Ultrasound Stimulation Inhibits Morphological Degeneration of Motor Endplates in the Denervated Skeletal Muscle of Rats

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ABSTRACT: Recovery of motor function after peripheral nerve injury requires treatment of the neuromuscular junction (NMJ), as well as the injured nerve and skeletal muscle. The purpose of this study was to examine the effects of ultrasound (US) stimulation on NMJ degeneration after denervation using a rat model of peroneal nerve transection. Twelve-week-old male Wistar rats were randomly assigned to 3 groups: US stimulation, sham stimulation, and intact. US or sham stimulation was performed on the left tibialis anterior (TA) muscle starting the day after peroneal nerve transection for 5 minutes daily under anesthesia. Four weeks later, the number and morphology of the motor endplates were analyzed to assess NMJ in the TA muscle. The endplates were classified as normal, partially fragmented, or fully fragmented for morphometric analysis. In addition, the number of terminal Schwann cells (tSCs) per endplate and percentage of endplates with tSCs (tSC retention percentage) were calculated to evaluate the effect of tSCs on NMJs. Our results showed that endplates degenerated 4 weeks after transection, with a decrease in the normal type and an increase in the fully fragmented type in both the US and sham groups compared to the intact group. Furthermore, the US group showed significant suppression of the normal type decrease and a fully fragmented type increase compared to the sham group. These results suggest that US stimulation inhibits endplate degeneration in denervated TA muscles. In contrast, the number of endplates and tSC and tSC retention percentages were not significantly different between the US and sham groups. Further investigations are required to determine the molecular mechanisms by which US stimulation suppresses degeneration.

KEYWORDS: Peripheral nerve, neuromuscular junction, terminal Schwann cell, peroneal nerve, denervation, pretzel

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Introduction

Peripheral nerve injury causes motor and sensory disturbances depending on the damaged nerve, which interfere with various daily activities. Peripheral nerves are capable of self-regeneration; however, if regeneration is delayed, motor dysfunction is likely to persist.[1,2] To prevent residual motor dysfunction, nerve tissue regeneration should be promoted as early as possible after peripheral nerve injury. Nerve suturing and autologous nerve transplantation are used to repair damaged peripheral nerves and promote regeneration. However, these effects are sometimes inadequate and do not lead to a complete functional recovery. One reason is that treatment of the peripheral nerve alone is insufficient for recovery from peripheral nerve injury. It has been reported that both the regeneration of nerve axons at the site of injury and reinnervation at the neuromuscular junction (NMJ) are important,[3] as the regeneration of nerve axons alone does not lead to recovery of motor function, even if the regenerated axons reach the innervating muscles.[4] Therefore, to restore motor function after peripheral nerve injury, a comprehensive approach to the NMJ and distal muscles, in addition to the nerve cell bodies and axons that make up the peripheral nervous system, is needed.[5]

An NMJ is the interface between motor nerve endings and skeletal muscles, and consists of motor nerve endings, motor endplates, and terminal Schwann cells (tSCs). Acetylcholine receptors (AChRs) are receptors for acetylcholine, which are released from motor nerve endings and innervated to cause muscle contraction. tSCs surround the motor nerve endings of NMJs and are involved in their formation and maintenance.[6]

NMJs are essential for maintaining motor function and muscle strength, and their morphology and number have been reported to contribute to efficient neuromuscular transmission.[7,8] After peripheral nerve injury, degeneration and changes in the number of endplates occur.[9] Normal endplates have a complex, pretzel-like morphology with numerous grooves in the postsynaptic membrane.[10] After peripheral nerve injury, they degenerate from the mature normal type to an immature fragmented type.[9] Additionally, endplates increased until the second week after transection and started to decrease from the fourth week in the tibialis anterior (TA) muscle of mice with transected peroneal nerve.[9] Furthermore, in rats with an injured sciatic nerve, the morphology of NMJs was maintained when the regenerated axons reached NMJs for a short period, eventually leading to motor function recovery. However, when the period was extended, NMJs degenerated, and motor function recovery was not achieved.[4] Therefore, inhibiting the degeneration and reduction of endplates after peripheral nerve injury, as described above, may be a means to restore motor function

before peripheral nerves regenerate and reach the NMJs. However, few studies have focused on the degeneration and reduction of endplates after peripheral nerve injury and few attempts have been made to suppress them.

