Short Title: MG NMJ electrophysiology

Full Title: Neuromuscular synapse electrophysiology in myasthenia gravis animal models

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Abstract

The neuromuscular junction (NMJ) forms the synaptic connection between a motor neuron and a skeletal muscle fibre. In order to achieve a sustained muscle contraction, this synapse has to reliably transmit motor neuronal action potentials onto the muscle fibre. To guarantee successful transmission even during intense activation of the NMJ, a safety factor of neuromuscular transmission exists. In the neuromuscular disorder myasthenia gravis (MG), autoantibodies are directed against acetylcholine receptors or, in the rarer variants, to other postsynaptic NMJ proteins. This causes loss of functional acetylcholine receptors, which compromises the safety factor of neuromuscular transmission, leading to the typical fatigable muscle weakness of MG. With intracellular micro-electrode measurement of (miniature) endplate potentials at NMJs in *ex vivo* nerve-muscle preparations from MG animal models these functional synaptic defects have been determined in much detail. This review will describe the electrophysiological events at the normal NMJ and the patho-electrophysiology at NMJs of animal models for MG.

Detailed electrophysiological investigation of synaptic signals at the neuromuscular junction (NMJ) has been instrumental to elucidate many basic aspects of synaptic transmission and to understand the pathophysiological mechanisms underlying neuromuscular synapthopathies. In this review we will describe the electrophysiology of the NMJ and the patho-electrophysiology of NMJs of animal models for the autoimmune neuromuscular disorder myasthenia gravis (MG).

Electrophysiological signals at the neuromuscular junction

Intracellular microelectrode recordings of synaptic electrophysiological signals at the NMJ can be made in muscle fibres of unfixed muscle specimens dissected from MG patients or experimental animals. For a more methodological description of such an electrophysiological analysis at the NMJ, the reader is referred to Ref. 1.[1]

The principal task of the NMJ is to transfer motor neuronal action potentials onto the skeletal muscle fibre in an exactly one-to-one fashion, irrespective of the neuronal firing frequency and duration. Only then, a properly sustained, tetanic muscle contraction can be guaranteed. Although this synaptic task of the NMJ seems a rather simple one, many highly complex structural and functional subcellular and molecular specializations are underlying. To understand the synaptic defects at the NMJ in the condition of MG it is essential to know its normal function, which we will briefly describe first.

The unitary signals of electrically excitable cells such as neurons and muscle cells are the action potentials. These all-or-none depolarizations are enabled by the existence of a resting membrane potential and the presence of several types of ion channels in the cell membrane. Action potentials are actively conducted over the membrane of excitable cells. Their rising phase is caused by Na^+ influx through voltage-gated Na_V channels. In the nodes of Ranvier of motor axons these are of the $Na_V1.6$ type while in skeletal muscle fibres these are of the $Na_V1.4$ type.[2,3] The motor neuronal action potential travels towards the nerve terminal in a jumpwise fashion via the axonal nodes of Ranvier, where the Na_V channels are present at high density and absence of myelin allows for Na^+ membrane flux. The decaying phase of action potentials is due to Na_V inactivation and opening of rectifying voltage-gated potassium channels. The small dimensions of mammalian motor axons (i.e. a diameter of only a few microns) preclude direct voltage measurements. In fact, much of what we know about ion fluxes and ion channel behaviour underlying axonal action potential generation and conduction has come from pioneering studies on the ~1 mm diametered giant motor axons of the squid.[4] When the neuronal action potential reaches the motor nerve terminal, it triggers

