

Severity of Myasthenia Gravis Influences the Relationship between Train-of-four Ratio and Twitch Tension and Run-down of Rat Endplate Potentials

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ABSTRACT

Background: Train-of-four ratio (TOFR) is often used to evaluate muscle relaxation caused by neuromuscular-blocking agents (NMBAs). However, it is unknown whether TOFR reliably correlates with the first twitch tension (T1) in patients with myasthenia gravis (MG). By using rat models of experimental autoimmune MG (EAMG), the authors verified the hypothesis that the severity of MG influences the relationship between TOFR and T1.

Methods: EAMG rats were divided into sham, moderate MG, and severe MG groups. Isometric twitch tension of the hemidiaphragm was elicited by phrenic nerve stimulation with and without use of the NMBA rocuronium to measure TOFR and T1, and run-down of endplate potentials was estimated in the three groups. Changes around the neuromuscular junction in EAMG rats were investigated by observation of electron micrographs.

Results: With similar attenuation of T1, TOFR was significantly ($n = 6$) different among the three groups in the presence of 50% inhibitory concentrations of rocuronium (IC50). Run-down in the sham group was significantly ($n = 8$) greater with exposure to IC50, whereas that in the severe MG group was statistically insignificant. Width of the primary synaptic cleft in the severe MG group was significantly ($n = 80$) greater than that in the other groups.

Conclusions: Severity of MG influences the relationship between TOFR and T1, together with changes in run-down of endplate potentials and those around the neuromuscular junction in rats. TOFR may, therefore, not be an accurate indicator of recovery from NMBAs in MG patients. (*ANESTHESIOLOGY* 2016; 124:369-77)

MYASTHENIA gravis (MG) is one of the most common autoimmune diseases compromising neuromuscular transmission, affecting 25 to 142 people per million.¹ MG patients are known to be highly sensitive to neuromuscular-blocking agents (NMBAs), necessitating cautious administration of NMBAs and adequate evaluation of muscle relaxation when general anesthesia with NMBAs is required for them.

Clinically, for monitoring neuromuscular function, the train-of-four ratio (TOFR: T_4/T_1 , where $T[X]$: Xth evoked twitch tension elicited by 2-Hz nerve stimulation) is preferred over T1 because it is the most precise indicator of neuromuscular function and because the value of T1 is unstable and control values of T1 before the administration of NMBAs are required for subsequent monitoring. Since the early 1970s, TOFR has been established as the monitoring standard for muscle relaxation induced by NMBAs,² with a predictable enough relationship between TOFR and T_1 ³⁻⁵ regardless of mean age, height, and weight.⁶ A TOFR of greater than 0.7 at the adductor pollicis muscle was considered the standard indicator of adequate recovery from NMBA-induced muscle relaxation.⁷ However, because an increased risk of aspiration caused by pharyngeal dysfunction was reported in patients with a

What We Already Know about This Topic

- Patients with myasthenia gravis are sensitive to pharmacologic neuromuscular blockade
- The train-of-four ratio is routinely used to monitor neuromuscular blockade; however, first twitch tension is infrequently used because it is difficult to measure
- The relationship between these two measures has not been defined in the context of myasthenia gravis

What This Article Tells Us That Is New

- A rat model was developed to study different severities of myasthenia gravis
- Phrenic nerve-stimulated diaphragmatic responses to a standardized rocuronium dose were recorded *in vitro*
- With increasing myasthenia gravis severity, train-of-four ratio and the first twitch tension became less reliable indicators of muscle strength during recovery from neuromuscular blockade, indicating that the evaluation of neuromuscular blockade by train-of-four ratio may overestimate the extent of recovery

TOFR less than 0.9 at the adductor pollicis muscle,⁸ both a TOFR of greater than 0.9 and recovery of T1 to 80 to 120% are considered indicative of adequate recovery from NMBA-induced muscle relaxation⁹ in normal patients. A few reports have, however, mentioned the adequate TOFR level in MG patients.

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Previous studies on TOFR and T1 in MG patients who received rocuronium (N.V. Organon, The Netherlands) and sevoflurane for general anesthesia did not discuss the relationship between TOFR and T1¹⁰ and did not mention the value of TOFR that denotes the adequate recovery from NMBA-induced muscle relaxation in MG patients. One case report described nonrecovery of T1 despite allowing adequate time after TOFR returned to control values with administration of sugammadex at the end of surgery in an MG patient,¹¹ suggesting that MG might alter the normal relationship between TOFR and T1. Reportedly, T1 recovery sometimes lags behind the recovery of TOFR when neuromuscular block is reversed with sugammadex¹²; hence, it is unclear whether MG influenced the relationship between TOFR and T1 in the case report. Moreover, the individual severity of MG varies considerably,¹³ with some patients occasionally showing a TOFR of less than 0.9 even before NMBA administration.¹⁴

We hypothesized that the severity of MG influences the relationship between TOFR and T1 and that the recovery of TOFR alone in MG patients might not be a sufficient indicator of recovery of NMBA-attenuated T1, unlike that in non-MG patients. To verify the hypothesis, we evaluated indirectly elicited twitch tension of the hemidiaphragm in rat models of MG of different severities, with and without rocuronium administration, by measuring both TOFR and T1. Furthermore, using a microelectrode method, we electrophysiologically investigated the mechanisms by which MG influences the relationship between TOFR and T1 and pathophysiologically clarified the changes around neuromuscular junctions (NMJs) damaged by MG-causing antibodies in rats.

Materials and Methods

The Animal Care and Use Committee of Sapporo Medical University (Sapporo, Hokkaido, Japan) approved the study protocol (No. 12-077). Female Lewis rats (weight: 92 to 103 g, age: 4 weeks; Charles River, Japan) were randomly divided into the following three groups: sham, moderate MG, and severe MG. The three experiments were performed under excessively deep oxygen–isoflurane anesthesia.

