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**14. ABSTRACT**

During the past year, we conducted experiments on animals in the 3-month delayed reinnervation groups (i.e., Del-NN, Del-ENF/NN, Del-SP/NN, Del-ANG, and Del-EEA) and 6-month denervation control. Three months after treatment, the extent of functional recovery of the treated TA muscles was examined by measuring toe spread distances and maximal muscle force. The extent of neuromuscular recovery was examined by quantifying regenerated axons, reinnervated motor endplates (MEPs), muscle weight, and denervated/atrophied muscle fibers. Our results showed that Del-ENF/NN and Del-SP/NN resulted in better muscle force recovery (72% and 70% of the control, respectively) than Del-NN alone (59%), Del-ANG (38%) and Del-EEA (43%). Tissue studies showed that Del-ENF/NN and Del-SP/NN resulted in better axonal regeneration, MEP reinnervation, and muscle mass preservation as compared with other delayed reinnervation groups used in this research. These findings suggest that focal injection of ENF and SP procedure are useful therapies for delayed muscle reinnervation.

**15. SUBJECT TERMS** Peripheral nerve injury, limb reinnervation, nerve-muscle-endplate grafting (NMEG), native motor zone (NMZ), axonal regeneration, axon-endplate connection, functional recovery

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## 1. INTRODUCTION:

Peripheral nerve injury (PNI) affects approximately 200,000 Americans each year. Muscle paralysis caused by PNI is usually managed by nerve repair, nerve grafting, nerve transfer, neuromuscular neurotization, tubulization techniques, and many others. Unfortunately, these methods result in poor functional recovery. Therefore, there is a pressing need to develop novel strategies for treating denervated muscle following PNI.

This project aims to determine the effectiveness of our newly developed surgical technique called "nerve-muscle-endplate grafting in the native motor zone (NMEG-NMZ)" for immediate and delayed limb muscle reinnervation. The outcomes of NMEG-NMZ were compared with those of standard end-to-end anastomosis (EEA) and autologous nerve graft (ANG) as well as denervation without treatment.

Our studies have demonstrated that NMEG-NMZ technique is feasible for limb reinnervation (*Mu L, et al. Limb muscle reinnervation with the nerve-muscle-endplate grafting technique: An anatomical feasibility study. Neurol Res Int 2021: Article ID 6009324, 7 pages*). Specifically, the tibialis anterior (TA) muscle denervated by resecting a segment of its innervating nerve (peroneal) can be reinnervated with a NMEG-NMZ from the lateral gastrocnemius muscle (GM-l). An NMEG pedicle containing a block of muscle (~8×6×4 mm), axon terminals, and a motor endplate (MEP) band with neuromuscular junctions was taken from the NMZ of the GM-l in continuity with its nerve branch. The efficacy of the NMEG-NMZ technique in limb reinnervation was examined using immediate and 3-month delayed reinnervation models. Three months after surgery, functional recovery of the TA treated with the NMEG-NMZ technique was evaluated by muscle force measurement and static toe spread analysis. Axonal regeneration, MEP reinnervation, and muscle mass were also evaluated.

Our experiments showed that in immediate reinnervation model NMEG-NMZ resulted in much better functional recovery (79% of the control) as compared with commonly used EEA (51%) (*Sobotka S, et al. Reinnervation of paralyzed limb muscle by nerve-muscle-endplate grafting technique. Neurosurgery 92:1091-1098, 2023.*). We also studied the beneficial effects of focal application of exogenous neurotrophic factors (ENF) (i.e., NGF/FGF-2) on the outcomes of NMEG-NMZ (NN). We found that NN/ENF resulted in 90% muscle force recovery of the treated TA, which is far superior to ANG (46%) and NMEG-NMZ surgery alone (79%) (*Mu L, et al. Focal application of neurotrophic factors augments outcomes of nerve-muscle-endplate grafting technique for limb muscle reinnervation. Journal of Reconstructive Microsurgery 2023, in press*). The average wet muscle weight was 87% and 52% of the control for muscles treated with NN/ENF and ANG, respectively. The mean number of the regenerated axons was 88% of the control for the muscles treated with NN/ENF, which was significantly larger than that for the ANG repaired muscles (39%). The average percentage of the reinnervated MEPs in the TA treated with NN/ENF (89%) was higher as compared with that in the ANG repaired TA (48%). These findings indicate that ENF enhances nerve regeneration and MEP reinnervation that further augment outcomes of NMEG-NMZ.

This final annual report represents the data from rats in the 3-month delayed reinnervation groups and provides a summary of the major findings from this project. Overall, NMEG-NMZ alone could restore muscle function (59% of the control) of the 3-month delayed treatment, but less well than immediate reinnervation with NMEG-NMZ (79%). However, we found that focal injection of NGF/FGF-2 and sensory protection (SP) have the potential to preserve muscle mass and MEPs, thereby improving outcomes of NMEG-NMZ for 3-month delayed reinnervation as described below.

## 2. KEYWORDS:

Peripheral nerve injury, limb reinnervation, nerve-muscle-endplate grafting (NMEG), native motor zone (NMZ), axonal regeneration, axon-endplate connection, functional recovery

## 3. ACCOMPLISHMENTS:

**What were the major goals of the project?**

**Aim 1:** Evaluate the efficacy of NMEG-NMZ for immediate reinnervation of denervated limb muscle, and determine the beneficial effects of nerve growth-stimulating methods (i.e., intraoperative brief ES and local administration of the ENFs NGF and FGF-2) on axonal regeneration and outcomes of NMEG-NMZ surgery. (100% completed).

**Aim 2:** Evaluate the efficacy of NMEG-NMZ for reinnervation of chronically denervated limb muscle, and assess the potential of specific therapeutic strategies for preservation of muscle mass and MEPs prior to NMEG-NMZ. (100% completed).

This research is to test our hypothesis that NMEG-NMZ technique would be effective for reinnervation of denervated limb muscle, and that the outcomes of NMEG-NMZ could be augmented by incorporating specific therapeutic strategies that enhance nerve regeneration as well as preserve muscle mass and MEPs in the target muscle. For NMEG-NMZ, we harvested an NMEG pedicle from lateral belly of the gastrocnemius muscle (GM-l) to reinnervate the experimentally denervated ipsilateral tibialis anterior (TA) muscle. Adjunctive therapies used in this research include intraoperative brief electrical stimulation (ES) and local application of exogenous neurotrophic factors (ENF). For comparison, we used standard nerve end-to-end anastomosis (EEA) and autologous nerve graft (ANG) as technique controls. We also use 3-month and 6-month denervation as controls.

**(a) Reinnervation models** include immediate and 3-mo delayed. Immediate reinnervation is performed immediately after TA nerve transection, while delayed reinnervation is carried out at the end of 3 months after TA nerve transection.

**(b) Animal groups:** The animals are randomly assigned to 12 groups (15 rats/per group), 6 immediate and 6 delayed reinnervation groups.

**Immediate (Imm) reinnervation groups:** (1) Imm-NMEG-NMZ (Imm-NN); (2) Imm-NN/ES; (3) Imm-NN/ENF; (4) Imm-EEA; (5) Imm-ANG; and (6) 3-month denervation.

**Delyed (Del) reinnervation groups:** (1) Del-NN; (2) Del-ENF/NN; (3) Del-sensory protection/NN (SP/NN); (4) Del-EEA; (5) Del-ANG; and (6) 6-month denervation.

**(c) Post-operative evaluations:** The experimental animals undergo post-operative evaluations after a 3-month recovery period. The post-operative evaluations include functional assessment (i.e., static toe spread analysis and muscle force measurement), neural studies (i.e., axonal regeneration and axon-MEP connections), and muscle analyses (i.e., muscle mass, fiber size, fiber type and myosin heavy chain composition). The data from this research are expected to provide evidence for the effectiveness of the NMEG-NMZ and adjunctive therapies for limb reinnervation.

**What was accomplished under these goals?**

► **Major Activities**

In the past year, we performed the following major activities.

● **Animal surgeries**

We performed 135 operations on 45 rats (3 operations/per rats) in 3 delayed reinnervation groups (15 rats/per group) that include: (1) Del-SP/NN; (2) Del-ANG; and (3) Del-EEA. In the delayed reinnervation groups, all treatments were given three months after denervation of the left TA. These surgical procedures were successfully performed.

● **Behavior motor functional evaluation**

Before muscle force measurement, all the experimental rats underwent static toe spread analysis to assess behavior motor functional recovery of the hind limbs. Static toe spread analysis was performed just before muscle force measurement at the end of the 3-month recovery period. For this functional testing, the rat was placed in an acrylic 40×20×20 cm container on a transparent base plate for observing footprints on the plantar view. A camera was positioned underneath the base plate to photograph the plantar surface of the rat hind limb paws. Images from the operated (O) and non-operated (N) footprints were randomly selected to measure the distance (mm) between the first and fifth toe spread (TS) and between the second and fourth intermediate toe spread (ITS). The mean values of TS and ITS in each group were computed and used for determining the TS and ITS factors (TSF and ITSF): TSF = (OTS – NTS)/NTS and ITSF = (OITS – NITS)/NITS. The ratios of the static sciatic index (SSI) were calculated using the following equation: SSI = (108.44 × TSF) + (31.85 × ITSF)–5.49. An index score of 0 is defined as normal and an index score of −100 indicates a complete functional loss.

● **Muscle force measurements**

Three months after muscle reinnervation, all animals underwent muscle force measurement to evaluate the extent of muscle force recovery. The force data from animals in Del-NN and Del-ENF/NN groups were collected in Y2 and analyzed in Y3. The force data for Del-SP/NN, Del-ANG and DeL-EEA groups were collected and analyzed in Y3. All data are summarized below (*see Key Outcomes*).

● **Tissue studies**

After muscle force measurement, the left experimental TA and right control for each rat were removed, measured, and prepared for tissue studies. The muscle samples were sectioned and stained using histological and immunohistochemical techniques. The major findings regarding muscle structure, axonal regeneration, and MEP reinnervation are summarized below (*see Key Outcomes*).

► **Specific Objectives for Delayed Reinnervation**

**Objective 1 –** Determine the degree of functional recovery of 3-month delayed (Del) reinnervation of the TA muscles treated by Del-NN, Del-ENF/NN, Del-SP/NN, Del-ANG, and Del-EEA.

**Objective 2 –** Determine the extent of axonal regeneration and axon-MEP connections in the TA muscles treated by 3-month delayed reinnervation.

**Objective 3 –** Document morphological and histological changes (i.e., muscle mass, fiber size, and fiber type and myosin heavy chain composition) in the delayed treated TA muscles.

► **Major Procedures**

The procedures for Del-NN and 6-mon-Den have been reported in Y2 annual report. Here, the procedures for Del-ENF/NN, Del-SP/NN, Del-ANG, and Del-EEA are given below.

● **Del-ENF injection/NN**

The rats in Del-ENF/NN group (n =15) received local injection of ENFs (i.e., NGF/FGF-2). Specifically, immediately after left TA denervation by resecting a 10-mm segment of its nerve, the NMZ of the denervated TA was injected with 0.5 ml of a mixture of NGF (100 ng/ml) and FGF-2 (100 μg/ml) biweekly for 3 months (**Fig. 1**), followed by NMEG-NMZ.

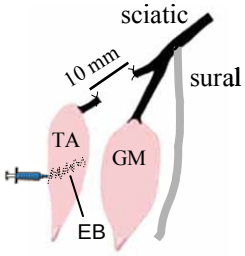



**Fig. 1.** Schematic showing injection of NGF/FGF-2 into the endplate band (EB) in the denervated TA muscle.

● **Del-SP/NN**

Immediately after transecting the left TA and sural nerves, the proximal end of the sural (sensory) nerve was anastomosed with the distal end of the transected TA nerve (i.e., sensory protection, SP) for three months to preserve muscle mass (**Fig. 2**), and then the sensory protected TA was reinnervated with NMEG-NMZ. The details regarding NMEG-NMZ procedures have been given in Y1 annual report.

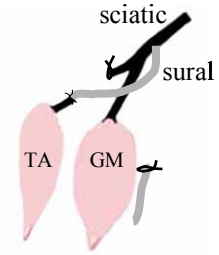



**Fig. 2.** Schematic showing sensory protection of the denervated TA muscle by suturing the proximal stump of the divided sural nerve to the distal stump of the TA nerve.

