Novel Sonomicrometry of Ex Vivo Diaphragm After Phrenic Nerve Injury: Role of Matrix Metalloproteinases

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KEY WORDS nicotinic acetylcholine receptor; neuromuscular junction; synaptic transmission; extracellular matrix; ultrasonic wave

ABSTRACT Extracellular matrix (ECM) proteins and their proteolytic enzymes, matrix metalloproteinases (MMPs), implicate in neuromuscular junctions (NMJs) function during development. However, their pathophysiological mechanisms in the diaphragm remain obscure, because a well-characterized ex vivo experimental system has still been lacking. In the study, we aim to develop a novel ex vivo method of sonomicrometry and evaluate validity of the method with a mouse diaphragm twitch after phrenic nerve injury. In an ex vivo experiment using phrenic nerve-injured mice, diaphragm twitch during electrical pulse stimulation of phrenic nerve was transiently suppressed on day 1. Recombinant MMPs administered in recording solution exerted dose-responsive suppression on the diaphragm twitch in normal mice tissue. Furthermore, gelatinolytic and immunoblot experiments were performed to evaluate MMPs' involvement and NMJs' insults. After nerve injury, (1) in vivo levels of MMPs were transiently upregulated at day 1 and (2) expressions of ECM proteins, agrin (nicotinic acetylcholine receptor stabilizer) and laminin, were transiently reduced at day 1 in the diaphragm. These alterations were cancelled by preinjection of the MMP inhibitor. In conclusion, MMPs hamper NMJ synaptic function in association with the impairment of ECM milieu. Our novel experimental method using ex vivo sonomicrometry is necessary for examining the molecular pathophysiolgy for the dysfunction of NMJs in the diaphragm. **Synapse 66:677–685, 2012.**

INTRODUCTION

Phrenic nerves drive respiration via diaphragmatic movement (Frazier and Revelette, 1991; Pacia and Aldrich, 1998). The nicotinic acetylcholine receptor (nAChR) serves as a modulator of synaptic transmission in the neuromuscular junctions (NMJs) of the diaphragm and evokes skeletal muscle twitch followed by relaxation. After phrenic nerve fibers are injured, for example, by trauma, respiration is unstable because of functional deficiency or paralysis of the diaphragm (Elefteriades et al., 2008). However, definitive characterization and treatment for this functional deficiency of the diaphragm have not been fully established (Bowerson et al., 2010; Lin et al., 2005).

Extracellular matrix (ECM) supports functional maintenance of NMJs. The ECM component includes laminin and agrin in the NMJ. Laminin and agrin are important for NMJ function associated with the signaling pathway inducing nAChR clustering (Reist et al., 1992; Weston et al., 2007) and synapse formation (Bezakova and Ruegg, 2003; Martin et al., 2005). Nerve injury results in ECM degradation of the syn-

Additional Supporting Information may be found in the online version of this article.

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Contract grant sponsor: Ministry of Education, Culture, Sports, Science and Technology of Japan; Contract grant number: 22390338

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Received 16 January 2012; Revision 24 February 2012; Accepted 27 February 2012

DOI 10.1002/syn.21552

Published online 5 March 2012 in Wiley Online Library (wileyonlinelibrary.com).

aptic site of NMJ (Irintchev et al., 1993). Therefore, protection of ECM from degradation after nerve injury may promote a rapid NMJ functional maintenance.

Conversely, matrix metalloproteinases (MMPs) are a proteolytic enzyme of ECM. MMPs are initially synthesized as inactive zymogen (i.e., MMPs precursors: pro-MMPs) (Harper et al., 1971). The pro-MMPs contain propeptide domain at carboxy terminals for keeping their enzymes inactive. Following the removal of propeptide domain, pro-MMPs are allowed to be secreted to extracellular milieu as active MMPs. Active form of MMPs (active MMPs) can degrade the ECM proteins. In addition, it is likely that MMPs secretion from presynaptic terminals of injured neurons induces proteolysis of ECM components for synaptic function (Ethell and Ethell, 2007). Because of these suggestions, in the study, we address a working hypothesis that the diaphragm functional deficiency by phrenic nerve injury is induced in the following processes: (1) phrenic nerve injury alters MMPs activities in the NMJ on diaphragm muscle, (2) secreted MMPs degrade typical ECM proteins such as laminin and agrin for synaptic transmission on diaphragmatic NMJ, (3) ECM degradation leads to NMJ functional impairment, (4) the NMJ functional impairment includes the reduction of acetylcholine (ACh) release from presynaptic terminals, and (5) the reduced ACh release results in dysfunction of diaphragm muscle including aberrant augment of a spontaneous twitch, which is likely to be muscle fasciculation (Connelly et al., 1992; Denys and Norris, 1979). It is necessary for the valid ex vivo simplified assay in NMJ to evaluate this hypothesis.

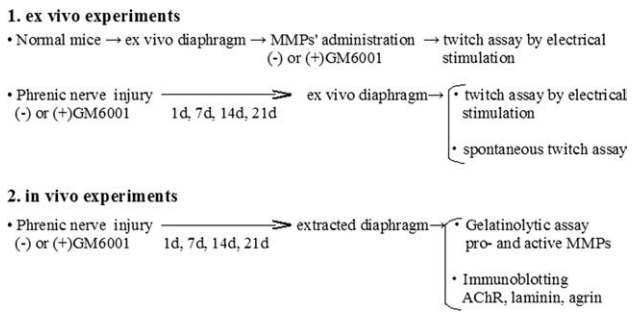

Fig. 1. Lines of experimental procedures.

Sonomicrometry has been well utilized to examine cardiac contraction (Sebag et al., 2005). Although in vivo models of diaphragmatic contraction using dogs (Newman et al., 1984; Wakai et al., 1994) and rabbits (Derrey et al., 2006; Zhan et al., 1995) monitored by sonomicrometry have been studied, the availability of an ex vivo experimental model would allow for quantitative evaluation of pharmacological effects without an influence from other organs.

In this study, we established a novel experimental method of ex vivo tracking of contraction of whole diaphragm tissues from mice and applied this method to a mouse model of phrenic nerve injury. We also performed immunoblotting analyses to examine temporal dynamics of ECM-related molecules in the NMJ from phrenic nerve injury. Our findings suggest the importance of the regulation of MMPs for maintenance of NMJs function in the diaphragm.

