examples of a healthy mouse (above) and a weak mouse (below). (c) Performance on the inverted screen at the end of the study in the three groups and mean + SEMs. At baseline all mice were able to sustain grip for 600 s without falling. Those mice that fell off the inverted screen before 600 s on two consecutive occasions are shown in the boxes. Kruskal–Wallis test and Dunn's multiple comparison test * p<0.05. (d) Serial titrations of sera binding to ^{125}I-recombinant human MuSK (mean + SEM), after subtraction of cpm precipitated by normal mouse serum. Three weak and three non-weak MuSK-mice were tested. Repeated ANOVA and Bonferroni post test **p<0.01; ***p<0.001.

Comparison of immunization with MuSK or Torpedo AChR

The main study repeated the immunization using 30 μg of MuSK and compared the results with 20 μg of Torpedo AChR using a similar protocol to that above but with mice sacrificed between 77 and 84 days. Towards the end of the study period, the weights of the MuSK-immunized mice (MuSK-mice) were significantly less than those of the AChR-immunized (AChR-mice) or PBS control (PBS-mice) groups (Fig. 1a). Objective weakness was apparent in six of the ten MuSK-mice and three of the nine AChR-mice (see Fig. 1b for examples), as demonstrated by a reduction in grip times on the inverted wire-mesh screen on two consecutive tests (mean value shown, Fig. 1c). At day 75, we tried to demonstrate bulbar involvement by measuring the amount of condensed milk drunk in 90 s, but although the results were lower in MuSK- and AChR-mice, particularly in the weak mice, they were not significantly different from the PBS-mice, largely because of considerable variations and low numbers in each group (Supplementary Fig. 2). For the results that follow, the weak mice will be described separately from those that did not show any clinical weakness.

Serum levels of MuSK-Abs two weeks after the second boost were significantly higher in the weak MuSK-mice compared with the non-weak MuSK-mice (Fig. 1d). The mice were sacrificed and individual muscle fibers (minimum ten per mouse) of the phrenic nerve hemidiaphragm were studied with microelectrodes. There were significant reductions in MEPP amplitudes, MEPP frequencies and EPP amplitudes between the five groups (Figs. 2a–c) with the weak AChR- and MuSK-mice having the lowest values. The MEPP frequencies were particularly low in MuSK-MG mice, more than could be accounted for by loss of MEPP amplitudes in the background (data not shown), and may reflect presynaptic changes which have been reported in MuSK-MG models (e.g. Cole et al., 2008; ter Beek et al., 2009). The quantal contents were significantly raised in the weak AChR-mice (Fig. 2d) but not in the MuSK-mice. There was an inverse correlation between quantal content and MEPP amplitude in the AChR-mice but no such correlation in the MuSK-mice (Figs. 2e,f).

To see whether the reduced MEPP amplitudes were associated with reduced numbers of AChRs, we used ^{125}I-α-bungarotoxin to measure the number of endplate AChRs. The numbers of AChRs were reduced in all four test groups compared to the PBS-mice (Fig. 3a), particularly the two weak AChR-mice (one hemidiaphragm was damaged and could not be tested), and overall there was a significant correlation between mean AChR numbers and the mean MEPP amplitudes for each animal (Fig. 3b).

Passive transfer

To see if the results of active immunization corresponded to those that might be found in patients, we used passive transfer with IgG purified from the plasma exchange samples of two MuSK-MG patients with high antibody titers, MuSK-MG-A (titer 78.1 nM) and MuSK-MG-B (titer 74.1 nM), and one AChR-MG patient (see Table 1 for details). These IgG preparations were chosen partly because they bound to mouse MuSK or AChR, respectively, and the MuSK IgGs reduced the clustering of mouse AChRs in C2C12 cells (data not shown).

Preliminary experiments were performed with 10 mg of IgG for five days, but there was only a marginal reduction in MEPP amplitudes (data not shown). However, following injection of 50 mg of IgG daily for five days, the antibody levels in sera from the MuSK-MG-A and B injected mice were very similar to those in the plasma