To inhibit degeneration and decrease of endplates, we implemented ultrasound (US) stimulation in this study. It has been reported that US stimulation promotes the proliferation and survival of Schwann cells in culture.[11] In addition, several research groups, including ours, have reported the effect of US stimulation on the promotion of peripheral nerve regeneration, especially on the thickening of the myelin sheath.[12,13] We hypothesized that US stimulation would promote the survival and proliferation of tSCs and inhibit the degeneration and reduction of the endplates after peripheral nerve injury. In experiments using mutant mice, removal of tSCs and Schwann cells before, during, and after NMJ development and maturation impaired the formation and maintenance functions of NMJs at each stage.[6] Additionally, the number of Schwann cells in the TA muscle decreased after peroneal nerve transection. At the same time, degeneration and reduction of NMJs have been observed.[9] Therefore, maintaining the number of tSCs in endplates after peripheral nerve injury may prevent endplate degeneration and reduction. This study aimed to determine the effects of US stimulation on the degeneration and reduction of the endplates after peripheral nerve injury.

Materials and Methods

Animals

Adult male Wistar rats (11-week-old) were placed in standardized cages (2 animals per cage) under a 12 hours light/dark cycle at 25°C on sawdust bedding and acclimatized for 1 week. The rats were allowed to move freely in their cages and were provided free access to food and water. They were randomly divided into 3 groups: intact (intact group, $n = 2$), sham stimulation (sham group, $n = 8$), and US stimulation (US group, $n = 8$). The sample size was determined according to a previous study.[12] No adverse events were observed, and all rats were included in the study.

Surgical procedure for peroneal nerve transection

The left peroneal nerve was transected in the US and sham groups according to a previously reported protocol.[9] Briefly, the rats were anesthetized with 5% isoflurane and 3 types of mixed anesthetic agents (0.1 mg/kg of medetomidine, 2.0 mg/kg of midazolam, and 2.5 mg/kg of butorphanol). The left peroneal nerve was exposed at the head of the fibula and transected approximately 3 mm from the TA. After transection, the distal nerve ending was reversed and sutured below the skin using 6-0 nylon sutures to prevent the nerve terminal from reinnervating the NMJ. The surgical incision was closed using 4-0 nylon sutures. Discontinuity of the peroneal nerve was confirmed during sacrifice (Supplemental Figure 1). All procedures were approved by the Institutional Animal Care and Use Committee of Kyoto University (approval number: Med Kyo 20027).

Ultrasound stimulation

All rats were anesthetized with 1% to 2% isoflurane. A US transducer was placed on the skin above the corresponding TA muscle after application of US transmission gel. US stimulation was started 1 day after the transection and performed every day using a portable ultrasonic treatment apparatus with an unfocused circular probe with an effective radiation area of 0.9 cm^2 and a 2.9 beam non-uniformity ratio (UST-770, ITO CO., LTD, Japan). The radiation parameters were as follows:140 mW/cm^2 intensity (spatial average and temporal average [SATA]), 1 MHz frequency, 1 kHz repeating frequency, 20% duty cycle, and 5 min/day treatment duration.[13] For the sham group, the power of the apparatus was turned off, and sham stimulation was performed. Four weeks after transection, the rats were sacrificed using an overdose of the 3 types of mixed anesthetic agents. The rats in the intact group did not receive US stimulation or surgery.