Experimental autoimmune MG (EAMG) was passively induced in the rats by IV administration of 1.2 or 1.8 mg/kg (in moderate and severe MG groups, respectively) intact monoclonal antibody 35 (mAb35, m-217; Sigma, Japan) dissolved in 1 ml of normal saline (NS), which is a rat immunoglobulin 1 that binds to the main immunogenic region of acetylcholine receptors (AChR). The sham group was treated with 1 ml of NS intravenously.

Clinical Assessment

After injection of NS (sham group) or mAb35 dissolved in NS (moderate and severe MG groups), MG severity was assessed on the basis of clinical symptoms and weight loss every 6 h in the three groups. The clinical symptoms were scored as follows: 0, no clinical symptoms; 1, first signs of grasp weakness after a few trials; 2, incomplete paralysis of

hind limbs; 3, hind limbs paralyzed and unable to stand; 4, moribund; and 5, dead,¹⁵ by the investigation assistant blinded to the treatment of the rats. The rats were sacrificed *via* deep oxygen–isoflurane anesthesia 60 h after injection of NS or mAb35.

Experiment 1: Measurements of Muscle Tension and Sensitivity to Rocuronium

The rats were dissected to obtain left phrenic nerve-hemidiaphragm preparations 36 h after injection of NS or mAb35 dissolved in NS, at which time the rats in the moderate and severe MG groups presented the most severe clinical symptoms. Strips of left hemidiaphragms (10 mm in width) that had been cut parallel to the muscle fibers were rapidly dissected with the attached phrenic nerve, central tendon, and rib cage intact. Each isolated strip was mounted vertically in a 27°C temperature-controlled tissue chamber (20 ml in volume) filled with modified Krebs solution¹⁶ bubbled with 95% O₂–5% CO₂ gas, the hemidiaphragms being fixed with the rib cage positioned inferiorly and suspended at the central tendon from a force-displacement transducer (Grass FT-03; Grass Instrument, USA) using a 3-0 silk suture. The phrenic nerve was positioned on wire bipolar platinum stimulating electrodes, and isometric twitch tension in the left hemidiaphragm was indirectly elicited by phrenic nerve stimulation using a stimulator (Grass-S-48; Grass Instrument) and a constant current unit under optimal preload (3 to 5 g) to deliver maximum twitch tension. Isometric twitch tension was recorded *via* the force transducer on a thermal chart recorder (WS-681G; Nihon-Kohden, Japan). Rocuronium was additively administered extracellularly every 10 min to the preparation from the bathing solution until complete muscle contraction was abolished. Twitch tension in the same preparation without rocuronium administration was defined as the control value. Competition data (IC[X]: X% inhibitory concentration for attenuation of T1, *n* = 6, respectively) were determined from a logistic sigmoidal dose–response model fitted to the concentration–twitch tension curves (% control). Using new hemidiaphragms, T1 (% control) was measured (*n* = 6) and TOFR was calculated (*n* = 6) 1 h after reaching the equilibrium state by administration of IC25, IC50, and IC75 of rocuronium.

Experiment 2: Electrophysiological Observations

Hemidiaphragm strips were dissected from new rats in all the groups by the prescribed method. Each isolated strip was pinned onto the silicon plastic base of a small chamber (3 ml in volume), which was continuously superfused (10 ml/min) with the Krebs solution. Spontaneous miniature endplate potential (MEPP; small depolarizations of the postsynaptic terminal caused by the release of a single acetylcholine vesicle from the motor nerve terminal into the NMJ), endplate potential (EPP; postjunctional potential induced by quantally released acetylcholine vesicles), and resting membrane potential (RMP) were assessed. MEPP, EPP, and RMP were

recorded from endplate regions on superficial muscle fibers of the strip with conventional intracellular microelectrodes (10 to 15 M Ω) filled with KCl (3.0M) using a current clamp amplifier (AxoClamp-2B; Axon Instruments, USA).¹⁷ The endplate regions were located with a microscope in the region around fine intramuscular nerve branches and were considered adequate when the rise time of the MEPP was less than 1 ms. MEPPs were recorded from fibers in which RMP was -65 mV or less in the three groups. EPPs were elicited by phrenic nerve stimulation using an electric stimulator and an isolator (SEN-3301 and SS-202J, respectively; Nihon-Kohden). To terminate nerve stimulation-induced generation of muscle action potentials and the subsequent muscle contractions, which disturb EPP recordings, the cut fiber preparation¹⁸ was performed on the muscle strips only when EPPs were measured. Data obtained from fibers for which RMP had depolarized by more than 8 mV were excluded from the analysis. EPPs were analyzed with an online computer analyzing system (MacLab/4s; Bio Research Center Co., Japan). EPPs recorded using normal modified Krebs solution (before rocuronium application) were defined as control values. After recording of baseline EPP, IC50 of rocuronium in modified Krebs solution was applied extracellularly to the preparations from the superfusing bathing solution, followed by recording of the amplitudes after stabilization of the effect of rocuronium, judged by the stability of the amplitudes.

After muscle contraction had ceased, a train of 50 EPPs elicited by supramaximal stimulation (0.05-ms square pulse, 2 Hz) of the phrenic nerve was recorded at intervals longer than 10 min. To standardize EPP amplitudes for a standard RMP, recorded EPP amplitudes were corrected for nonlinear summation using the formula $[EPP_{CR} = EPP_{UR} \times (70 - RVP) / (RMP - RVP - EPP_{UR})]$,¹⁹ where EPP_{CR} and EPP_{UR} were the corrected and uncorrected EPP amplitudes, respectively, and RVP was the reversal potential of the endplate membrane, that is, -5 mV.¹⁸ The quantal content (QC) was calculated using the formula $[QC = (EPP_{CR} / VEPP)^2]$,²⁰ where VEPP was the variance of the 50 corrected EPP amplitudes in the train.²¹ The run-down of EPPs was estimated as the ratio of $EPP_{21-50amp} / EPP_{1amp}$, where EPP_{1amp} is the amplitude of the first EPP (EPP_1) and $EPP_{21-50amp}$ is the amplitude of the averaged EPP from the 21st to the 50th EPPs (EPP_{21-50}).