MATERIALS AND METHODS

Experimental procedure

Lines of experimental procedures are summarized in Figure 1. Each subject is described in the following sections. A number of mice used in the following experiments are summarized in Table I.

TABLE I. Number of mice used in this study

				Phrenic nerve injury			
Experiment	Figure	MMP inhibitor	Sham-operated	Day 1	Day 7	Day 14	Day 21
Ex-vivo diaphragm twitch	Fig 3	−GM6001	4	5	6	5	4
		+GM6001	5	5	6	5	4
	Fig 4	Recombinant MMP	0 μg/ml	0.5 μg/ml		1.0 μg/ml	1.0 μg/ml+GM6001
		rMMP-2	4	5		4	6
		rMMP-9	4	7		6	5
				Phrenic nerve injury			
		MMP inhibitor	Sham-operated	Day 1	Day 7	Day 14	Day 21
Gelatinolytic assay	Fig 5	−GM6001	4	7	5	4	4
		+GM6001	4	6	5	3	4
				Phrenic nerve injury			
		MMP inhibitor	Sham-operated	Day 1	Day 7	Day 14	Day 21
Immunoblot	Fig 6	−GM6001	4	7	5	3	4
		+GM6001	4	6	5	3	4

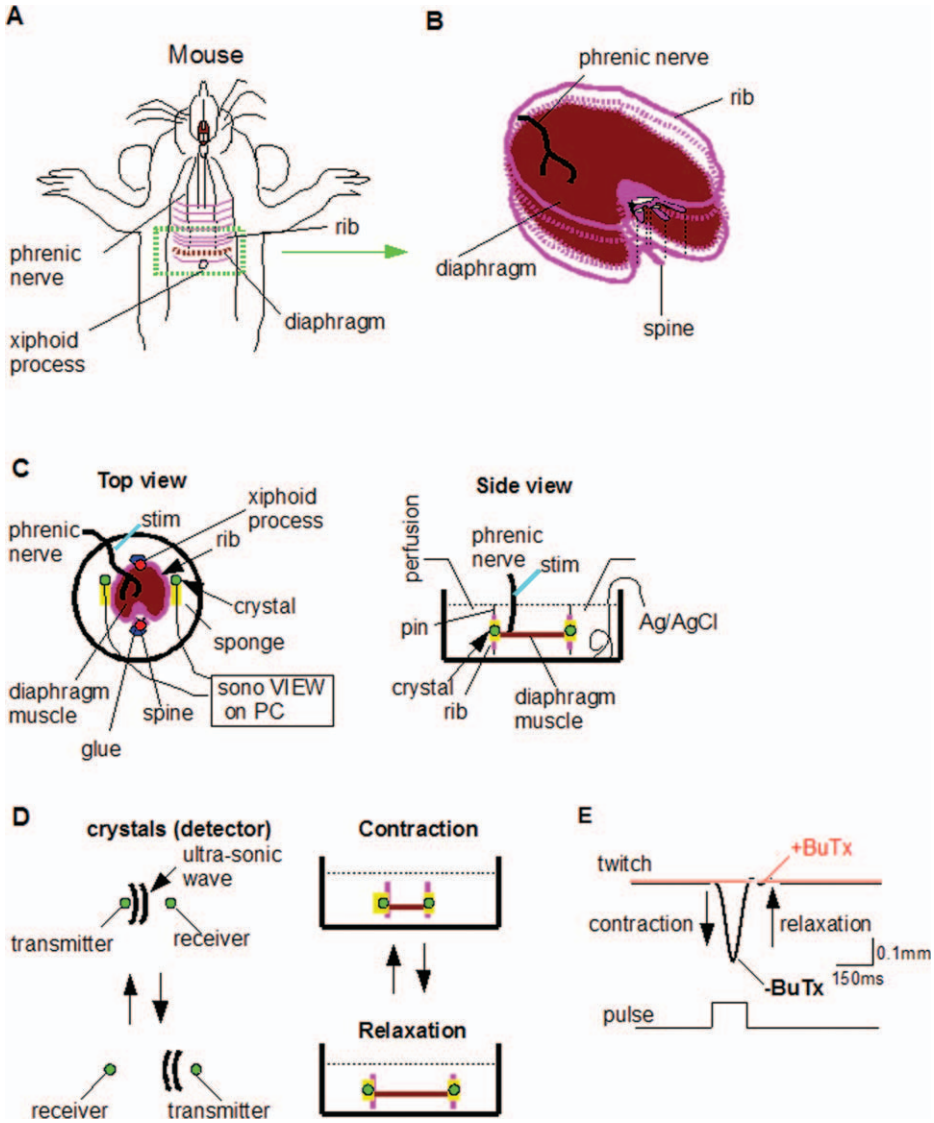

Fig. 2. Sonomicrometric measurement of diaphragm muscle twitch following phrenic nerve stimulation. **A**: Region of harvesting of ex vivo diaphragm from mice (green rectangle). **B**: Higher magnification of region harvested in A. **C**: Schema of experimental procedure for generating diaphragm twitch. Top view: mouse diaphragm with ribs was fixed with glue to a plastic culture dish at the caudal side of xiphoid process and spine. Side view: diaphragm twitch length was measured by crystals attached to ribs with pins. Sponges wrapped the wires connected to crystals and were utilized to attach the side of ribs with pins. The ribs are not fixed to the dish and are free to move. Stim: electrical pulse stimulation (1–10 mA, 150-ms duration) of the phrenic nerve fiber. Ag/AgCl: reference electrode. Perfusion: perfusion of tyrode solution. **D**: Rationale of sonomicrometry. Crystals: position detectors for transmitting and receiving ultrasonic waves. **E**: Representative traces of diaphragm twitch. Bungarotoxin (BuTx) 1.0 μM abolished the twitch. Scale bars: 150 ms, 0.1 mm.

