Targeting the safety factor for neuromuscular transmission to treat myasthenia gravis

G Lorenzo Odierna PhD[1] and
William D Phillips PhD[,2]

[1]Tasmanian School of Medicine, University of Tasmania, Hobart, Tasmania, Australia
[2]School of Biomedical Sciences, The University of Queensland, St Lucia, Queensland, Australia. Email: bill.phillips@uq.edu.au

ABSTRACT

In myasthenia gravis autoantibodies attack the postsynaptic membrane of the neuromuscular junction and cause fatiguing weakness that can wax and wane. Weakness occurs when the safety factor for neuromuscular transmission becomes marginal, meaning that the (postsynaptic) endplate potential is no longer sufficient to reliably trigger action potentials in the muscle fiber. Cholinesterase inhibitor drugs provide temporary relief by increasing the endplate potential amplitude, but additional symptomatic treatment options are needed. Here we review our recent experience in early preclinical testing of candidate compounds. Using an *ex vivo* mouse nerve-muscle contraction assay, followed by endplate potential recordings, we examined the effects of cannabinoids. Our findings highlighted the potentially confounding effects of dimethylsulfoxide (DMSO) when used as a solubilizing agent. They also demonstrate the need to take synaptic homeostasis into account, which can otherwise distort or mask the effects of bioactive agents upon neurotransmission. In all, our studies taught us some hard lessons: pitfalls for the basic scientist seeking to develop a candidate drug.

Keywords: neuromuscular junction disease; myasthenia gravis; experimental myasthenia, synaptic homeostasis

Introduction

In myasthenia gravis (MG) autoantibodies target proteins in the postsynaptic membrane of the neuromuscular junction (NMJ). They reduce the efficacy of neuromuscular transmission by several different pathophysiological mechanisms (reviewed by Huijbers et al. 2022). To understand how impaired neuromuscular transmission leads to weakness it is useful to first briefly review the structure and function of the healthy NMJ.

NMJ structure and function

Under the microscope the human NMJ looks a bit like a bunch of grapes. The motor axon branches to form several terminal swellings, called boutons (Fig 1A, boutons in red). The presynaptic membrane of each bouton is aligned above a portion of the postsynaptic (muscle) membrane that is rich in acetylcholine receptors (AChRs, green labelling). Enlarged under the electron microscope, the postsynaptic membrane is seen to have many deep infoldings. Each infolding marks out a potential site of neurotransmission (Fig 1B). The minimal synaptic unit consists of synaptic vesicles docked on the presynaptic membrane and primed to release their cargo of acetylcholine (Fig 1C). Release of a single such quantum of acetylcholine produces a membrane depolarization known as a miniature end plate potential (MEPP, amplitude ~1mV). MEPPs are thought to occur due to spontaneous release of primed synaptic vesicles and are used as a measure of quantal amplitude.

The endplate potential (EPP) is caused by synchronized release of many such quanta. Every action potential in the motor axon triggers the opening of a small number of voltage-gated calcium channels that are tethered to each primed vesicle. Calcium ions diffuse in through these open channels to produce a brief, local plume of ionic calcium that binds to sensor proteins on the vesicle, triggering exocytosis of acetylcholine. The estimated number of vesicles released to produce the EPP is referred to as 'quantal content'. The EPP activates voltage-gated sodium channels that are concentrated at the base of the postjunctional folds (Fig 1C). The amplitude of the EPP is normally more than sufficient to initiate a muscle action potential, but not so in MG (Fig 1D). Acetylcholinesterase within the synaptic cleft (Fig 1C) rapidly terminates the EPP by breaking down the acetylcholine (MacIntosh et al. 2006; Plomp et al. 2015).

The safety factor and its limitations

The *safety factor for neuromuscular transmission* is typically about two-fold, meaning that synaptic signalling is twice as strong as is needed to trigger an action potential in the muscle fiber (Wood and Slater 2001). In myasthenia gravis, postsynaptic sensitivity to acetylcholine is impaired, causing a reduction in the amplitude of the EPP, and consequently the safety factor. There is some natural (impulse to impulse) variability in the amplitude of the EPP, so when MG reduces the safety factor to unity (approximately 1.0), many nerve impulses will fail to trigger a postsynaptic action potential (Fig 1D; Elmqvist et al. 1964).