opening of voltage-gated Ca^{2+} channels which are of the $Ca_V2.1$ type (Fig. 1).[5] These channels are present at so-called active zones, which are presynaptic molecular complexes where structural proteins keep functional proteins in optimal position to control neurotransmitter release.[6] $Ca_V2.1$ channels are anchored at active zones by binding to structural presynaptic and synaptic cleft molecules such as bassoon, CAST/erc2 and laminin-β2, either to the pore-forming subunit or to accessory subunits.[7,8] About 2.5 active zones are present in every μm^2 of presynaptic membrane, implying the presence of ~800 active zones per nerve terminal, at least in the mouse.[9] Action potential-induced opening of the presynaptic $Ca_V2.1$ channels results in Ca^{2+} influx into the motor nerve terminal along its steep concentration gradient (i.e. extracellular ~2 mM, intracellular ~200 nM). The ensuing increase of cytoplasmatic Ca^{2+} concentration in the vicinity of the active zone activates a complex of molecules which orchestrate exocytosis of synaptic vesicles that are filled with the neurotransmitter ACh. The principal components of this complex are the Ca^{2+} sensor synaptotagmin and the SNARE proteins synaptobrevin, SNAP-25 and syntaxin. Synaptotagmin and synaptobrevin are present in the membrane of the synaptic ACh vesicles, while SNAP-25 and syntaxin are embedded in the presynaptic membrane. Together (and modulated by many factors), these proteins govern the docking and controlled exocytosis of transmitter containing vesicles, leading to neurotransmitter release into the synaptic space.[10] Each ACh vesicle contains a 'quantum' of ~10,000 ACh molecules. Per nerve impulse, a number of these quanta (the 'quantal content') is released simultaneous. The quantal content is dependent on many factors, such as age, species, muscle type etc. Human nerve terminals, which are small (~100 μm^2), have a quantal content of ~20, while the larger mouse nerve terminals (~250 μm^2) release ~50 ACh quanta per nerve impulse.[11,12] Roughly, the quantal content is determined by the absolute number of active zones present in a motor nerve terminal, as each active zone has a more or less fixed chance of ~10% of releasing an ACh quantum upon $Ca_V2.1$ channel activation by an action potential.[9,13] In addition to the action potential-evoked simultaneous exocytosis of multiple ACh quanta, now and then (roughly once per second at the mouse NMJ) there is exocytosis of a single ACh quantum. This is a spontaneous process which can presumably be regarded as a spillage of quanta from the very large pool of ACh vesicles. Electrophysiological experiments using the specific $Ca_V2.1$ blocker ω-Agatoxin-IVA showed that the rate of spontaneous uniquantal ACh release is for ~50% dependent on $Ca_V2.1$ opening.[14]

Once quanta are exocytosed, ACh molecules diffuse into the ~50 nm wide synaptic cleft were a large proportion of the neurotransmitter is degraded by basal lamina-anchored acetylcholinesterase (AChE), limiting the lateral spread of ACh away from the releasing active zone. Remaining ACh can then bind to the muscle ACh receptors (AChRs), localized at high density (~10,000/μm^2) on the tops of the typical postsynaptic folds (Fig. 1.). Skeletal muscle fibres express nicotinic AChRs.[15-17] These are ligand-gated ion channels that consist of five transmembrane subunits (i.e. two α1, a β1, a δ and an ε subunit) in the adult situation. Embryonic NMJ AChRs or AChRs that appear on the extrasynaptic muscle fibre surface after denervation have a γ subunit instead of the ε subunit. The binding of an ACh molecule to each of the binding pockets formed by the two α1 subunits with one of their neighbouring subunits causes conformational changes which result in opening of the ion pore.[15] AChRs have a single channel conductance of ~60 pS and their opening at physiological condition results in influx of mainly Na^+ ions, as well as some efflux of K^+. A few percent of the net inward ion current through the muscle AChR is carried by Ca^{2+} ions.[18] Together, the multiple ACh quanta released by a neuronal action potential open thousands of AChRs and the resulting net influx of positive charge causes a local depolarization of the postsynaptic membrane. This is the endplate potential (EPP), which has a duration of a few ms and is terminated when ACh becomes degraded by AChE. The amplitude of an EPP varies between species, muscle type, age etc., and lies mostly between 15 and 30 mV. The purpose of the EPP is to cause opening of many voltage-gated $Na_V1.4$ channels, which reside in the bottom of the postsynaptic folds. This results in the upstroke of an action potential, which will travel away from the NMJ in two directions and will subsequently trigger T-tubular mechanisms that elicit muscle fibre contraction. While the EPPs are directly functional, the spontaneous release of single ACh quanta leads to a miniature EPPs (MEPPs) of ~0.3-1.5 mV (Fig. 2), which are too small to trigger a muscle fibre action potential. In the electrophysiological analysis of the MG NMJ, the MEPP amplitude can be used as an indicator for the density of functional AChRs (see below). In addition, their uniquantal amplitude is used in quantal content calculations. To this end, the amplitude of the multiple quanta-based EPP is divided by that of the mean uniquantal MEPP, measured at the same NMJ.[1]

