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ORIGINAL ARTICLE

Inhibiting Matrix Metalloproteinases Protects Evoked Electromyography Amplitudes and Muscle Tension in the Orbicularis Oris Muscle in a Rat Model of Facial Nerve Injury

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Abstract

Facial nerve injury results in degradation of the neuromuscular junction (NMJ) and blocks neurotransmission between the pre- and postsynaptic structures, which are separated by a synaptic cleft. Matrix metalloproteinases (MMPs), enzymes that degrade and modify the extracellular matrix, play critical roles in regulating NMJ remodeling. We previously demonstrated that MMP1, MMP2, MMP3, MMP7, and MMP9 are overexpressed in facial nerve-innervated orbicularis oris muscle after facial nerve injury in a rat model. In the present study, the MMP inhibitor prinomastat was administered to rats after facial nerve injury. The MMP levels, agrin expression, and muscle-specific kinase (MuSK) phosphorylation were evaluated. Variations in evoked electromyography (EEMG) amplitude were also recorded. Compared with the control group, MMP expression in the orbicularis oris after facial nerve injury was significantly reduced in the prinomastat group. Inhibition of MMP expression maintained agrin expression and MuSK phosphorylation; the NMJ morphology was also protected after the injury. Moreover, prinomastat treatment sustained EEMG amplitude and muscle tension after the injury. These findings indicate that inhibiting MMPs can protect the function and morphology of the NMJ and demonstrate the need for protection of the NMJ at early stages after facial nerve injury.

Key Words: Extracellular matrix metalloproteinases, Facial nerve injury, Neuromuscular junction, Orbicularis oris, Prinomastat.

INTRODUCTION

Evoked electromyography (EEMG) is widely used during otolaryngology-head and neck surgery to detect the location and function of the facial nerve and reduce the incidence of iatrogenic nerve injury (1, 2). Patients with pre-existing facial nerve injury exhibit a decrease in EEMG responses during general anesthesia (3). Monitoring the EEMG of the facial nerve is, therefore, critical to avoid secondary facial nerve injury during facial nerve repair and decompression. Our previous studies revealed that EEMG amplitude of the orbicularis oris was decreased and was less sensitive to nondepolarizing muscle relaxants after facial nerve injury (4–6). Subsequently, we found that this phenomenon may be correlated with degradation of the neuromuscular junction (NMJ), which requires a complex exchange of signals between motor neurons and skeletal muscle fibers, leading to the accumulation of postsynaptic proteins (7).

The formation and aggregation of postsynaptic membrane nicotinic acetylcholine receptors (nAChR) are hallmarks of NMJ maturation, a process closely associated with electrical signal transmission between presynaptic and postsynaptic surfaces in the NMJ (8). The presynaptic released acetylcholine binding to the postsynaptic nAChR generates the endplate potential, which dominates the production of EEMG responses in skeletal muscle (6). The NMJ normally has a pretzel-like morphology; however, this morphology is remodeled after facial nerve injury and the NMJ becomes fragile with an indistinct profile. Moreover, the nAChR is significantly upregulated at both synaptic and nonsynaptic sites in the orbicularis oris after facial nerve injury (9, 10).

The NMJ organizer agrin is secreted from motor neurons and has a major role in the formation and maintenance of NMJ (11). Neural agrin activates muscle-specific kinase (MuSK) tyrosine phosphorylation to promote nAChR aggregation at the NMJ (12, 13); however, facial nerve injury can result in the downregulation of agrin, which decreases MuSK phosphorylation (10).

Numerous studies have reported that matrix metalloproteinases (MMPs) affect the NMJ after nerve injury (14, 15). Enzymatic activity, protein abundance, and gene expression of MMPs are markedly increased following various physiological stimuli and pathological insults. Synaptic plasticity, which refers to activity-dependent modifications of synaptic strength and efficacy, is a fundamental feature of the nervous system that allows adaptation to and recovery from various

insults. MMPs, which are involved in the regulation of cell structure, play an important role in stabilizing and remodeling the cell matrix (16) as well as the NMJ (17).

MMPs are upregulated in the NMJ after nerve injury (18). By deleting *MMP3*, the agrin level in the NMJ was maintained after nerve injury in mice, and the nAChR level at the NMJ was significantly higher than in wild-type mice (15). MMP3 and other types of MMPs also play an important role in regulating NMJ remodeling after nerve injury. MMP-1, MMP-2, and MMP-9 overexpression were found following skeletal muscle injury (19) and MMP-2 plays a critical role in skeletal muscular atrophy; moreover, inhibiting MMP-2 expression can prevent NMJ remodeling during inflammatory myopathies. Thus, NMJ remodeling may be prevented by the blockade of MMPs.