Tissue preparation for TA muscle

After overdose sacrifice, the rats were transcardially perfused with 0.9% saline wash until the liver was cleared of blood, and then perfused with 4% paraformaldehyde (PFA). After 30 minutes of rest, the left TA muscle was excised from the hind limb, post-fixed in 4% PFA for 24 hours, and dehydrated in 30% sucrose for 48 hours at 4°C. The muscles were then embedded in tragacanth gum on corks and frozen in liquid nitrogen-cooled 2-methylbutane. The muscles were stored at −80°C until use.

Immunohistochemistry

The collected TA muscle was sliced in the longitudinal direction, and frozen sections (20 µm thickness) were prepared every 300 µm. To evaluate endplates (AChR clusters) in NMJs, frozen sections were incubated with alpha-bungarotoxin, Alexa Fluor™ 647 conjugated (α-BTX, 1:1000; B35450, Invitrogen, USA) for 1 hour at room temperature. α-BTX binds specifically to AChRs in the postsynaptic membrane. The frozen sections were warm-bathed in antigen activation solution (HistoVT One, 06380-05, Nacalai Tesque, Japan) at 70°C for 20 minutes, blocked at room temperature for 1 hour (Blocking One Histo, 06349-64, Nacalai Tesque, Japan), and incubated overnight at 4°C in polyclonal rabbit Anti-S100 ready-to-use (S100, IR504, DAKO, Denmark) to evaluate the tSCs in NMJs. S100 is expressed in the cytoplasm of Schwann cells in the peripheral nervous system, and can be used to label Schwann cells. The sections were then incubated with secondary antibodies Alexa Fluor 594 goat anti-rabbit IgG (1:200; 853501, Invitrogen, USA), α-BTX (1:500), and Cellstain DAPI solution (1:5000; 340-07971, FUJIFILM Wako, Japan) for 1 hour at room temperature. After staining, all sections were mounted using the PermaFluor™ aqueous mounting medium (TA-030-FM, Thermo Scientific, USA).

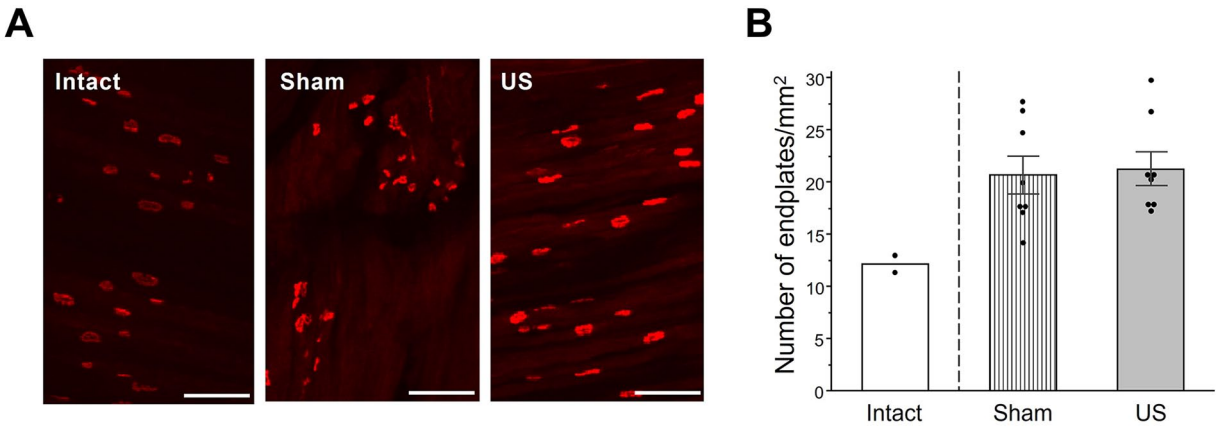

Figure 1. Number of motor endplates: (A) representative endplates (α-BTX, red) 4 weeks after the peroneal nerve transection in intact, sham (denervation with sham stimulation), and US (denervation with US stimulation) groups. Scale bar: 100 μm and (B) mean number of endplates per mm^2. Intact group (n = 2), sham group (n = 8), US group (n = 8). Error bars indicate standard error.