Preparation of diaphragm

All animal experiments were performed under the guidelines of the Animal Care and Use Committee of Osaka University Medical School and the animal care guidelines of Kyoto University. Mice were purchased from Nihon SLC (Hamamatsu, Japan) and were euthanized under deep anesthesia. The phrenic nerve and diaphragm with five round-shaped rib bones remaining tightly attached (Figs. 2A and 2B) were isolated with the spine and xiphoid process and maintained in an ice-cold tyrode solution including NaCl (137), KCl (2.7), $NaHCO_3$ (11.9), NaH_2PO_4 (0.42), $MgCl_2$ (0.49), $CaCl_2$ (1.8), glucose (11.1), all in mM, and ascorbic acid (1 mg/ml), adjusted to pH 7.2–7.4. For stable recording, the whole diaphragm including the phrenic nerve was maintained for 30 min before use in 25°C tyrode solution into which a gas mixture of 95% O_2 and 5% CO_2 was bubbled. Diaphragm viability was evaluated by eliciting successful contraction by direct electrical stimulation of the diaphragm muscle under imposed excess current (>50 mA) before and after the recording (Supporting Information file 1).

Sonomicrometry

Piezoelectric crystals were attached to the sides of both the right and left ribs with pins (Fig. 2C, side view). The bottoms of the thoracic vertebra and xiphoid process, to which the ribs were connected, were glued to a 35-mm culture dish, and both the right and left ribs, to which the crystals were attached, were allowed to move freely. Perfusion of tyrode solution (2–3 ml/min) at 37.0°C ± 1°C was performed to maintain electrical excitability of the phrenic nerve and diaphragm muscle. We used a sonomicrometric device (sonoLab; Sonometrics Corp., London, Ontario, Canada) to measure the real-time degree of the diaphragmatic contraction with the pair of small piezoelectric transducers as described previously (Newman et al., 1984; Wakai et al., 1994). In brief, the change in distance between sample points can be measured with a transmitter and receiver (the two piezoelectric crystals) by transmission of ultrasonic waves (Figs. 2D and 2E). The spine and xiphoid process were fixed, and diaphragmatic movement was detected via sideways movement of the ribs attached to the crystals as applied in a modification of a previously reported method (Newman et al., 1984).

To electrically stimulate the phrenic nerve, a monopolar stimulation electrode (0.6-mm diameter, stainless steel) was placed on phrenic nerve fibers at 20 mm above the NMJ. To elicit a spontaneous diaphragm twitch, only one depolarizing pulse was applied for brief depolarization of the diaphragm muscle followed by no electrical stimulation. Spontaneous twitch incidence was determined by downward deflection of the sonomicrometry trace (indicating diaphragmatic contraction).

Exogenous administration of MMPs was provided to determine the dose-responsive effects of MMPs in prepared diaphragm tissue from normal mice. To analyze the effects of MMPs on the diaphragm twitch, recombinant MMPs were administered to the tyrode solution. For recombinant MMPs (rMMPs, 0–1.0 μg/ml), rMMP-2 (human recombinant active MMP-2, Cat# PF023, Calbiochem, Darmstadt, Germany) and rMMP-9 (human recombinant active MMP-9, Cat# PF140, Calbiochem) were used. The data were analyzed with sonoVIEW software (Sonometrics Corp.).

Phrenic nerve injury and MMP inhibitor injection

At 10 weeks old, all mice were deeply anesthetized by intraperitoneal injection of pentobarbital (45.0 mg/kg) and were placed in a homeothermic blanket to keep the rectal temperature at 37.0°C ± 1°C throughout the operation. After the bilateral phrenic nerves were exposed and visualized under a light microscope, nerve injury was performed on phrenic nerves for 30 s with forceps frozen in liquid nitrogen until use. The mice can keep breathing because of intact intercostal nerves during the operation. To evaluate the effects of MMPs on aberrant diaphragm twitch, the following experiment was performed: GM6001 (Calbiochem, Darmstadt, Germany), a broad-spectrum MMP inhibitor (1.0 mg/ml, diluted in phosphate-buffered saline), was injected intraperitoneally to infuse the drug through whole diaphragm 1 h before phrenic nerve injury. In the sham-operated mice, a similar procedure was followed but without performing nerve injury. We prepared two kinds of shams corresponding to the nerve-injured untreated group and the GM6001-treated group, independently. Bungarotoxin, nAChR antagonist, applied in recording solution was used to confirm that the twitch was activated by nAChR-dependent synaptic transmission. After phrenic nerve injury, the ex vivo tissue models were prepared at days 1, 7, 14, and 21 and followed by sonomicrometric recordings.

Gelatin zymography

Diaphragm muscles collected from mice 1, 7, 14, and 21 days after the phrenic nerve injury with or without the injection of GM6001 were homogenized in a lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10% glycerol, 0.5% SDS, 1.5 mM $MgCl_2$, 1 mM EDTA, and a protease inhibitor cocktail (03969-21, Nacalai Tesque, Kyoto, Japan) and analyzed by gelatin zymography as described previously (Wang et al., 2010) using SDS-PAGE (10% polyacrylamide containing 1.0 mg/ml gelatin). The amount of the sample loaded was normalized against the amount of tissue as estimated by bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific, Waltham, MA) of tissue lysate. Each band was identified by a position marker: pro-MMP-2 (i.e., gelatinase precursor, 72 kDa), active MMP-2 (62 kDa), pro-MMP-9 (92 kDa), and active MMP-9 (84 kDa). Gelatinolytic band intensities were analyzed by Image-J software.

Immunoblotting

After homogenizing, a diaphragm tissue was collected from mice 1, 7, 14, and 21 days after the phrenic nerve injury with or without injection of GM6001, and the amount of protein in each sample was determined by standard BCA assay. The proteins were separated by SDS-PAGE and analyzed by immunoblot assay as described previously (Wang et al., 2010). The primary antibodies used were anti-nAChR (mouse monoclonal; BD Bioscience, Franklin Lakes, NJ), anti-agrin (mouse monoclonal; Assay Designs, Plymouth Meeting, PA), and anti-laminin (rabbit polyclonal; Sigma-Aldrich, St. Louis, MO). Immunoreactive band intensities were analyzed by Image-J software.

Statistical analysis

Values are shown as means ± SEM. Statistical significance of all differences was assessed by Student *t*-test. For all statistical analyses in this study, a value of $P < 0.05$ indicates statistical significance.