Experiment 3: Electron Microscopic Observation

The left hemidiaphragms of new rats from all groups were handled for thin electron microscopic observation using the modified method.²² The fixed left hemidiaphragms were carefully trimmed into 10 to 12 grafts 1×2 mm in length, containing cholinesterase-positive endplates. Thin sections (less than 50 nm thickness) that were stained with uranyl acetate were examined under an electron microscope at 80 kV (JEM-1400; JEOL, Japan). We took two to five pictures of the motor endplate in each graft at $\times 15,000$ to

$\times 40,000$ magnification. Finally, we obtained more than 20 electron micrographs of motor endplates from each hemidiaphragm. We used the electron micrographs to observe the condition of the nerve terminal area and for measuring the width of the primary synaptic cleft of the NMJ and the size of synaptic vesicles in the nerve terminal using standard stereological methods.²³ We performed measurements in at least 80 NMJs in each group to calculate these parameters.

Statistics

Sample sizes were decided based on previous experience.^{17,22} Differences among the sham, moderate MG, and severe MG groups in the effects of rocuronium on T1 were evaluated by using competition analysis data (IC50 and HillSlope at IC50), which were determined from a logistic sigmoidal dose-response model fitted to the rocuronium concentration-response curves with the computer program Prism 5 (GraphPad, Inc., USA). Data are shown as medians, means, or means \pm SD. Data were analyzed by using Friedman test followed by Wilcoxon test, Kruskal-Wallis test followed by Dunn test, two-way repeated-measures ANOVA with *post hoc* (Tukey) testing, one-way ANOVA with Tukey testing, and two-tailed paired *t* test. A *P* value of less than 0.05 or 0.017 (Bonferroni correction) was considered significant.

Results

Clinical Assessment

After mAb35 injections, EAMG rats showed clinical symptoms of weakness, fatigability, and weight loss. Clinical symptom scores ($n = 10$ in each group; fig. 1), which were maximal at 36 h after mAb35 injection and began to decrease at 42 to 60 h, were significantly higher at 18 to 36 h after mAb35 injection in the severe MG group than those in the other groups ($P < 0.017$), whereas the scores in the moderate and severe MG groups were significantly higher than those in the sham groups ($P < 0.01$, each). Body weight ($n = 10$ in each group) in the moderate MG group ($-15.7 \pm 2.1\%$) significantly decreased from the original body weight at 36 h after mAb35 injection when compared with that in the sham group ($P < 0.01$), and body weight in the severe MG group ($-21.5 \pm 2.7\%$) significantly decreased more than that in the other groups ($P < 0.01$, each). In the sham group, however, body weight increased ($3.2 \pm 1.2\%$) from the original body weight at 36 h after NS injection.

Experiment 1

T1 values without administration of rocuronium were 25.6 ± 10.2 , 8.9 ± 3.5 , and 5.6 ± 1.9 g (mean \pm SD) in the sham, moderate MG, and severe MG groups, respectively. Four data values, in which preload was under 3 g, were excluded. The dose-response curve ($n = 6$ in each group; fig. 2) of rocuronium for indirectly elicited twitch tension in the moderate MG group was significantly shifted leftward when compared with the sham group ($P < 0.01$), and

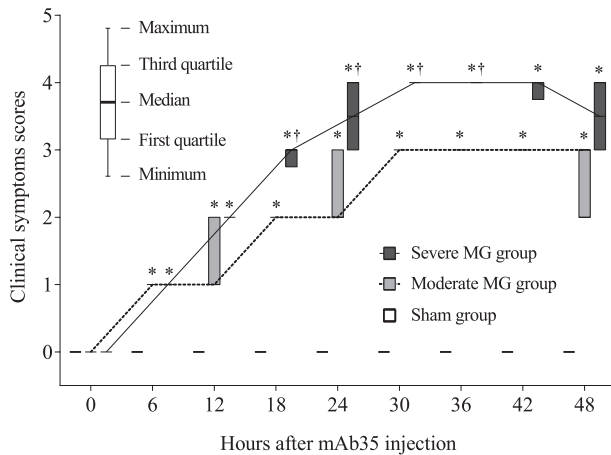


Fig. 1. Clinical symptom scores after injection of intact monoclonal antibody 35 (mAb35). The score in the severe myasthenia gravis (MG) group was significantly higher at 18 to 36 h after mAb35 injection than that in the moderate MG group ($P < 0.017$), and the scores in the moderate and severe MG groups were significantly higher at 6 to 48 h after mAb35 injection than that in the sham group ($P < 0.01$, each). The clinical symptoms were expressed as follows: 0, no clinical symptoms; 1, first signs of grasp weakness after a few trials; 2, incomplete paralysis of hind limbs; 3, hind limbs paralyzed and unable to stand; 4, moribund; and 5, dead. $n = 10$ in each group. * $P < 0.01$ versus sham group, † $P < 0.017$ versus the moderate MG group. Data were analyzed using Friedman test followed by Wilcoxon test to evaluate the significant effect of time and Kruskal-Wallis test followed by Dunn test to compare the three groups at each time point with Bonferroni correction for multiple comparisons.