Quantitative analyses

Three frozen sections were randomly selected from the superficial, intermediate, and deep regions of the thin-sectioned TA muscle in the longitudinal direction. Immunohistological staining was performed on these sections, and images of the α-BTX-positive AChR clusters were obtained using a confocal laser scanning microscope (FLUOVIEW FV10i-O, OLYMPUS, Japan). The images were analyzed using NIH ImageJ (https://imagej.nih.gov/ij/).[14,15] To evaluate the number of endplates, α-BTX-positive AChR clusters were counted, and the mean value per unit area was calculated. More than 100 endplates per TA sample were randomly selected for analysis to evaluate the endplate morphology. Following previous studies,[16] α-BTX-positive AChR clusters were classified into 3 types: normal (consisting of less than 5 fragments and multiple perforations), partially fragmented (consisting of 5 or more fragments or a single fragment and no perforation), and fully fragmented (consisting of granular and mottled fragments) (Supplemental Figure 2A), and the percentage of each type in the total number of α-BTX-positive AChR clusters was calculated. More than 50 endplates per TA sample were analyzed to evaluate tSCs. The number of tSCs per endplate and percentage of endplates with tSCs (tSC retention percentage) were calculated. The number of tSCs was assessed based on the number of DAPI-positive nuclei matched to α-BTX-positive AChR clusters and S100-positive Schwann cells (Supplemental Figure 2B).[16] All quantitative analyses were confirmed by 2 trained evaluators (AI and YA), without blinding.

Statistical analyses

Data are presented as mean ± standard error. All data were assessed for normal distribution using the Shapiro–Wilk normality test and for homoscedasticity using Levene's test. Statistically significant differences between the US and sham groups were detected using Student's t-test or Wilcoxon rank-sum test if the data were not normally distributed. The significance level was set to 5%. All statistical analyses were performed using JMP Pro 15 software (SAS Institute, USA).

Results

Number of endplates

Representative α-BTX-positive AChR cluster images are shown in Figure 1A. There was no significant difference in the number of endplates between the US and sham groups (21.3 ± 1.62 in the US group and 20.7 ± 1.78 in the sham group, P = .81) (Figure 1B). In addition, there appeared to be more endplates in both groups than in the intact group (12.1 ± 0.81) 4 weeks after transection.

Endplate morphology

Representative morphologies of the endplates in each group are shown in Figure 2A. After peroneal nerve transection, the morphology of endplates degenerated in both the US and sham groups. Compared to the intact group, both the US and sham groups showed a trend toward a decrease in the normal type and an increase in the partially and fully fragmented types (Figure 2B-D). The US group had significantly more normal-type endplates than the sham group (52.2% in the US group versus 46.0% in the sham group, P = .02) (Figure 2B). Additionally, the US group had significantly fewer fully fragmented endplates than the sham group (11.1% in the US group versus 16.4% in the sham group, P = .02) (Figure 2D). There was no significant difference between the US and sham groups in the partially fragmented type (36.5% in the US group versus 38.0% in the sham group, P = .62) (Figure 2C). These results indicate suppression of the normal type decrease and a fully fragmented type increase in the US group.