Determinants of the safety factor

The safety factor depends upon multiple features of the healthy NMJ. On the presynaptic side the high quantal content at rest is thought to depend upon hundreds of synaptic vesicles that are primed and ready to release
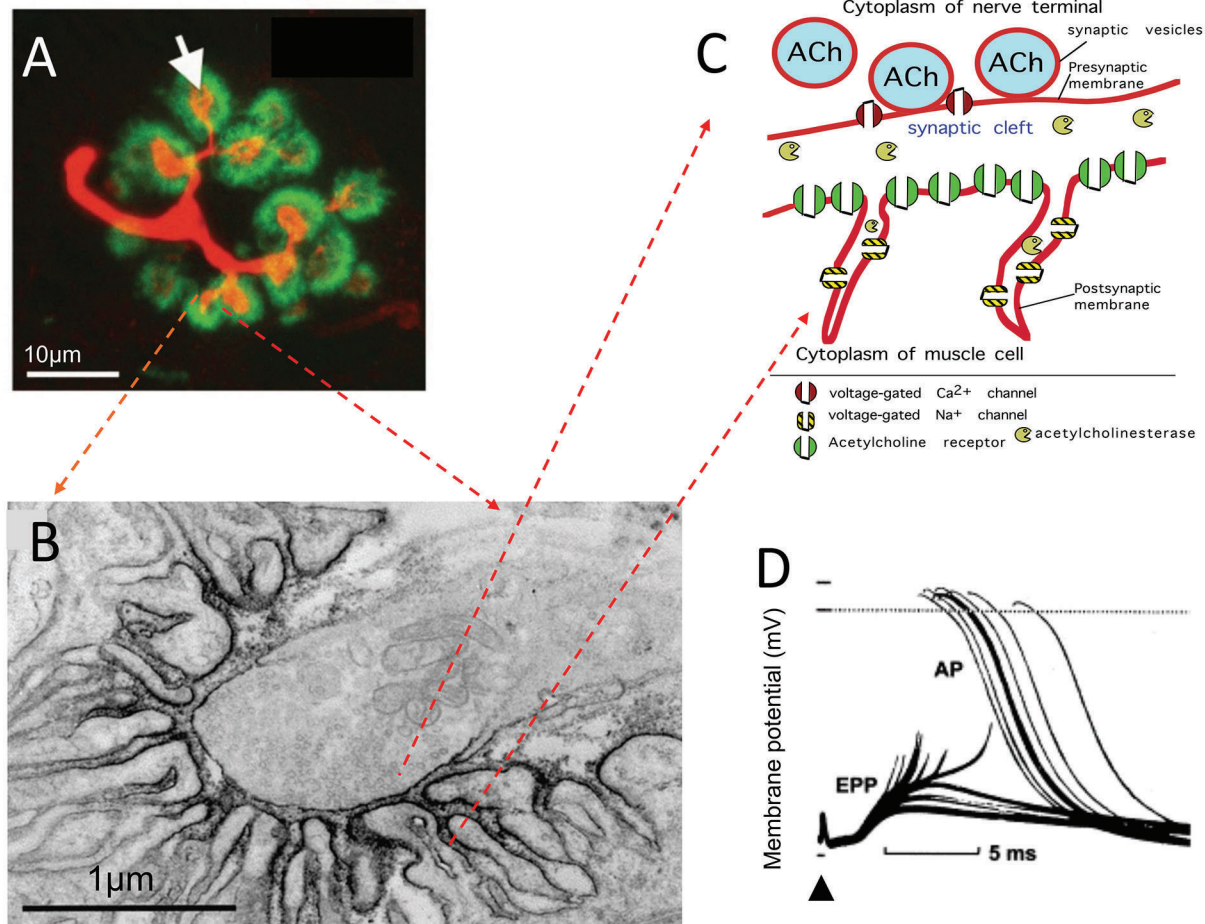
Fig 1. Structure and (dys-)function of the human NMJ. (A) Immunofluorescent image of a human NMJ. Each presynaptic terminal bouton (red, arrow points to one bouton) is aligned above AChR-rich postsynaptic membrane (green; modified from Ding et al 2022; Creative Commons Attribution License, CC-BY 4.0). **(B)** Each bouton forms the core of a 3D calyx-like structure as can be seen from this transverse electron microscope image. The AChRs are concentrated near the tips of the postjunctional folds (dark staining) separated from the overlying presynaptic membrane by the narrow synaptic cleft (modified from Ohno et al. 2002; RightsLink licence # 5493930960934). **(C)** Cartoon representation of a single presynaptic acetylcholine release site aligned above a postsynaptic membrane infolding. **(D)** Multiple superimposed recordings of EPPs and action potentials recorded from a muscle biopsy of an MG patient. Every nerve stimulus should trigger the all-or-none action potential (AP) but in this myasthenic muscle the reduced amplitude of the EPPs often fails to reach the required threshold (figure modified from Elmqvist et al 1964; RightsLink license # 5493921030479).