Muscle fibres have a depolarization threshold for action potential firing which is depending on the density and electrophysiological characteristics of $Na_V1.4$ channels. Two factors assure successful synaptic transmission at the NMJ: 1) a high $Na_V1.4$ density at the NMJ causes a local lowering of the firing threshold, facilitating muscle fibre action potential

induction by EPPs[19-23]; 2) in spite of this relatively low firing threshold, EPPs are generally much larger than the minimal amplitude to reach the threshold.[12,24] This safety factor in neuromuscular transmission amounts ~2-3 (at rodent NMJs, at human NMJs it is ~2).[25] While such a safety factor might seem superfluous this is not the case. It protects the NMJ against transmission failure when quantal content (and thus also the EPP) runs down during intense synaptic activity (Fig. 2B). The underlying reason is that some neuro-exocytotic factors become limiting. EPP rundown can be ~20-30% during trains of physiological activity of the NMJ, which can be at rates of 20-100 Hz.[26] The rundown of EPPs at human NMJs is somewhat more prominent than in rodent NMJs, by ~40%.[27] Only by having a considerable safety factor can the neuromuscular transmission at the NMJ exactly follow the motor neuronal firing pattern. In this way sustained tetanic contraction is warranted (Fig. 2C).

Aberrant electrophysiology at the neuromuscular junction in myasthenia gravis

The electrophysiological problem at the NMJ in MG is that the EPPs are smaller than in normal NMJs. During prolonged synaptic activity they can even become of subthreshold amplitude so that they can no longer trigger muscle fibre action potentials (Fig. 2B). Thus, there is a substantial reduction of the safety factor of neuromuscular transmission at MG NMJs. Due to slight variability of EPP amplitude during prolonged activity, a myasthenic NMJ in a critical state with peri-threshold EPPs will have intermittent transmission failures. This phase will progress into a more permanent synaptic failure state after some time of activity due to ongoing EPP rundown. If many NMJs in the skeletal muscles of MG patients are in such a state of having progressive block of NMJ transmission, this leads to the typical symptoms of fatigable muscle weakness. If EPPs are too small already from the start of NMJ activity to reach the firing threshold, the connected muscle fibre will not contract at all. This will contribute to a degree of permanent muscle weakness.

How do the EPPs at MG NMJs become reduced in amplitude? The answer is loss of postsynaptic sensitivity for the neurotransmitter ACh due to reduced density of functional AChRs. The great majority (~85%) of MG patients has auto-antibodies (IgG1 and IgG3 type) which are directed against postsynaptic AChRs at NMJs. The effect of these autoantibodies is three-fold: 1) removal of AChRs due to cross-linking and subsequent internalization; 2) functional AChR block and 3) the activation of complement with formation of membrane-attack complex that causes focal lysis.[28,29] Together this results in the two main electrophysiological deficits which both contribute to reduction of the safety factor of neuromuscular transmission: 1) the EPPs become small due to the loss of a proportion of the

functional AChRs and 2) the firing threshold becomes elevated due to reduction of Nav1,4 channel density due to the complement-mediated focal membrane damage at the postsynaptic NMJ (Fig. 2).