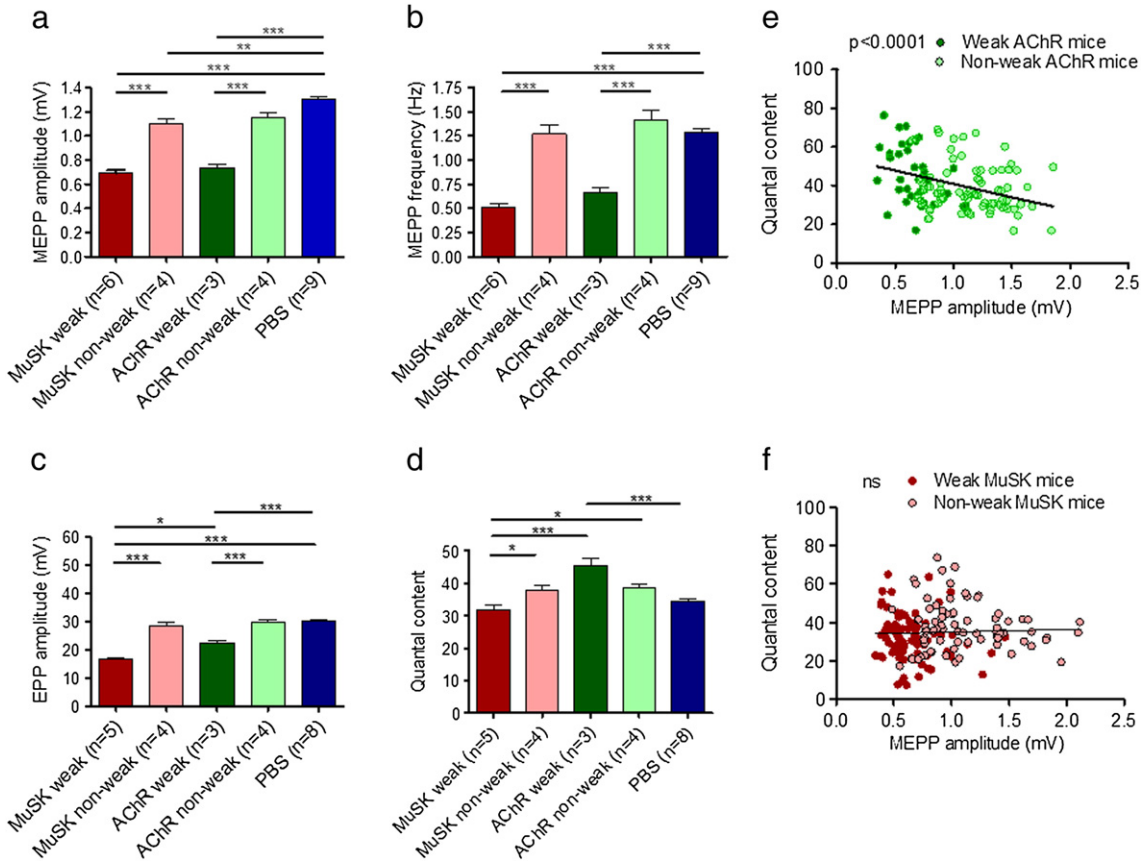

Fig. 2. Effect of active immunization on electrophysiology of neuromuscular transmission.Individual data from each muscle fiber for each group (number of mice given below each column) examining (a) MEPP amplitude, (b) MEPP frequency, (c) EPP amplitude and (d) quantal content. Mean + SEM and significant differences (Kruskal–Wallis test with Dunn's multiple comparison test) are shown for each measurement: *p<0.05; **p<0.01; ***p<0.001. Correlation between MEPP amplitude and quantal content in (e) AChR-mice and (f) MuSK-mice. Analysis performed by linear regression.