Prinomastat is a potent, orally active MMP inhibitor with IC_{50} of 79, 6.3, and 5.0 nM for MMP1, MMP3, and MMP9, respectively (20). Prinomastat is a second-generation MMPs inhibitor shown to inhibit tumor growth and angiogenesis (21, 22); however, it is unclear whether prinomastat can protect the NMJ from degradation after facial nerve injury.

In the present study, prinomastat was administered to rats to determine whether inhibiting MMPs can protect the function and morphology of the NMJ in the orbicularis oris after facial nerve injury.

MATERIALS AND METHODS

Experimental Animals

A total of 60 male Sprague-Dawley (SD) rats (weight 150–180 g) were purchased and raised at the Experimental Animal Center of Shanghai General Hospital. All animal procedures and care were conducted in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals and approved by the Institutional Animal Use and Care Committee of Shanghai Jiao Tong University.

Reagents

The following antibodies and reagents were used: Goat antiagrin antibody (AF550, R&D Systems, Minneapolis, MN), rabbit polyclonal anti-MMP1 (ab51074, Abcam, Cambridge, UK), rabbit polyclonal anti-MMP2 (ab92536, Abcam), rabbit polyclonal anti-MMP3 (ab53015, Abcam), rabbit polyclonal anti-MMP7 (PA5-79684, Invitrogen, Waltham, MA), rabbit monoclonal anti-MMP9 (ab76003, Abcam), rabbit polyclonal anti-GAPDH (ab9485, Abcam), MMP3 inhibitor prinomastat (HY-12170A, MedChemExpress, Princeton, NJ), phospho-tyrosine mouse mAb (P-Tyr-100, Cell Signaling Technology, Danvers, MA), rabbit polyclonal anti-MuSK (GTX25619, GenTex, Zeeland, MI), AlexaFluor α-bungarotoxin (B13422, Life Technologies, Carlsbad, CA), AlexaFluor594-labeled donkey antigoat secondary antibody (A11058, Invitrogen) AlexaFluor594 donkey antirabbit (A21207, Invitrogen), and AlexaFluor488 α-bungarotoxin (B13422, Life Technologies).

Experimental Design

The SD rats were randomly divided into an experimental group, a control group and an unoperated group with 20 rats in each group. Each group was further divided into 4 subgroups consisting of 5 rats each, in which the EEMG monitoring and muscle tension assessments were performed on days 0, 3, 7, and 14 after facial nerve injury. Animals were anesthetized by intraperitoneal injection of pentobarbital (40 mg/kg). The left-sided facial nerve injury was induced using the crush injury model as previously described (23). Using microvascular hemostatic forceps (W40340; Shanghai Medical Instruments Co, Shanghai, China), a standard crush of the facial nerve was performed according to the Sunderland method, in which crushing 3 buckles for 60 seconds produces a grade IV injury. Loss of the blink reflex and vibrissae orientation was observed during or after surgery. The experimental group was then treated with an intraperitoneal injection of prinomastat solution (30 mg/kg) once a day (prinomastat group). The control group was treated with the same amount of normal saline.

EEMG Recording

At the designated time point in the protocol for each group, the rats were anesthetized with an intraperitoneal injection of pentobarbital (40 mg/kg). Bilateral orbicularis oris muscles innervated by the facial nerve were exposed. The EEMG was simultaneously recorded for the muscle using a bioelectric signal processing system (model SMUP-PC, Jide Experimental Instrument Factory, Shanghai, China). Briefly, a recording pin electrode was inserted into the muscle and connected to the system. The stimulating electrode was placed at the distal end of the injured facial nerve to prevent the electric current from contacting the injured area of the facial nerve. The nerves were stimulated with a single supramaximal train of rectangular pulses (4 V, duration 0.2 ms). The pulse was repeated 4 times at 2.0 Hz at 2-second intervals and the mean EEMG amplitude of the corresponding muscle was calculated.

Muscle Tension Recording

"Indirect electrical stimulation-evoked twitch tension" was recorded with an "MPA Multiple Channel Biological Signal Analysis System" (provided by Shanghai General Hospital, School of Medicine, Shanghai Jiao Tong University). The proximal end of the nerve was stimulated with a supramaximal train of rectangular pulses (intensity, 15 V; duration, 0.05 ms; frequency, 0.1 Hz). The pulse was repeated 3 times at 5-second intervals, and the mean twitch tension was calculated.