AChR myasthenia gravis animal model electrophysiology

The knowledge of these patho-electrophysiological mechanisms and the underlying autoimmunity at the NMJ in MG has come in large part from animal model studies. The first animal model for MG dates from 1973 when active immunization of rabbits was performed.[30] The animals were injected with a purified AChR fraction derived from electric organs of *Electrophorus electricus*, an eel species. After about one month, the rabbits developed flaccid muscle weakness. Similar to MG patients, the symptoms of muscle weakness improved when the animals were treated with neostigmine, an acetylcholinesterase inhibitor that increases the available ACh at the NMJ. Anti-eel AChR antibodies were detected in the rabbit serum. No *ex vivo* detailed electrophysiological measurements were performed at NMJs of these rabbits. Instead, electromyography during 40 Hz repetitive nerve stimulation was performed and showed a decrement of the compound muscle action potential that more or less stabilized at ~40% after the fourth nerve stimulus. This is indicative of progressive NMJ transmission block, as also observed in MG patients in diagnostic clinical electromyographical testing. The decrement was corrected by neostigmine injection, further demonstrating NMJ dysfunction as the underlying culprit.

In the same era, additional studies (re-)produced active immunization MG models in guinea pigs, rabbits, rats and rhesus monkeys, using eel AChR but also AChR derived from the ray *Torpedo californica* or *marmorata*.[31-33] The models were termed 'experimental autoimmune MG' (EAMG). They showed fatigable muscle weakness and decrement of the compound muscle action potential, which could be normalized by acetylcholinesterase inhibitors. Another line of direct evidence for failure of NMJ transmission due to AChR block as cause in MG were rat studies in which α-toxin from the Formosan cobra, a known AChR blocker, was injected. This caused MG-like decrement of compound muscle action potentials which could be normalized by an acetylcholinesterase inhibitor.[34] Another crucial finding, providing proof of the pathogenicity of MG autoantibodies, was that passive transfer to mice of purified IgG from MG patients induced pathophysiological effects at the NMJ.[35] This was the first study to show with *ex vivo* electrophysiological analyses using an intracellular micro-electrode that MEPPs at MG mouse NMJs had small amplitudes (~30% of normal), indicating lowered AChR density. Similarly, the first microelectrode studies of

NMJs in active immunization EAMG rats also clearly revealed small MEPPs.[36,37] Collectively, these early studies provided the first proof that the pathophysiological mechanism in MG is a decrease in AChR density due to auto-antibody attack at the NMJ and that a reduced MEPP amplitude forms the electrophysiological hallmark. Further passive transfer studies in which mice, rats and guinea pigs were injected with rat AChR monoclonal antibodies, were in line with these earlier studies as they confirmed MEPP reductions.[38,39]

The second electrophysiological factor which contributes to safety factor reduction in MG is the elevation of the muscle fibre firing threshold. This is due to the complement-mediated postsynaptic membrane damage. It results not only in AChR density reduction, but also in removal of postsynaptic $Na_V1.4$ channels from the bottom of the postsynaptic membrane folds, where they are enriched.[21] In NMJs of a rat passive transfer MG model and in human MG biopsy NMJs it was shown that AChR antibodies indirectly cause reduced Nav1.4 density via complement activation and thereby elevate the firing threshold at the NMJ by ~10 mV.[22,40] The AChR antibodies do not directly alter the electrophysiological features of the remaining $Na_V1.4$ channels.[40]

In our own laboratory we developed non-immunological rat and mouse models for AChR MG by chronic injection of low doses of α-bungarotoxin, a near-irreversible blocker of AChRs.[41,42] With careful dosing this produces controllable mild muscle weakness. The NMJ electrophysiology could be studied in much detail, also because in the 1990s the pharmacological tools μ-conotoxin-GIIIA and -B became available which can be used to selectively block $Na_V1.4$ channels of the muscle fibre and thus prevent muscle fibre action potentials. In this way, EPPs can be measured without being disturbed by action potentials and the contraction these cause.[43] From micro-electrode studies in the α-bungarotoxin-induced rat MG model it appeared that the myasthenic NMJ tries to compensate the loss of AChR density by increasing the presynaptic ACh release per nerve impulse.[43,44] This homeostatic increase of quantal content was confirmed in human AChR MG NMJs.[11] The quantal content level of individual NMJs is inversely related to their myasthenic severity (i.e. as indicated by the reduction in MEPP amplitude). Therefore, local retrograde signalling is likely taking place. Multiple candidates for post- and presynaptic factors and the retrograde signals involved have been hypothesized, but the exact mechanism causing the compensatory increase of ACh release is not elucidated yet.[44] An increased size of the presynaptic pool of ACh vesicles available for release might be of importance.[45] The observed compensatory homeostatic reaction of the motor nerve terminal to the postsynaptic loss of functional AChR density might offer protection against synaptic failure of the myasthenic NMJs, especially in