Number of tSCs and tSC retention percentage

After peroneal nerve transection, both the US and sham groups showed a decreasing trend in tSC number and retention percentage compared with the intact group. However, there was no significant difference between the US and sham groups (tSC number: 2.7 ± 0.01 in the intact group, 1.1 ± 0.15 in the sham group, and 1.4 ± 0.25 in the US group, P = .43; tSC retention

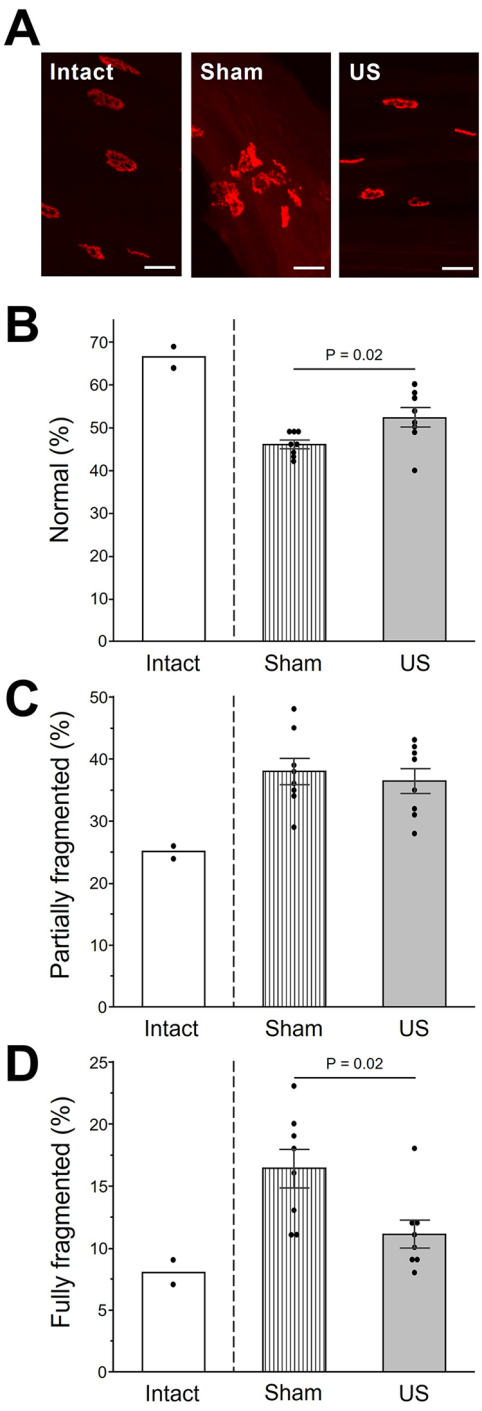

Figure 2. Morphology of motor endplates. (A) representative endplate morphology 4 weeks after the peroneal nerve transection in intact, sham (denervation with sham stimulation), and US (denervation with US stimulation) groups. Scale bar: 20 μm. (B-D) Proportion of normal, partially fragmented, and fully fragmented types in the total number of endplates. Intact group ($n=2$), sham group ($n=8$), US group ($n=8$). Error bars indicate standard error.

percentage: 98.0% in the intact group, 51.3% in the sham group, and 62.5% in the US group, $P=.25$) (Figure 3A and B). For a further detailed examination, the number of tSCs and retention percentage in the normal-type endplates were analyzed, and no significant differences were found between the 2 groups (number of tSCs: 1.1 ± 0.19 in the sham group versus 1.6 ± 0.33 in the US group, $P=.43$; tSC retention percentage: 50.9% in the sham group versus 63.2% in the US group, $P=.26$) (Supplemental Figure 3A and B).

Discussion

The present study demonstrated the effects of US stimulation in an attempt to inhibit changes that occur in the endplate after peripheral nerve injury. Our results revealed that the percentage of endplate degeneration was significantly lower in the US group than in the sham group, confirming that degeneration was suppressed by US stimulation, although tSC number and retention percentage were not significantly different between the US and sham groups.

In the present study, the number of endplates 4 weeks after peroneal nerve transection was higher in both the US and sham groups than in the intact group. Li et al reported that the number of endplates temporarily increased 2 weeks after mouse peroneal nerve transection and decreased after 4 weeks.[9] Similarly, a marked increase in the number of AChRs in the plasma membrane has been reported within 3 days after nerve injury.[17] There is a possibility that the 4 weeks after transection evaluated in this study was the time when endplates temporarily increased before beginning to decrease. Hence, changes in the number of endplates should be investigated over a longer period.