RESULTS

Ex vivo measurement of diaphragm twitch by sonomicrometry

We established an experimental method with sonomicrometry to determine diaphragm twitch pattern (Supporting Information file 2). In our method, when the diaphragm contracts, both ribs are forced to move according to diaphragm contraction length, because the mice ribs are fixed only at the xiphoid process and the spine. As a consequence, the movement of the diaphragm can be tracked by two crystals, one a transmitter and the other a receiver, which are tightly attached to the side of ribs (Figs. 2C and 2D). The SonoVIEW software detects the distance between crystals over a continuous time course. When the distance between crystals becomes shorter during diaphragmatic contraction (Fig. 2D), the twitch trace (Fig. 2E, baseline) deflects downward, followed by diaphragm relaxation for which the trace deflects upward to the baseline. Thus, this method allows measurement of real-time diaphragm twitch during electrical pulse stimulation of phrenic nerve fibers conducting to NMJs in the diaphragm. Diaphragm twitch recorded in response to stimulation (Fig. 2E, black trace) was abolished by 1.0 μM bungarotoxin, nAChR antagonist (Fig. 2E, red trace). This result suggests that diaphragm twitch is evoked by NMJ excitation and strengthens the validity of this experimental assay.

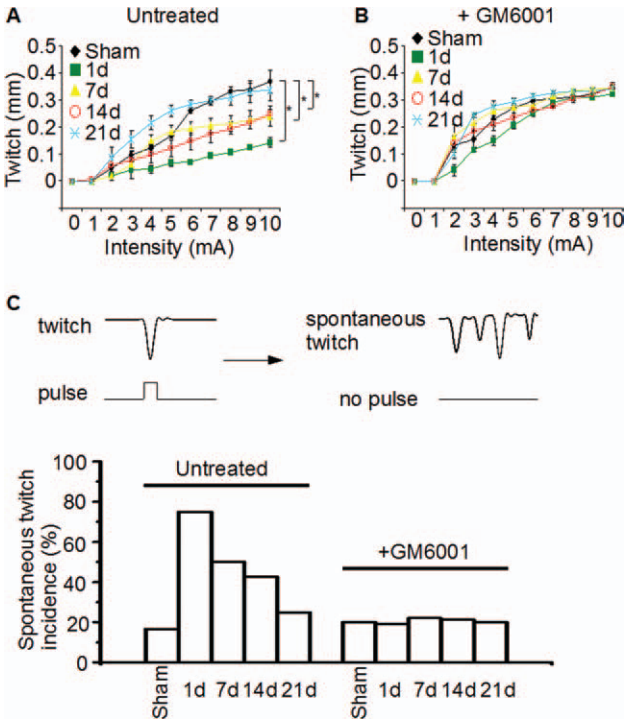

Fig. 3. Phrenic nerve injury induces diaphragm twitch deficiency via matrix metalloproteinases (MMPs). **A**: Reduced diaphragm twitch after phrenic nerve injury. **B**: Cancellation of diaphragm twitch deficiency by GM6001 (1.0 mg/ml). **C**: Average rate of incidence of spontaneous diaphragm twitch in mice. Scheme: Left trace, brief depolarization of diaphragm by a single pulse (5 mA, 150-ms duration); right trace, spontaneous twitch was initiated and continued for several minutes without continual electrical stimulation of the phrenic nerve. Untreated: without GM6001 treatment. Bars represents mean ± SEM; untreated: sham, $n = 4$, phrenic nerve injury, day 1: $n = 5$, day 7: $n = 6$, day 14: $n = 5$, and day 21: $n = 4$; GM6001: sham, $n = 5$, phrenic nerve injury, day 1: $n = 5$, day 7: $n = 6$, day 14: $n = 5$, and day 21: $n = 4$. *Significantly different from Sham, day 1 ($P = 0.026$), day 7 ($P = 0.047$), and day 14 ($P = 0.049$).

MMP-2 and -9 cause transient functional deficiency of the diaphragm after phrenic nerve injury

When the NMJ was stimulated in steps by pulses of increasing amperage (1–10 mA, 1-mA steps), diaphragm twitch length became gradually longer (Fig. 3A; sham-operated, black rectangles), indicating that the diaphragm uses cable properties via the NMJs. Next, we performed a phrenic nerve injury, and 1 day after the injury, the twitch amplitude was decreased at every electrical pulse stimulation intensity delivered to the phrenic nerve (Fig. 3A, green squares). The twitch amplitudes gradually recovered to half that of the sham-operated amplitude by 7 (0.22 ± 0.02 mm, 10-mA stimulation: yellow triangle) and 14 days (0.24 ± 0.03 mm, 10-mA stimulation: purple open circle), and the amplitude at 21 days after nerve injury was similar to that of the sham-operated mice (Fig. 3A, blue asterisks). These findings suggest that phrenic nerve injury abrogates diaphragm twitch transiently. Next, to determine whether MMPs are involved in the diaphragm twitch functional deficiency, GM6001 (MMP inhibitor) was injected in vicinity to the diaphragm 1 h before phrenic nerve injury. GM6001 cancelled the transient reduction of diaphragm twitch after the phrenic nerve injury (Fig. 3B).

Transient lacking of synaptic transmission by nerve injury causes diaphragm fasciculation (Denys and Norris, 1979). We found that the spontaneous twitch was induced after delivery of a single electrical pulse in the recording solution and lasted for several minutes followed by cessation of electrical stimulation. The average rate of incidence of spontaneous diaphragm twitch was calculated between each mouse group. The incidence transiently increased in the mice after nerve injury compared with the sham-operated mice, followed by return to the sham-operated level (Fig. 3C, untreated group [no GM6001]). How-