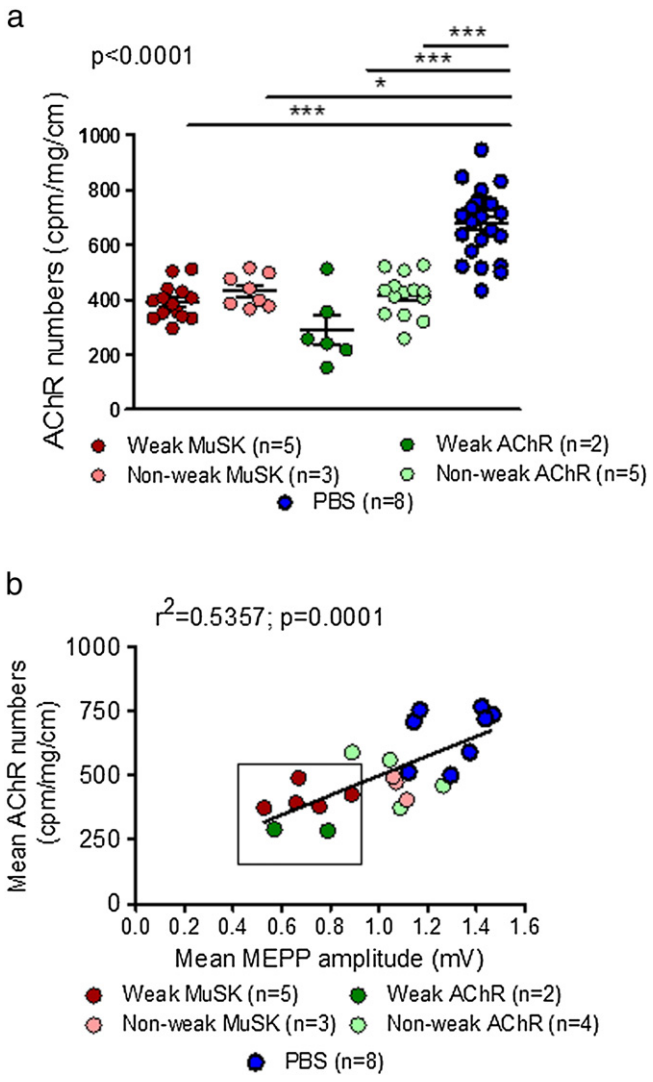

Fig. 3. Effect of active immunization on AChR numbers.(a) AChR numbers were measured by ^{125}I-α-bungarotoxin binding to endplate specific regions of the muscle. The results are shown as cpm/mg/cm (mean + SEM) bound to endplate-containing muscle fiber bundles (2–3 for each mouse) after subtraction of non-endplate binding. Kruskal–Wallis test with Dunn's multiple comparison test ***p<0.001. (b) Correlation between mean MEPP amplitude and mean AChR numbers for each mouse with analysis by linear regression. Weak mice are shown in the box (b).

sample from the two patients (Fig. 4a). Notably, however, these levels were five to ten-fold lower than those in the actively immunized mice (Fig. 1d) and there was no weight loss or overt weakness in any of the mice. Despite this, a significant reduction in MEPP amplitudes was found in MuSK-MG-A IgG and AChR-MG IgG injected, and to a lesser extent MuSK-MG-B IgG injected, mice (Fig. 4b). The EPP amplitudes were also significantly reduced but MEPP frequencies were not significantly altered (data not shown). As with the active immunization in weak-MuSK-mice, the quantal contents in MuSK-IgG injected mice were not different from control values, but were reduced compared with those in mice receiving AChR-MG IgG (Fig. 4c). There was also a modest reduction in AChR numbers in the MuSK-MG-A injected mice compared with controls, and a significant positive correlation between MEPP amplitude and AChR numbers in all mice examined (Figs. 5a,b), but there were no significant correlations between quantal contents and MEPP amplitudes (Supplementary data — Fig. 3).

Discussion

The results of this study address the electrophysiology of MuSK-MG and how it differs from AChR-MG. We found that not all MuSK or AChR immunized mice became weak reflecting the greater safety factor for neurotransmission in mice (Wood and Slater, 2001), but those that did had markedly reduced MEPP amplitudes, reduced MEPP frequencies and reduced EPP amplitudes, associated with reduced AChR numbers. However, whereas the quantal contents were raised in weak AChR immunized mice they were normal in weak MuSK immunized mice. Similar trends were found in mice injected with two MuSK-MG IgGs, although serum levels of human MuSK antibodies were approximately five times lower than the mouse MuSK antibodies in actively immunized mice. Overall we propose that the weakness seen in both MuSK-immunized and MuSK-MG IgG injected mice may be due not only to a reduction in postsynaptic AChR numbers, with the resulting reduction in MEPP and EPP amplitudes, but also to a lack of a presynaptic compensatory increase in quantal release which is seen in AChR-MG. The role of MuSK in the retrograde signaling that results in these changes needs to be examined further.

It has previously been reported that AChR numbers are not deficient in MuSK-MG (Shiraishi et al., 2005), suggesting that the antibodies must have some indirect effect on neuromuscular transmission, but these observations were in the limb muscles, which show less marked clinical and single fiber electromyographic changes (Farrugia et al., 2006); the AChR numbers and morphology in the more affected patients' muscles were not examined. Although there have been no systematic electrophysiological studies on

Table 1
Patients whose IgG antibodies were used in passive transfer experiments.

