Myasthenia gravis with antibodies to muscle-specific kinase : clinical characteristics, epidemiology, and immunological aspects

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VIII

Pre- and postsynaptic neuromuscular junction abnormalities in MuSK myasthenia

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Abstract

Autoantibodies to muscle-specific kinase (MuSK) can cause myasthenia gravis (MG). The pathophysiological mechanism remains unknown. We report in vitro electrophysiological and histological studies of the neuromuscular junction in a MuSK MG patient. Low levels of presynaptic acetylcholine release and small miniature endplate potentials were found. This combination of pre- and postsynaptic abnormalities was supported by histology, revealing partially denervated postsynaptic areas, and some degeneration of postsynaptic folds. Results suggest that anti-MuSK antibodies reduce the stability of muscle-nerve contact.

Introduction

Autoimmunity against the neuromuscular junction leads to muscle weakness in myasthenia gravis (MG). Although the majority of patients are seropositive for autoantibodies against the postsynaptic acetylcholine receptor (AChR), a minority (<6%) have autoantibodies against muscle-specific kinase (MuSK). [54,157] MuSK is a transmembrane tyrosine kinase localised at the postsynaptic specialisation of the muscle cell membrane. [65,158] In conjunction with the proteins LRP4 [73,74] and Tid1, [79] it is involved in the development and maintenance of the neuromuscular junction, in particular the clustering of AChRs in the postsynaptic membrane through tyrosine phosphorylation, and in the localisation of acetylcholinesterase (AChE) in the basal lamina of the synaptic cleft through interaction with its anchoring protein ColQ. [65, 80] Although the molecular mechanism by which MuSK/LRP4/Tid1 regulate neuromuscular junction formation and maintenance has become increasingly clear, [159,160] the pathogenic role of anti-MuSK antibodies and the pathophysiological mechanisms by which they may cause muscle weakness in MuSK MG are less well understood. The high disease specificity of anti-MuSK antibodies, the correlation of their titre with muscle weakness severity, [161] and the experimental demonstration in myotubes that these antibodies are capable of reducing MuSK-dependent AChR clustering, [54,158] all suggest a pathogenic role. However, no clear histological abnormalities consistent with a disturbance of neuromuscular synaptic function have been found in biopsies of affected muscles. In particular, a reduction of AChR density was not found in morphological studies, in contrast to positive control tissue from AChR MG patients. [125,126] Despite these findings, a reduction in the amplitude of miniature endplate potentials (MEPPs) was observed in the only MuSK MG biopsy to date in which neuromuscular synaptic function was studied with microelectrode methods. [126] MEPPs are the unitary postsynaptic responses resulting from spontaneous presynaptic release of single acetylcholine (ACh) quanta and their amplitude is largely determined by the density of functional AChRs. MEPP amplitude reduction is an established electrophysiological hallmark of neuromuscular junctions of AChR MG patients. [162] In view of the apparent inconsistency between electrophysiological and morphological observations at biopsied MuSK MG neuromuscular junctions and the very limited data reported so far, it is important to study function and morphology in additional MuSK MG patients. In this study we describe in vitro electrophysiological and histological aberrations of the neuromuscular junction in a patient with acquired MuSK MG.

Methods

Muscle biopsy

Parasternal intercostal muscle tissue was obtained under local anaesthesia using lidocain as part of a study of seronegative MG performed at the University Medical Center of Utrecht in 1994 and 1995. Approval from the local medical ethics committee and informed consent from the patient were obtained. The histological examination took place at the same hospital. Immediately after obtaining the biopsied material, part of the biopsy (a few centimetres long) was transported in 250 ml of pre-oxygenated Ringer's medium to the Leiden University Medical Center for in vitro electrophysiology studies.