● **Del-ANG**

Del-ANG was used as a technique control. Three months after left TA nerve transaction, a 10-mm segment of the TA nerve was resected and the gap defect was bridged with left sural nerve (**Fig. 3**). Three months after ANG, the rats in this group underwent muscle force measurement and then the TA muscles on both sides were removed and prepared for tissue studies.

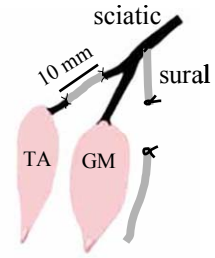



**Fig. 3.** Schematic of ANG procedure. Note that a 10-mm segment of the TA nerve gap defect is bridged with the same length of sural nerve.

**● Del-EEA**

Del-EEA was used as a technique control. Three months after TA nerve transection, both ends of the transected TA nerve were anastomosed to reinnervate the denervated muscle.

**► Key Outcomes**

The results from the rats in immediate reinnervation groups have been presented in Y1 and Y2 annual reports and the major findings have been published in scientific journals.

The data from the rats in 3-month delayed reinnervation groups have been collected, analyzed, and summarized below.

**● Functional Recovery**

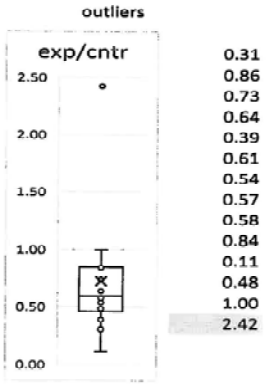
**─ Maximal muscle force:** The force data from the animals in Del-NN, Del-ENF injection/NN, Del-SP/NN, Del-ANG, and Del-EEA are given below.

Our studies have shown that NMEG-NMZ results in 79% muscle force recovery of the immediately reinnervated TA muscle, which is far superior to commonly used nerve repair methods. Unfortunately, muscle force recovery of NMEG-NMZ when it is delayed by 3 months falls to 59% due to muscle atrophy and MEP degeneration (**Fig. 4**). The proposed research aims to improve the success of NMEG-NMZ to treat 3-month delayed limb reinnervation by testing different treatment combinations such as Del-ENF injection/NN and Del-SP/NN. Del-ENF/NN and Del-SP/NN resulted in 72% and 70% force recovery, respectively. These results are better than those obtained from Del-EEA (43%) and Del-ANG (38%).

| | | musle force | |
|---|---|---|---|
| rat# | 3m_NMEG cnt | | exp/cntr |
| 1 | 3 | 9.79 | 0.31 |
| 2 | 5 | 5.8 | 0.86 |
| 3 | 5.4 | 7.4 | 0.73 |
| 4 | 5.23 | 8.21 | 0.64 |
| 5 | 3.04 | 7.89 | 0.39 |
| 6 | 5.33 | 8.72 | 0.61 |
| 7 | 1.86 | 3.47 | 0.54 |
| 8 | 3.22 | 5.65 | 0.57 |
| 9 | 3.02 | 5.25 | 0.58 |
| 10 | 3.1 | 3.71 | 0.84 |
| 11 | 0.985 | 8.65 | 0.11 |
| 13 | 4.5 | 9.342 | 0.48 |
| 14 | 6.25 | 6.27 | 1.00 |
| average | **3.841** | **6.935** | **0.588** |
| std | 1.560 | 2.071 | 0.239 |
| median | 3.22 | 7.4 | 0.58 |
| SE | 0.450 | 0.598 | 0.069 |

#15 no record
#12 outlier of exp/cntr

z = 3.1798. p=0.00148
Wilcoxon signed-rank test




| rate of forces exp/cnt |
|---|
| 0.31 |
| 0.86 |
| 0.73 |
| 0.64 |
| 0.39 |
| 0.61 |
| 0.54 |
| 0.57 |
| 0.58 |
| 0.84 |
| 0.11 |
| 0.48 |
| 1.00 |
| 2.42 |

**Fig. 4.** Maximal muscle force from operated and control TA muscles of the rats in the Del-NN group. Average maximal muscle force level on the operated side was 59% of muscle force on the control side (p=0.00148).

— **Static toe spread analysis (STSA):** STSA was used for assessing limb function after treatments. Our data showed that after muscle denervation ENF injection into the NMZ (Del-ENF/NN) and Del-SP for 3 months and followed by NN resulted in better toe spread recovery (76% and 73% of the control, respectively) (**Fig. 5**) as compared with Del-NN alone (65%), Del-EEA (50%), and Del-ANG (46%), respectively.

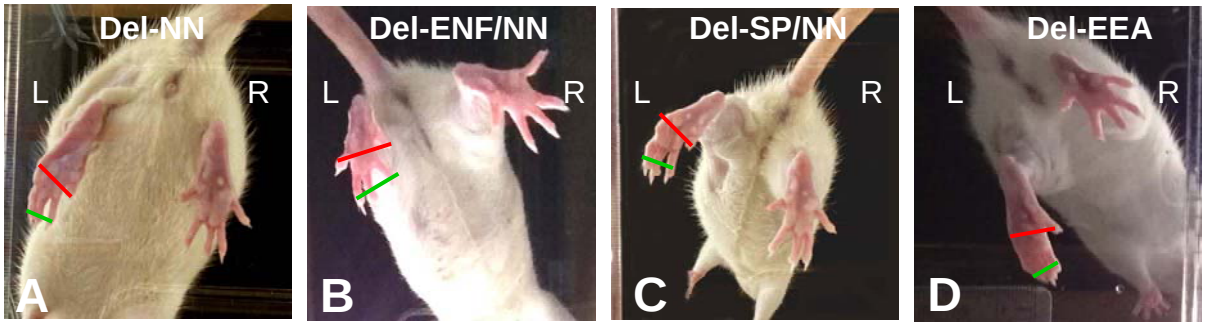



**Fig. 5.** Comparison of hind limb footprints between left (L) operated and right (R) non-operated sides in the rats with Del-NN (**A**), Del-ENF/NN (**B**), Del-SP/NN (**C**), and Del-EEA (**D**). Note that Del-ENF/NN resulted in better toe spread recovery as compared with other treatments.

● **Muscle Recovery**

The TA muscles on both sides were removed and weighed at the end of experiments for each rat. Our results showed that Del-ENF/NN and Del-SP/NN resulted in better muscle weight recovery (83% and 78% of the control, respectively) (**Table 1**) as compared with Del-NN alone (71%) (**Table 2**), and much better than Del-EEA (58%), Del-ANG (42%), and 6-month denervation (26%).

**Table 1.** Wet weight of TA muscles for Del-ENF/NN group (n = 15)

| Animal no. | Body weight, g | Left TA g | Right TA g | Ratio L/R |
|---|---|---|---|---|
| 1 | 418 | 0.54 | 0.65 | 0.83 |
| 2 | 356 | 0.37 | 0.66 | 0.56 |
| 3 | 341 | 0.56 | 0.65 | 0.86 |
| 4 | 431 | 0.64 | 0.67 | 0.96 |
| 5 | 360 | 0.54 | 0.61 | 0.89 |
| 6 | 355 | 0.73 | 0.62 | 1.18 |
| 7 | 399 | 0.58 | 0.69 | 0.84 |
| 8 | 360 | 0.48 | 0.58 | 0.83 |
| 9 | 399 | 0.64 | 0.72 | 0.89 |
| 10 | 333 | 0.66 | 0.80 | 0.83 |
| 11 | 445 | 0.42 | 0.60 | 0.70 |
| 12 | 402 | 0.46 | 0.63 | 0.73 |
| 13 | 402 | 0.47 | 0.68 | 0.69 |
| 14 | 507 | 0.58 | 0.65 | 0.89 |
| 15 | 475 | 0.54 | 0.69 | 0.78 |
| Average | 399 | 0.55 | 0.66 | **0.83** |

L, left; R, right.

**Table 2.** Wet weight of TA muscles for Del-NN group (n = 14)

| Animal no. | Body weight, g | Left TA g | Right TA g | Ratio L/R |
|---|---|---|---|---|
| 1 | 395 | 0.53 | 0.75 | 0.71 |
| 2 | 419 | 0.64 | 0.82 | 0.78 |
| 3 | 493 | 0.60 | 0.88 | 0.68 |
| 4 | 409 | 0.52 | 0.62 | 0.84 |
| 5 | 431 | 0.55 | 0.61 | 0.90 |
| 6 | 378 | 0.42 | 0.69 | 0.61 |
| 7 | 415 | 0.38 | 0.69 | 0.55 |
| 8 | 372 | 0.48 | 0.67 | 0.72 |
| 9 | 395 | 0.46 | 0.65 | 0.71 |
| 10 | 397 | 0.46 | 0.69 | 0.67 |
| 11 | 367 | 0.57 | 0.65 | 0.88 |
| 12 | 319 | 0.45 | 0.67 | 0.67 |
| 13 | 350 | 0.46 | 0.74 | 0.62 |
| 14 | 349 | 0.42 | 0.65 | 0.65 |
| Average | 392 | 0.50 | 0.70 | **0.71** |

L, left; R, right.

Figure 6 shows gross appearance and muscle volumes of the treated TA muscles and contralateral controls in Del-NN (**Fig. 6A**), Del-ENF/NN (**Fig. 6B**), Del-SP/NN (**Fig. 6C**), and 6-month-Den (**Fig. 6D**) groups. H&E stained cross-sections showed that the TA muscles treated with Del-ENF/NN (**Fig. 6B'**) and Del-SP/NN (**Fig. 6C'**) exhibited better preservation of muscle structure and myofiber morphology with less fiber atrophy and connective tissue hyperplasia as compared with Del-NN (**Fig. 6A'**), Del-ANG and Del-EEA treated TA (data not shown) as well as 6-month denervated TA (**Fig 6D'**). These findings demonstrated that prior NMEG-NMZ (NN) surgery ENF injection into the NMZ and SP treatments could maintain muscle mass to certain levels. The combination of the NMEG-NMZ surgery with the ENF and/or SP resulted in better preservation of the muscle mass as compared with Del-NN alone and commonly used ANG and EEA.

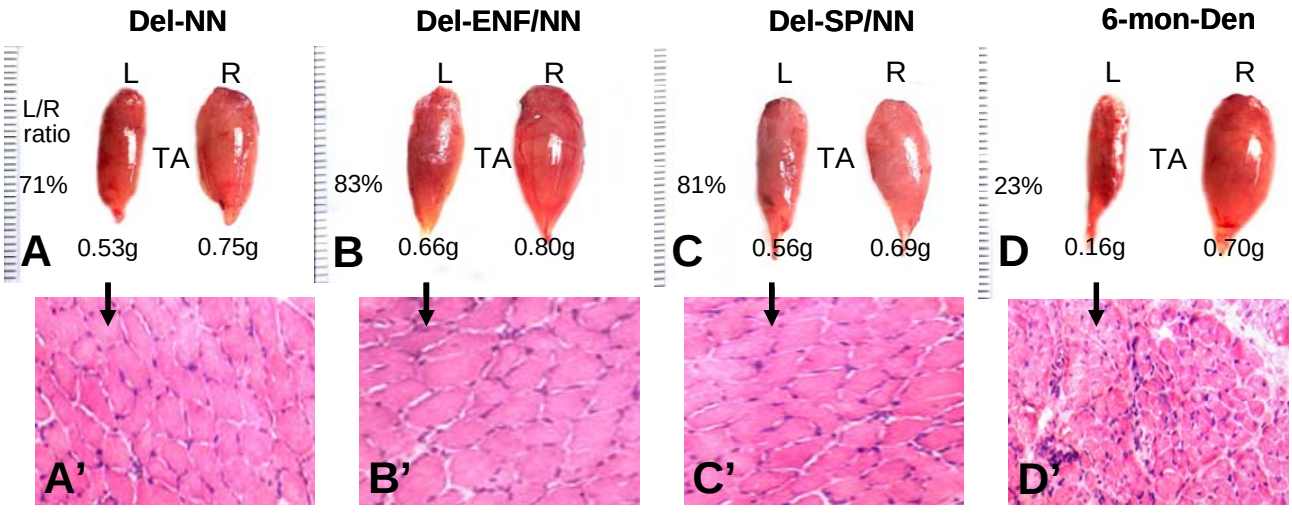



**Fig. 6.** Muscle masses and myofiber morphology of the rat left (L) TA muscles treated with Del-NN (**A**), Del-ENF/NN (**B**), Del-SP/NN (**C**) and 6-mon-Den (**D**). Note that the muscle masses of the left TA muscles treated with Del-ENF/NN and Del-SP/NN are larger than those treated with Del-NN alone and 6-month denervation. H&E stained cross sections from the left treated muscles (**A'-D'**) showed that Del-ENF/NN (**B'**) and Del-SP/NN (**C'**) treated muscles exhibited better preservation of muscle structure with less fiber atrophy as compared with Del-NN (**A'**) and 6-month denervation (**D'**).

