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Short communication

3,4-Diaminopyridine improves neuromuscular transmission in a MuSK antibody-induced mouse model of myasthenia gravis

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ABSTRACT

This study investigated the effect of 3,4-diaminopyridine (3,4-DAP), a potent potentiator of transmitter release, on neuromuscular transmission *in vivo* in a mouse model of myasthenia gravis (MG) caused by antibodies against muscle-specific kinase (MuSK; MuSK-MG) and *ex vivo* in diaphragm muscle from these mice. 3,4-DAP significantly improved neuromuscular transmission, predominantly by increasing acetylcholine (ACh) release, supporting presynaptic potentiation as an effective treatment strategy for MuSK-MG patients who have defective transmitter release. In MuSK-MG, we suggest that only low-dose acetylcholinesterase (AChE) inhibitors be used to avoid side effects, and we propose that 3,4-DAP may be effective as a symptomatic therapy.

1. Introduction

Myasthenia gravis (MG) is caused by autoantibodies against postsynaptic membranes at neuromuscular junctions (NMJs), leading to failure of neuromuscular transmission mediated by acetylcholine (ACh) and clinical symptoms of ptosis, fatigue and muscle weakness. While the majority of MG cases (~90%) have antibodies against ACh receptors (AChRs; AChR-MG), MG caused by antibodies against muscle-specific kinase (MuSK; MuSK-MG) is frequently severe and requires emergent and aggressive therapy to manage respiratory distress (Vincent et al., 2008).

The therapeutic protocol for MG includes symptomatic and immunosuppressive treatments. In general, first-line symptomatic treatment is required in most patients until immunosuppressive treatment is effective. The strategy of symptomatic drugs is to improve neuromuscular transmission by increasing presynaptic transmitter release and potentiating postsynaptic effects. Acetylcholinesterase (AChE) inhibitors, which could potentiate postsynaptic effects, are generally effective for most AChR-MG patients. However, MuSK-MG patients are frequently unresponsive to these drugs or develop cholinergic crisis, characterized by increasing muscle weakness that causes dysphagia and respiratory insufficiency (Evoli et al., 2003; Sanders et al., 2003; Hatanaka et al., 2005; Evoli et al., 2008). In addition, MuSK-MG patients receiving AChE inhibitors may show abnormal patterns of repetitive firing to low-frequency motor nerve stimulation via electromyography (EMG). The emergence of repetitive firing indicates an increased sensitivity to ACh (Punga et al., 2006), which may result from the interference of MuSK with accumulation of AChE in synaptic basal lamina of NMJs (Cartaud et al., 2004). Recently, our animal model in which 100% of mice develop experimental autoimmune MG (EAMG) after immunization with MuSK protein reproduced the same EMG patterns showing hypersensitivity to ACh as MG patients. These mice also exhibited decreased levels of AChE and AChE-anchoring protein collagen Q at postsynaptic membranes, revealing the mechanism by which AChE inhibitor treatment exacerbates MuSK-MG symptoms *in vivo* (Mori et al., 2012).

Animal models of EAMG are integral for developing and assessing appropriate medications for patients afflicted with MuSK-MG. The current study focused on improving neuromuscular transmission in MG by increasing transmitter release. Specifically, we determined whether 3,4-diaminopyridine (3,4-DAP), a potent potentiator of transmitter release, could improve neuromuscular transmission *in vivo* in mice with MuSK-EAMG and *ex vivo* in diaphragm muscle from these mice.

2. Materials and methods

2.1. Immunization of mice

All procedures were approved by the Animal Care and Use Committee of Tokyo Metropolitan Geriatric Hospital and Institute of

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Gerontology. Female A/WySnJ mice aged 8 weeks or older (The Jackson Laboratory) were anesthetized and injected with 20 μg MuSK emulsified with complete Freund's adjuvant (CFA) on day 0, then boosted with 20 μg MuSK emulsified with incomplete Freund's adjuvant (IFA) on day 14. Recombinant MuSK protein was prepared as described previously (Mori et al., 2012). Control mice were injected with PBS and CFA on day 0, then boosted with PBS and IFA on day 14.

2.2. EMG

Changes in compound muscle action potential (CMAP) were determined as described previously (Mori et al., 2012). Decrement was calculated as the percent amplitude change between the first CMAP and the smallest CMAP that were evoked by a train of 10 impulses. If the amplitude of the first CMAP was also the smallest, the decrement was designated as 0%. 3,4-DAP (Tokyo Kasei) was freshly prepared in PBS and administered at 8 mg/kg, i.p. A typical mouse weighing 20 g received 100 μl of 1.6 mg/ml 3,4-DAP. EMG was performed 20 min later.

2.3. Ex vivo electrophysiology

Membrane potentials and miniature endplate potentials (MEPPs) were recorded using a specimen composed of left phrenic nerve and hemi-diaphragm muscle as described previously (Mori et al., 2012). To measure evoked endplate potentials (EPPs), μ-conotoxin GIIIB (1 μM final concentration, Peptide Institute) was applied to suppress muscle contraction, and the phrenic nerve was stimulated with supramaximal voltage at 0.7 Hz. 3,4-DAP was applied to the specimen-immersed chamber (100 μM final concentration), and synaptic events were recorded 20 min later. Amplitudes of EPPs and MEPPs were standardized to a membrane potential of −75 mV. Quantal content was calculated by using the values of mean MEPP amplitude, mean EPP amplitude and membrane potential in the same muscle fiber in the formula described previously (McLachlan and Martin, 1981). A total of 8–15 NMJs were assessed from each mouse.