their contents in response to a nerve impulse. On the postsynaptic side, the normal high quantal amplitude (approximately 1mV) depends upon the dense packing of AChRs at the tips of the postjunctional membrane folds. The deep membrane infoldings of the human NMJ (Fig 1B) funnel synaptic currents from the AChRs to the voltage-gated sodium channels at the base of these folds (Fig 1C). In MG, antibody-mediated loss of AChRs and widening of the synaptic cleft reduce the amplitudes of both the MEPP and the EPP. Complement-mediated damage to the postjunctional folds can also raise the threshold for an action potential (Ruff and Lennon 2008; for a recent review

see Huijbers et al. 2022). Both of these changes reduce the safety factor.

Neuromuscular transmission decay

Neuromuscular transmission is vulnerable to fatigue. Muscle contraction force is controlled, in large part, by the frequency of nerve impulses relayed from nerve to muscle through the NMJ (MacIntosh et al. 2006). This can become a problem because during every train of nerve impulses the EPP amplitude declines due to a decline in quantal content ('synaptic depression'; Kamenskaya et al. 1975). During sustained, high-frequency neuromuscular transmission

the decay in quantal content is explained by progressive depletion of the pool of primed synaptic vesicles on the presynaptic membrane (Wang et al. 2016). The rate of vesicle depletion can also be influenced by cholinergic and purinergic autoreceptors on the nerve terminal (Santafe et al. 2015; Sanabria et al. 2022). In healthy muscle, a large safety factor (at rest) ensures that the EPP continues to trigger postsynaptic muscle action potentials despite the natural decay in quantal content during each impulse train. It remains uncertain whether the healthy NMJ ever fails in living, behaving animals. In conditions such as MG, where the safety factor becomes marginal, the intrinsic property of synaptic depression is expressed as fatiguing failure of the muscle action potential (in one muscle fiber after another).

A few difficult lessons about translation

Drugs such as pyridostigmine enhance EPP amplitude by slowing the breakdown of acetylcholine in the synaptic cleft. As first line treatment for MG they provide immediate relief. They can also help minimize corticosteroid dosage when treating chronic MG. However, a substantial subset of patients report loss of efficacy with pyridostigmine, and adverse side effects are common (Remijn-Nelissen et al. 2022). It might be possible to overcome these limitations if we could find a novel drug that would improve the safety factor by targeting a different component of the NMJ. Being new to preclinical translation work we thought we were onto something when a pilot study in our lab suggested that cannabinoids might have the potential to restore EPP amplitude in a mouse model of MG (Morsch et al. 2018). With funding from the Lambert Initiative for Cannabinoid Research we undertook a follow-up study to clarify the pharmacology and mechanism of cannabinoid action at the mouse NMJ. We wanted to see if a cannabinoid therapeutic could be developed.