	AChR-MG	MuSK-MG-A	MuSK-MG-B
Age at onset	26	25	6
Gender	M	F	F
Symptomatic weakness at presentation	Bulbar, facial, limb	Ocular, bulbar, facial and respiratory muscle weakness	Ocular, respiratory and limb muscle weakness
Neurophysiology assessment (muscles tested)	RNS: significant decrement (anconeus, abductor digiti minimi)	SFEMG: moderate jitter (abductor digiti minimi)	RNS: normal (abductor digiti minimi and abductor pollicis brevis)
Tensilon test	Not done	Positive	Positive
Response to initial plasma exchange	Good	Good	Good
Subsequent treatment	Cs, Az, IVIG	PEx, IVIG, Cs, Az	IVIG, Cs, Az, Mx
Clinical course	One further relapse required IVIG. Has remained well on treatment	Symptomatic weakness every 9/12 requiring PEx despite long term IST	One further relapse following cessation of IS treatment. Now on long term IST
Thymectomy	Good response (pharmacological remission)	No response	Not done

Abbreviations:
RNS = repetitive nerve stimulation (at 3 Hz unless stated), SFEMG = single fiber electromyography, PEx = plasma exchange, IVIG = intravenous immunoglobulin, Cs = corticosteroids, Az = azathioprine, Mx = methotrexate, IST = immunosuppressive therapy.

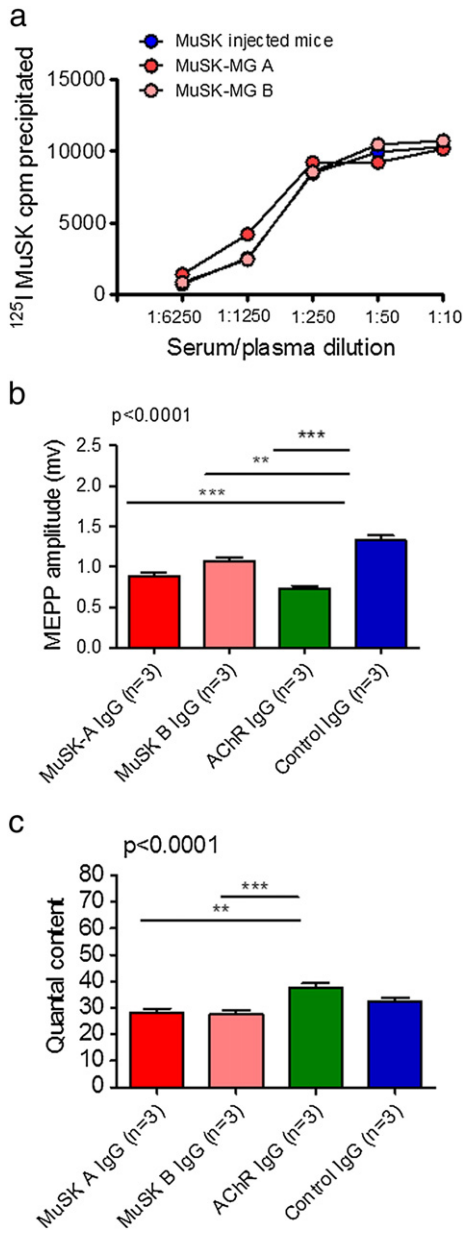

Fig. 4. Passive transfer of human IgG to mice.(a) Serial titrations of sera binding to ^{125}I-recombinant human MuSK, after subtraction of cpm precipitated by mouse receiving normal IgG. Results from individual endplate data (mean + SEM) for each group (three mice, > 10 endplates in each) examining (b) MEPP amplitude and (c) quantal content. Kruskal–Wallis test with Dunn's multiple comparison test $^{**}p<0.01$; $^{***}p<0.001$.

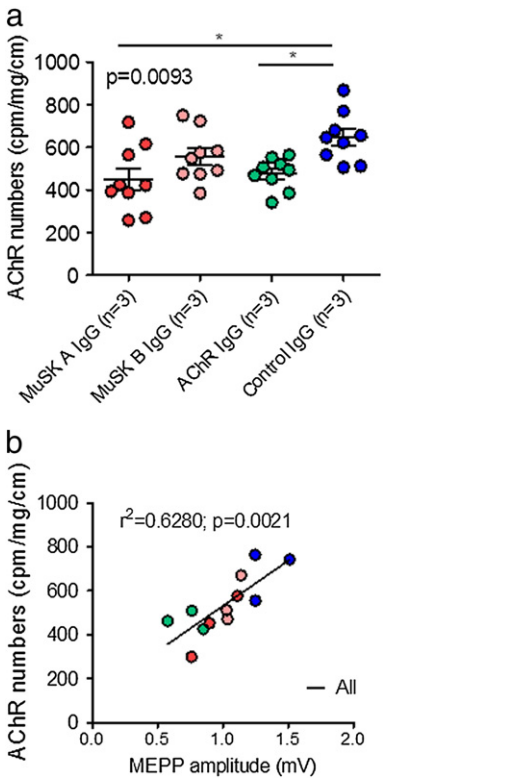

Fig. 5. Effect of passive transfer on AChR numbers.(a) AChR numbers were measured by ^{125}I-α-bungarotoxin binding (mean + SEM) to endplate specific regions of the muscle. Kruskal-Wallis test with Dunn's multiple comparison test * $p<0.05$. (b) Correlation between MEPP amplitude and AChR numbers for individual mice with analysis by linear regression.

patients' muscle biopsies in vitro, a recent study found both a reduction in MEPP frequency and in quantal content in one MuSK-MG intercostal biopsy compared with those typically found in their AChR-MG patients (Niks et al., 2011); as here, the reduction in MEPP amplitude was comparable in MuSK-MG and AChR-MG biopsies. Their results in that patient, therefore, were not dissimilar from our findings in both active and passive models.