Furthermore, no significant difference in the number of endplates between the US and sham groups was observed. This result suggests that US stimulation does not affect the number of endplates. Various proteins are involved in the formation of NMJs and the accumulation of AChRs. For example, Agrin, secreted from motor nerve endings, binds to its receptor Lrp4, activates MuSK, and forms AChRs in the NMJ.[18-20] The relationship between these proteins and US stimulation has not been reported and requires further investigation.

In this study, degeneration of the endplate of the TA muscle was observed 4 weeks after peroneal nerve transection. Compared to the intact group, the number of mature endplates (normal type) decreased and the number of immature endplates (partially and fully fragmented type) increased in both the US and sham groups. Furthermore, compared to the sham group, the US group showed an inhibition of the decrease in normal endplates and an inhibition of the increase in fully fragmented endplates. These results suggest that the endplate of the TA muscle degenerates due to peroneal nerve transection and that US stimulation may inhibit this degeneration. We hypothesized that US stimulation would promote the proliferation and morphological maintenance of tSCs, which in turn would maintain the morphology of the endplates. However, the number and retention percentage of tSCs did not differ significantly between the US and sham groups. In addition, no significant differences were found in the endplates of the normal type, whose degeneration was suppressed in the US group. These results suggest that factors other than the tSC number and retention percentage may have maintained the endplate morphology in the US group.

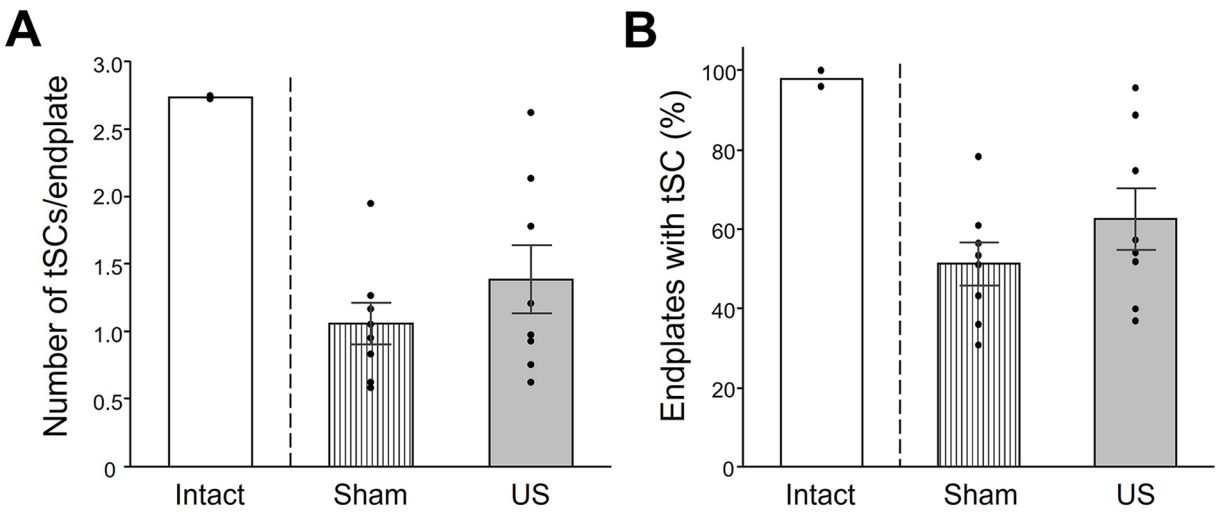

Figure 3. Number of tSCs and percentage of endplates with tSCs: (A) number of tSCs/endplate and (B) percentage of endplates with tSCs. Intact group ($n=2$), sham group ($n=8$), US group ($n=8$). Error bars indicate standard error.