Western Blot

Total protein was extracted from muscle tissue in RIPA lysis buffer supplemented with a protease/phosphatase inhibitor cocktail (Beyotime, Shanghai, China). Protein concentration was measured using a BCA assay. Lysates of 20 μg were then subjected to electrophoresis using the NuPAGE Novex Gel system (Thermo Fisher Scientific, Waltham, MA) for SDS-PAGE. Anti MMPs antibody, anti-GAPDH antibody, and HRP-coupled secondary antibodies were used. Signals

were visualized with chemiluminescent substrate and densitometric analysis was performed with Image-pro plus.

Immunofluorescence Staining

Samples were fixed in cold acetone (4°C) for 10 minutes after immediate separation. The muscles were immersed in 30% sucrose solution for 2 hours at 4°C to dehydrate and then frozen in liquid nitrogen. Serial 15-mm-thick transverse sections were sliced using a cryostat microtome system (Leica, Heidelberg, Germany) at approximately −25°C. After permeabilization with 1% Triton X-100 for 20 minutes, the specimens were blocked with 10% normal donkey serum for 30 minutes at room temperature and then incubated with primary antibodies in 10% normal donkey serum overnight at 4°C. Next, the samples were incubated with secondary antibodies and AlexaFluor488 conjugated α-bungarotoxin for 1 hour, which was used to label the NMJ. Finally, the sections were mounted and images were captured under a confocal laser microscope (TCS SP8; Leica). Sections treated without primary antibodies were used as controls.

Immunoblotting

The muscles were harvested from rats, lysed in modified RIPA buffer (Beyotime Biotechnology), and precleared before coupling with MuSK. After the lysates were incubated with anti-MuSK antibody for pulldown assays to evaluate MuSK phosphorylation, the antigen complex was precipitated with protein A/G Sepharose beads (20422, Thermo Fisher Scientific). The pellets were washed 3 times in lysis buffer. Proteins (40 µg) were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to PVDF membranes. After blocking with 2% BSA/TBS-T, the membranes were incubated at 4°C overnight and proteins were detected using a secondary antibody. The proteins were reprobed with anti-MuSK antibody to identify equal loading of the MuSK protein.

Statistical Analysis

The density (IOD/area) of fluorescence was calculated using Image-Pro Plus software. Graph Pad Prism 7.0 software (GraphPad, San Diego, CA) was used to count and analyze the data, and Photoshop (2020) to create the figures. The protein levels in the orbicularis oris muscle before and after administration were compared using unpaired t-test. A p-value <0.05 was considered statistically significant.

RESULTS

Prinomastat Inhibited MMP Overexpression in the Orbicularis Oris Muscle after Facial Nerve Injury

The orbicularis oris muscle was isolated from rats and Western blot was used to identify MMP expression in both groups. After facial nerve injury, MMP1, MMP2, MMP3, MMP7, and MMP9 were overexpressed in the control group (Fig. 1). The prinomastat group exhibited lower MMP1,

MMP2, MMP3, MMP7, and MMP9 expression levels on day 3 after facial nerve injury than the control group (Fig. 1). On days 7 and 14 after facial nerve injury, the MMP expression levels in the muscle were compared between the 2 groups; the prinomastat group had consistently lower expression levels of all MMPs than the control group (Fig. 1).

Prinomastat Protected the Agrin Expression Level in Orbicularis Oris after Facial Nerve Injury

Immunofluorescence was used to detect the agrin expression level in the NMJ in both groups. Based on fluorescence density analysis, the agrin expression levels on days 3 and 14 after facial nerve injury were significantly higher in the prinomastat group than in the control group (0.0556 ± 0.01305 vs 0.0657 ± 0.01219; 0.0517 ± 0.01314 vs 0.0643 ± 0.01316, $p < 0.05$, $n = 20$, Fig. 2). The difference in the agrin expression level between control and prinomastat groups was the greatest on day 7 after facial never injury (0.0502 ± 0.01131 vs 0.0601 ± 0.01272, $p < 0.05$, $n = 20$, Fig. 2). Moreover, in the prinomastat group, the NMJ in the orbicularis oris exhibited almost complete morphology and most retained the typical pretzel shape. In the control group, the NMJ became fragmented, with blurred boundaries, and lost the typical pretzel shape.