● **Axonal Regeneration**

Neurofilament (NF) staining of the muscle sections showed intramuscular regenerated axons in the TA muscles treated with Del-NN (**Fig. 7A**), Del-ENF/NN (**Fig. 7B**), Del-SP/NN (**Fig. 7C**), Del-ANG (**Fig. 7D**), and Del-EEA (data not shown).

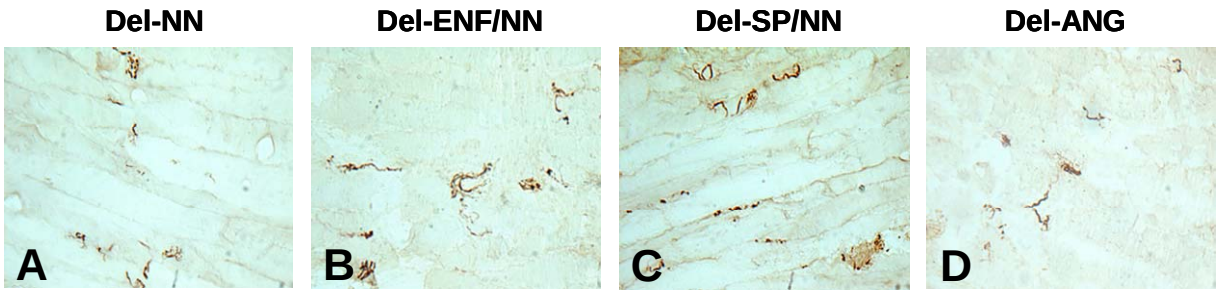



**Fig. 7.** Photomicrographs of muscle sections immunostained for NF from the left TA muscles treated with Del-NN (**A**), Del-ENF/NN (**B**), Del-SP/NN (**C**) and Del-ANG (**D**), showing regenerated axons (darkly stained threads and dots) in the treated muscles.

Our studies showed that Del-ENF/NN and Del-SP/NN resulted in better axonal regeneration as compared with Del-NN alone and technique controls (i.e., Del-ANG and Del-EEA). Del-ANG and Del-EEA resulted in poor axonal regeneration. The intramuscular axonal density (**Table 3**) was assessed by measuring the number of the NF-immunoreactive (NF-ir) axons and the area fraction of the axons within a section area (1.0-mm$^2$) with public domain ImageJ software (v. 1.45s; NIH, Bethesda, Maryland).

**Table 3.** Count and %area of regenerated axons in the left tibialis anterior (TA) treated with Del-ENF/NN and right control muscles in rats (n = 15)

| Animal no. | Left TA | | Right TA | | Ratio (L/R) | |
|---|---|---|---|---|---|---|
| | Count | %Area | Count | %Area | Count | %Area |
| 1 | 625 | 0.599 | 936 | 0.923 | 0.668 | 0.649 |
| 2 | 582 | 0.556 | 837 | 0.915 | 0.695 | 0.608 |
| 3 | 729 | 0.694 | 977 | 0.896 | 0.746 | 0.775 |
| 4 | 578 | 0.633 | 931 | 0.879 | 0.621 | 0.720 |
| 5 | 882 | 0.895 | 899 | 0.916 | 0.981 | 0.977 |
| 6 | 669 | 0.729 | 925 | 0.983 | 0.723 | 0.742 |
| 7 | 524 | 0.638 | 798 | 0.826 | 0.657 | 0.772 |
| 8 | 498 | 0.555 | 1026 | 0.987 | 0.485 | 0.562 |
| 9 | 609 | 0.593 | 884 | 0.913 | 0.689 | 0.650 |
| 10 | 713 | 0.696 | 948 | 0.896 | 0.752 | 0.777 |
| 11 | 523 | 0.502 | 823 | 0.828 | 0.635 | 0.606 |
| 12 | 489 | 0.548 | 981 | 0.955 | 0.498 | 0.574 |
| 13 | 510 | 0.576 | 992 | 0.959 | 0.514 | 0.601 |
| 14 | 522 | 0.619 | 899 | 0.988 | 0.581 | 0.627 |
| 15 | 623 | 0.703 | 944 | 0.911 | 0.660 | 0.772 |
| Average | 605 | 0.636 | 920 | 0.918 | **0.660** | 0.694 |

L = left; R = right.

The mean count of regenerated axons in the TA muscles treated with Del-ENF/NN is shown in **Table 3**. Individual data from other groups are not shown. Our studies showed that Del-ENF/NN and Del-SP/NN resulted in significantly more regenerated axons (66% and 60% of the control, respectively) as compared with Del-NN (55%), Del-EEA (36%) and Del-ANG (28%) and. These findings suggest that focal injection of NEFs (i.e., NGF/FGF-2) and SP procedure have the potential for enhancing axonal regeneration for delayed muscle reinnervation.

● **Innervated MEPs**

Double fluorescence staining was used to label innervated (with visible axon attachments) and non-innervated (without visible axon attachments) MEPs in the treated and control TA muscles. Our results showed that the average percentage of the innervated MEPs in the Del-ENF/NN (77%) and Del-SP/NN (72%) was higher as compared with that in the Del-NN (69%), Del-EEA (46%), and Del-ANG (34%) treated TA muscles (**Fig. 8**). These findings are consistent with the extent of functional recovery, suggesting that reinnervation of the denervated MEPs is important for functional restoration.

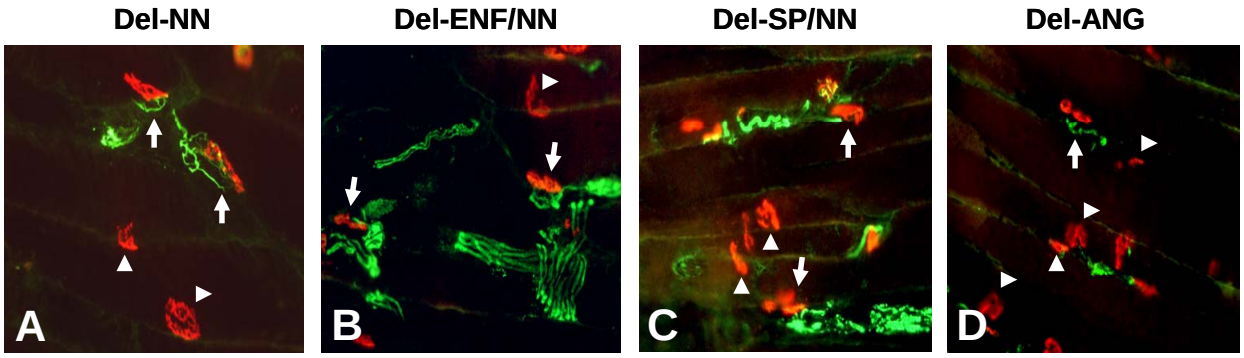



**Fig. 8.** Immunostained sections of the TA muscles treated with Del-NN (**A**), Del-ENF/NN (**B**), Del-SP/NN (**C**), and Del-ANG (**D**), showing MEPs (red) and their innervating axons (green). Arrows indicate innervated MEPs with visible axon attachments, while arrowheads indicate non-innervated MEPs without visible axon attachments. 200x.

Our studies on the 3-month delayed reinnervation demonstrated that NMEG-NMZ resulted in better outcomes as compared with the commonly used EEA and ANG nerve repair methods. However, the outcomes of the NMEG-NMZ technique for 3-month delayed reinnervation were less optimal as compared with those of immediate NMEG-NMZ reinnervation. This is due, at least in part, to the denervation-induced muscle and MEP loss. For optimal outcomes of NMEG-NMZ and other reinnervation methods, more work is needed to reveal specific strategies to rescue muscle from atrophy and prevent MEP degradation following nerve injuries. Our studies showed that NGF/FGF-2 focal injection to the NMZ of the denervated muscle or sensory protection (SP) has the potential to maintain muscle mass and MEPs. Preserving muscle mass and MEPs after muscle denervation is critical for subsequent successful NMEG-NMZ reinnervation. Our findings suggest that focal application of NGF/FGF-2 and SP procedure could maintain muscle mass and MEPs, thereby improving the outcomes of the NMEG-NMZ in 3-month delayed reinnervation.

► **Project Summary**

This project was proposed to determine the efficacy of the NMEG-NMZ (NN) technique for immediate (Imm) and 3-month delayed (Del) reinnervation of denervated TA limb muscle. We also studied the beneficial effects of adjunct therapies (i.e., focal application of NGF/FGF-2, ES and SP) on the outcomes of NMEG-NMZ. **Table 4** below summarizes the key findings from work conducted over the entire research period.

**Table 4.** Extent of functional and neuromuscular recovery of the treated TA muscles in rats.

| Groups | Muscle force recovery (%) | Muscle weight L/R ratio | Axon counts L/R ratio | Innervated MEPs (%) |
|---|---|---|---|---|
| Imm-NN | 79 | 0.86 | 0.76 | 83 |
| Imm-NN/ENF | 90 | 0.87 | 0.88 | 89 |
| Imm-NN/ES | 84 | 0.85 | 0.83 | 85 |
| Imm-EEA | 51 | 0.71 | 0.46 | 59 |
| Imm-ANG | 46 | 0.52 | 0.39 | 48 |
| 3-month Den | – | 0.26 | – | – |
| Del-NN | 59 | 0.71 | 0.55 | 69 |
| Del-ENF/NN | 72 | 0.83 | 0.66 | 77 |
| Del-SP/NN | 70 | 0.78 | 0.60 | 72 |
| Del-EEA | 43 | 0.58 | 0.36 | 46 |
| Del-ANG | 38 | 0.42 | 0.28 | 34 |
| 6-month Den | – | 0.26 | – | – |

● **Conclusions**

Our studies allow us to make the following conclusions.

First, *immediate* reinnervation procedures (i.e., Imm-NN, Imm-NN/ENF, Imm-NN/ES, Imm-ANG, and Imm-EEA) resulted in better functional and neuromuscular recovery as compared with *delayed* reinnervation methods (i.e., Del-NN, Del-ENF injection/NN, Del-SP/NN, Del-ANG, and Del-EEA).

Second, Imm-NN and Del-NN resulted in more favorable outcomes compared to commonly used Imm- and Del-EEA and ANG, respectively. The NMEG-NMZ could be an alternative option to manage denervated limb muscles caused by traumatic nerve injuries.

Third, the outcomes of NMEG-NMZ reinnervation technique can be augmented by adjunct therapies (i.e., ENF, SP and ES). After muscle denervation, focal injection of NGF/FGF-2 into the NMZ or sensory protection (SP) for three months could preserve muscle mass and MEPs that favor subsequent delayed NMEG-NMZ reinnervation and improve outcomes.

Finally, more work is warranted to determine the effectiveness of the combined strategy (i.e., NMEG-NMZ plus adjunct therapies) for long-term (≥ 6 months) delayed limb reinnervation.

Overall, the delayed muscle reinnervation is a major clinical challenging issue. Our further studies will focus on improving outcomes of the NMEG-NMZ technique for long-term (≥ 6 months) delayed muscle reinnervation.

**What opportunities for training and professional development has the project provided?**

Nothing to report.

**How were the results disseminated to communities of interest?**

Nothing to report.

**What do you plan to do during the next reporting period to accomplish the goals?**

This is the final report.

***4.*** **IMPACT:**

**What was the impact on the development of the principal discipline(s) of the project?**

Analyses of the data obtained from animals with 3-month delayed reinnervation showed that SP and NGF/FGF-2 injection into the NMZ of denervated muscle could preserve muscle mass and MEPs that help improve outcomes of subsequent NMEG-NMZ surgery.

**What was the impact on other disciplines?**

Nothing to report.

**What was the impact on technology transfer?**

Nothing to report.

**What was the impact on society beyond science and technology?**

Nothing to report.