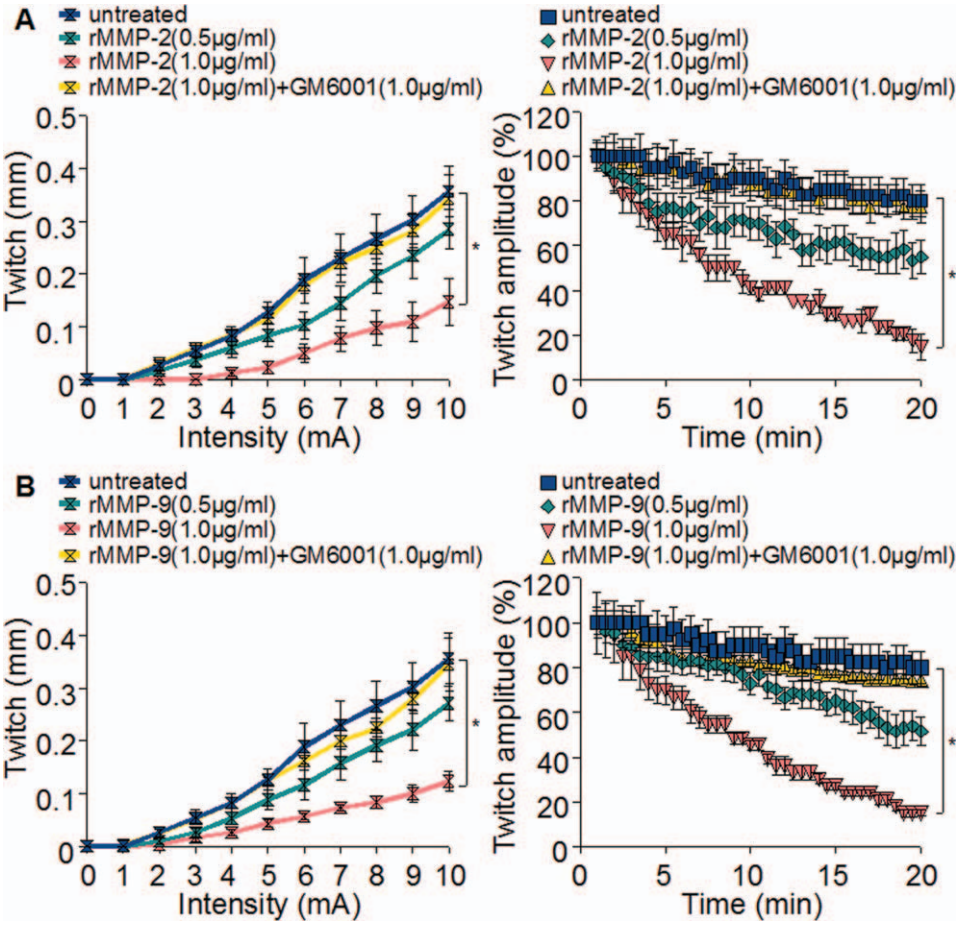

Fig. 4. Dose-responsive effects of ex vivo recombinant matrix metalloproteinases (rMMPs) on the suppression of diaphragm muscle twitch. Left panels: Both rMMP-2 (**A**) and rMMP-9 (**B**) increased thresholds of cable properties in diaphragm twitch during stepwise increase in electrical pulse stimulation of the phrenic nerve (1–10 mA intensity, 150-ms duration). rMMPs were administered at 5 min before recording. Right panels: Decrease in amplitude of diaphragm twitch during phrenic nerve stimulation (10-mA intensity, 150-ms duration, and 30-s intervals for 20 min) by rMMP-2 (A) and rMMP-9 (B). Twitch amplitude (%) was calculated with dividing by first evoked twitch amplitude. Bars represents mean ± SEM; untreated group, $n = 4$; rMMP-2 (0.5 μg/ml)-treated group, $n = 5$; rMMP-2 (1.0 μg/ml)-treated group, $n = 4$; rMMP-9 (0.5 μg/ml)-treated group, $n = 7$; rMMP-9 (1.0 μg/ml)-treated group, $n = 6$; rMMP-2 (1.0 μg/ml) + GM6001-treated group, $n = 5$; rMMP-9 (1.0 μg/ml) + GM6001-treated group. *Significantly different from untreated: left panels: A, rMMP-2 ($P = 0.043$); B, rMMP-9 ($P = 0.016$) and right panels: A, rMMP-2 ($P = 0.037$); B, rMMP-9 ($P = 0.016$).

ever, an increase in the incidence rate after nerve injury was prevented by pretreatment with GM6001 (Fig. 3C, +GM6001 group). Altogether, these results suggest that inhibition of MMPs is effective in the prevention of diaphragm functional deficiency after phrenic nerve injury.

Dose-responsive inhibitory effects of rMMP-2 and rMMP-9 on diaphragm twitch

We performed ex vivo experiments prepared from normal mice to test whether administration of MMPs had a detrimental effect directly on NMJ-driven muscle twitch. The diaphragm twitch was recorded 30 min after the addition of rMMP-2 (final: 0, 0.5, and 1.0 μg/ml) or rMMP-9 (final: 0, 0.5, and 1.0 μg/ml) to the recording solution (Fig. 4). Diaphragm twitches were suppressed in a higher dose of rMMP-2 (Fig. 4A, left panel, red trace) and rMMP-9 (Fig. 4B, left panel, red trace). Time course analyses indicated that diaphragm twitches in the presence of rMMP-2 and -9 reduced more significantly than sham-operated control at 5 min after the onset of recording and followed by the gradual enhancement of the reduction over time until the end of recording (Figs. 4A and 4B, right panels). In addition, the diaphragm twitch reduction by rMMP-2 and/or -9 was cancelled by GM6001 (Figs. 4A and 4B). Taken together, these findings suggest that both rMMP-2 and -9 affect diaphragm twitch directly in a dose-responsive manner.