US stimulation has been reported to promote the expression of various growth factors, including fibroblast growth factors (FGF).[21] In particular, Ito et al reported that FGF18, a member of the FGF family, is involved in the maintenance of NMJs. In mice lacking FGF18, the NMJ groove in the diaphragm was reduced, and a simple structure of the NMJ was formed.[22] The relationship between US stimulation and the molecular mechanisms involved in NMJ morphology has been less well reported and requires further investigation.

Many previous studies have shown that US stimulation affects Schwann cells,[11,12,21] which differs from our finding that US stimulation did not affect the tSC number and retention. One possible cause is the influence of US stimulation conditions such as stimulation intensity. Chang and Hsu reported that Schwann cells in artificial nerve conduits were activated at an intensity of 40 mW/cm^2 (SATA).[12] However, there are no reports on the optimal US stimulation intensity for tSCs in endplates, and further investigation is needed.

This study had some limitations. Firstly, due to the limited sample size in the intact group, no meaningful statistical comparisons could be made between the intact and experimental groups. Although such comparisons were not the primary objective of this study, the reader should take this into account when interpreting the results. Second, the study did not elucidate the molecular mechanism by which US stimulation suppresses endplate degeneration. Future studies should not only evaluate tSCs but also examine the effects of US stimulation on growth factor expression and mechanotransduction. Third, the results of this study did not reveal changes in the endplates caused by US stimulation over time. Lastly, even if regenerating axons reach the NMJ while inhibiting its degeneration, it is not clear whether motor function would be improved in the US group compared to the sham group. Experiments using old mice have reported that age-related degeneration of the NMJ cannot be an indicator of impaired neuromuscular transmission,[23,24] and the correlation between NMJ degeneration and motor function remains controversial. However, it has not been tested whether suppressing endplate degeneration after peripheral nerve injury facilitates better motor function recovery and muscle reinnervation. Therefore, it is desirable to evaluate the relationship between changes in the endplates over time, motor function, and nerve reinnervation.

Conclusion

In this study, we examined the effects of US stimulation on the motor endplates following peripheral nerve injury. US stimulation was found to inhibit endplate degeneration. In contrast, under the stimulation conditions of the present study, US stimulation did not affect the number of endplates or tSCs and their retention 4 weeks after peroneal nerve transection.

Author Contributions

Design of the work: AI and YA; acquisition and analysis of data: AI, YA, Hideki K; interpretatin of data: AI YA, Hideki K and Hiroshi K; drafted the article: AI and YA; revised article: Hideki K and Hiroshi K; All authors have read and agreed to the published version of the manuscript.

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Significance Statement

- Ultrasound stimuli inhibited the degeneration of motor endplates in the rat peroneal transection model.
- Our results indicate that this effect is not related to the number of terminal Schwann cells or their retention percentage.

Supplemental Material

Supplemental material for this article is available online.