mild myasthenic conditions. On the other hand, the increased quantal content is associated by a more intense rundown during high rate activity.[11,43] This is possibly due to neuro-exocytotic factors becoming limiting sooner due to the higher demand. Thus, extra EPP rundown at MG NMJs might partly counterpoise a beneficial effect of increased quantal content.

MuSK- and LRP4 myasthenia gravis animal model electrophysiology

More recent MG animal modelling studies have focussed on the rarer variants of MG, with autoantibodies against the postsynaptic NMJ proteins muscle-specific kinase (MuSK) and low-density lipoprotein receptor-related protein 4 (LRP4). MuSK MG and LRP4 MG have some clinical and pharmacological features distinct from AChR MG, including the regional distribution pattern of the muscle weakness. For more detailed information the reader is referred to Ref. 28.[28] MuSK and LRP4 are transmembrane proteins which are involved in the neuronal agrin-dependent embryonic formation of AChR clusters and their maintenance in later phases.[46,47] The recently generated active- and passive immunization mouse models for MuSK and LRP4 MG have been very well-characterized with detailed *ex vivo* electrophysiological investigations (Table 1).[48-55] Many electrophysiological similarities exist with the active- and passive immunization and toxin-induced models for AChR MG. In particular, the MEPPs are reduced in amplitude and frequency, and the EPPs are reduced in amplitude and have extra rundown during high-rate synaptic activity. A decrement of muscle action potentials has been found in electromyographical investigations of weak animals. Morphologically, there is clear fragmentation, dispersal and density reduction of AChR clusters in both the MuSK and LRP4 MG model NMJs. The only (but very distinct and important) difference from AChR MG in the electrophysiological analyses is that MuSK- and LRP4 MG model NMJs do not show increased quantal content. Rather, some studies have even shown a somewhat decreased quantal content, as compared to normal control NMJs. In more detailed NMJ analyses of MuSK MG mouse model electrophysiology it has also been shown that no inverse relationship exists between MEPP amplitude and quantal content at individual NMJs, indicating the absence of a synaptic homeostatic response to the reduced postsynaptic neurotransmitter sensitivity.[55,56, JJ Plomp, unpublished data] Similarly, absence of a compensatory increase in quantal content was shown in the few electrophysiological NMJ studies in human MuSK MG muscle biopsies published so far.[27,57] Collectively, these studies suggest that MuSK and/or LRP4 are in some way involved in the mechanism underlying synaptic homeostasis at the myasthenic NMJ, possibly as postsynaptic sensing (co-)factor or as a source of retrogradely acting signalling molecules, e.g. by shedding of extracellular

fragments.[58,59] Further electrophysiological and other types of studies will be needed to elucidate their exact roles.

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Table 1.

	normal	**AChR MG**	**MuSK MG**	**LRP4 MG**
CMAP decrement	no	yes	yes	yes
MEPP amplitude	~1 mV	reduced	reduced	reduced
MEPP frequency	~1 /s	same or reduced	reduced	reduced
single EPP amplitude	~30 mV	reduced	reduced	reduced
high-rate EPP rundown	~20-30%	exaggerated	exaggerated	?
Quantal content	~30-50	increased	same or reduced	reduced
For references see e.g.	11,50,51,54-56	11,55	50,51,55,56	54

Table legend

Comparison of the main neuromuscular electrophysiological features of mouse models of AChR, MuSK and LRP myasthenia gravis variants. CMAP = compound muscle action potential; EPP = endplate potential; MEPP = miniature EPP.

Figure legends

Figure 1. Schematic drawing of the neuromuscular junction and the localization of the key ion channels in the pre- and postsynaptic membrane.