## 5. CHANGES/PROBLEMS:

**Changes in approach and reasons for change**

Nothing to report.

**Actual or anticipated problems or delays and actions or plans to resolve them**

Nothing to report.

**Changes that had a significant impact on expenditures**

Nothing to report.

**Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

**Significant changes in use or care of human subjects**

Nothing to report.

**Significant changes in use or care of vertebrate animals**

Nothing to report.

**Significant changes in use of biohazards and/or select agents**

Nothing to report.

**6. PRODUCTS:**

- **Publications, conference papers, and presentations**

**Journal publications.**

Three papers resulting from the work under this award have been published as shown below.

(1) Mu L, Chen J, Li J, Sobotka S, Nyirenda T. Limb muscle reinnervation with nerve-muscle-endplate grafting technique: An anatomical feasibility study. Neurology Research International 2021, Article ID 6009342, 7 pages, https://doi.org/10.1155/2021/6009342
acknowledgement of federal support (yes).

(2) Sobotka S, Mu L, Chen J, Li J, Nyirenda T. Reinnervation of paralyzed limb muscle by nerve-muscle-endplate grafting technique. Neurosurgery 92:1091-1098, 2023.
acknowledgement of federal support (yes).

(3) Mu L, Chen J, Sobotka S, Li J, Nyirenda T. Focal application of neurotrophic factors augments outcomes of nerve-muscle-endplate grafting technique for limb muscle reinnervation. Journal of Reconstructive Microsurgery (in press), 2023.
acknowledgement of federal support (yes).

**Books or other non-periodical, one-time publications.**

Nothing to report.

**Other publications, conference papers and presentations**.

Nothing to report.

- **Website(s) or other Internet site(s)**

Nothing to report.

- **Technologies or techniques**

Nothing to report.

- **Inventions, patent applications, and/or licenses**

Nothing to report.

- **Other Products**

Nothing to report.

## 7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

**What individuals have worked on the project?**

| | | |
|---|---|---|
| Liancai Mu | PI | (6 cal. mos) He has performed animal surgeries, data interpretation, and written manuscripts and reports. |
| Jingming Chen | Research Associate | (12 cal. mos) She has performed animal surgeries, tissue staining, and data collection. |
| Jing Li | Research Tech | (7.2 cal. mos) He has performed tissue photographing, image analysis, and data collection. |
| Stanislaw Sobotka | Associate Scientist | (2.4 cal. mos) He has performed electrophysiological recordings, data analyses, and manuscript writing. |
| Themba Nyirenda | Biostatistician | (0.6 cal. mos) He has performed data analyses and involved in manuscript preparation. |

**Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Nothing to report.

**What other organizations were involved as partners?**

Nothing to report.

## 8. SPECIAL REPORTING REQUIREMENTS

**COLLABORATIVE AWARDS: QUAD CHARTS:**

A quad chart has been updated and submitted with attachments.

## 9. APPENDICES:

**Publications**

(1) Mu L, Chen J, Li J, Sobotka S, Nyirenda T. Limb muscle reinnervation with nerve-muscle-endplate grafting technique: An anatomical feasibility study. Neurology Research International 2021, Article ID 6009342, 7 pages, https://doi.org/10.1155/2021/6009342
Acknowledgement of federal support (yes).

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Acknowledgement of federal support (yes).

(3) Mu L, Chen J, Sobotka S, Li J, Nyirenda T. Focal application of neurotrophic factors augments outcomes of nerve-muscle-endplate grafting technique for limb muscle reinnervation. Journal of Reconstructive Microsurgery (in press), 2023.
Acknowledgement of federal support (yes).

## Reinnervation of Paralyzed Limb muscle by Nerve-Muscle-Endplate Grafting Technique

W81XWH2010195

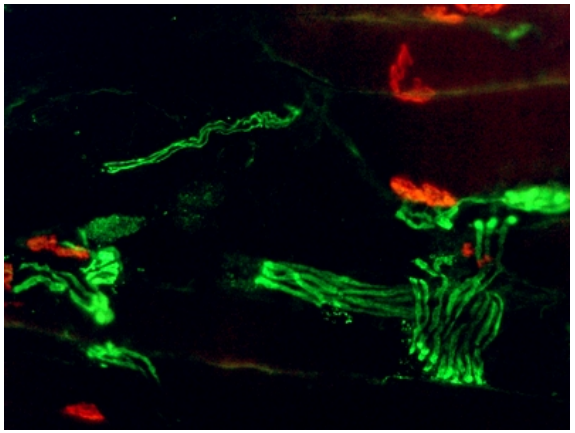
**PI:** Liancai Mu, MD, Ph.D **Org:** Hackensack Meridian Health - CDI **Award Amount: $1,686,264**

### Study/Product Aim(s)

- **Aim 1:** Evaluate the efficacy of NMEG-NMZ technique for immediate reinnervation of denervated limb muscle, and determine the beneficial effects of nerve growth stimulation methods (i.e., brief nerve stimulation and ENF) on axonal regeneration and outcomes of NMEG-NMZ.
- **Aim 2:** Evaluate the efficacy of NMEG-NMZ technique for reinnervation of chronically denervated limb muscle, and assess the potential of specific therapies (i.e., sensory protection, and intramuscular injection of neurotrophic factors) for preservation of muscle mass and endplates prior to NMEG-NMZ surgery.

### Approach

- Microsurgical procedures (NMEG-NMZ; sensory protection; nerve end-to-end anastomosis control; and denervation control).
- Physiological evaluation (muscle force measurement).
- Neural studies (nerve regeneration and axon-endplate connections).
- Muscle studies (fiber types, fiber size, muscle mass).



Image, showing MEPs (red) and their innervating axons (green) in a TA muscle treated with Del-ENF/NN.

### Timeline and Cost

| Activities / CY | 1 | 2 | 3 |
|---|---|---|---|
| Microsurgical procedures | | | |
| Physiological evaluation | | | |
| Neural studies | | | |
| Muscle studies & data analyses | | | |
| **Estimated Budget ($K)** | **$515k** | **$586k** | **$585k** |

**Updated:** (08/2/2023)

**Goals/Milestones**

**CY1 Goal** – Microsurgery and functional evaluations.

☑ Perform surgeries and some muscle force measurement.

**CY2 Goals** – Microsurgery, functional evaluations, and tissue studies.

☑ Perform surgeries and physiological testing.

☑ Perform neural and muscle studies.

**CY3 Goal** – Tissue studies, data collection, and data analyses.

☑ Examine intramuscular axonal regeneration and axon-endplate connections.

☑ Investigate myosin heavy chain-based fiber types, fiber size and muscle mass.

**Comments/Challenges/Issues/Concerns**

None.

**Budget Expenditure to Date**

Projected Expenditure: **$1,686,264**

Actual Expenditure: **$1,686,264**

Hindawi
Neurology Research International
Volume 2021, Article ID 6009342, 7 pages
https://doi.org/10.1155/2021/6009342

*Research Article*

# Limb Muscle Reinnervation with the Nerve-Muscle-Endplate Grafting Technique: An Anatomical Feasibility Study

**Liancai Mu**,[1] **Jingming Chen**,[1] **Jing Li**,[1] **Stanislaw Sobotka**,[1,2] **and Themba Nyirenda**[1]

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Academic Editor: Mamede de Carvalho



*Background*. Peroneal nerve injuries results in tibialis anterior (TA) muscle paralysis. TA paralysis could cause "foot drop," a disabling condition that can make walking difficult. As current treatment methods result in poor functional recovery, novel treatment approaches need to be studied. The aim of this study was to explore anatomical feasibility of limb reinnervation with our recently developed nerve-muscle-endplate grafting (NMEG) in the native motor zone (NMZ). *Methods*. As the NMEG-NMZ technique involves in nerves and motor endplates (MEPs), the nerve supply patterns and locations of the MEP bands within the gastrocnemius (GM) and TA muscles of rats were investigated using Sihler's stain and whole-mount acetylcholinesterase (AChE) staining, respectively. Five adult rats underwent TA nerve transaction. The denervated TA was reinnervated by transferring an NMEG pedicle from the ipsilateral lateral GM. At the end of a 3-month recovery period, maximal muscle force was measured to document functional recovery. *Results*. The results showed that the TA was innervated by the deep peroneal nerve. A single MEP band was located obliquely in the middle of the TA. The GM was composed of two neuromuscular compartments, lateral (GM-l) and medial (GM-m), each of which was innervated by a separate nerve branch derived from the tibial nerve and had a vertically positioned MEP band. The locations of MEP bands in the GM and TA muscles and nerve supply patterns demonstrated that an NMEG pedicle can be harvested from the GM-l and implanted into the NMZ within the TA muscle. The NMEG-NMZ pilot study showed that this technique resulted in optimal muscle force recovery. *Conclusion*. NMEG-NMZ surgery is feasible for limb reinnervation. Specifically, the denervated TA caused by peroneal nerve injuries can be reinnervated with a NMEG from the GM-l.

## 1. Introduction

Peripheral nerve injuries (PNIs) to the extremities and resultant muscle paralysis are a major source of chronic disabilities which limit the opportunities to work and diminish quality of life [1]. Although a number of surgical procedures have been used to restore motor function following PNIs [2], the currently available nerve repair surgeries result in poor functional recovery [2–4] due primarily to insufficient axonal regeneration and a failure to reinnervate the denervated motor endplates (MEPs) in the target muscle [5–7]. Therefore, there is a pressing need for new methods to improve outcomes.

We developed a novel surgical technique called the nerve-muscle-endplate grafting (NMEG) technique for muscle reinnervation [8]. The ideal is that a denervated muscle could be reinnervated by transplanting an NMEG pedicle from a neighboring donor muscle. An NMEG pedicle is composed of a donor nerve branch and a block of muscle that contains numerous MEPs and nerve terminals. In our neck muscle model, an NMEG was harvested from sternohyoid muscle and implanted to an MEP-free area in the ipsilateral denervated sternomastoid muscle [8]. As MEP reinnervation of a denervated muscle is critical for motor recovery [6, 7], we modified the procedures by implanting the NMEG pedicle to the native motor zone (NMZ) of the target muscle that contains an MEP band and nerve terminals. This NMEG-NMZ is based on the rationale that denervated MEPs in the NMZ are preferential sites for reinnervation. Studies showed that, after nerve injury and/or

direct nerve implantation, regenerating axons preferentially make synaptic contact at the original MEPs [9–15]. Unlike other nerve repair methods, NMEG-NMZ provides an abundant source of nerve terminals that favor axonal regeneration. As the NMEG pedicle is implanted to the NMZ of the target muscle, this facilitates rapid axon-MEP connections. We have demonstrated that NMEG-NMZ results in better functional recovery (82% of the control) [16] than NMEG implantation to an MEP-free area in the target muscle (67%) [8]. However, it remains unknown if the NMEG-NMZ technique is effective for limb reinnervation.

The purpose of this study was to determine the anatomical feasibility of transferring an NMEG from the gastrocnemius muscle (GM) to reinnervate the ipsilateral denervated tibialis anterior (TA) muscle in a rat model.

## 2. Materials and Methods

*2.1. Animals.* In this study, ten hind limbs of adult female Sprague Dawley rats (Charles River Laboratories, MA) were obtained after completion of other experiments. The nerve supply patterns and the locations of MEP bands in the GM and TA muscles were studied. In addition, five rats were used in our pilot study to determine the surgical feasibility and functional outcome. These animal studies were ethically reviewed and approved by the Institutional Animal Care and Use Committee prior to the onset of experiments. All animals were handled in accordance with the *Guide for Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication no. 85–23, revised 1996).