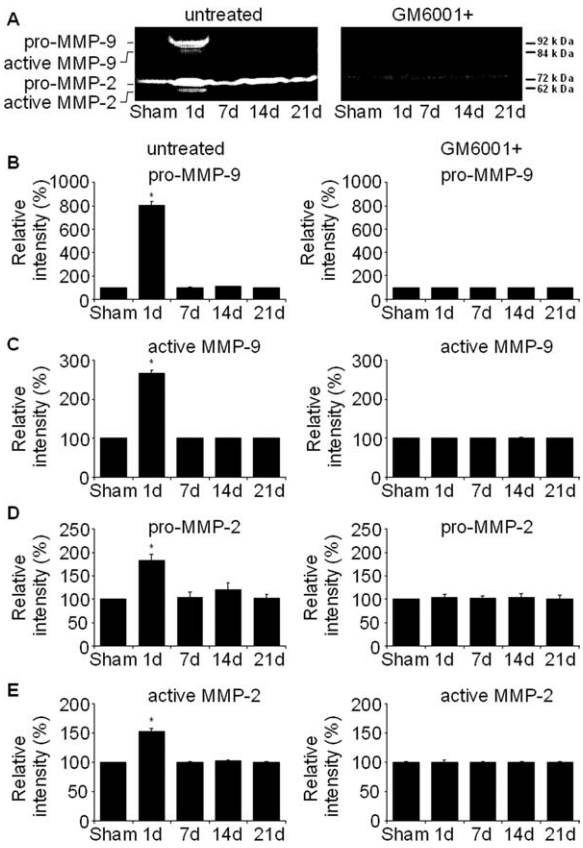

Fig. 5. Transient upregulation of in vivo matrix metalloproteinases (MMPs) gelatinolytic activity in diaphragm muscle after phrenic nerve injury. **A**: Gelatin zymography of the proteins of diaphragm muscle in mice subjected to phrenic nerve injury, GM6001-treated mice and sham-operated mice with or without the administration of GM6001. Positions of the pro-MMP-2 (72 kDa), active MMP-2 (62 kDa), pro-MMP-9 (92 kDa), and active MMP-9 (84 kDa) bands are indicated. The amount of sample loaded was normalized against the amount of tissue, as estimated by BCA assay of tissue lysate. Each sample was exposed to the same level of UV light. **B**–**E**: Densitometric relative quantification of gelatinase bands. Normalization of band intensities between each sample was performed by dividing against sham-operated mice. Pro- and active MMP-9 and pro- and active MMP-2 were upregulated 1 day after phrenic nerve injury and returned to the basal levels by day 7 against in sham-operated mice. In GM6001-treated mice, temporal alteration of gelatinolytic immunoreactivity was apparently not significant. Bars represents mean ± SEM; sham, $n = 4$ each for with or without the administration of GM6001; phrenic nerve injury alone, $n = 7$ for day 1, $n = 5$ for day 7, $n = 4$ for day 14, and $n = 4$ for day 21; GM6001-treated mice, $n = 6$ for day 1, $n = 5$ for day 7, $n = 3$ for day 14, and $n = 4$ for day 21. Statistical difference of each sample was determined against sham-operated mice. *Significantly different from Sham, pro-MMP-9: day 1 ($P = 0.004$), active MMP-9: day 1 ($P = 0.014$), pro-MMP-2: day 1 ($P = 0.016$), and active MMP-2: day 1 ($P = 0.047$).

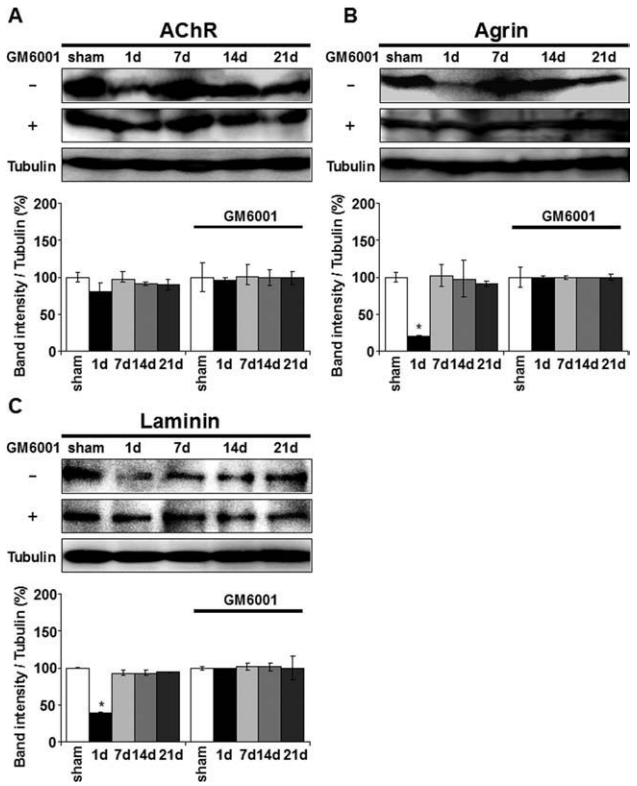

Fig. 6. Transient degradation of expression of proteins to support neuromuscular junction function in the diaphragm after phrenic nerve injury. **A**–**C**: Temporal pattern of protein expression in the diaphragm in phrenic nerve-injured mice with or without GM6001 treatment and the corresponding sham-operated mice. Lysates (200 μg) from diaphragmatic tissues were subjected to immunoblot assay. α-Tubulin was used as a loading control. For semiquantitative analyses, average band intensity for each immunoreactive band was stored and calculated by Image-J, and the value was then divided by the α-tubulin band intensity. Bars represents mean ± SEM; sham, $n = 4$ each for with or without the administration of GM6001; phrenic nerve injury alone, $n = 7$ for day 1, $n = 5$ for day 7, $n = 4$ for day 14, and $n = 4$ for day 21; GM6001-treated mice, $n = 6$ for day 1, $n = 5$ for day 7, $n = 3$ for day 14, and $n = 4$ for day 21. Statistical difference of each sample was determined against sham-operated mice. *Significantly different from Sham for B, agrin: day 1 ($P = 0.001$) and C, laminin: day 1 ($P = 0.001$).

Transiently increased gelatinolytic activity of in vivo MMP-2 and -9 in the diaphragm after phrenic nerve injury

To determine the temporal dynamics of in vivo MMP-2 and MMP-9 underlying NMJ function, we examined endogenous levels of MMPs gelatinolytic activity. One day after phrenic nerve injury, gelatinolytic bands were clearly found at 92 kDa (pro-MMP-9), 84 kDa (active MMP-9), 72 kDa (pro-MMP-2), and 62 kDa (active MMP-2) (Fig. 5A, left panel). We performed densitometric analyses in each mouse line. The pro-MMP-9 (Fig. 5B) and pro-MMP-2 (Fig. 5D) lines showed drastic enhancement of gelatinolytic activities 1 day after phrenic nerve injury. In addition, the active forms of MMP-9 (Fig. 5C) and MMP-2 (Fig. 5E) showed transient augmentation as well. Their transient increases at day 1 returned to sham-operated levels by day 7 and later after nerve injury. These alterations were cancelled in the mice undergoing preinjection of GM6001 (Figs. 5A–5D; right panel). Our findings suggest that the functional impairment of NMJs in diaphragms following phrenic nerve injury is closely linked to the transient upregulation of MMPs.