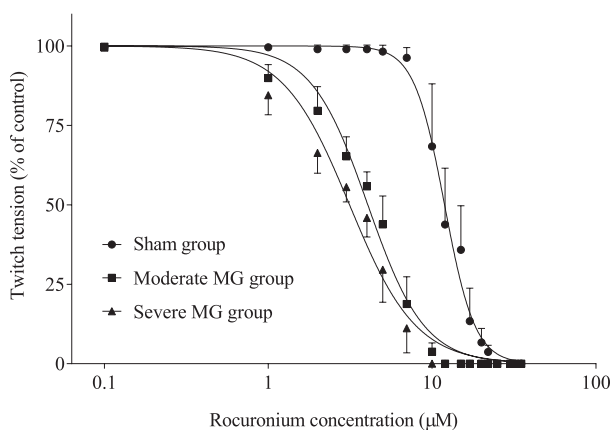


Fig. 2. Dose-response curves of rocuronium for indirectly elicited twitch tension in the three groups. The dose-response curve in the moderate myasthenia gravis (MG) group was significantly shifted leftward, more than that in the sham group ($P < 0.01$), and the curve in the severe MG group was significantly shifted further leftward than those in the other groups ($P < 0.01$, each). Data are shown as means \pm SD, $n = 6$ in each group. Data were analyzed by using two-way repeated-measures ANOVA with the Tukey test.

the curve in the severe MG group was significantly shifted further leftward when compared with the other groups ($P < 0.01$, each). The HillSlope at IC50 of rocuronium in the moderate MG group was significantly smaller than that in the sham group ($P < 0.01$), and the HillSlope in the severe MG group was significantly smaller than those in both the sham and the moderate MG groups ($P < 0.01$, each, data not shown). The IC25, IC50, and IC75 of rocuronium in the moderate MG group (2.62, 4.05, and 6.27 μM , respectively) were significantly smaller ($P < 0.01$, $n = 6$, each) than those in the sham group (9.59, 12.11, and 15.30 μM , respectively), and those in the severe MG group (1.96, 3.23, and 5.32 μM , respectively) were significantly smaller ($P < 0.01$, $n = 6$, each) than those in the other groups. T1 (% control) decreased to the same extent with administration of IC25, IC50, and IC75 of rocuronium in the three groups and was not significantly different among the three groups (data not shown). TOFR ($n = 6$ in each group; fig. 3) was significantly smaller in the moderate and severe MG groups than in the sham group without rocuronium administration and decreased as the concentration of rocuronium increased in all three groups. TOFRs with IC25 and IC50 of rocuronium in the moderate MG group were significantly higher than those in the sham group ($P < 0.01$) and those in the severe MG group were significantly higher than those in the other two groups ($P < 0.01$, each).

Experiment 2

Amplitude of MEPP ($n = 30$ in each group; fig. 4A) in the moderate MG group was significantly smaller than that in the sham group. Furthermore, the amplitude in the severe MG group was significantly smaller than those in the other groups ($P < 0.01$, each). However, frequencies of MEPP

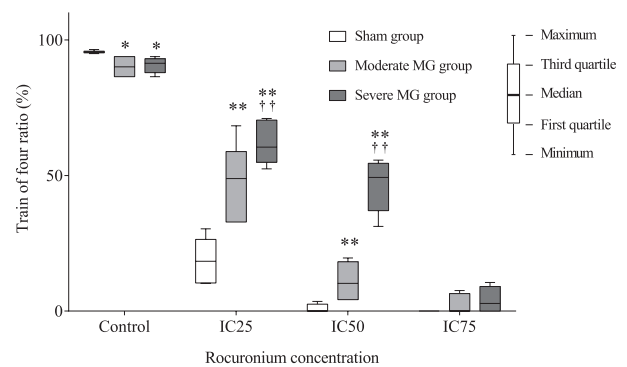


Fig. 3. Train-of-four ratio with administration of rocuronium. Train-of-four ratio was significantly smaller in the moderate and severe myasthenia gravis (MG) groups than in the sham group at control and was significantly different among the three groups with 25% inhibitory concentration (IC25) for attenuation of first evoked isometric twitch tension and IC50 of rocuronium. $n = 6$ in each group. * $P < 0.05$ versus sham group, ** $P < 0.01$ versus sham group, † $P < 0.01$ versus the moderate MG group. Data were analyzed by using two-way repeated-measures ANOVA with the Tukey test.

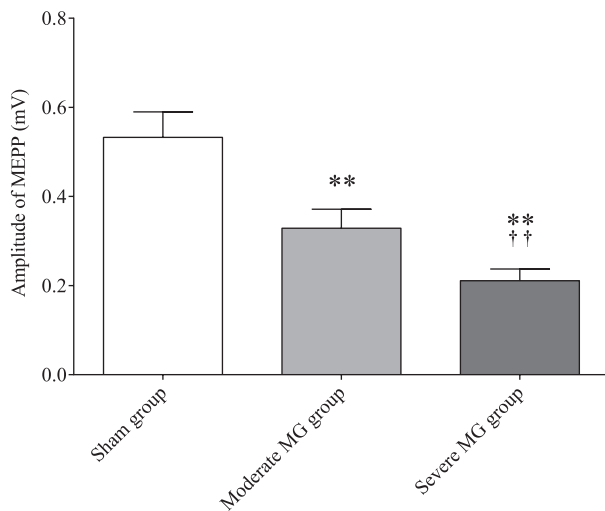
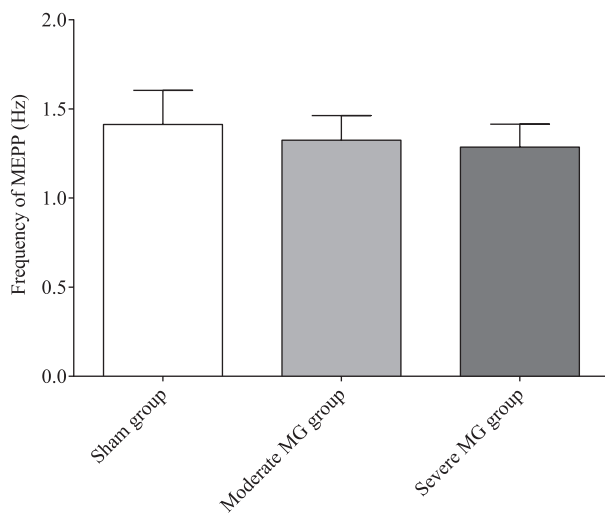
A Amplitude of MEPP**B Frequency of MEPP**