Endplate electrophysiology

The in vitro electrophysiology studies were performed as described previously. [162] In brief, muscle fibers in Ringer's medium containing 2 mM Ca^{2+} and 1 mM Mg^{2+} at 26-28°C were impaled with a glass microelectrode, connected to standard electrophysiological and digitising equipment. In the MuSK MG biopsy, synaptic signals were recorded from a sample of 28 endplates. At each endplate, 4-26 MEPPs were recorded, from which the mean endplate value was calculated. At each of the 28 studied endplates, 28-42 endplate potentials (EPPs) were recorded during 0.3-Hz electrical stimulations of an intramuscular nerve branch through a suction stimulation electrode, after allowing for some depolarisation to prevent muscle fiber action potentials (at resting membrane potentials of around -65 and more positive, EPPs at most human muscle fibers no longer trigger an action potential [162], presumably due to Na^+ channel inactivation). The mean resting membrane potential during EPP recordings was -67.2 mV. At each endplate a mean 0.3-Hz EPP amplitude was calculated from the amplitudes of the recorded EPPs. Whenever possible, EPPs were also recorded at 3- and 30-Hz nerve stimulation. Mean MEPP and EPP amplitudes at each endplate were normalised to a standard resting membrane potential of -75 mV and EPPs were corrected for nonlinear summation. The quantal content, which is the number of ACh quanta released per nerve stimulus, was calculated directly by dividing the normalised and corrected mean 0.3-Hz EPP amplitude by the normalised mean MEPP amplitude at each endplate. From the mean endplate values of each electrophysiological parameter at the 28 sampled endplates, an overall biopsy mean was calculated. Control data were obtained from biopsies of 4 non-neurological-diseased subjects (referred to as "controls") and 6 patients with AChR MG, [162] supplemented by the unpublished results of 17 more AChR MG patients and 3 more controls.

Endplate histology

Part of the biopsy specimen was pinned out to prevent shrinkage and fixed in a periodate-lysine-paraformaldehyde solution. For electron microscopy, part of this material was refixed in 2% glutaraldehyde solution, postfixed in 1% osmium tetroxide solution, dehydrated and embedded in Epon 812. Endplate-containing regions were located in semithin sections stained with toluidine blue. Ultrathin sections from selected regions were contrasted with uranyl acetate followed by lead citrate and viewed using an electron microscope (1200 EX. JEOL Ltd., Tokyo, Japan). Another part of the specimen was freshly frozen with isopentane cooled in liquid nitrogen. Longitudinal 50-µm-thick cryostat sections were stained with a silver-choline esterase method for intramuscular nerve fibers and endplates. [163]

Results

The patient

In 1994, a 29-year-old woman experienced fluctuating ptosis and diplopia. Symptoms improved upon administering neostigmine. Oral pyridostigmine was unsuccessful, causing only gastrointestinal side effects and muscle trembling. Anti-AChR antibodies were negative. Two months later, she experienced progressive dysphagia, dysarthria and weight loss. She became dyspnoeic after exercise and had to support her head when doing the housekeeping and her jaw when chewing. Neurological examination also revealed mild proximal weakness of her arms and legs. Anti-AChR antibodies were absent again. Tube feeding was initiated. Spirometry showed a reduced vital capacity (71% of predicted) and a very low maximal inspiratory pressure (MIP, 21% of predicted). No compound muscle action potential decrement was found upon repetitive stimulation of the ulnar, facial and accessory nerve. Stimulated single-fiber electromyography of the orbicularis oculi muscles revealed increased jitter and blocking. Neostigmine led to slight improvement of the MIP (35% of predicted), but also to generalised fasciculations and muscle cramps. Nine months after onset, an intercostal muscle biopsy was performed. She had then been off all medication for 3 months. After the biopsy, prednisone treatment resulted in marked clinical improvement. In 2002, a relapse occurred with neck extensor weakness, diplopia, and mildly impaired swallowing and chewing. At this point, anti-MuSK antibodies were found that could also be demonstrated in stored serum from 1994. No antibodies to the AChR or voltage-gated calcium channels were found. To exclude a genetic cause, the *MuSK* gene was sequenced, revealing three known single-nucleotide polymorphisms (rs1784573, rs2766999 and rs578430), but no mutations in the coding sequence.

In vitro electrophysiology

The mean MEPP amplitude of the MuSK MG biopsy was 0.33 ± 0.02 mV; that is, approximately 50% smaller than the group mean value of the controls (and below the lower limit of the range of their individual biopsy mean values), but within the range of biopsy mean values from AChR MG patients (Table 8.1).