Dynamics of MMPs influence ECM environment in the diaphragm after phrenic nerve injury

To examine the effects of MMPs on constituent proteins of the ECM environment that are involved in NMJ function, we performed immunoblot assay for the diaphragm following phrenic nerve injury using specific antibodies related to neurotransmission. First, nAChR expression was slightly reduced 1 day after nerve injury (Fig. 6A). Second, we examined agrin, a marker for nAChR clustering signals associated with ECM protein components (Bentzinger et al., 2005; Reist et al., 1992; Weston et al., 2007). Agrin expression was significantly reduced 1 day after nerve injury (Fig. 6B). Third, laminin, a component of basal laminae in the NMJ, supports nAChR clustering by agrin (Wu et al., 2010). Laminin expression was significantly reduced at day 1 after nerve injury (Fig. 6C). Finally, altered expression of all of these proteins was cancelled by preinjection of GM6001 (Figs. 6A–6C, GM6001 groups). These findings suggest that upregulation of transient MMPs after nerve injury triggers the onset of disruption of ECM-related proteins that support synaptic transmission in the NMJ.

DISCUSSION

The purposes of this study were to develop a novel method to track ex vivo diaphragm twitch and to determine the role of MMPs in the protein degradation for maintenance of NMJs function using a mouse model of phrenic nerve injury. Although several reports suggest the usefulness of sonomicrometry, almost all studies are limited to the in vivo model of cardiac movement (Sebag et al., 2005), and only a few reports address its use with the diaphragm (Fiz et al., 2004; Newman et al., 1984; Platt et al., 1998). In this study, dissected diaphragm tissue was used under controllable experimental conditions (i.e., temperature and reagent administration in the recording solution). We showed successful detection of diaphragm twitch during electrical pulse stimulation of the phrenic nerve (Fig. 2). Our findings suggest that sonomicrometry is useful for evoked movement under controllable experimental conditions in dissected tissues consisting of phrenic nerve and NMJs in diaphragm muscle.

Our ex vivo experiments revealed that rMMPs in recording solution directly inhibit muscle twitch by electrical stimulation in a dose-responsive manner (Fig. 4). In addition, this result may reflect the in vivo results of transient reduction of diaphragm twitch (Fig. 3) in conjunction with upregulation of MMP-2 and -9 (Fig. 5) in diaphragm tissue after phrenic nerve injury. Finally, we confirmed that these effects are cancelled by MMP inhibitors, GM6001. GM6001 is widely utilized to inhibit MMPs. The specificity of GM6001 is not so rigid; however, its sensitivity is higher in MMP-2 and -9 than other MMPs (Saghatelian et al., 2004). In addition, although another metalloproteinase, which is suppressed by GM6001, might recently be suggested (Saghatelian et al., 2004), no evidence has been addressed for the involvement of another factor for diaphragm twitch dysfunction after phrenic nerve injury. In any case, our findings, at least, suggest that MMP-2 and -9 are critical for a harmful effect on normal physiological activity of diaphragm twitch in NMJ.

What is the functional role of MMPs after nerve injury? Several reports indicate that MMPs cause structural alterations of NMJ (VanSaun et al., 2003) and regulate agrin clearance from basal laminae (VanSaun and Werle, 2000) during NMJ development. In addition, in pathological condition, MMP-2 and -9 are specific substrates of agrin and impair normal neuronal functions (Agrawal et al., 2006). Because MMP-2 and -9 are mainly upregulated after nerve injury (Platt et al., 2003), MMP-2 and -9 predominantly exert effects on the NMJ after phrenic nerve injury. These reports suggest that MMPs have an impact on degradation of laminin and agrin, affecting ECM milieu. Agrin is well known to function as a mediator of synaptic transmission in the NMJ (Gautam et al., 1996; Hilgenberg et al., 2006; Martin et al., 2005). Then, it is possible that the downregulation of agrin in response to MMP activation disturbs synaptic transmission in conjunction with the instability of AChR. In addition, the fact that MMPs disrupt laminin in the nerve (Gautam et al., 1996) implies that it would also occur in the NMJ. Laminin and its binding protein potentially support normal synaptic transmission (Knight et al., 2003) and muscle contraction (Bogdanik et al., 2008; Han et al., 2009; Patton et al., 2001), and it would be acceptable that the dysfunction of the diaphragm following phrenic nerve injury is partly depending on the degradation of lamina, induced by MMPs in the NMJ. In this study, we successfully demonstrate the transient degradation of agrin and laminin (Fig. 6) via upregulation of MMPs on the diaphragm muscle (Fig. 5) after the phrenic nerve injury. These findings are consistent with our model that increased secretion of MMPs in the NMJ after phrenic nerve injury disrupts a repertoire of proteins in the ECM environment and thereby impairs diaphragmatic functions.

Our novel experimental method using sonomicrometry is necessary for examining the pathophysiological function of NMJs in the diaphragm. The method shows a novel aspect of the physiological role of MMPs and highlights their molecular and pathophysiological roles in the diaphragm. Our combined findings suggest a novel therapeutic potential for inhibition of MMP-2 and -9 in the acute prevention of respiratory dysfunction caused by phrenic nerve injury.

ACKNOWLEDGMENTS

The authors thank Mr. Stipp for critical reading of the manuscript and correction of English sentences.

REFERENCES

Agrawal S, Anderson P, Durbeej M, van Rooijen N, Ivars F, Opdenakker G, Sorokin LM. 2006. Dystroglycan is selectively cleaved at the parenchymal basement membrane at sites of leukocyte extravasation in experimental autoimmune encephalomyelitis. J Exp Med 203:1007–1019.

Bentzinger CF, Barzaghi P, Lin S, Ruegg MA. 2005. Overexpression of mini-agrin in skeletal muscle increases muscle integrity and regenerative capacity in laminin-alpha2-deficient mice. FASEB J 19:934–942.

Bezakova G, Ruegg MA. 2003. New insights into the roles of agrin. Nat Rev Mol Cell Biol 4:295–308.

Bogdanik L, Framery B, Frolich A, Franco B, Mornet D, Bockaert J, Sigrist SJ, Grau Y, Parmentier ML. 2008. Muscle dystroglycan organizes the postsynapse and regulates presynaptic neurotransmitter release at the Drosophila neuromuscular junction. PLoS One 3:e2084.

Bowerson M, Nelson VS, Yang LJ. 2010. Diaphragmatic paralysis associated with neonatal brachial plexus palsy. Pediatr Neurol 42:234–236.

Connelly NR, Silverman DG, Brull SJ. 1992. Temporal correlation of succinylcholine-induced fasciculations to loss of twitch response at different stimulating frequencies. J Clin Anesth 4:190–193.