Fig. 4. Influence of the severity of myasthenia gravis (MG) on miniature endplate potential (MEPP) at baseline. The amplitudes of MEPP (A) were significantly different among the three groups. The frequency of MEPP (B) was not significantly different among the three groups. Data are shown as means \pm SD, $n = 30$ or 8 in each group (A or B, respectively). ** $P < 0.01$ versus sham group, †† $P < 0.01$ versus moderate MG group. Data were analyzed by using one-way measures ANOVA with the Tukey test.

($n = 8$ in each group; fig. 4B) were not significantly different among the three groups ($n = 8$, each). RMP was increased at the time of EPP (fig. 5A, an example of EPP) measurement but not significantly different among the sham, moderate MG, and severe MG groups (-40 ± 3.5 , -42 ± 4.8 , and -43.1 ± 4.9 mV, respectively, $n = 8$, each). Twenty-eight data points, where the microelectrode had shifted off muscle during the measurement, and 19 data points, in which the RMP had depolarized by more than 8 mV, were excluded. The train of 50 EPPs at 2 Hz displayed an initial run-down

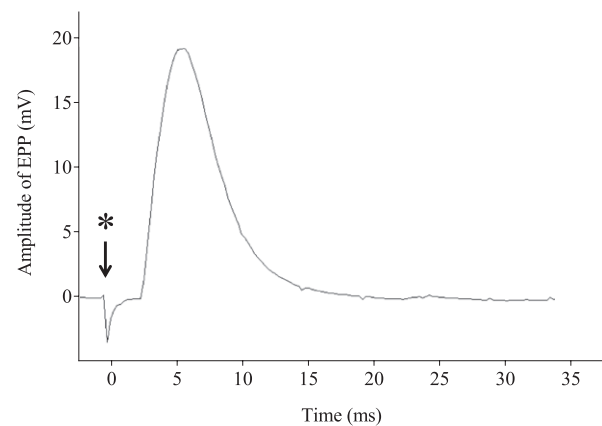
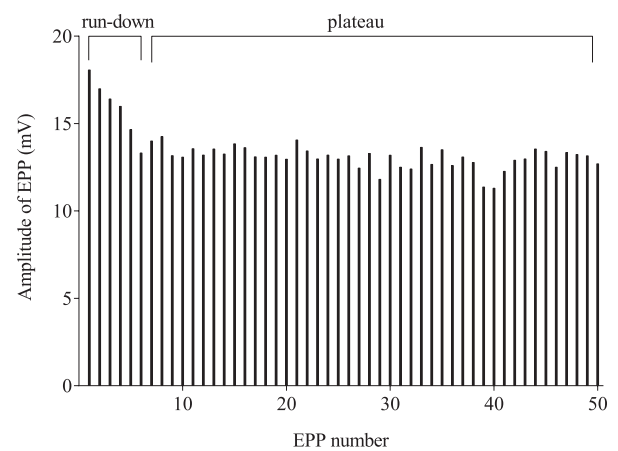
A Raw data of EPP**B Train of 50 EPPs**

Fig. 5. An example of endplate potential (EPP) indirectly elicited by phrenic nerve stimulation (A) and a train of 50 EPPs at 2 Hz (B) from an isolated rat diaphragm. The amplitudes of recorded EPPs were approximately 10 to 20 mV (in the sham group). The train of EPPs displayed an initial run-down (usually in the first 6 to 10 EPPs in the train) followed by a plateau. *Stimulus artifact.

(in the first 6 to 8 EPPs in the train) followed by a plateau in all three groups (fig. 5B).

EPP_1 amp and EPP_{21-50} amp / EPP_1 amp ($n = 8$ in each group; fig. 6, A and B) without rocuronium administration in the moderate and severe MG groups were significantly smaller than those in the sham group ($P < 0.01$, each; fig. 6, A and B). EPP_1 amp was significantly decreased by administration of IC50 of rocuronium in all three groups ($P < 0.01$, each). EPP_{21-50} amp / EPP_1 amp was significantly decreased by administration of IC50 of rocuronium in the sham and moderate MG groups ($P < 0.01$, each) but not in the severe MG group. QC of EPP_1 and EPP_{21-50} (QC₁ and QC₂₁₋₅₀, respectively, $n = 8$ in each group; fig. 6, C and D) without rocuronium administration in the moderate MG group was significantly smaller than those in the sham group; moreover, their values in the severe MG group were significantly smaller than in the other groups ($P < 0.01$, each). QC₁ and