Table 8.1 In vitro electrophysiology in MuSK MG and controls

	MEPP			EPP			Quantal content
	Amplitude (mV)	Risetime (0-100%, ms)	Frequency (min^{-1})	Amplitude (mV)	Risetime (0-100%, ms)	Decay Tau (ms)	Quantal content
Patient biopsy mean ± SE	**0.33 ± 0.02**	**2.0 ± 0.02**	**2.60 ± 0.33**	**5.78 ± 0.59**	**2.3 ± 0.11**	**5.5 ± 0.26**	**18.8 ± 1.7**
AChR MG group mean ± SE	**0.21 ± 0.02** (0.11-0.43)	**2.1 ± 0.13** (1.2-3.8)	**3.84 ± 0.35** (1.02-9.01)	**9.65 ± 0.60** (5.32-14.94)	**2.9 ± 0.14** (1.6-4.6)	**7.5 ± 0.55** (4.7-16.7)	**58.3 ± 3.7** (35.8-84.5)
Controls group mean ± SE	**0.63 ± 0.08** (0.38-1.05)	**2.7 ± 0.20** (1.9-3.5)	**4.05 ± 0.54** (1.48-6.14)	**12.89 ± 1.50** (8.87-16.84)	**2.9 ± 0.16** (2.5-3.6)	**5.2 ± 0.58** (3.5-7.6)	**28.4 ± 1.7** (24.0-35.3)
Selcen mean ± SE	**0.35 ± 0.02**						**31 ± 5.8**
Controls by Selcen	**1.00 ± 0.025**						**31 ± 1**

In vitro electrophysiology data for the MuSK MG patient (biopsy mean was calculated from mean values of parameters at n = 28 endplates), AChR MG patients (group mean from biopsy means of n = 23 biopsies, range of biopsy means shown in parentheses), and healthy controls (group mean from biopsy means of n = 7 biopsies, range of biopsy means shown in parentheses). The last two rows include data published from a MuSK MG patient recorded by Selcen *et al*. [126] Data expressed as mean ± SE (range of biopsy means).

The amplitude of EPPs evoked by 0.3-Hz nerve stimulation was 5.8 ± 0.6 mV; that is, a 55% reduction compared with the group mean value of controls (Figure 8.1A). The mean MEPP frequency was rather low (2.6 ± 0.33 min^{-1}), but still fell within the ranges of AChR MG patients and controls. The EPP rise time was slightly smaller than the lower limit of the control range, but fell within the AChR MG range. The EPP decay time constant was in the range of AChR MG and controls. Mean quantal content was 18.8 ± 1.7; that is, 34% lower than the group mean of the controls, 68% lower than the group mean of AChR MG biopsies, and clearly below the lower limits of the ranges of the individual biopsy mean values of both groups. Upon repetitive 3-Hz nerve stimulation at normal resting membrane potentials (about -80 mV), some endplates showed intermittent subthreshold EPPs and muscle fiber action potentials triggered by suprathreshold EPPs, as well as delayed action potentials triggered by just-above-threshold EPPs (Figure 8.1B). High-rate stimulation (30 Hz) at normal resting membrane potential resulted in an increasing occurrence of subthreshold EPPs during the stimulation (Figure 8.1C). The 30-Hz EPP recordings at somewhat depolarised fibers (*i.e.*, at membrane potentials where EPPs no longer triggered

muscle fiber action potentials) showed that the amplitude of the EPP declined during the stimulus train to 54 ± 3.6% (n = 28 endplates) of its initial value (Figure 8.1D), which is close to the control group mean value (60 ± 4.5%; n = 4 biopsies), but less pronounced than the EPP decline level of AChR MG patients (group mean 42 ± 1.4%; n = 23 biopsies), although being just within its range of individual biopsy mean values (29-55%).