From mechanism to preclinical translation

The first thing we learned to appreciate was the need for a bioassay to quickly assess the effect of various compounds on the safety factor. Animal models of MG previously used EPP recordings (Morsch et al. 2018). They provide detailed mechanistic information about quantal synaptic transmission, but they are very time consuming and require fairly extensive replication (n=8 preparations). This makes them impractical for screening multiple compounds. Instead, we employed an *ex vivo* mouse phrenic nerve-hemidiaphragm muscle contraction preparation. A train
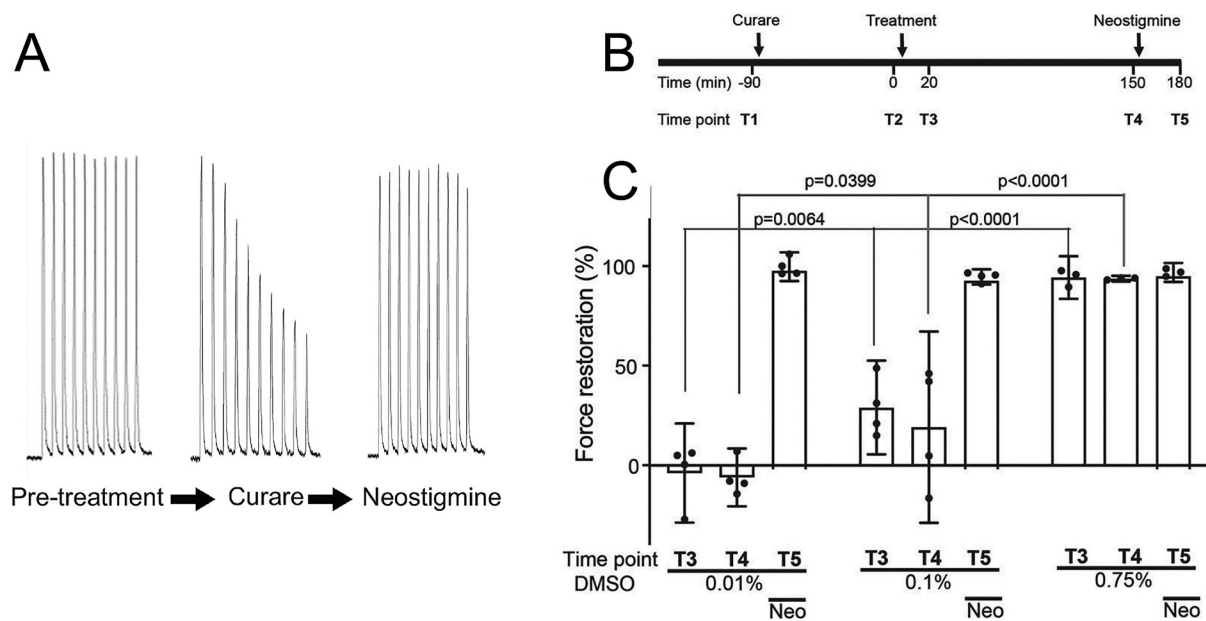
Fig 2. **A muscle contraction assay to assess the effect of compounds on the safety factor**. **(A)** Contraction force recordings from an isolated section of mouse diaphragm muscle. A train of ten nerve stimuli (3/second) yielded ten twitch contractions of equal force (Pre-treatment). After adding 700 nM tubocurarine (Curare), a decrement in the train reflected progressive failure of neuromuscular transmission. Further addition of the acetylcholinesterase inhibitor drug, neostigmine (Neo), restored consistent twitch force. **(B)** Timeline for a typical assay run. **(C)** Quantitation of force restoration. We measured the degree to which DMSO reversed the curare-induced decrement at the indicated timepoints after adding the test compound (T3=20 min, T4=150 min). Symbols show results from replicate preparations. Bars show means and 95% confidence intervals (P values produced from two-way ANOVA with Tukey multiple comparisons post-test; figure modified from Odierna and Phillips 2021; © 2021 – IOS Press).

of ten stimuli to the nerve (3/sec) normally produces ten brief twitch contractions, all the same amplitude. To mimic myasthenic conditions we used tubocurarine to block the majority of the postsynaptic AChRs. The resulting drop in safety factor became evident as a progressive decrement in twitch force during each train of ten stimuli: analogous to the decrement in the compound muscle action potential in myasthenic muscles (Fig 2A; Plomp et al. 2015). We then measured the percentage decrement in the force from the first (unaffected) twitch to the last twitch in the train to assess the potential of various compounds to restore the safety factor.