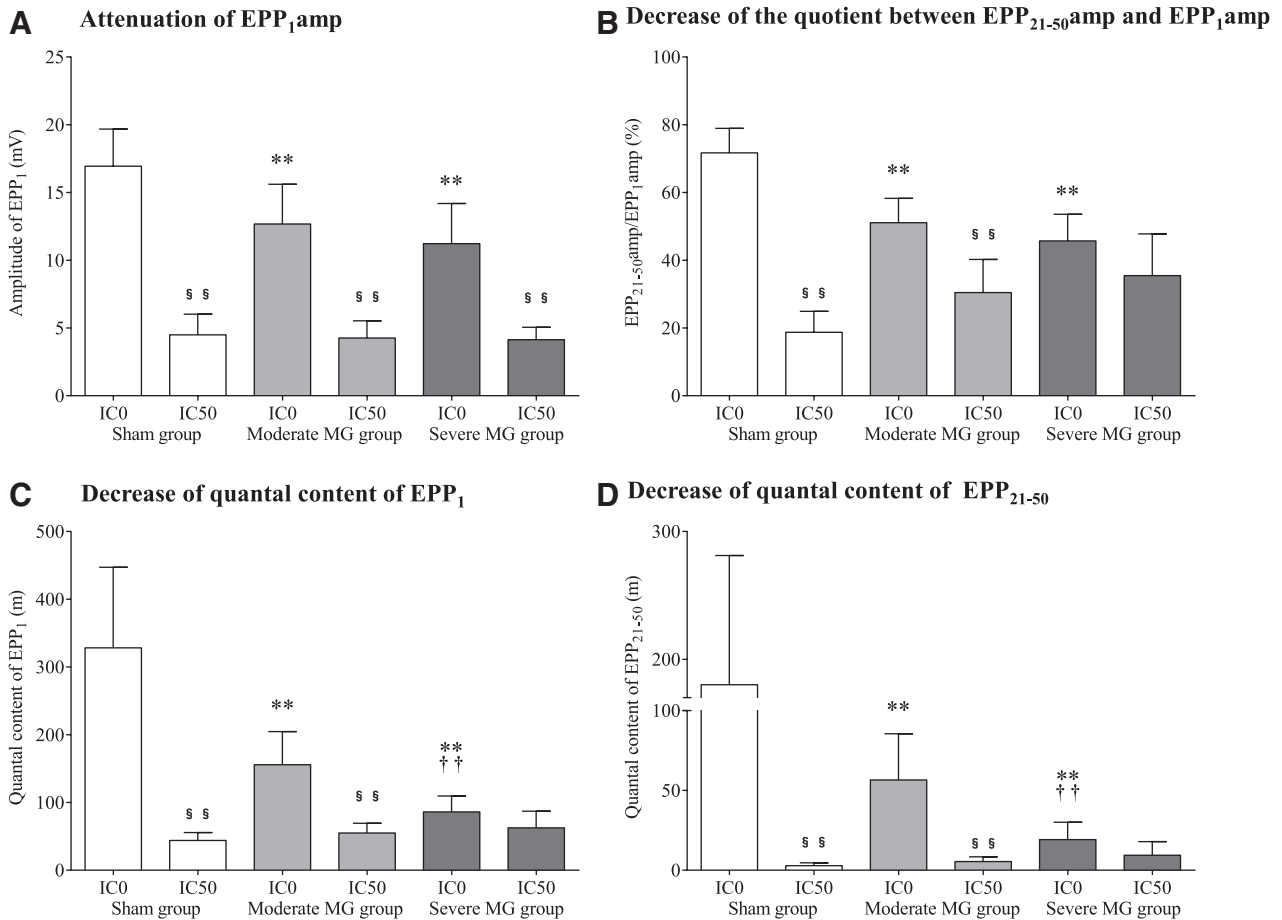


Fig. 6. Influence of the severity of myasthenia gravis (MG) on endplate potential (EPP). Amplitude of the first EPP (EPP₁amp; A) and the quotient between the averaged amplitude from the 21st to the 50th EPPs (EPP₂₁₋₅₀amp) and EPP₁amp, as an indication of EPP run-down (B), were significantly smaller at 0% inhibitory concentration (IC0) for first evoked isometric twitch tension of rocuronium in the moderate and severe MG groups than in the sham group. Quantal content of EPP₁ (QC₁; C) and EPP₂₁₋₅₀ (QC₂₁₋₅₀; D) were significantly different at IC0 of rocuronium among the three groups. EPP₁amp (A) was significantly decreased by administration of IC50 of rocuronium in the three groups (A), whereas indicators of EPP run-down (B), QC₁ (C), and QC₂₁₋₅₀ (D) were significantly decreased in the sham and moderate MG groups by administration of IC50 of rocuronium. Data are shown as means \pm SD, $n = 8$ in each group. ** $P < 0.01$ versus sham group at IC0 of rocuronium, †† $P < 0.01$ versus moderate MG group at IC0 of rocuronium, §§ $P < 0.01$ versus each group at IC0 of rocuronium. Data were analyzed by using two-way repeated-measures ANOVA with the Tukey test among three groups and two-tailed paired t test with Bonferroni correction between IC0 and IC50 in each group.

QC₂₁₋₅₀ with administration of IC50 of rocuronium in the sham and moderate MG groups, but not in the severe MG group, were significantly decreased when compared with those without rocuronium administration ($P < 0.01$, each).

Experiment 3

We observed more than 80 electron micrographs of junctional regions from four rats in each group. Simplification of the postsynaptic region caused by the degeneration of postsynaptic folds was observed at the NMJ of the hemidiaphragm in the EAMG rats (fig. 7, A–C). The width of the primary synaptic cleft in the moderate MG group was significantly greater than that in the sham group ($P < 0.01$) and that in the severe MG group was significantly greater than those in the other groups ($P < 0.01$, each; fig. 7D).

The size of the synaptic vesicles was not significantly different among the sham, moderate MG, and severe MG groups ($1,497.0 \pm 391.2$, $1,485.2 \pm 423.1$, and $1,410.5 \pm 411.4 \mu\text{m}^2$, respectively, $P < 0.01$; fig. 7E).