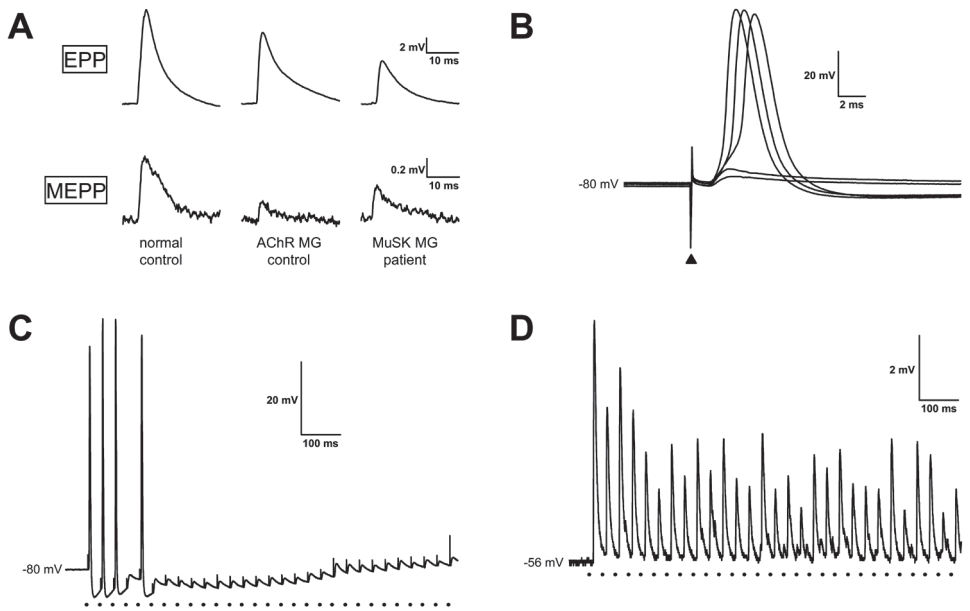

Figure 8.1 In vitro electrophysiology in MuSK MG
Electrophysiological signals recorded in vitro at neuromuscular synapses of the MuSK MG patient. For clarity, stimulus artifacts have been removed. (A) Representative examples of low-rate (0.3 Hz) evoked EPPs and spontaneous MEPPs. (B) Five superimposed example traces of responses recorded in the MuSK MG biopsy during 3-Hz stimulation. Two bare, apparently subthreshold, EPPs are shown and three muscle fiber action potentials, apparently triggered by suprathreshold EPPs, with variable delay relative to the moment of stimulation (black triangle). (C) Only the first few evoked EPPs trigger a muscle fiber action potential during high-rate (30 Hz) nerve stimulation (indicated by black dots). (D) Example trace of the rundown of EPPs at an endplate during 30-Hz stimulation.

Histology

Four endplates were available for analysis. One showed a postsynaptic area that was partially denuded of nerve terminal area and degenerated secondary postsynaptic folds (Figure 8.2A). Two other endplates had preserved postsynaptic areas, but were not covered by a nerve terminal (Figure 8.2B), although nearby one of these areas a preterminal nerve ending could be seen, surrounded by a Schwann cell, imposing as a nerve sprout. Z-disk streaming indicated myofibrillar degeneration (Figure 8.2C). One endplate appeared normal. Light microscopy revealed evidence of increased branching of intramuscular nerves (Figure 8.2D).