2.4. Statistics

Group differences between control and MuSK-injected mice were analyzed by either unpaired *t*-tests or Mann–Whitney *U*-tests. Paired *t*-tests were used to analyze the effects of 3,4-DAP treatment in EMG experiments. One-way ANOVAs were used to assess parameters of synaptic events from *ex vivo* electrophysiology experiments. Statistical significance was set at $P<0.05$.

3. Results

3.1. 3,4-DAP improves neuromuscular transmission

About two weeks after treatment with recombinant MuSK protein (see Materials and methods), all five A/WySnJ mice exhibited MG-like phenotypes, including weight loss and muscle weakness. In addition, while EMG recordings from control gastrocnemius muscle in response to 3-Hz repetitive nerve stimulation showed no abnormal CMAP decrements (defined as >10%) (0.26 ± 0.14%; range 0–0.76%) (Fig. 1A and D), MuSK-injected gastrocnemius muscle showed significant decrements (24.0 ± 2.62%; range 16.6–32.4%) (Fig. 1B and D), indicating neuromuscular transmission failure. Again, the amplitude of the first CMAP in MuSK-injected mice (69.8 ± 7.24 mV) was significantly decreased relative to controls (102.6 ± 3.75 mV) (Fig. 1E). A single injection of 3,4-DAP (8 mg/kg) to MuSK-injected mice reversed the CMAP decrease (3.45 ± 1.51%; range 0.6–8.9%; Fig. 1C and F) and significantly increased the amplitude of the first CMAP from 12.0 mV to a maximum of 34.7 mV (25.4 ± 3.93 mV; Fig. 1G). Similarly, 3,4-DAP also significantly increased the first CMAP amplitude in control mice (15.3 ± 3.45 mV) (data not shown). These results demonstrate that 3,4-DAP improved neuromuscular transmission in MuSK-injected

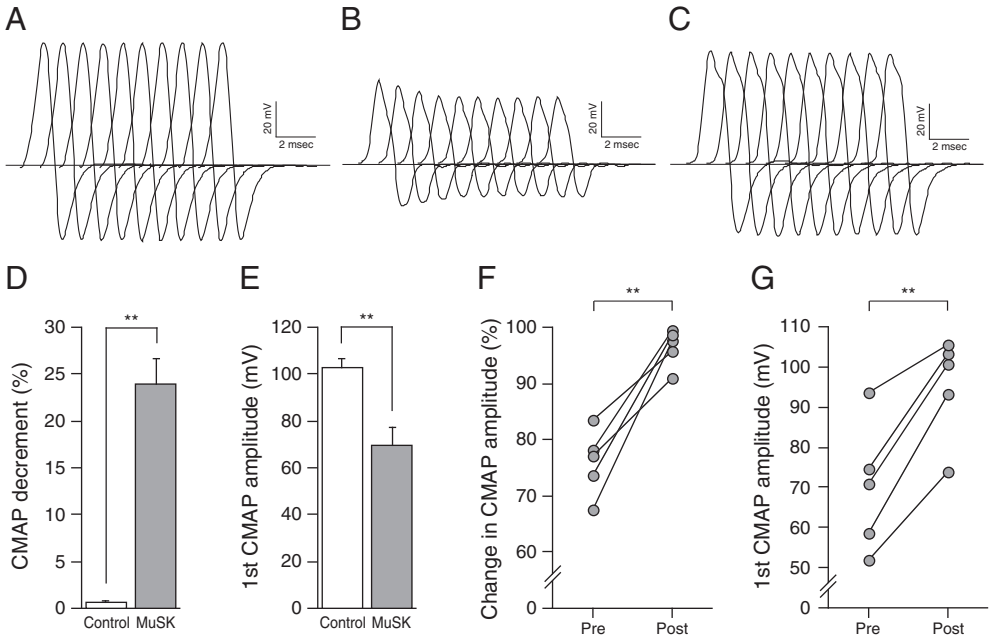

Fig. 1. Effect of 3,4-DAP on CMAP decrement.Representative EMG traces from gastrocnemius muscles of control mice (A) and MuSK-injected mice before (B) and after (C) treatment with 3,4-DAP. MuSK-injected mice exhibited significant CMAP decrement (D) and reduction in the amplitude of the first CMAP (E) (n = 5 mice/group). $^{**}P<0.01$ by Mann–Whitney *U*-test (D) or unpaired *t*-test (E). (F) Changes in CMAP decrement before and after 3,4-DAP administration in MuSK-treated mice. 3,4-DAP significantly improved CMAP decrement (84.1 ± 7.2%; range, 60.7 to 95.4%). (G) Changes in the first CMAP amplitude before and after 3,4-DAP administration in MuSK-treated mice. 3,4-DAP significantly increased CMAP amplitude (39.1 ± 7.5%, range, 12.8 to 59.3%). $^{**}P<0.01$ (paired *t*-test).