Denys EH, Norris FH Jr. 1979. Amyotrophic lateral sclerosis. Impairment of neuromuscular transmission. Arch Neurol 36: 202–205.

Derrey S, Verin E, Laquerriere A, de Barros AB, Lacoume Y, Freger P, Marie JP. 2006. Restoration of diaphragmatic function after diaphragm reinnervation by inferior laryngeal nerve; experimental study in rabbits. Respir Res 7:17.

Elefteriades J, Singh M, Tang P, Siegel MD, Kenney B, Pandey A, Kopf GS. 2008. Unilateral diaphragm paralysis: Etiology, impact, and natural history. J Cardiovasc Surg 49:289–295.

Ethell IM, Ethell DW. 2007. Matrix metalloproteinases in brain development and remodeling: Synaptic functions and targets. J Neurosci Res 85:2813–2823.

Fiz JA, Jane R, Torres A, Morera J, Galdiz B, Gea J, Grassino A. 2004. Non-invasive monitoring of diaphragmatic timing by means of surface contact sensors: An experimental study in dogs. BMC Pulm Med 4:8.

Frazier DT, Revelette WR. 1991. Role of phrenic nerve afferents in the control of breathing. J Appl Physiol 70:491–496.

Gautam M, Noakes PG, Moscoso L, Rupp F, Scheller RH, Merlie JP, Sanes JR. 1996. Defective neuromuscular synaptogenesis in agrin-deficient mutant mice. Cell 85:525–535.

Han R, Kanagawa M, Yoshida-Moriguchi T, Rader EP, Ng RA, Michele DE, Muirhead DE, Kunz S, Moore SA, Iannaccone ST, Miyake K, McNeil PL, Mayer U, Oldstone MB, Faulkner JA, Campbell KP. 2009. Basal lamina strengthens cell membrane integrity via the laminin G domain-binding motif of alpha-dystroglycan. Proc Natl Acad Sci USA 106:12573–12579.

Harper E, Bloch KJ, Gross J. 1971. The zymogen of tadpole collagenase. Biochemistry 10:3035–3041.

Hilgenberg LG, Su H, Gu H, O'Dowd DK, Smith MA. 2006. Alpha3-Na^+/K^+-ATPase is a neuronal receptor for agrin. Cell 125: 359–369.

Irintchev A, Salvini TF, Faissner A, Wernig A. 1993. Differential expression of tenascin after denervation, damage or paralysis of mouse soleus muscle. J Neurocytol 22:955–965.

Knight D, Tolley LK, Kim DK, Lavidis NA, Noakes PG. 2003. Functional analysis of neurotransmission at beta2-laminin deficient terminals. J Physiol 546:789–800.

Lin PT, Andersson PB, Distad BJ, Barohn RJ, Cho SC, So YT, Katz JS. 2005. Bilateral isolated phrenic neuropathy causing painless bilateral diaphragmatic paralysis. Neurology 65:1499–1501.

Martin AO, Alonso G, Guerineau NC. 2005. Agrin mediates a rapid switch from electrical coupling to chemical neurotransmission during synaptogenesis. J Cell Biol 169:503–514.

Newman S, Road J, Bellemare F, Clozel JP, Lavigne CM, Grassino A. 1984. Respiratory muscle length measured by sonomicrometry. J Appl Physiol 56:753–764.

Pacia EB, Aldrich TK. 1998. Assessment of diaphragm function. Chest Surg Clin N Am 8:225–236.

Patton BL, Cunningham JM, Thyboll J, Kortesmaa J, Westerblad H, Edstrom L, Tryggvason K, Sanes JR. 2001. Properly formed but improperly localized synaptic specializations in the absence of laminin alpha4. Nat Neurosci 4:597–604.

Platt RS, Kieser TM, Easton PA. 1998. Eliminating ultrasonic interference from respiratory muscle EMG. Respir Physiol 112:203–213.

Platt CI, Krekoski CA, Ward RV, Edwards DR, Gavrilovic J. 2003. Extracellular matrix and matrix metalloproteinases in sciatic nerve. J Neurosci Res 74:417–429.

Reist NE, Werle MJ, McMahan UJ. 1992. Agrin released by motor neurons induces the aggregation of acetylcholine receptors at neuromuscular junctions. Neuron 8:865–868.

Saghatelian A, Jessani N, Joseph A, Humphrey M, Cravatt BF. 2004. Activity-based probes for the proteomic profiling of metalloproteases. Proc Natl Acad Sci USA 101:10000–10005.

Sebag IA, Handschumacher MD, Ichinose F, Morgan JG, Hataishi R, Rodrigues AC, Guerrero JL, Steudel W, Raher MJ, Halpern EF, Derumeaux G, Bloch KD, Picard MH, Scherrer-Crosbie M. 2005. Quantitative assessment of regional myocardial function in mice by tissue Doppler imaging: Comparison with hemodynamics and sonomicrometry. Circulation 111:2611–2616.

VanSaun M, Werle MJ. 2000. Matrix metalloproteinase-3 removes agrin from synaptic basal lamina. J Neurobiol 43:140–149.

VanSaun M, Herrera AA, Werle MJ. 2003. Structural alterations at the neuromuscular junctions of matrix metalloproteinase 3 null mutant mice. J Neurocytol 32:1129–1142.

Wakai Y, Leevers AM, Road JD. 1994. Regional diaphragm shortening measured by sonomicrometry. J Appl Physiol 77:2791–2796.

Wang H, Imamura Y, Ishibashi R, Chandana EP, Yamamoto M, Noda M. 2010. The Reck tumor suppressor protein alleviates tissue damage and promotes functional recovery after transient cerebral ischemia in mice. J Neurochem 115:385–398.

Weston CA, Teressa G, Weeks BS, Prives J. 2007. Agrin and laminin induce acetylcholine receptor clustering by convergent, Rho GTPase-dependent signaling pathways. J Cell Sci 120:868–875.

Wu H, Xiong WC, Mei L. 2010. To build a synapse: Signaling pathways in neuromuscular junction assembly. Development 137:1017–1033.

Zhan WZ, Farkas GA, Schroeder MA, Gosselin LE, Sieck GC. 1995. Regional adaptations of rabbit diaphragm muscle fibers to unilateral denervation. J Appl Physiol 79:941–950.