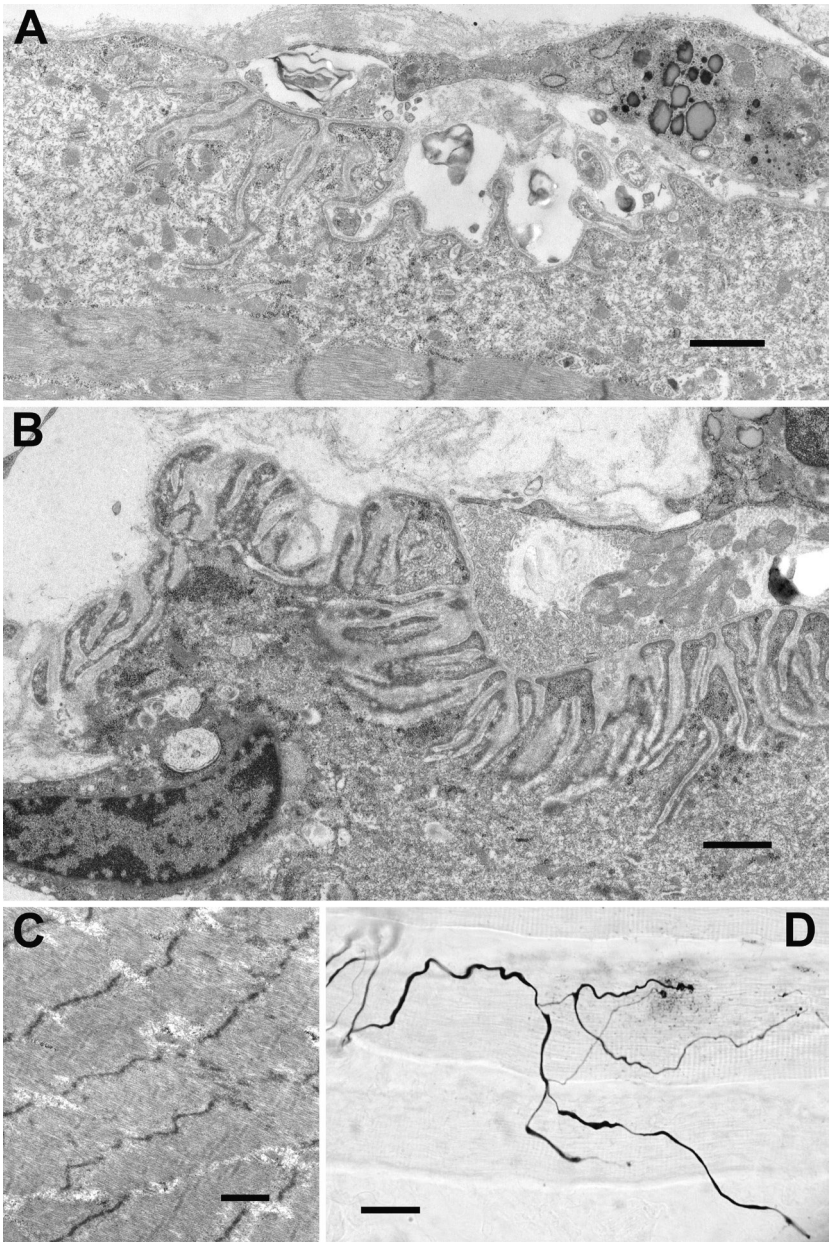

Figure 8.2 Histology in MuSK MG

Histological studies of the intercostal muscle biopsy. (A) Electron micrograph of a small profile of a nerve terminal (centre left) next to an area with degenerating postsynaptic folds. A Schwann-cell (upper right) contains abundant lipofuscin. (B) A nerve terminal (right) adjacent to a denuded area with conserved secondary clefts. Terminals contain translucent areas, sometimes with lipid remnants. (C) Z-disk streaming compatible with myofibrillar degeneration, which is also seen in the lower left corner of A. Bars in A, B and C = 1µm. (D) Light micrograph of silver-choline esterase staining for nerve fibers and endplates showing increased branching of intramuscular nerve fibers. Bar = 20µm.

Discussion

The course of symptoms, with spontaneous fluctuations and a response to immunosuppressive therapy, is consistent with that of an acquired autoimmune disorder of neuromuscular transmission. This could be confirmed by single-fiber electromyography in vivo, revealing an increased jitter and blocking, and by the presence of anti-MuSK antibodies in serum. Nine months after onset of symptoms, the biopsy was taken from a clinically affected muscle, as judged from the respiratory symptoms and the low spirometry values. At that time, no immunosuppressive therapy had been given. The patient had also not been treated with acetylcholinesterase inhibitors in the prior months.

In vitro electrophysiology showed small MEPPs of about half the control size. This is compatible with a postsynaptic defect, and has been unambiguously demonstrated at biopsied neuromuscular junctions of AChR MG patients. [162,164,165] Reduced MEPPs were found in another electrophysiological study of intercostal muscle endplates of a MuSK MG patient, [126] and in a preliminary animal study using active immunisation of mice with the extracellular domain of human MuSK. [157] Finally, passive immunisation of mice using serum obtained from anti-AChR-antibody-negative MG patients who later proved to be positive for anti-MuSK antibody, also led to a reduction of MEPP amplitude. [51,157] Thus, MEPP amplitude reduction seems to emerge as a general MuSK MG feature.