REFERENCES

1. Kobayashi J, Mackinnon SE, Watanabe O, et al. The effect of duration of muscle denervation on functional recovery in the rat model. *Muscle Nerve*. 1997;20:858-866.
2. Palispis WA, Gupta R. Surgical repair in humans after traumatic nerve injury provides limited functional neural regeneration in adults. *Exp Neurol*. 2017;290:106-114.
3. Vannucci B, Santosa KB, Keane AM, et al. What is normal? Neuromuscular junction reinnervation after nerve injury. *Muscle Nerve*. 2019;60:604-612.
4. Sakuma M, Gorski G, Sheu SH, et al. Lack of motor recovery after prolonged denervation of the neuromuscular junction is not due to regenerative failure. *Eur J Neurosci*. 2016;43:451-462.
5. Lu C-Y, Santosa KB, Jablonka-Shariff A, et al. Macrophage-derived vascular endothelial growth factor-A is integral to neuromuscular junction reinnervation after nerve injury. *J Neurosci*. 2020;40:9602-9616.
6. Barik A, Li L, Sathyamurthy A, Xiong WC, Mei L. Schwann cells in neuromuscular junction formation and maintenance. *J Neurosci*. 2016;36:9770-9781.
7. Fambrough DM, Drachman DB, Satyamurti S. Neuromuscular junction in myasthenia gravis: decreased acetylcholine receptors. *Science*. 1973;182:293-295.
8. Mori S, Koshi K, Shigemoto K. The important role of the neuromuscular junction in maintaining muscle mass and strength. *J Phys Fit Sports Med*. 2014;3:111-114.
9. Li L, Yokoyama H, Kaburagi H, et al. Remnant neuromuscular junctions in denervated muscles contribute to functional recovery in delayed peripheral nerve repair. *Neural Regen Res*. 2020;15:731-738.
10. Marques MJ, Conchello JA, Lichtman JW. From plaque to pretzel: fold formation and acetylcholine receptor loss at the developing neuromuscular junction. *J Neurosci*. 2000;20:3663-3675.
11. Tsuang YH, Liao LW, Chao YH, et al. Effects of low intensity pulsed ultrasound on Rat Schwann cells metabolism. *Artif Organs*. 2011;35:373-383.
12. Chang CJ, Hsu SH. The effects of low-intensity ultrasound on peripheral nerve regeneration in poly(DL-lactic acid-co-glycolic acid) conduits seeded with Schwann cells. *Ultrasound Med Biol*. 2004;30:1079-1084.
13. Ito A, Wang T, Nakahara R, et al. Ultrasound therapy with optimal intensity facilitates peripheral nerve regeneration in rats through suppression of pro-inflammatory and nerve growth inhibitor gene expression. *PLoS One*. 2020;15:e0234691.
14. Rasband WS. *Image J*. National Institutes of Health; 1997-2012. Accessed June 7, 2022. http://imagej.nih.gov/ij/
15. Schneider CA, Rasband WS, Eliceiri KW. NIH image to ImageJ: 25 years of image analysis. *Nat Methods*. 2012;9:671-675.
16. Snyder-Warwick AK, Satoh A, Santosa KB, Imai SI, Jablonka-Shariff A. Hypothalamic sirt1 protects terminal Schwann cells and neuromuscular junctions from age-related morphological changes. *Aging Cell*. 2018;17:e12776.
17. Merlie JP, Isenberg KE, Russell SD, Sanes JR. Denervation supersensitivity in skeletal muscle: analysis with a cloned cDNA probe. *J Cell Biol*. 1984;99:332-335.
18. McMahan UJ, Horton SE, Werle MJ, et al. Agrin isoforms and their role in synaptogenesis. *Curr Opin Cell Biol*. 1992;4:869-874.
19. Kim N, Stiegler AL, Cameron TO, et al. Lrp4 is a receptor for agrin and forms a complex with MuSK. *Cell*. 2008;135:334-342.
20. Zhang B, Luo S, Wang Q, Suzuki T, Xiong WC, Mei L. Lrp4 serves as a coreceptor of agrin. *Neuron*. 2008;60:285-297.
21. Ren C, Chen X, Du N, et al. Low-intensity pulsed ultrasound promotes Schwann cell viability and proliferation via the GSK-3β/β-catenin signaling pathway. *Int J Biol Sci*. 2018;14:497-507.
22. Ito K, Ohkawara B, Yagi H, et al. Lack of FGF18 causes abnormal clustering of motor nerve terminals at the neuromuscular junction with reduced acetylcholine receptor clusters. *Sci Rep*. 2018;8:434.
23. Willadt S, Nash M, Slater CR. Age-related fragmentation of the motor endplate is not associated with impaired neuromuscular transmission in the mouse diaphragm. *Sci Rep*. 2016;6:24849.
24. Willadt S, Nash M, Slater C. Age-related changes in the structure and function of mammalian neuromuscular junctions. *Ann N Y Acad Sci*. 2018;1412:41-53.