Two main active immunization models have been described previously (Jha et al., 2006; Shigemoto et al., 2006), but they focused on morphological changes rather than the underlying electrophysiology of neuromuscular transmission. They found fragmentation of AChR clusters (Jha et al., 2006; Shigemoto et al., 2006) and alterations in nerve terminal structure and branching (Cole et al., 2008; Jha et al., 2006) which we saw at some endplates (data not shown). We established an active immunization model of MuSK-MG in order to understand better the electrophysiology and to compare with immunization against AChR. The MEPP amplitudes are dependent on the density and number of AChRs, which are typically reduced in MG (Fambrough et al., 1973; Ito et al., 1978). The reduction in AChR numbers in weak MuSK-MG mice is therefore likely responsible for the reduced MEPP and EPP amplitudes. There was also a quite marked reduction in MEPP frequencies; that could have been caused partly by loss of very small MEPPs in the "noise" of the recordings but, judging by the frequency distribution of MEPP amplitudes (data not shown), the proportion of MEPPs that were likely to have been lost was small (<20%) even in the weak MuSK-mice. The reduced MEPP frequency would therefore support the existence of some abnormality of presynaptic function. Of clearer significance, there was an increase in the quantal content in the weak AChR-mice that was not found in the MuSK-mice. A similar increase was previously found in patients with AChR-MG, and in mice immunized with AChR, and is thought to represent a presynaptic compensatory mechanism (Plomp et al., 1995). Our results confirm those findings in AChR-mice and support the existence of retrograde signaling from the post- to pre-synaptic membranes which may be responsible for the increased quantal release. The mechanism by which this occurs is not clear, but since the weak MuSK-MG mice did not show the increase in quantal content, MuSK may be involved in this compensatory mechanism, and MuSK antibodies may interfere with this aspect of MuSK function.

A recent study looked at the bulbar selectivity of MuSK-MG (Punga et al., 2011). Immunized mice developed marked generalized muscle weakness and bulbar dysfunction. There was a lower level of mRNA for MuSK in masseter muscle than in the axial or limb muscles with upregulation of MuSK mRNA expression after immunization against MuSK. These results appear to differ from the unexpected five-fold greater MuSK protein expression at the neuromuscular

junctions of masseter compared with gastrocnemius that we found in preliminary results, and a direct comparison of the protein and mRNA expression needs to be done.

To our knowledge, this is the first attempt to compare the results of active immunization with passive transfer of human autoantibodies. The results suggest that active immunization provides a useful model of the human disease, both in the changes observed in MuSK-MG injected and MuSK-mice and also in the modest but significant differences in the findings between the MuSK and AChR mice. Although the results were less robust in the MuSK-MG IgG mice, to those from active immunization, the antibody levels achieved in the mice sera were lower than those achieved with active immunization. Previous passive transfer studies have not examined the electrophysiology of transmission but have concentrated on morphological changes that suggest a loss of AChRs and also of a loss of apposition between pre- and postsynaptic structures after 8 days of injections (Cole et al., 2008), or the regeneration of neuromuscular junctions after notexin which was impaired in comparison with control IgG injected endplates (ter Beek et al., 2009). Studies in patients subsequently identified as being MuSK-Ab positive (AV unpublished data) also suggested some presynaptic effects in addition to the expected reduction in MEPP amplitudes (Burges et al., 1994). Overall, results in both active and passive transfer models of MuSK-MG demonstrate that there are defects in the function of the presynaptic nerve terminals as well as in the numbers of postsynaptic AChRs, and that these additional problems may explain some of the disease severity. Since MuSK is not known to be present in the presynaptic nerve terminal, further experiments are indicated to explore this potentially important aspect of MuSK function.

Acknowledgments

We are very grateful to Richard and Letizia Richardson and the Patrick Berthoud Trust for support (SV). Additional support came from the Medical Research Council (JC) and the Muscular Dystrophy Campaign (RW).

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.expneurol.2012.01.025.

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