In another active immunisation study rabbits were immunised with an extracellular MuSK portion. Muscle weakness clearly developed and AChR density, as determined with fluorescence labelling, was greatly reduced. [166] However, such a reduction was not found in human morphological studies, [125,126] or in the passive transfer study of mice, as determined by radiolabelling. [51] This is a puzzling finding and suggests an effect of antibody binding to MuSK on AChR function.

Importantly, whereas in AChR MG an increase in quantal content can be observed as a compensatory presynaptic mechanism resulting from retrograde signalling from muscle fiber to nerve terminal, [162,165] the biopsy mean quantal content at the endplates of our study patient was 68% below that of the group mean of AChR MG patients, well below the lower limit of the range of biopsy means within this group, and even lower than in controls. In the MuSK MG patient described by Selcen *et al*., no reduced quantal content was found. [126] The quantal content in that study was calculated with the "variance method" (*i.e.*, from fluctuations of EPP amplitudes in preparations that were treated by d-tubocurarine to lower EPPs to subthreshold amplitude), instead of with the "direct method" without pharmacological manipulation, as applied here. The variance method is known to yield overestimated quantal content values, [162,167] and thus the value calculated by Selcen *et al*. may actually have been somewhat lower. Another important difference between the Selcen *et al*. study and our study is that the biopsy of their patient was taken later in the course of the disease, during a period

of relatively stable clinical symptoms, and in the presence of muscular atrophy, unlike the present case presented. In their accompanying morphological analyses, the presynaptic and postsynaptic neuromuscular junction ultrastructure seemed preserved.

A presynaptic defect might in theory result from a direct action of anti-MuSK antibodies on the presynaptic nerve terminal. However, neuronal presence of MuSK is unlikely,[61] although it cannot be completely excluded.[168] It is more likely that the autoimmune attack on MuSK in the postsynaptic membrane interferes, either directly or indirectly, with synaptic homeostatic and stabilisation pathways, with ensuing disturbance of retrograde signalling causing the presynaptic alterations. The observed pre- and postsynaptic defects at endplates in the MuSK MG biopsy led to failure of the neuromuscular transmission as indicated by small EPPs that became subthreshold, especially at high-rate stimulation of the motor nerve. The histological data accompanying our in vitro electrophysiological analyses must be regarded as qualitative and do not allow firm conclusions, in view of the large variability between endplates and the low number of observations we were able to make, due to the limited material. However, the observation of endplates (partially) lacking nerve terminals in combination with degeneration of postsynaptic folds and myofibrils supports the combination of pre-and postsynaptic abnormalities found in the microelectrode studies. Reduced MEPP amplitudes and quantal content have also been described in amyotrophic lateral sclerosis.[169] In patients with this disorder, a dysfunction of the neuromuscular transmission can be found in vivo, and has been attributed to a combined effect of nerve degeneration and regeneration, with a small size of nerve terminals, and a conduction block in immature axons. Histological studies show (partially) denuded postsynaptic areas with a relatively intact structure of the folds, similar to some of the junctions shown herein.[170]

Disturbances in the apposition between pre- and postsynaptic neuromuscular junction structures have been demonstrated in mice after passive immunisation with patient anti-MuSK IgG.[171] These mice showed a myasthenic phenotype, substantiated by a decreased density of AChRs in fluorescence-labelling studies and a decrement of compound muscle action potentials in repetitive nerve stimulation electromyography. Unfortunately, no in vitro electrophysiological analysis was performed at the neuromuscular junctions.

In conclusion, our electrophysiological and histological data show not only postsynaptic defects at the neuromuscular junction of a MuSK MG patient, but also demonstrate presynaptic disturbances. It is highly likely that these neuromuscular synaptic deficits are caused by the circulating anti-MuSK antibodies and underlie the muscle weakness of the patient. Anti-MuSK antibodies may reduce the stability of the muscle-nerve contact, leading to a process of nerve retraction and regeneration.

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