The difficult problem presented by bioactive solvents

The second thing that became clear to us was that cannabinoids are very hydrophobic. A solubilizing agent such as dimethylsulfoxide (DMSO) or ethanol is needed to prepare a stock solution from the powdered compound. We found that quite a high molar ratio of DMSO to cannabinoid (a few hundred to one) was needed to prevent the drug from precipitating when the stock solution was subsequently diluted into physiological saline. In practice, a final concentration of 10 μM cannabinoid could only be achieved by including a final concentration of 0.1% DMSO (v/v). Presumably, DMSO forms amphipathic shells around the (hydrophobic) cannabinoid molecules. We are uncertain how the interaction with DMSO might affect the biochemical actions of cannabinoids.

The third thing we discovered was that the real active ingredient for restoring safety factor in our bioassay was the DMSO, not cannabinoids. At a concentration of 0.01% DMSO had no detectable effect on the contraction force decrement, but at concentrations of 0.1% and 0.75% DMSO produced a dose-dependent restoration of force (Fig 2C). We tested two different dual CB1/ CB2 cannabinoid receptor agonists (CP 55,940 and WIN 55,212-2) using the minimum necessary concentration of DMSO to keep them in solution. For each set of experiments, the DMSO component of the treatment was sufficient to explain the observed force restoration. On the contrary, we found that cannabinoids had a negative effect on the safety factor. Follow up contraction experiments using selective agonists suggested that the delayed negative effect of the cannabinoids was mediated by the CB1 receptor, but not the CB2 receptor. Our findings eliminated cannabinoids as potential therapeutic agents to treat MG. Instead, we learned about how organic solvents and cannabinoids affect nerve-muscle function and some challenges facing early preclinical drug development.

Synaptic homeostasis: adapt the assay to the disease context

The NMJ doesn't give up easily. When myasthenic autoantibodies cause a reduction in quantal amplitude, the nerve terminal tries to compensate by increasing quantal content (Plomp et al. 1992, 1995). Acute partial blockade of postsynaptic AChRs can trigger a rapid compensatory increase in the pool of readily-releasable (primed) synaptic vesicles in the nerve terminal (Wang et al. 2016). Our combined electrophysiology results certainly demonstrated this response. From a total of 24 muscle preparations, the average MEPP amplitude was 1.09mV, the mean EPP was 17.7mV, and the mean quantal content was 17.9. When muscles were bathed in 500 nM tubocurarine, the MEPP amplitude fell by 82%, but the (evoked) EPP declined less due to a compensatory 43% increase in quantal content (Odierna and Phillips 2021, supplementary). This illustrates the adaptive presynaptic response that might help mitigate neuromuscular transmission failure in some situations where quantal amplitude is reduced. Evidently, in symptomatic MG patients a gross reduction in quantal amplitude overwhelms the capacity of the nerve terminal to compensate effectively.