Prinomastat Treatment Sustained the MuSK Phosphorylation Level after Facial Nerve Injury

Neural agrin can intensify MuSK phosphorylation, which is reflected by the ability of the muscle to cluster nAChR at the NMJ. We characterized the extent of MuSK phosphorylation to detect whether prinomastat can protect the downstream effects of agrin. Immunoprecipitation was obtained using MuSK antibody, and probing with phosphotyrosine antibodies revealed a 96-kDa band representing phosphorylated MuSK. When comparing the MuSK phosphorylation between control and prinomastat groups, the MuSK phosphorylation level gradually decreased from day 3 to day 14 in the control group, but the prinomastat group had a steady MuSK phosphorylation level (day 3: 65.2 ± 6.1 vs 75.2 ± 6.9; day 7: 44.5 ± 5.6 vs 65.1 ± 7.2, $p < 0.05$, $n = 3$; day 30: 65.1 ± 5.3 vs 80.6 ± 7.1, $p < 0.05$, $n = 3$, Fig. 3A, B). Synaptic-specific nAChR expression is a process that is closely associated with MuSK phosphorylation; therefore, nAChR expression was evaluated in both groups. The endplates were labeled with α-bungarotoxin, which selectively binds to the nAChR α1 subunit. Immunostaining revealed that nAChR expression decreased on days 7 and 14 after facial nerve injury in the control group, while the prinomastat group had relatively sustained nAChR expression level after facial nerve injury (Fig. 4A, B).

Inhibiting MMP Overexpression Maintained EEMG Amplitudes after Facial Nerve Injury

Compared to the control group, the NMJs in the prinomastat group exhibited more normal and complete morphology with the typical pretzel motor endplate. We found no significant change of NMJ in the unoperated group. The EEMG is a
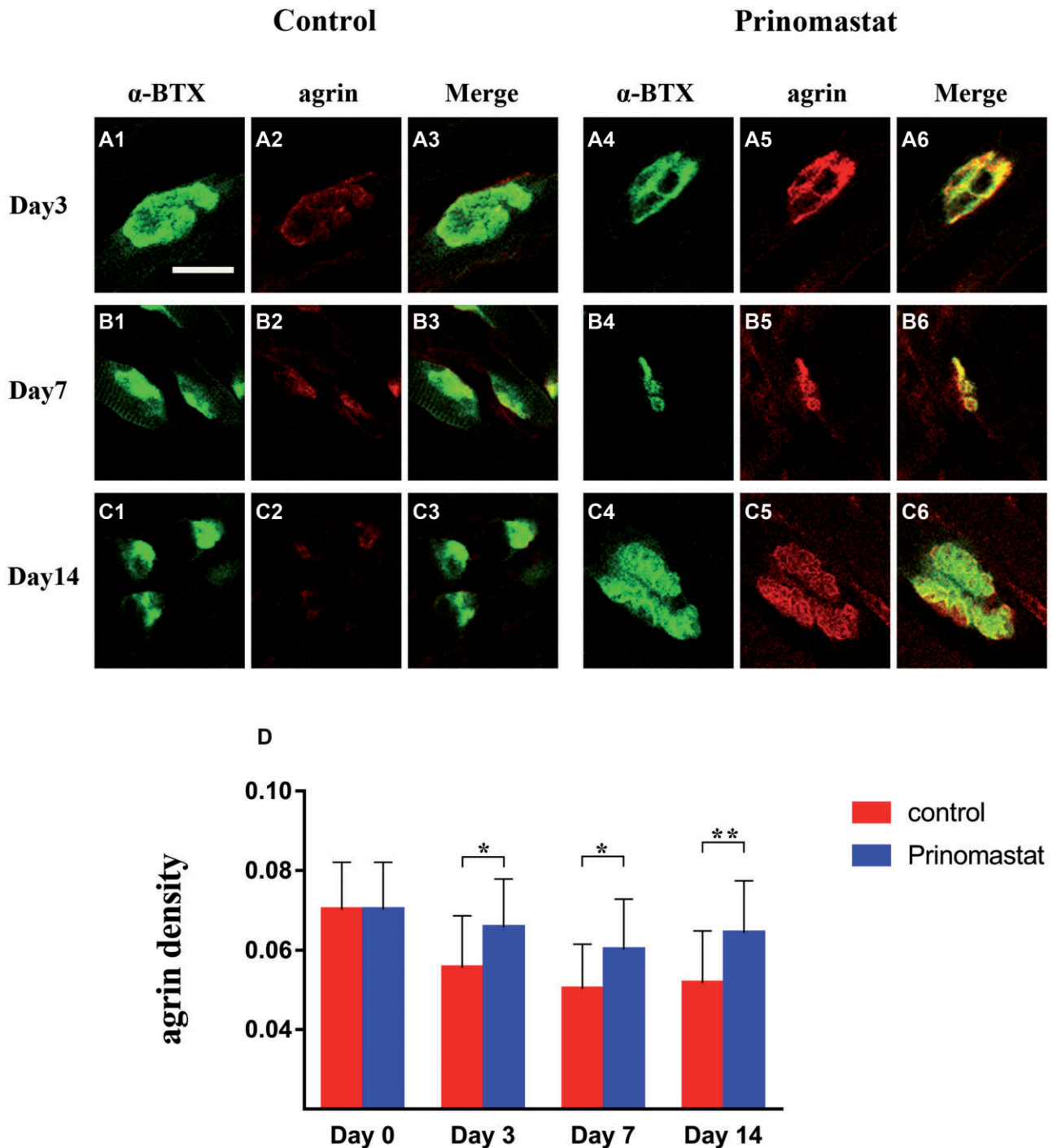
FIGURE 1. (A1–A6, B1–B6, C1–C6) Immunostaining for MMP3 of the orbicularis oris in control and prinomastat groups on days 3, 7, and 14 after facial nerve injury. **(D)** Western blot for MMP1, MMP2, MMP3, MMP7, and MMP9 in the orbicularis oris in prinomastat and control groups. MMP1, MMP2, MMP3, MMP7, and MMP9 expression levels in control and prinomastat groups at days 0, 3, 7, and 14 after facial nerve injury. **(E)** Bar chart of MMP1, MMP2, MMP3, MMP7, and MMP9 at days 0, 3, 7, and 14 after facial nerve injury in the prinomastat group. **(F)** Bar chart of MMP1, MMP2, MMP3, MMP7, and MMP9 at days 0, 3, 7, and 14 after facial nerve injury in the control group. The error bars represent SD; scale bar: 20 μm.