*2.2. Sihler's Stain.* Five fresh left legs of rats were removed and processed with Sihler's stain, a whole-mount nerve staining technique, to map out branching and distribution patterns of the sciatic nerve and its branches. The details regarding Shiner's stain have been given in our previous publications [17, 18]. In brief, the legs were fixed for 3 weeks in 10% unneutralized formalin; macerated and depigmented for 2 weeks in 3% potassium hydroxide (KOH) solution; decalcified for 2 week in Sihler's solution I (one part glacial acetic acid, one part glycerin, and six parts 1% aqueous chloral hydrate) with several changes; stained for 3 weeks in Sihler's solution II (one part stock Ehrlich's hematoxylin, one part glycerin, and six parts 1% aqueous chloral hydrate); and destained for 3 hr in Sihler's solution I. The legs were washed in running tap water for 1 hr between the aforementioned staining steps. The stained legs were then rinsed for 1 hr in 0.05% lithium carbonate solution to darken the nerves, cleared for 3 days in 50% glycerin, and finally, preserved for 4 weeks before microdissection in 100% glycerin with a few thymol crystals for transparency. After transillumination by a xenon light source (model 610; Karl Storz, Endoscopy-America, Culver City, CA), the stained limb muscles were dissected under a dissecting microscope (TYP 3555110; Wild, Heerbrugg, Switzerland) with 10–30x magnification using microsurgical instruments. The nerves supplying the calf muscles were traced from the main trunk of the sciatic nerve to its major branches and terminations within individual calf muscles. Finally, the dissected specimens were photographed with a Nikon camera (model D5300; Nikon, Japan) under transillumination from a xenon light source (P-Frame A-5A, Taiwan).

*2.3. Whole-mount AChE Staining.* Five entire GM and TA muscles on the left side were removed from rat legs. The muscles were treated with whole-mount AChE staining to locate the MEP band as described in our previous publications [18, 19]. Briefly, the entire TA and GM muscles were fixed for 2 hr in 10% phosphate-buffered formalin; washed in 0.1 M phosphate buffer (PB) at pH 7.4 and pH 6.0 for 15 min in each; incubated in stock solution (cupric sulfate 150 mg, glycerin 190 mg, magnesium chloride 500 mg, maleic acid 900 mg, 4% sodium hydroxide 15 ml, 40% sodium sulfate (anhydrous) 85 ml, and acetylthiocholine iodide 100 mg) at pH 6.0 and 37°C for 2 hr; rinsed in 40% sodium sulfate (anhydrous) for 15 min; washed for 15 min in distilled water (DW); immersed for 15 min in 20% potassium ferricyanide; washed in DW for 60 min; and preserved in 50% glycerin for 3 days. The stained muscles were transilluminated by a xenon light source, dissected under a dissecting microscope (TYP 3555110, Wild), and photographed with a Nikon camera (model D5300; Nikon) under transillumination from a xenon light source (P-Frame A-5A).

*2.4. Surgical Feasibility Pilot Study.* After determining the nerve supply patterns and the location of the MEP bands within the TA and GM muscles, we performed NMEG-NMZ surgery in five rats under general anesthesia as described in our previous publications [8, 16]. First, the left TA was denervated by excising a 10 mm segment of its nerve. Both ends of the nerve were ligated to prevent nerve regeneration. Second, an NMEG pedicle containing a block of muscle ($\sim 8 \times 6 \times 4$ mm), axon terminals, and a MEP band with neuromuscular junctions was harvested from the NMZ of the left lateral GM in continuity with its nerve branch. Third, a muscular defect, with dimensions similar to the NMEG pedicle, was made in the NMZ of the left denervated TA. Finally, the NMEG pedicle was embedded into the TA defect and sutured with 10–0 nylon. At the end of the 3-month recovery period, the maximal muscle force of the TA muscles on both sides was measured as described [8, 16] to document functional recovery.

## 3. Results

*3.1. Calf Muscles.* Figure 1 shows the calf muscles and major branches of the sciatic nerve in the rat hind limb. The calf muscles include GM, soleus, flexor hallucis longus (FHL), flexor digitorum longus (FDL), tibialis posterior (TP), TA, and extensor digitorum longus (EDL). The GM is composed of two neuromuscular compartments (NMCs), lateral (GM-l) and medial (GM-m).

The TA is a fusiform muscle located in the anterior part of the leg. It arises from the lateral tibia, and its tendon inserts on the medial foot. Along with fibularis tertius, EDL and EHL, it comprises the anterior (extensor) compartment of the leg. TA lies medial to EDL, which makes it the most medial muscle in the anterior compartment of the leg.

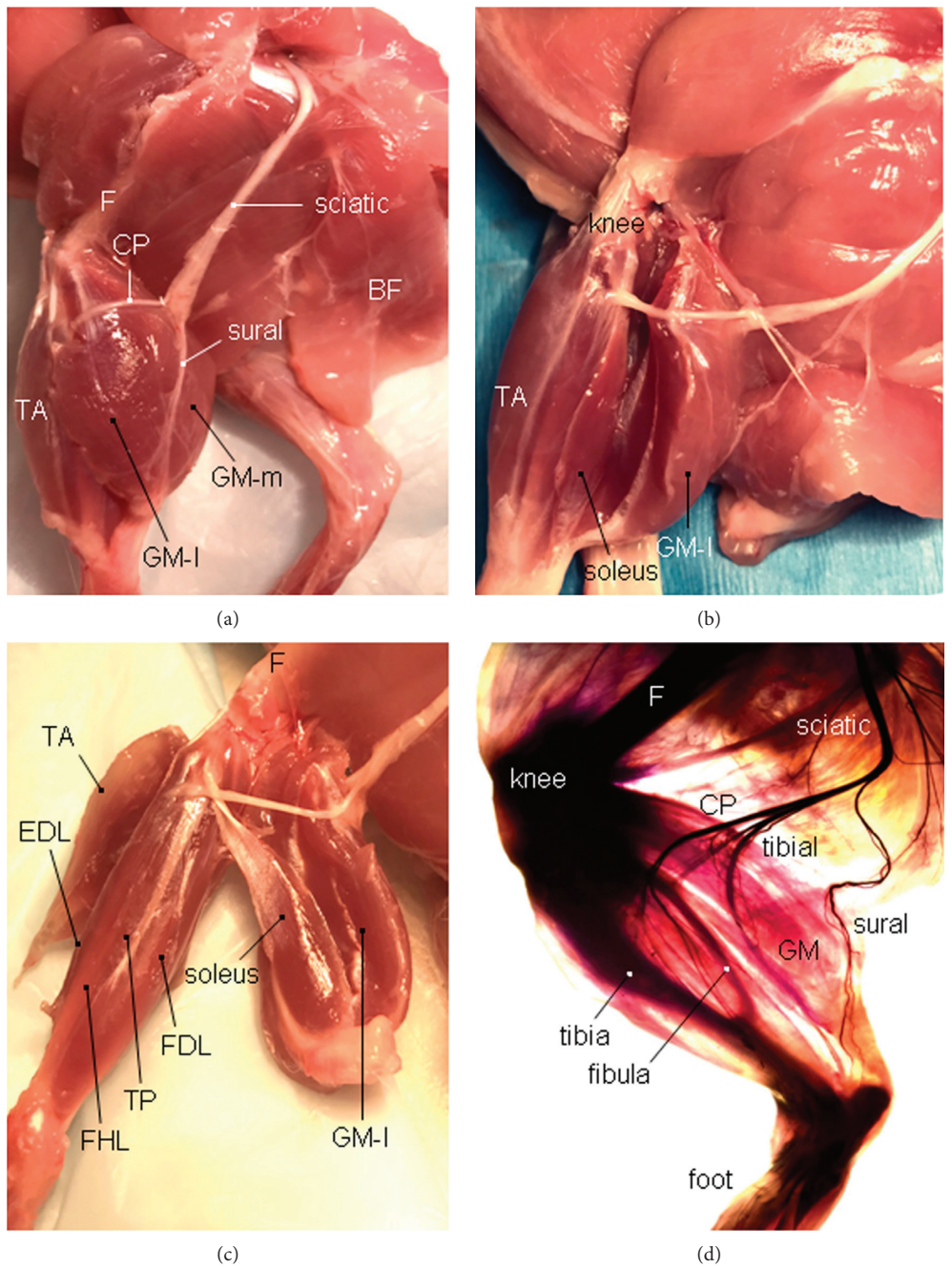



Figure 1: Calf muscles and their innervation of the rat left hind limb. (a) The calf muscle group of the left hind limb is exposed after the biceps femoris (BF) is reflected laterally. CP, common peroneal nerve; F, femur; TA, tibialis anterior muscle; GM-l, lateral compartment of the GM; and GM-m, medial compartment of the GM. (b) Left GM is detached and separated from the soleus muscle. (c) The separated calf muscles, including GM, soleus, flexor digitorum longus (FDL), flexor hallucis longus (FHL), and tibialis posterior (TP) muscle innervated by the tibial nerve, as well as the TA and extensor digitorum longus (EDL) innervated by the deep peroneal nerve. F, femur. (d) A rat left hind limb processed with Sihler's stain without microdissection, showing anatomical relationships among calf muscles, innervating nerves, and bone structures.

*3.2. Branching and Distribution of Sciatic Nerve.* Sihler's stain (Figures 1(d) and 2) showed that the sciatic nerve is divided into three major branches: common peroneal nerve (CP), tibial nerve, and sural nerve (sensory). The CP winds around the neck of the fibula and divides into a superficial and a deep branch. The deep peroneal nerve (DPN) innervates the TA and EDL in the anterior compartment of the leg.

The tibial nerve innerves the GM, soleus, FHL, FDL, and muscles in the foot (Figure 2). Specifically, tibial nerve gives off three branches, the first branch (the thinnest one) to the GM

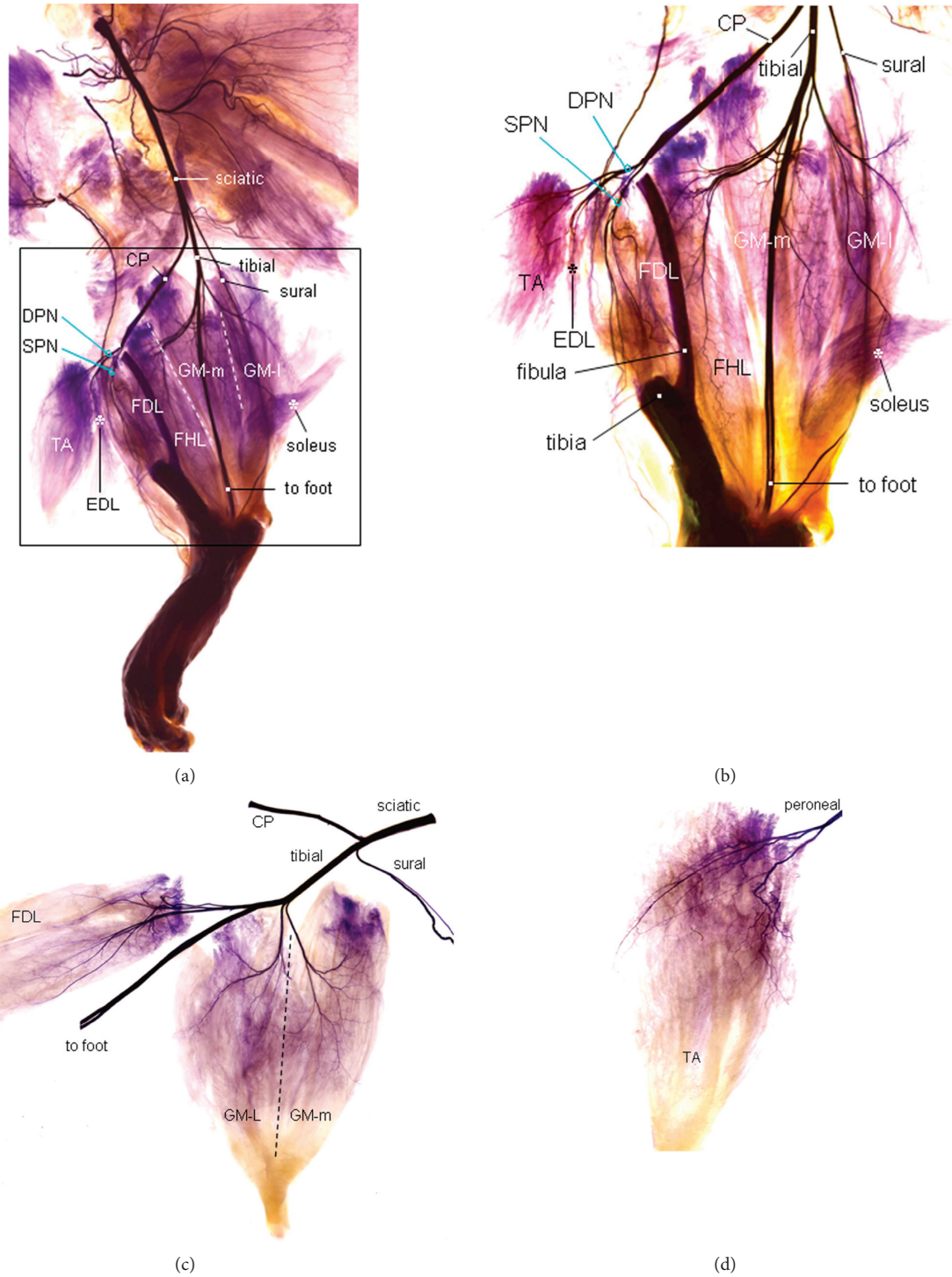
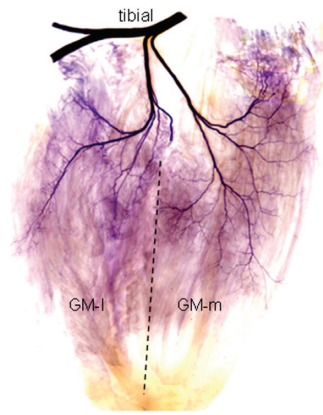



Figure 2: Continued.