Discussion

The rat passive EAMG model is the representative animal model of MG since the 1970s^{24–26}; however, a few studies have demonstrated details about muscle relaxation, NMBAs, or both using the model. One study, in which prolonged muscle relaxation caused by NMBAs was observed in EAMG rats as well as in human MG patients,²⁷ did not mention the influence of severity of MG on the process of recovery from muscle relaxation or adequate ways for evaluation of recovery although these are important questions for MG patients who

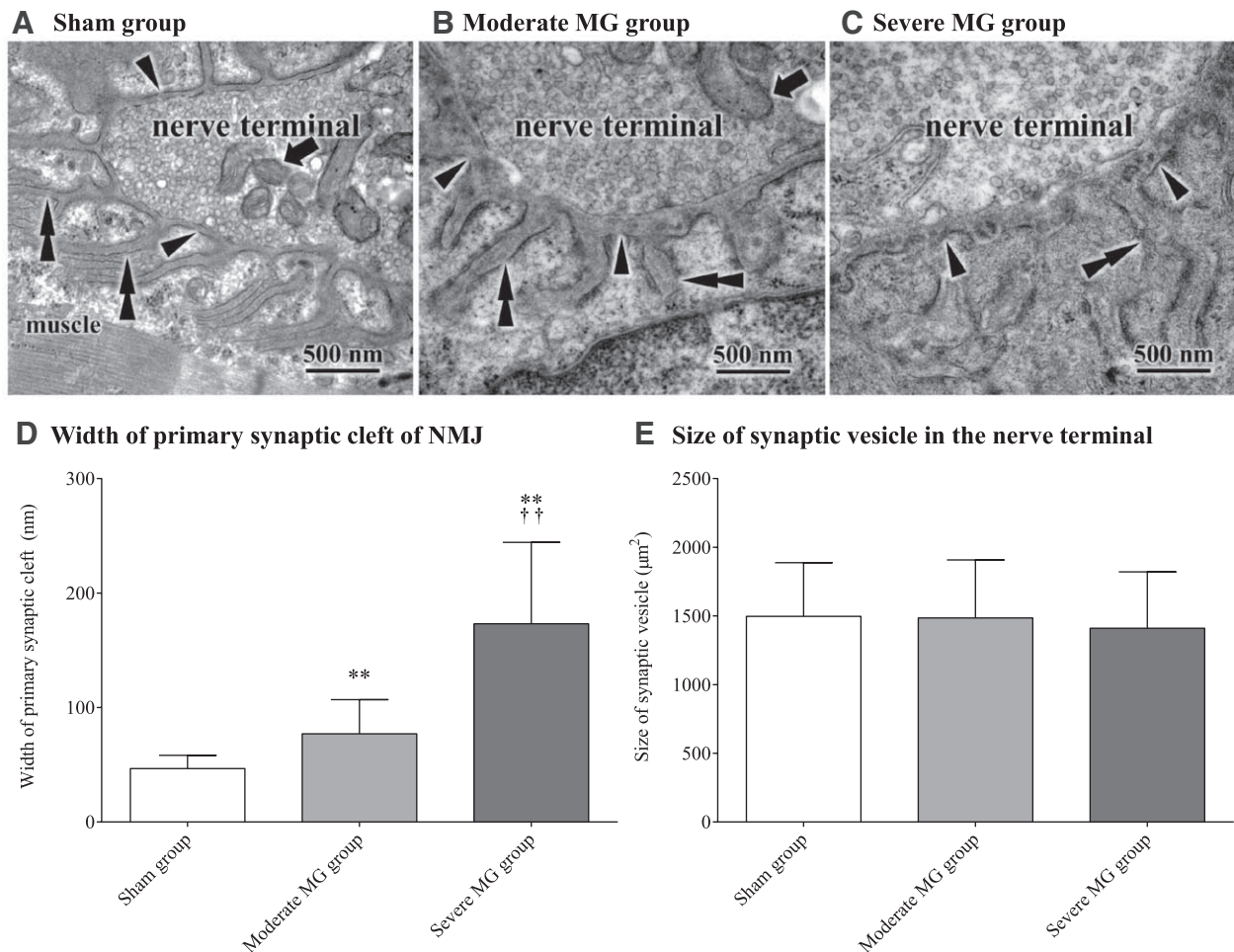


Fig. 7. Electron micrographs of the neuromuscular junction (NMJ) from the left hemidiaphragm of rats with increasing severity of myasthenia gravis (MG). NMJs from the hemidiaphragms of rats in each group were observed by electron microscopy (A–C). The narrow space between the nerve terminal and junctional fold is the primary synaptic cleft (arrowheads). Secondary synaptic clefts (double arrowheads) are present among the junctional folds. Acetylcholine receptors are located in high density in the surface of junctional folds, at the area of contact with the primary synaptic cleft. The mitochondria (arrows) exist in nerve terminals. Simplification of the postsynaptic region caused by degeneration of postsynaptic folds was observed at the NMJ of rats with moderate and severe MG (B and C, respectively). The width of the primary synaptic cleft (D) was significantly different among the three groups. The size of the synaptic vesicle (E) in the nerve terminal was not significantly different in the three groups. Data are shown as means \pm SD derived from 80 junctional regions of four rats from each group. ** $P < 0.01$ versus sham group, †† $P < 0.01$ versus moderate MG group. Data were analyzed by using one-way measures ANOVA with the Tukey test.