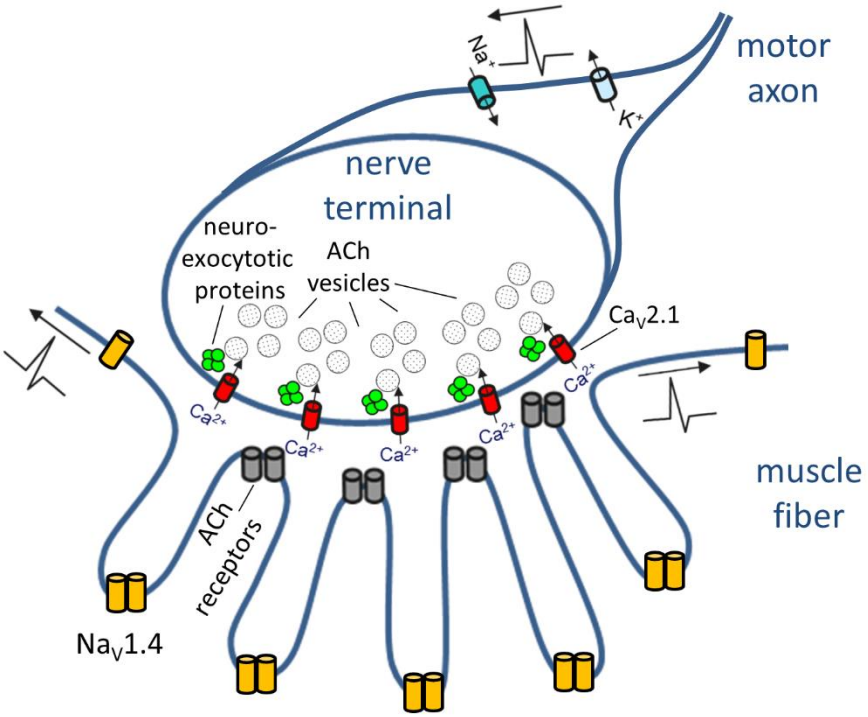
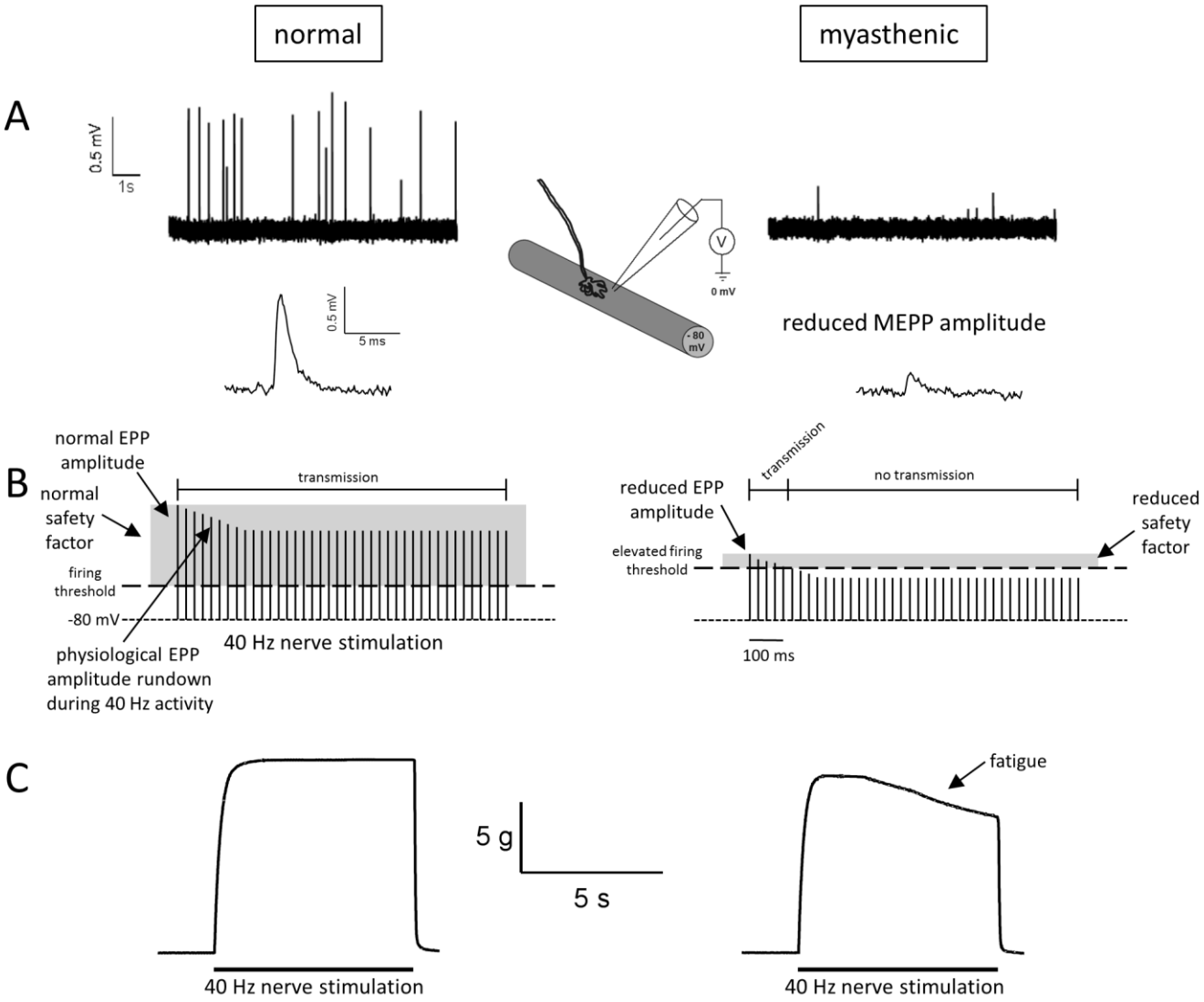