FIGURE 2. (A–C) Immunostaining for agrin in the NMJ of the orbicularis oris in control and prinomastat groups on days 3, 7, and 14 after facial nerve injury. **(D)** Graph of agrin density at days 3, 7, and 14 after facial nerve injury in the control and prinomastat groups. The error bars represent SD. α-bungarotoxin: α-BTX. Scale bar: 20 μm; **p < 0.01, *p < 0.05.

continuation of endplate potentials (EPPs) that are produced by the NMJ, and the EEMG current relies on complete function of the NMJ. Therefore, EEMG amplitude of orbicularis oris was compared between the control and prinomastat groups to determine whether prinomastat can protect the function of orbicularis oris after facial nerve injury. The unoperated group showed

a steady EEMG amplitude after facial nerve injury. The EEMG amplitude was decreased in control and prinomastat groups after facial nerve injury. However, the amplitude did not differ between control and prinomastat groups on day 3 after facial nerve injury (8.0 ± 1.12 vs 8.8 ± 1.11; $p > 0.5$, n = 8; Fig. 4). The EEMG amplitude was significantly decreased on
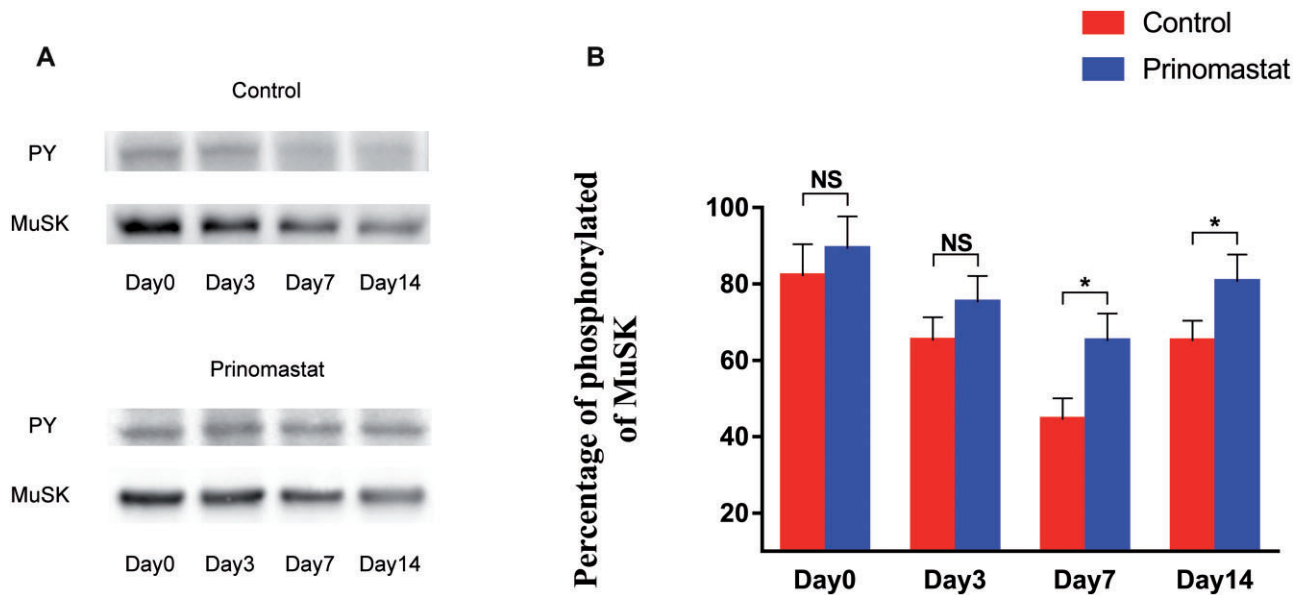
FIGURE 3. (A) Sample blot showing the tyrosine-phosphorylated MuSK (PY) band and the MuSK band in the control and prinomastat groups. The band was approximately 96 kDa. **(B)** Graph of the percentage of phosphorylated MuSK relative to total MuSK in the control and prinomastat groups. Error bars represent SD. NS: nonsignificant, *p < 0.05.