(e)

Figure 2: A Sihler's stained rat hind limb, showing the major branches of the sciatic nerve and their distribution in the calf muscles. (a) Low-power view of the Sihler's stained left hind limb after microdissection, 4x. The sciatic nerve is divided into three major branches: CP, tibial, and sural (sensory). The peroneal nerve gives off branches to innervate TA and EDL. The tibial nerve gives off branches to supply the GM, soleus, FHL, FDL, and muscles in the foot. The GM-l and GM-m are outlined by white dotted lines. (b) Magnification of the boxed region in A showing the intramuscular branching and distribution patterns of the peroneal and tibial nerves. The upper part of the tibia was removed. The deep peroneal nerve (DPN) supplies branches to the TA and EDL. The tibial nerve gives off three branches, first branch to the GM, second branch to the foot, and third branch to the FHL and FDL. Also, the GM nerve branch gives off two secondary branches, one innervating the GM-l and soleus and another supplying the GM-m. SPN, superficial peroneal nerve, 12x. (c–e) High-power view of the branching of the sciatic nerve and intramuscular innervation of the tibial and peroneal nerves, 16x.

and soleus, the second branch (the thickest one) to the foot, and the third branch to the FHL and FDL. GM-l and GM-m are innervated by separate nerve branches derived from the tibial nerve. The nerve branch to the GM-l gives off a branch to innervate the soleus muscle (Figures 2(a) and 2(b)).

*3.3. MEP Bands within the TA and GM Muscles.* The MEP band is formed by numerous neuromuscular junctions. Figure 3 shows the MEP bands within the TA and GM muscles. The MEP band within the TA is located obliquely in the middle of the muscle (Figure 3(a)). In the GM, each of the GM-l and GM-m compartments has its own MEP band which is vertically located (Figure 3(b)).

*3.4. Surgical Feasibility of NMEG-NMZ in Limb Reinnervation.* The NMZs within the GM and TA muscles were delineated based on the locations of MEP bands and their innervating nerve terminals (Figure 4(a)). Our NMEG-NMZ pilot study showed that an NMEG pedicle can be harvested from the NMZ of the GM-l and transplanted to the NMZ of the TA (Figure 4(b)). In the rats with NMEG-NMZ surgery ($n = 5$), the average muscle force of the reinnervated TA recovered up to 81% of the contralateral control. These findings suggest that if the TA is denervated following peroneal nerve injury, the NMEG-NMZ technique could be an option to treat "foot drop" caused by TA paralysis.

## 4. Discussion

We investigated the branching and distribution of the sciatic nerve and NMZs within the TA and GM muscles in the rat. This anatomical study on the nerve supply patterns and locations of MEP bands in the TA and GM muscles allows us to identify their NMZs for NMEG-NMZ surgery. Since the GM-l lies adjacent to the TA, an NMEG pedicle from the NMZ of the GM-l could reach to the NMZ of the TA without difficulty. Our pilot study showed that NMEG-NMZ resulted in promising functional recovery three months after limb muscle reinnervation.

TA is the dorsiflexor of the foot and plays a critical role in walking. Paralysis of the TA caused by CP or DPN injuries or lesions results in foot drop, a disabling condition that can make walking difficult and lead to frequent falls.

Traditional treatment modalities include use of an ankle-foot orthosis, tendon surgery, and nerve repair. Tendon transfer surgery is often used to treat foot drop with mixed results [20, 21]. For example, all or a part of the healthy posterior tibial tendon is transferred to the dorsum of the foot for restoring foot dorsiflexion. However, the foot drop tendon transfer surgery results in weak ankle dorsiflexion [22].

Nerve repair [23], nerve grafting [24], and nerve transfer [25–28] are commonly used to manage sciatic and peroneal nerve injuries and lesions. Unfortunately, 64% of repair and grafting of the sciatic nerve [29] and 46–54% of the common peroneal nerve palsies [23, 24, 29] fail to restore functional dorsiflexion. Nerve transfer procedures such as a tibial nerve branch to the deep peroneal nerve [26–28] or a bundle of nerves supplying the soleus and lateral GM to the deep peroneal nerve [25] have been used to treat TA paralysis after peroneal and/or sciatic nerve injuries, which have had mixed results. Therefore, there is a great need to develop new approaches for foot drop treatment.

Poor motor recovery after PNIs and nerve repair is due primarily to insufficient axonal regeneration and failure to reinnervate the denervated MEPs in the target muscle. In

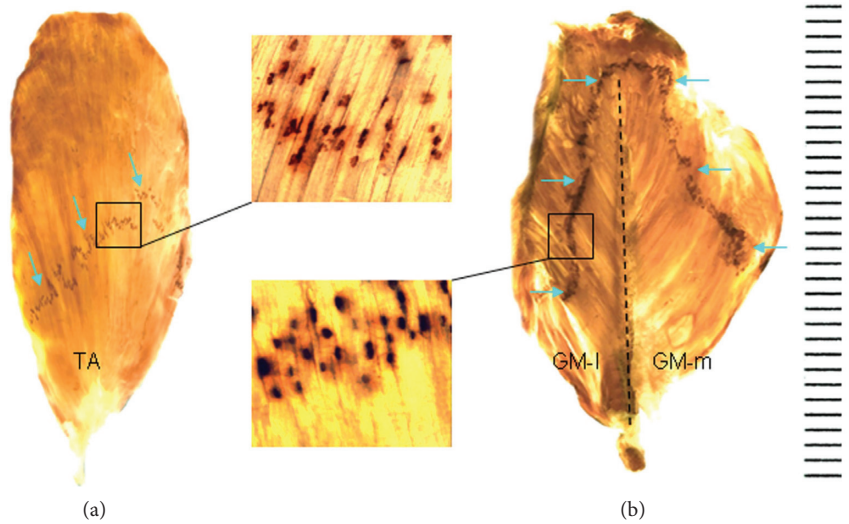



Figure 3: Locations of the MEP bands within the TA and GM as demonstrated by whole-mount AChE staining. (a) The MEP band (arrows) with numerous neuromuscular junctions (black dots) within the TA is located obliquely in the middle of the muscle. (b) Each of the GM-l and GM-m compartments in the GM has its own MEP band (arrows). The vertical dished line in the GM indicates midline.

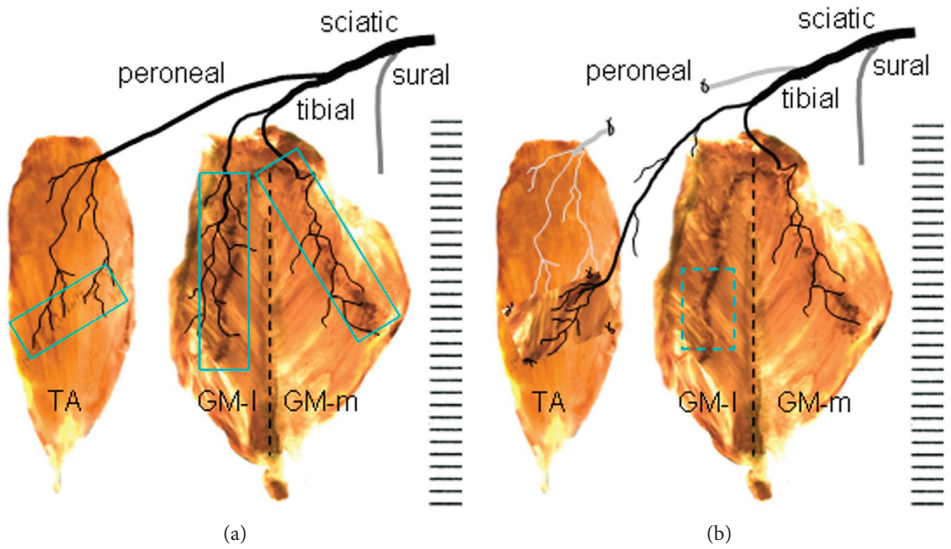



Figure 4: (a) Native motor zones (NMZs) within the TA and GM muscles (boxed regions) as demonstrated by Sihler's stain and AChE staining. (b) TA denervation and NMEG-NMZ transplantation. The TA is denervated by resecting a segment of the peroneal nerve. The denervated TA is treated with the NMEG-NMZ technique. An NMEG pedicle with a nerve branch is harvested from the NMZ of the GM-l (boxed region) and implanted to the NMZ of the denervated TA muscle.

response to this, we developed the NMEG. NMEG-NMZ is a recently developed novel surgical technique that targets NMZ for rapid MEP reinnervation, thereby leading to favorable functional recovery. Transplanting an NMEG from GM-l to the NMZ of the TA muscle is anatomically and surgically feasible and could offer several advantages to current treatment options. First, NMEG-NMZ provides an abundant source of nerve terminals that favor axonal regeneration. Second, as an NMEG pedicle is implanted directly to the MEP zone, NMEG-NMZ physically shortens regeneration distances and favors rapid axon-MEP connections. Finally, NMEG has ample pedicle-recipient muscle interfaces, which provide enough space for axonal regeneration at multiple points in the implanted NMEG pedicle and grow across the interfaces to reach the target.

This study showed that transferring an NMEG pedicle from GM-l to the NMZ of the TA can be used to treat TA paralysis caused by CP or DPN injuries. Further experimental studies are needed to evaluate the efficacy of the NMEG-NMZ technique for limb muscle reinnervation.

## Data Availability

All data of this study are available upon request from the first author Liancai Mu.

## Ethical Approval

In conducting research using animals, the investigators adhered to the laws of the United States and regulations of the Department of Agriculture. This protocol was approved by the USAMRMC Animal Care and Use Review Office (ACURO) for the use of rats.

## Disclosure

Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the Department of Defense.

## Conflicts of Interest

The authors have no conflicts of interest to declare.

## Acknowledgments

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# Reinnervation of Paralyzed Limb Muscle by Nerve-Muscle-Endplate Grafting Technique

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**BACKGROUND:** We have developed a novel reinnervation technique called nerve-muscle-endplate grafting in the native motor zone (NMEG-NMZ). However, it remains unknown whether the NMEG-NMZ is effective for limb reinnervation.

**OBJECTIVE:** To evaluate the efficacy of the NMEG-NMZ in limb muscle reinnervation.

**METHODS:** Forty-five adult rats were divided into 3 groups: NMEG, end-to-end anastomosis (EEA, technique control), and denervation control (DC). The left tibialis anterior muscle was denervated by resecting its nerve. For NMEG-NMZ, the denervated tibialis anterior was reinnervated by transferring a NMEG pedicle from the lateral gastrocnemius muscle. Three months after surgery, static toe spread analysis was performed for all rats and muscle force was measured for the rats treated with NMEG and EEA. Muscle weight, myofiber morphology, regenerated axons, and reinnervated motor endplates in the treated muscles were also quantified and compared with those in the DC group.

**RESULTS:** NMEG-NMZ technique resulted in better muscle force recovery (79% of the control) compared with EEA (51% of the control, $P = .048$). Toe spread analysis in NMEG-NMZ reinnervated muscles showed static sciatic index = −16.8, whereas −41.4 in EEA, $P < .0001$). The average weight of the NMEG-NMZ reinnervated muscles (86%) was greater than those of the EEA treated (71%) and DC (26%) muscles (all $P < .0001$). The mean count of the regenerated axons in the muscles with NMEG-NMZ was 76% of the control, which was larger than that in the muscles with EEA (46%), $P < .0001$.