Figure 2. Electrophysiological signals at the myasthenic neuromuscular junction (NMJ). With intracellular micro-electrode measurements the spontaneous miniature endplate potentials (MEPPs) and nerve-stimulation evoked endplate potentials (EPPs) can be recorded from the muscle fibre near the NMJ. (A) Examples of severely reduced MEPP amplitude at a myasthenic NMJ from a passive transfer myasthenia gravis mouse model, indicating reduced ACh receptor density due to the autoantibody attack. In the top row pictures ten recorded sweeps of 1 s duration have been superimposed. Bottom row show exemplary MEPPs at magnified time scale. (B) Schematic illustration of the reduced safety factor at the myasthenic NMJ (right), as compared to a normal control NMJ (left). The low safety factor is caused by the combination of EPPs being reduced due to loss of ACh receptors and an elevated firing threshold of the muscle fibre due to loss of postsynaptic $Na_V1.4$ channels resulting from complement-mediated focal membrane damage. The physiological rundown of EPPs during high-rate activity brings the EPPs under the firing threshold at the myasthenic NMJ, and muscle fibre action potentials are no longer triggered. At the normal NMJ, EPPs remain supra-threshold in spite of their amplitude rundown, resulting in continuous successful synaptic transmission. (C) Recorded tetanic contraction of *ex vivo* mouse diaphragm muscles upon 40 Hz nerve stimulation. The muscle from the normal mouse (left) shows sustained contraction, while the muscle from a passive transfer myasthenia gravis mouse (right) shows fatigable weakness. Apparently, a proportion of the muscle fibres in this muscle has an NMJ with progressive block of neuromuscular transmission due to EPPs becoming subthreshold, such as depicted in (B).


normal
myasthenic
A
0.5 mV
1s
0.5 mV
5 ms
V
0 mV
- 80 mV
reduced MEPP amplitude
B
normal EPP amplitude
normal safety factor
transmission
firing threshold
-80 mV
physiological EPP amplitude rundown during 40 Hz activity
40 Hz nerve stimulation
transmission
no transmission
reduced EPP amplitude
reduced safety factor
elevated firing threshold
100 ms
C
5 g
5 s
fatigue
40 Hz nerve stimulation
40 Hz nerve stimulation