Homeostatic plasticity at the NMJ is triggered by increased quantal amplitude

While curare reduced the quantal amplitude, DMSO had the opposite effect. The mechanism by which 0.1% DMSO increased the MEPP amplitude is not certain. At very high concentrations (>1%) DMSO can inhibit acetylcholinesterase, but cholinesterase inhibition would prolong the EPP duration whereas 0.75% DMSO did not (Odierna and Phillips 2021). Irrespective of the mechanism of action, the increase in MEPP amplitude after addition of 0.1% DMSO was not accompanied by the expected rise in EPP amplitude (Fig 3A). A compensatory fall in quantal content prevented any increase in EPP (Odierna and Phillips 2021). This suggests that the homeostatic response can also work in the opposite direction: reducing quantal release in response to an acute rise in quantal amplitude. Interestingly, in the presence of tubocurarine (where MEPP amplitude was 20% of its normal value), DMSO did not provoke a compensatory reduction in quantal content. Under such myasthenic-like conditions, addition of 0.1% DMSO elicited increases in the amplitudes of both the MEPP and the EPP (Fig 3B). There was no opposing reduction in quantal content (Fig 3C, compare filled circles to open circles). Together these results suggest that an increase in quantal amplitude only triggers a compensatory reduction in quantal content if the MEPP amplitude exceeds its normal, physiological level. The results are consistent with the idea that the MEPP has a physiological set point value, below or above which the homeostat will be triggered (Ribchester and Slater 2018). This has practical implications for testing of new drugs to restore safety factor in MG. Their effect upon the myasthenic NMJ must be assessed under myasthenic-like conditions, where MEPP amplitude is suppressed, so that homeostatic compensation will not mask potential positive effects.
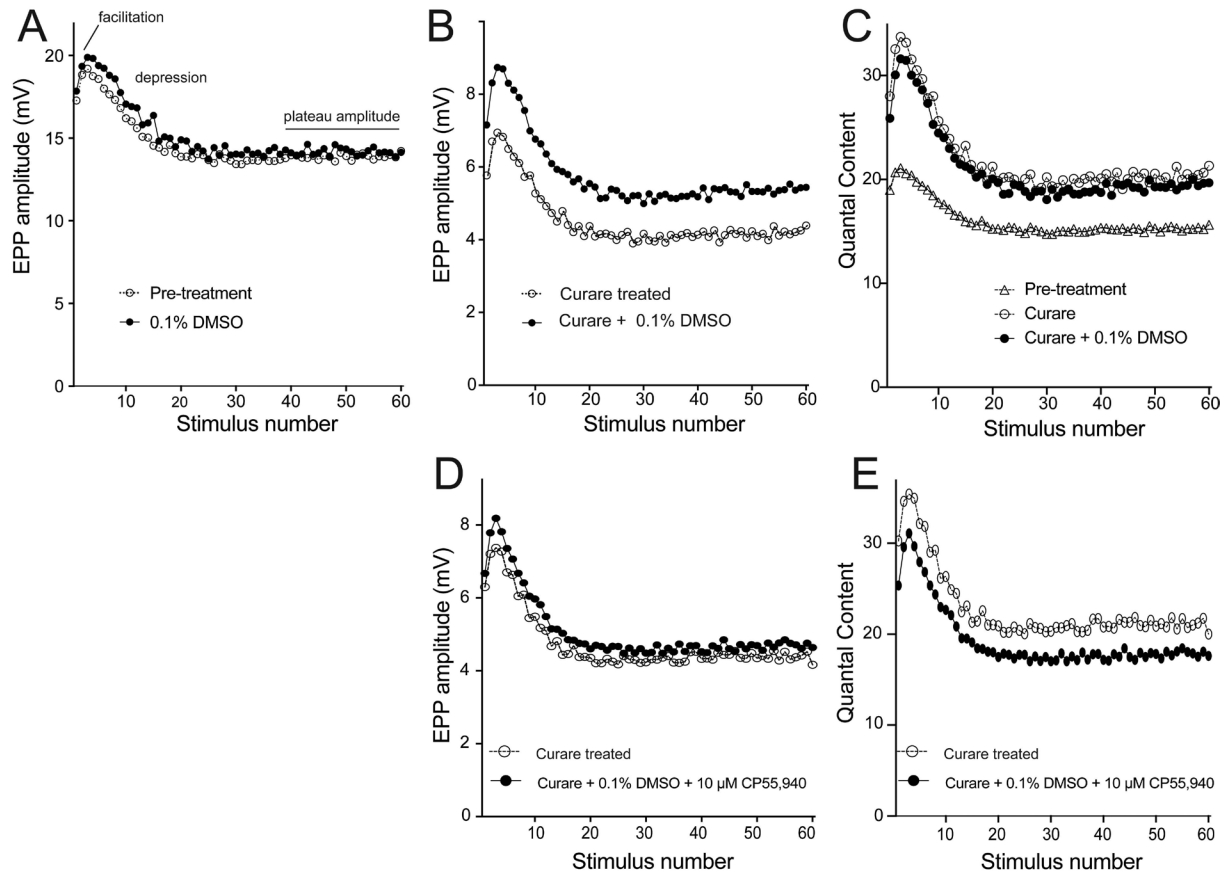
Fig 3. Changes in EPP amplitude and quantal content during trains of 60 nerve stimuli at 40/second. (A) In the absence of curare, EPP amplitude underwent an initial brief facilitation followed by synaptic depression in response to stimulation at 40Hz. Similar results were found with and without 0.1% DMSO. **(B)** Under myasthenia-like conditions (the presence of 500 nM tubocurarine), 0.1% DMSO caused a marked increase in EPP amplitude (note the different amplitude scale compared to panel A). **(C)** Quantal content estimates for the experiments depicted in panels A and B. **(D)** EPP amplitudes in the presence of curare (open circles) are compared to results after treatment with the combination of curare plus 0.1% DMSO and 10 µM CP 55,940 (closed circles). **(E)** Quantal content estimates for the experiments depicted in panel D. Note that our EPPs were not corrected for non-linear summation. In each panel symbols represent the means for n=8 mouse phrenic nerve-hemidiaphragm preparations (modified from Odierna and Phillips 2021; © 2021 – IOS Press).