are prone to developing perioperative respiratory depression. Therefore, an advanced EAMG rat model, representing more severe clinical symptoms of MG than the traditional model, was produced in this study to evaluate the influence of severity of MG on the relationship between TOFR and T1. We injected 1.5-fold (1.8 mg/kg) or 2-fold (2.4 mg/kg) greater amounts of mAb35 than the traditional dosage²⁸ (1.2 mg/kg) of mAb35 in the preliminary study to induce more severe EAMG. Rats who received 1.8 mg/kg of mAb35 survived and presented with severe muscle weakness and weight loss, indicating that their symptoms were more severe than those of traditional MG model rats, which was enough to be statistically different, whereas doses of 2.4 mg/kg of mAb35 were fatal, resulting in death within 24 to 36 h. Therefore, we administered 1.8 mg/kg or the traditional dose

of 1.2 mg/kg of mAb35 to create the severe and moderate MG groups, respectively. Both groups presented the highest score of clinical symptoms within 30 to 42 h after mAb35 injection, with recovery 48 h after mAb35 injection. Therefore, the rats were considered to be an adequate MG model 36 h after mAb35 injection in this study. Rats in the severe MG group were more sensitive to rocuronium, and they showed significantly higher clinical symptom scores than those in the moderate MG group 36 h after mAb35 injection, suggesting that both the EAMG models used in our study might be useful for other animal studies in any field in which the influence of severity of MG is investigated.

We administered additional rocuronium doses every 10 min until twitch tension reached a state of equilibrium. Attenuation of TOFR and T1 by administration of

rocuronium was measured in the three groups to evaluate the influence of the severity of MG on their interrelationship. With the application of IC25 and IC50 of rocuronium, TOFR in the severe MG group was significantly attenuated, though to a lesser extent, than those in the other groups, whereas an IC25 and IC50 dose of rocuronium attenuated T1 to the same extent in all three groups, suggesting that TOFR is attenuated less than T1 by rocuronium administration in the severe MG group. Therefore, our hypothesis, recovery of TOFR might not accurately reflect recovery of T1 in severe MG patients, was confirmed. This is the first report describing the indicators of neuromuscular function with rocuronium in MG and the influence of severity of MG on the relationship between TOFR and T1. However, some NMBAs attenuate acetylcholinesterase activity, which is involved in the relationship between T1 and TOFR, the strength of the effect varying according to the type of relaxant.²⁹ Thus, the observed results may differ with different NMBAs.

Presynaptic and postsynaptic function at the NMJ was electrophysiologically investigated without rocuronium in the three groups to evaluate the mechanisms of the change in the relationship between TOFR and T1 without rocuronium in MG. We found that amplitude of MEPP, reflecting postsynaptic function that strongly influences T1, was significantly smaller in the severe MG group than in the other groups, whereas frequency of MEPP, reflecting presynaptic function that strongly influences TOFR, was not significantly different among the three groups without rocuronium. Other studies reported that amplitude of MEPP was smaller in the MG than in the non-MG group and that the frequency of MEPP was almost the same in both groups, but without mentioning their relationship to the severity of MG.^{24,30} Our results suggest that neuromuscular function is more severely damaged with increasing severity of MG, together with attenuation of postsynaptic but not presynaptic function. This might indicate that attenuation of T1 is greater than that of TOFR without rocuronium administration in severe MG patients, such that TOFR might not be as accurate an indicator of neuromuscular function in severe MG when compared with T1.

Furthermore, the effect of severity of MG on presynaptic and postsynaptic function with rocuronium administration was evaluated by measurement of EPPs and its calculation results, run-down of EPPs ($EPP_{21-50}amp / EPP_1amp$), and QC after rocuronium administration in the three groups because measurement of MEPPs, which have a low amplitude similar to that of noise, could not be established. EPP_1amp , attenuation of which reflects postsynaptic function, was significantly reduced by administration of IC50 of rocuronium in all three groups. By contrast, $EPP_{21-50}amp / EPP_1amp$ and QC, attenuation of which reflects presynaptic function, were not significantly reduced in the severe MG group unlike the other groups. Therefore, postsynaptic function at the NMJ seemed to be more easily affected by

administration of rocuronium than presynaptic function in the severe MG group, suggesting that this is the mechanism by which T1 is attenuated more than TOFR by rocuronium administration in the severe MG group because postsynaptic function influences T1 and presynaptic function influences TOFR.

Neuromuscular junctions were evaluated pathophysiologically by electron microscopy to assess whether postsynaptic function at the NMJ is damaged more easily than presynaptic function with and without rocuronium in the severe MG group. Simplification of the postsynaptic region caused by degeneration of the postsynaptic folds was observed at the NMJs in the hemidiaphragms from moderate and severe MG groups. Expansion in the width of primary synaptic cleft strongly influences the inhibition of postsynaptic function. The width of the primary synaptic cleft in the severe MG group was significantly greater than those in other groups, indicating that postsynaptic function was affected more easily than presynaptic function with and without rocuronium in the severe MG group. Impairment of neuromuscular transmission by anti-AChR antibodies results from three different mechanisms: direct binding to AChR, increased degradation of AChR, and complement-induced damage to the NMJ. Complement-induced damage to the NMJ occurs only in the postsynaptic region unlike the other two mechanisms.³¹ These facts indicate that the differences between postsynaptic function and presynaptic function in MG are caused by complement-induced damage in postsynaptic region, supporting our result that widening of primary synaptic clefts at postsynaptic regions was considered to influence their differences.

In conclusion, the severity of MG influences the relationship between TOFR and T1, together with changes in the run-down of EPPs caused by widening of primary synaptic clefts at postsynaptic regions in rats. TOFR was attenuated less than T1 by administration of rocuronium in severe MG patients, indicating that the use of TOFR as an indicator of muscle strength during recovery from neuromuscular blockade in severe MG patients might result in overestimation of the extent of neuromuscular recovery and also that the currently used definition of sufficient recovery, which includes a TOFR of greater than 0.9 and recovery of the T1 response to 80 to 120%, should be used carefully.

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Competing Interests

The authors declare no competing interests.

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