day 7 in the control group compared with the prinomastat group (6.6 ± 1.15 vs 8.5 ± 1.22; $p < 0.01$, n = 8; Fig. 4). Subsequently, the EEMG amplitude progressively recovered in both groups. However, the prinomastat group had a greater EEMG amplitude compared with the control group (7.4 ± 1.13 vs 9.0 ± 1.21; $p < 0.05$, n = 8; Fig. 4).

Prinomastat Treatment Preserved Muscle Tension after Facial Nerve Injury

Muscle contraction is associated with nerve electrical impulses. To some extent, the EEMG the amplitude reflects muscle tension. We measured tension in the orbicularis oris muscle in vivo after facial nerve injury and found that prinomastat attenuated the injury-induced muscle tension decrease on days 3, 7, and 14 (Fig. 4H–K).

DISCUSSION

NMJ remodeling and muscular atrophy are significant consequences of traumatic nerve injury (24). The NMJ normally assumes a pretzel-like morphology that is remodeled after injury (10). NMJ degradation may facilitate healthy motor nerve terminals to construct a new NMJ. As injured nerve is repaired, the NMJ is remodeled at the original position. We propose that the degradation of an altered NMJ is indispensable for building a new NMJ. However, even if there is complete repair of a nerve trunk, motor endplate and muscle function cannot be fully restored (4–6).

MMPs are enzymes that degrade and modify the extracellular matrix. They cleave matrix proteins, including the NMJ organizer agrin, which contributes to maintaining the maturation and function of the NMJ (17, 25). Muscle-derived MMPs collapse the NMJ resulting in a block of neurotransmission after

denervation (14, 15, 26). However, nerve injury results in overexpression of MMPs, which may play a negative role in NMJ remodeling after nerve trunk repair.

Prinomastat, a second-generation MMP inhibitor and a nonpeptidomimetic, is a low-molecular weight molecule designed to bind the MMP zinc-binding site. Prinomastat is a potent, orally active MMPs inhibitor with IC_{50} of 79, 6.3, and 5.0 nM for MMP1, MMP3, and MMP9, respectively. It is bioavailable after oral administration and possesses favorable drug-like physiochemical properties (27–29). The sources of MMPs are very wide, and many cells such as vascular endothelial cells, macrophages, and fibroblasts can produce and secrete MMPs (30). Considering pharmacodynamics and economic benefits, prinomastast was administrated into rats intraperitoneally and when compared with the control group, prinomastat effectively inhibited MMP1, MMP2, MMP3, MMP7, and MMP9 expression in orbicularis oris on days 3, 7, and 14 after facial nerve injury.

Next, we investigated whether prinomastat could protect agrin expression in the NMJ of the orbicularis oris after facial nerve injury. Neural agrin, released by the presynaptic surface, aggregates at the NMJ and can be cleaved by MMPs (15). Agrin was stained and α-bungarotoxin was used to locate the NMJ and detect agrin expression. When the density of immunofluorescence was compared between the prinomastat and control groups, the prinomastat group had higher agrin expression compared with the control group from days 3 to 14. These results indicate that prinomastat can maintain agrin expression in the NMJ of the orbicularis oris muscle by inhibiting MMP expression after facial nerve injury.