**CONCLUSION:** NMEG-NMZ technique has unique advantages and is superior to EEA for muscle reinnervation and functional recovery.

**KEY WORDS:** Nerve-muscle-endplate grafting, Muscle reinnervation, Peripheral nerve injury, Anterior tibialis muscle, Gastrocnemius muscle, Tetanic force measurement

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Traumatic injuries to the sciatic nerve result in leg muscle paralysis which is a predominant source of chronic disabilities. In the United States, there are 200 000 cases of peripheral nerve injuries (PNIs) annually,[1] and an estimated $300 billion is spent annually on care for disabled Americans.[2,3] At present, multiple surgical interventions have been used for restoring motor function after PNIs.[4] Unfortunately, nerve reconstruction results in poor functional recovery[5,6] because fewer nerve fibers are able to pass through the coaptation site and reach the target muscle.[7,8] Unsatisfactory motor recovery has been attributed mainly to insufficient axonal regeneration and poor motor endplate (MEP) reinnervation in the target muscle.[9,10] Therefore, a great need exists to develop novel therapies for improving surgical outcomes.

More recently, we developed the nerve-muscle-endplate grafting (NMEG) technique for muscle reinnervation[11] and performed a series of related studies[12-14] in a rat neck muscle model. The underlying concept is that a denervated muscle after

**ABBREVIATIONS: AChE,** acetylcholinesterase; **AChR,** acetylcholine receptor; **DC,** denervation control; **EEA,** end-to-end anastomosis; **GM,** gastrocnemius muscle; **GM-l,** lateral compartment of GM; **GM-m,** medial compartment of GM; **H&E,** hematoxylin and eosin; **IACUC,** Institutional Animal Care and Use Committee; **ITS,** intermediary toe spread; **ITSF,** ITS factor; **MEP,** motor endplate; **NF,** neurofilament; **NF-ir,** NF immunoreactive; **NITS,** nonoperated side intermediate toe spread; **NMEG,** nerve-muscle-endplate grafting; **NMZ,** native motor zone; **NN,** NMEG-NMZ; **NTS,** nonoperated side toe spread; **OITS,** operated side intermediate toe spread; **OTS,** operated side toe spread; **PBS,** phosphate-buffered saline; **PNI,** peripheral nerve injury; **SSI,** static sciatic index; **STSA,** static toe spread analysis; **TA,** tibialis anterior; **TS,** toe spread; **TSF,** TS factor.

PNIs can be reinnervated by transferring an NMEG pedicle from a neighboring intact muscle. Our studies have demonstrated that native motor zone (NMZ) is an ideal region for muscle reinnervation because it contains numerous neuromuscular junctions and nerve terminals. Implantation of an NMEG from a donor muscle to the NMZ within a recipient denervated muscle[14] results in better outcomes as compared with NMEG implantation to a MEP-free area.[11] However, it remains unknown whether the NMEG-NMZ technique is effective for limb reinnervation.

Clinically, paralysis of tibialis anterior (TA) muscle caused by peroneal nerve injuries results in foot drop, a disabling condition that can make walking difficult and lead to frequent falls. Foot drop is usually treated with tendon transfer with mixed results.[15-17] Nerve repair,[18] nerve grafting,[19] and nerve transfer[20-23] are commonly used to manage sciatic and peroneal nerve injuries. Unfortunately, approximately half of the patients with sciatic nerve repair and grafting[24] and peroneal nerve repair[18,19] fail to restore functional dorsiflexion.

We hypothesized that NMEG-NMZ may be a useful treatment option to restore motor function of the denervated TA muscle. We performed anatomic feasibility study on the limb muscle reinnervation by the NMEG-NMZ and showed that an NMEG pedicle can be taken from the lateral gastrocnemius muscle (GM-l) to reinnervate TA muscle.[25]

The purpose of this study was to assess the efficacy of the NMEG-NMZ technique for TA reinnervation after PNIs in a rat model.

## METHODS

### Animals and Experimental Groups

Forty-five 3-month-old female Sprague-Dawley rats (Charles River Laboratories) were used. The rats were assigned into 3 groups (15 rats/per group): NMEG-NMZ (NN group), end-to-end anastomosis (EEA group), and denervation control (DC group). These studies were reviewed and approved by the Institutional Animal Care and Use Committee. All animals were handled in accordance with the *Guide for Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996).

### Surgical Procedures

Animals were anesthetized by an intraperitoneal injection of a mixture of ketamine (80 mg/kg) and xylazine (5 mg/kg). Under aseptic conditions, an incision was made in the posterior thigh and calf of the hind limb to expose the TA and GM and the 3 major branches of the sciatic nerve.

For NMEG-NMZ, the left TA was denervated by resecting a 10-mm segment of its nerve, and the cut ends were ligated. The NMZs in the left TA and GM were outlined according to the locations of the MEP band and muscular nerve branches (Figure 1A) as described.[25] An NMEG containing a block of muscle (~8 × 6 × 4 mm), an intact donor nerve branch with feeding vessels and numerous nerve terminals, and an MEP band with numerous neuromuscular junctions were taken from the NMZ of the GM-l. A muscular defect with the same dimensions as the NMEG pedicle was made in the NMZ of the left denervated TA muscle. The NMEG was then embedded into the TA defect and sutured with 4 10-0 nylon (Figure 1B). After surgery, the wound was closed with 4-0 prolene.

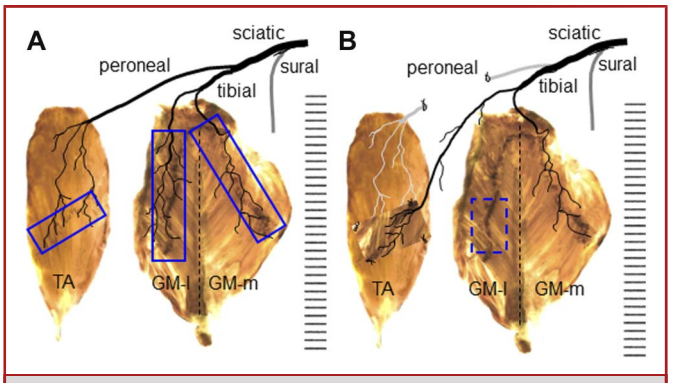


**FIGURE 1.** **A**, *NMZs (boxed regions) within the left rat TA muscle and GM as demonstrated by Sihler stain that shows nerve supply patterns and whole-mount AChE staining which localizes motor endplate bands with numerous neuromuscular junctions (black dots).* **B**, *TA denervation and NMEG-NMZ transplantation. The TA is denervated by resecting a segment of peroneal nerve. The denervated TA is reinnervated with the NMEG-NMZ technique. An NMEG pedicle containing a block of muscle tissue and a nerve branch is harvested from the NMZ of the lateral compartment of GM muscle (GM-l) (boxed region) and implanted to the NMZ of the denervated TA muscle. GM, gastrocnemius muscle; GM-l, lateral compartment of GM; GM-m, medial compartment of GM; NMEG, nerve-muscle-endplate grafting; NMZ, native motor zone; TA, tibialis anterior (Printed with permission from Figure 4 in the article by Mu et al[25]).*

For EEA nerve repair (technique control), the left TA nerve was transected and both cut ends were anastomosed with 2 interrupted 10-0 nylon microsutures. For denervation control, a 10-mm segment of the TA nerve was removed and both cut ends were ligated.

### Postoperative Evaluations

#### *Static Toe Spread Analysis*

Static toe spread analysis (STSA) described by Bervar[26] was performed just before muscle force measurement because it is a useful method for evaluating limb motor function.[27-30] For STSA, the rat was placed in an acrylic 40 × 20 × 20 cm container on a transparent base plate for observing footprints on the plantar view. A camera was positioned underneath the base plate to photograph the plantar surface of the rat hind limb paws. The operated (O) and nonoperated (N) footprints were randomly selected to measure the distance (mm) between 1 and 5 toe spread (TS) and between 2 and 4 intermediary toe spread (ITS). The mean values of TS and ITS in each group were computed and used for determining the TS and ITS factors (TSF and ITSF): TSF = (OTS − NTS)/NTS and ITSF = (OITS − NITS)/NITS. The ratios of the static sciatic index (SSI) were calculated using the following equation: SSI = (108.44 × TSF) + (31.85 × ITSF) − 5.49.[26] An index score of 0 is defined as normal, and an index score of −100 indicates a complete functional loss.[26]

TSF: toe spread factor; ITSF: intermediate toe spread factor; OTS: operated side toe spread; NTS: nonoperated side toe spread; OITS: operated side intermediate toe spread; NITS: non-OITS.

#### *Maximal Tetanic Force Measurement*

At the end of the 3-month recovery period, muscle force of the TA on both sides was measured as described.[11-14] In brief, the anesthetized rat was placed in prone position on a platform, the distal tendon of the TA was

severed, and the muscle was dissected proximally up to the level of its origin. The transected TA tendon was tied with a 4-0 suture and connected to force transducer attached to a servomotor lever arm of the Dual-Mode Lever System (305B-LR; Aurora Scientific Inc.). The nerves examined were stimulated (0.2 s train of 0.2 ms bipolar pulses at 200 pulses/s with current 0.01-5 mA). To avoid muscle fatigue, 1-minute breaks were used between nerve stimulations. During muscle force measurement, the examined muscle and nerve were bathed with warm mineral oil. Collected data were analyzed offline with DIAdem 11.0 software (National Instruments).

### *Muscle Sample Preparation*

At the end of the experiments, both TA muscles in each rat were removed and weighed. Each muscle was divided into 2 segments: superior one-third and inferior two-thirds. The muscle samples were frozen in melting isopentane cooled with dry ice. The superior segments were cut transversly (10 µm thick) and stained for routine hematoxylin and eosin (H&E) staining. The inferior segments were cut horizontally (50 µm thick) and immunostained for detecting axons and MEPs.

### *Neurofilament (NF) Staining*

Some horizontal sections were immunostained with a monoclonal antibody against phosphorylated NF (sternberger monoclonal incorporated anibody 31 [SMI-31]; Covance Research Products) as described.[14] In brief, the sections were blocked with 2% bovine serum albumin for 30 minutes, incubated with primary antibody SMI-31 (1:800) at 4 °C overnight, incubated for 2 hours with the biotinylated secondary antibody (anti-mouse, 1:1000, Vector), and treated with a Vectastain avidin-biotin complex kit (1:1000 avidin-biotin complex Elite, Vector).

For a given muscle, 3 stained sections were randomly selected to count NF immunoreactive (NF-ir) axons. The stained sections were viewed under a Zeiss photomicroscope (Axiophot-1; Carl Zeiss) and photographed using a universal serial bus 3.0 digital microscope camera (Infinity 3-3URC; Lumenera Corp.). The density of the intramuscular axons was evaluated by counting the axons within a section area (1.0 $mm^2$) using ImageJ (v. 1.45s; NIH).

### *Double Fluorescence Staining*

Intramuscular axons and MEPs in the TA were labeled as described in our publication.[31] In brief, some horizontal sections were (1) fixed for 20 minutes at 4 °C in Zamboni fixative, (2) blocked for 30 minutes in 1.5 T buffer containing 4% normal goat serum, (3) incubated overnight at 4 °C with primary antibody SMI-31 to label axons (Covance Research Products Inc), and (4) incubated for 2 hours both with a secondary antibody (goat anti-mouse antibody conjugated to Alexa 488) and with α-bungarotoxin conjugated with Alexa 596 (Invitrogen Corporation).

The stained sections were photographed. For each muscle sample, at least 100 labeled MEPs were randomly selected to determine the percentages of the innervated (visible axon attachment) and noninnervated (no visible axon attachment) MEPs.

### Statistical Analysis

STSA data, force values, muscle weights, and axon and MEP counts of the operated and nonoperated TA muscles in each rat were computed and compared. The *t*-test (unpaired or paired as appropriate) was used for comparison of normally distributed data. The Mann-Whitney *U* test (for not paired comparisons) or the Wilcoxon signed-rank test (for paired comparisons) was used for comparison of nonparametric data. Type I error at $P < .05$ (2-tailed) was treated as statistically significant. All comparisons were made with SAS 9.4 software or with online statistics calculators.

## RESULTS

### Toe Spread Recovery

The animals in the NN group had better toe spread recovery than those in the EEA (Figure 2A) and DC groups. Figure 2B shows the statistical difference ($P < .0001$, unpaired *t*-test) in SSI scores between NN (−16.84; SD = 4.16) and EEA (−41.4; SD = 13.46) groups.