Effects of cannabinoids on quantal neuromuscular transmission

The homeostatic response seen with DMSO had the potential to mask any beneficial effects of our candidate drugs. To avoid this, we simulated myasthenic conditions in subsequent electrophysiology experiments by including 500 nM tubocurarine in the bath solution. In this way we then tested the effects of a potent dual CB1/CB2 receptor agonist, CP 55,940 (Odierna and Phillips 2021). In the presence of curare, 0.1% DMSO increased both MEPP and EPP amplitudes (Fig 3B). In contrast, the combination of 10 µM CP 55,940 with 0.1% DMSO raised the amplitude of the MEPP by 24% (attributed to the DMSO component) but there was no significant increase in the EPP amplitude (Fig 3D). In these experiments the opposing fall in the quantal content could be attributed to the CP 55,940 component (Fig 3E).

Previous studies have described differing, often contradictory, effects of cannabinoid receptor agonists on MEPP amplitude and quantal content (up, down, or no effect; reviewed in Ge et al. 2020). The seeming inconsistency of the earlier studies might be explained by differences in the specific cannabinoids and concentrations, the solubilizing agents, and muscle preparations. In any event, the large sample sizes we employed (n=8 preparations) give us some confidence that the effects of DMSO and CP 55,940 that we recorded should at least be reproducible. Consistent with our contraction findings, our electrophysiology results under myasthenic conditions suggest that the DMSO-induced increase in quantal amplitude was opposed by the effects of the cannabinoid, which acted to reduce the quantal content. A presynaptic CB1 receptor-mediated

reduction in quantal release at the mammalian NMJ would be consistent with the known actions of cannabinoids upon transmitter release at some synapses in the CNS (Wilson and Nicoll 2001; Kano 2014). These findings show that neurobiological experiments testing the synaptic effects of cannabinoids can be confounded if they include DMSO as a solubilizing agent at concentrations as low as 0.1% (v/v).

Summary

Neuromuscular transmission is generally a safe bet. The safety factor measures the degree to which synaptic signalling exceeds the minimal required to activate the muscle fiber. A healthy safety factor (two or more) ensures the reliability of the NMJ during physiological (tetanic) muscle contractions. In MG the safety factor becomes marginal, and subclinical disease can quickly progress to frank weakness. New drugs to restore a strong safety factor are needed. Development of such drugs will require fast assays that mimic the impaired safety factor at the myasthenic NMJ. Many candidate compounds are hydrophobic, requiring amphipathic solubilizing agents. However, agents such as DMSO have the potential to mask and distort the effects of candidate compounds on synaptic function, in ways that must be taken into account. Finally, a better understanding of the mechanisms behind synaptic homeostasis at the NMJ may also reveal new therapeutic candidates.

Acknowledgements

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