Formation of the NMJ is characterized by aggregation of nAChR at synaptic sites, a process closely associated with MuSK phosphorylation (31). MuSK is a downstream factor expressed at low levels in myoblasts cultured in vitro; animal
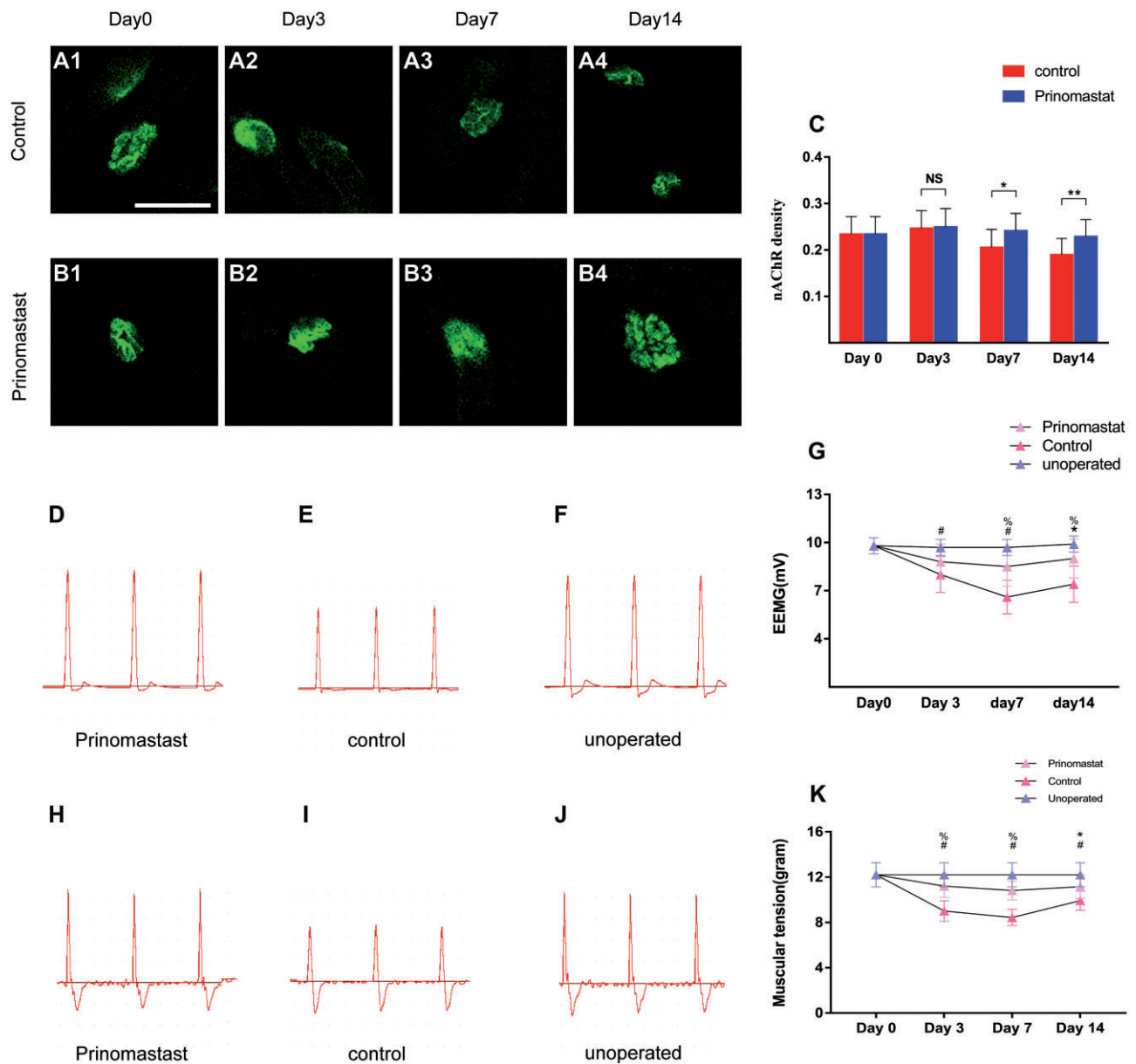
FIGURE 4. (A, B) Immunostaining for nAChR in the orbicularis oris in control and prinomastat groups on days 0, 3, 7, and 14. **(C)** Graph of nAChR. **(D–F)** EEMG amplitude values of the orbicularis oris on day 7 after facial nerve injury in the prinomastat, control, and unoperated groups. **(G)** Graph showing the EEMG amplitude of the orbicularis oris 0, 7, and 14 days after facial nerve injury in the control and prinomastat groups. The error bars represent SD. **(H–J)** Muscle tension in the orbicularis oris days 7 after facial nerve injury in the prinomastat, control, and unoperated groups. **(K)** Graph showing the muscle tension of the orbicularis oris 0, 7, and 14 days after facial nerve injury in the control and prinomastat groups. The error bars represent SD. Scale bar: 20 μm. NS: nonsignificant, [#]$p < 0.01$, control group versus unoperated group, [%]$p < 0.01$, control group versus prinomastat group, [*]$p < 0.05$, control group versus prinomastat group.

experiments have shown that MuSK is specifically expressed in developing muscles during the early embryonic stage, but that MuSK levels decrease significantly in mature muscle (26). The ability of MuSK to aggregate nAChR is dependent on a tyrosine phosphorylation modification of its membrane region (32). Neural agrin activates MuSK phosphorylation to promote nAChR expression at the NMJ. Subjects with *agrin* or *MuSK* mutations die at birth due to disrupted neuromuscular function (31). We previously demonstrated downregulation of agrin

resulted in progressively reduced MuSK phosphorylation after facial nerve injury (10). In the present study, prinomastat was proven to maintain MuSK phosphorylation level after facial nerve injury. We hypothesized that inhibiting MMPs protected agrin expression in the NMJ and sustained the MuSK phosphorylation level after facial nerve injury. Additionally, when the morphology of the motor endplate was compared under a fluorescence microscope, the NMJ in the prinomastat group had a more normal and complete morphology, with a typical pretzel motor endplate. In

contrast, the motor endplates in the control group were fragmented, blurred, and they lost the pretzel shape.

The EEMG reflects nerve transduction and transmission in the NMJ. The EEMG is a continuation of endplate potentials that are produced by the postsynaptic surface of the NMJ; the EEMG current relies on complete function of the NMJ. Facial nerve injury remodels the NMJ of orbicularis oris and obstructs transmission in the NMJ (33). Unlike the neurotransmission that is completely blocked after denervation, peripheral nerve injury still results in the retention of some neurotransmission that can maintain EEMG monitoring (4). In the present study, the stimulating electrode was placed at the distal end of the injured facial nerve to prevent the electric current from contacting the injured area of the facial nerve. The EEMG amplitude of the orbicularis oris was recorded and decreased on day 3 after facial nerve injury in both groups; no significant difference was observed between the control and prinomastat groups. On day 7 after nerve injury, the EEMG amplitude in the control group decreased whereas in the prinomastat group, the orbicularis oris exhibited sustained EEMG amplitude. The same result was observed on day 14 after injury, which is considered to be a long-term nerve injury. The facial nerve injury decreased the EEMG amplitude of the orbicularis oris in both groups, and inhibiting MMPs with prinomastat maintained the EEMG amplitude of the orbicularis oris innervated by the injured facial nerve.

Muscle tension determines the capability for muscle contraction. A previous study demonstrated that muscle twitch tension decreased after facial nerve injury (10). Inhibiting MMPs protected against decreased neurotransmission after facial nerve injury, where effective neurotransmission is a prerequisite for muscular contraction. Muscle contraction is associated with electrical nerve impulses; however, the contractility of skeletal muscles an also be affected by muscle-related factors, such as the intracellular concentration of Ca^{2+}. Our study found that prinomastat preserved muscle tension after facial nerve injury. Effective electrical transmission generates myoelectric potential, which to some extent determines muscle tension. However, it is not yet clear whether inhibiting MMPs expression facilitates calcium uptake of skeletal muscle.

In summary, prinomastat significantly reduced MMP expression levels in the NMJ of the orbicularis oris after facial nerve injury. Prinomastat administration sustained the agrin level in the NMJ and maintained the function and morphology of the NMJ in the orbicularis oris. Prinomastat treatment prevented the EEMG amplitude from decreasing after facial nerve injury. The presynaptic quantal release of acetylcholine also maintains the EEMG amplitude (34), but it is not clear whether MMPs can regulate the presynaptic release of acetylcholine or reabsorption of acetylcholine. We will investigate this in future work. Nerve injury-induced degeneration and dysfunction of the NMJ are an unsolved problem that can result in disastrous complications such as myasthenia and amyotrophy, and more researches are needed to determine how to protect function and morphology perioperatively.

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