### Muscle Force Recovery

In both NN and EEA groups, the averaged maximal muscle force of the reinnervated TA was significantly lower than that of the contralateral side (Wilcoxon signed-rank test, $P = .034$ and $P = .0038$, respectively, Figure 3A, upper graph). The rates of experimental to control side were significantly larger in the NN group (0.79) than in EEA (0.51, Mann–Whitney *U* test, $P = .048$, lower graph).

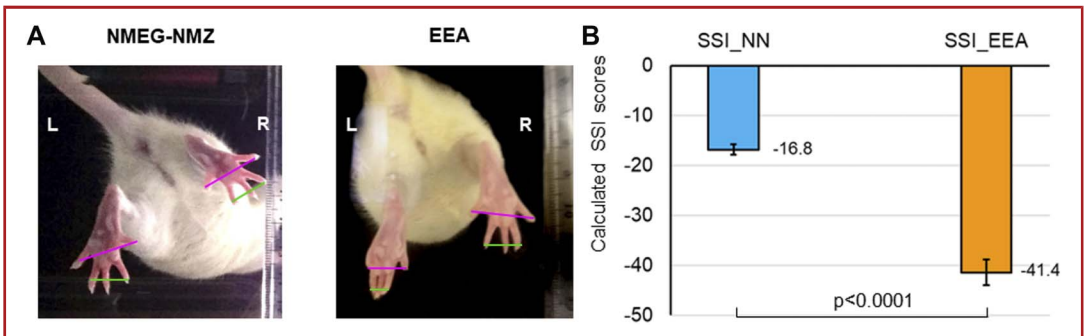


**FIGURE 2.** **A**, *Comparison of hind limb footprints between L operated and R nonoperated sides in the rats with NMEG-NMZ and EEA.* **B**, *Average SSI scores show strong significant difference in SSI scores between NN and EEA groups. Note that NMEG-NMZ resulted in more optimal motor recovery of the operated side as compared with EEA. Error bars represent standard error. EEA, end-to-end anastomosis; L, left; NMEG, nerve-muscle-endplate grafting; NMZ, native motor zone; NN, NMEG-NMZ; R, right; SSI, static sciatic index.*

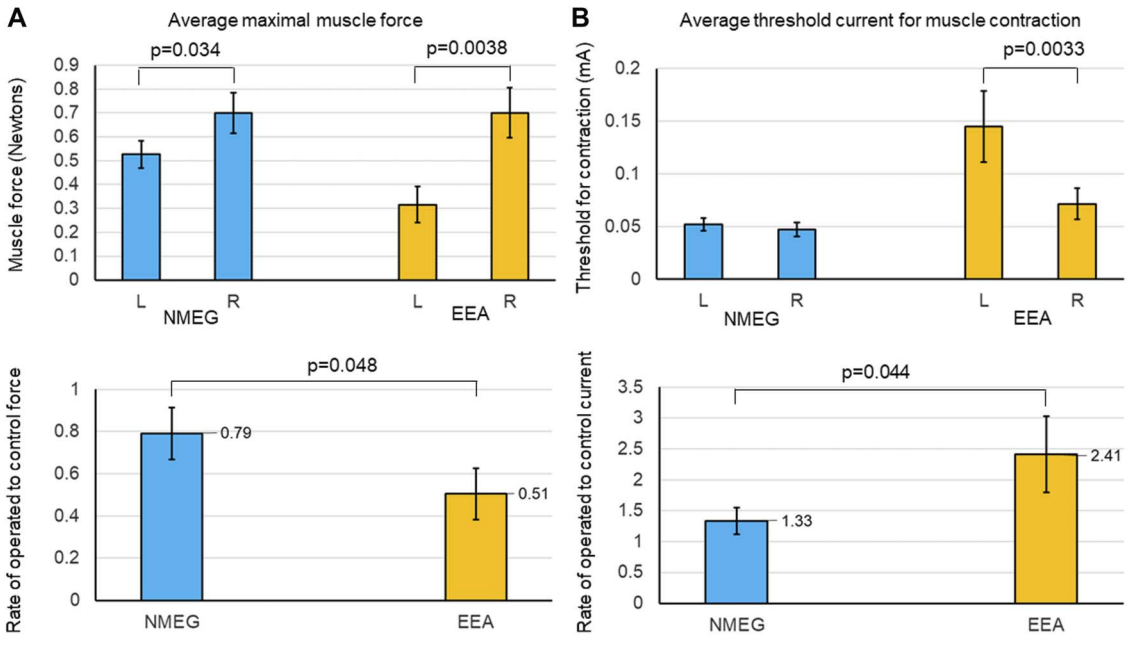


**FIGURE 3.** *Analyses of muscle force and stimulation threshold data obtained from operated and nonoperated tibialis anterior muscles in NN and EEA groups.* **A**, *Top graph, group averages of maximal muscle force; bottom graph, the rate of experimental/control muscle force.* **B**, *Top graph, group averages of stimulation threshold—minimal current, which produces muscle contraction; bottom graph, the rate of experimental/control stimulation threshold. EEA, end-to-end anastomosis; L, left; NMEG, nerve-muscle-endplate grafting; NMZ, native motor zone; NN, NMEG-NMZ; R, right.*

In the NN group, the stimulation threshold current on the operated side was not significantly different from that on the contralateral side (Wilcoxon signed-rank test, $P > .05$, Figure 3B, upper graph). However, in the EEA group, the stimulation threshold on the operated side was significantly larger than that on the contralateral side (Wilcoxon signed-rank test, $P = .0033$). The mean rates of experimental to control side thresholds were significantly smaller in the NN group (1.33) than EEA (2.41, Mann–Whitney $U$ test, $P = .044$).

### Muscle Mass, Wet Weight, and Myofiber Morphology

The NMEG-NMZ–treated TA had larger size and exhibited less fiber atrophy compared with the EEA treated and denervated TA (Figure 4A).

The mean weight of the operated TA side was lower than that of the control in each of the comparison groups (all $P < .0001$, paired $t$-test, Figure 4B). The average weight ratio of the operated to control side was 0.859 for the NN group, significantly smaller 0.709 for the EEA group and much smaller 0.257 for DC (all $P < .0001$, unpaired t-test).

### Axonal Regeneration and MEP Reinnervation

The axon counts of TA muscles in NN and EEA groups are illustrated in Figure 5A. The NMEG-NMZ–treated TA exhibited more extensive axon regeneration than the EEA-treated muscle. Double fluorescence staining showed the innervated and noninnervated MEPs.

Figure 5B shows significantly higher mean ratio of axon counts in the NMEG-NMZ–treated TA (76% of control) than that in the EEA-treated muscle (45.7%; $P < .0001$, unpaired $t$-test). Similarly strong statistical difference was observed for area fractions ($P < .0001$, unpaired $t$-test).

## DISCUSSION

To the best of our knowledge, this is the first study to use NMEG-NMZ technique for limb reinnervation in a rat model. There are several key findings. First, NMEG-NMZ is surgically feasible for limb muscle reinnervation in the rat. Second, NMEG-NMZ resulted in better functional recovery as compared with EEA. Third, NMEG-NMZ resulted in greater percentages of the regenerated axons and reinnervated MEPs than the EEA. Finally, NMEG-NMZ–reinnervated muscles had a larger wet weight than EEA-treated and denervated muscles.

The NMEG-NMZ technique has unique advantages that contribute to the satisfactory outcomes. First, there are numerous nerve terminals in the implanted NMEG pedicle, thereby facilitating axonal regeneration. Second, the regenerating axons from the implanted NMEG pedicle easily reach and reinnervate the denervated

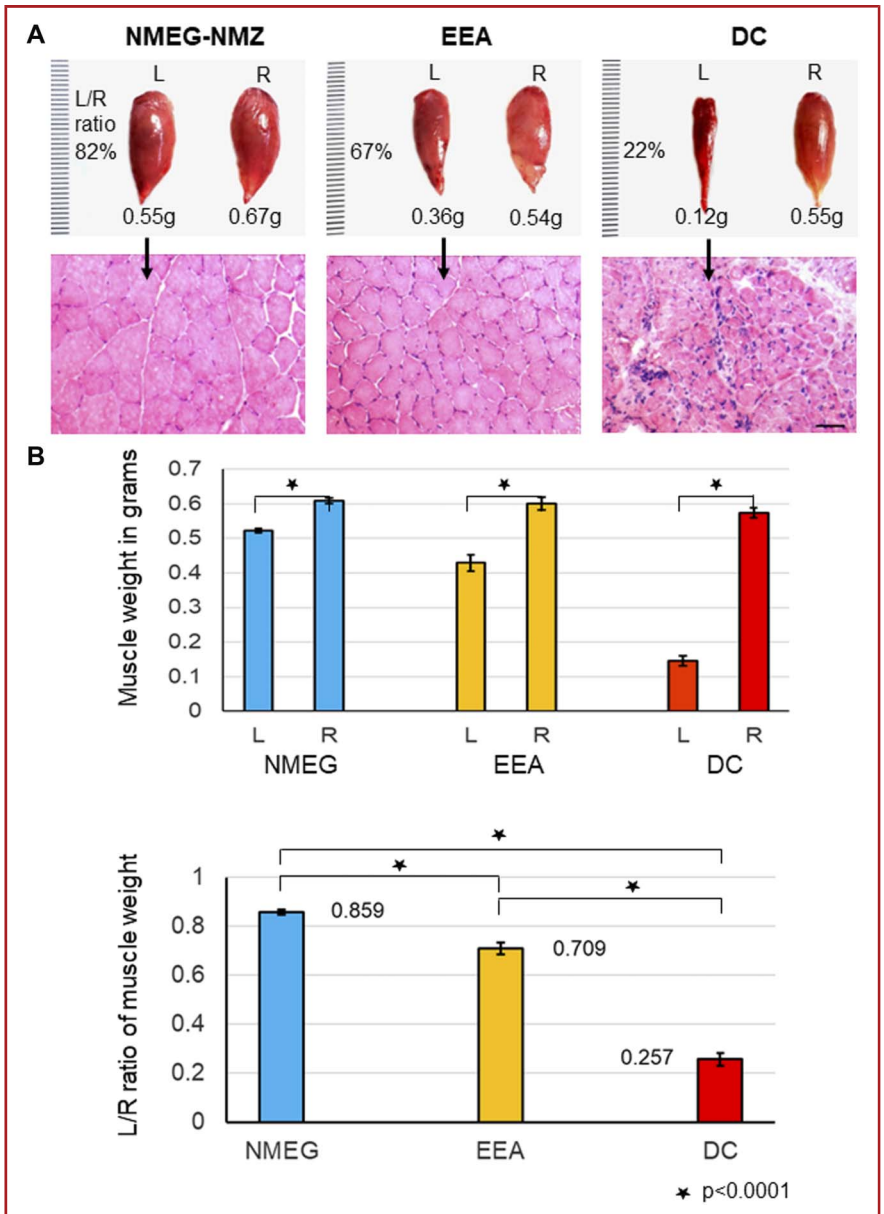


**FIGURE 4.** **A**, *Top graph, representative images of the L reinnervated and denervated TA muscles and R contralateral controls from rats in NN (rat #1), EEA (rat #15), and denervation control (rat #6) groups, showing gross appearance and muscle mass. Graph below, hematoxylin and eosin–stained cross-sections of the TA muscles reinnervated with NMEG-NMZ and EEA as well as denervated TA. Note that NMEG-NMZ reinnervated TA exhibited very good preservation of myofiber morphology with less fiber atrophy as compared with EEA reinnervated and denervated muscles. Bar = 50 μm.* **B**, *Top graph, shows mean weight of the operated TA was lower than that of the control in each of comparison groups (all P < .0001, paired t-test). Graph below, the average weight ratio of the operated to control side was 0.859 for the NN group, significantly smaller 0.709 for the EEA group, and much smaller 0.257 for DC (all P < .0001, unpaired t-test) indicating that NMEG-NMZ–treated muscles resulted in significantly better muscle weight recovery than EEA-treated and denervated muscles. DC, denervation control; EEA, end-to-end anastomosis; L, left, MEPs, motor endplates; NMEG, nerve-muscle-endplate grafting; NMZ, native motor zone; NN, NMEG-NMZ; R, right; TA, tibialis anterior.*

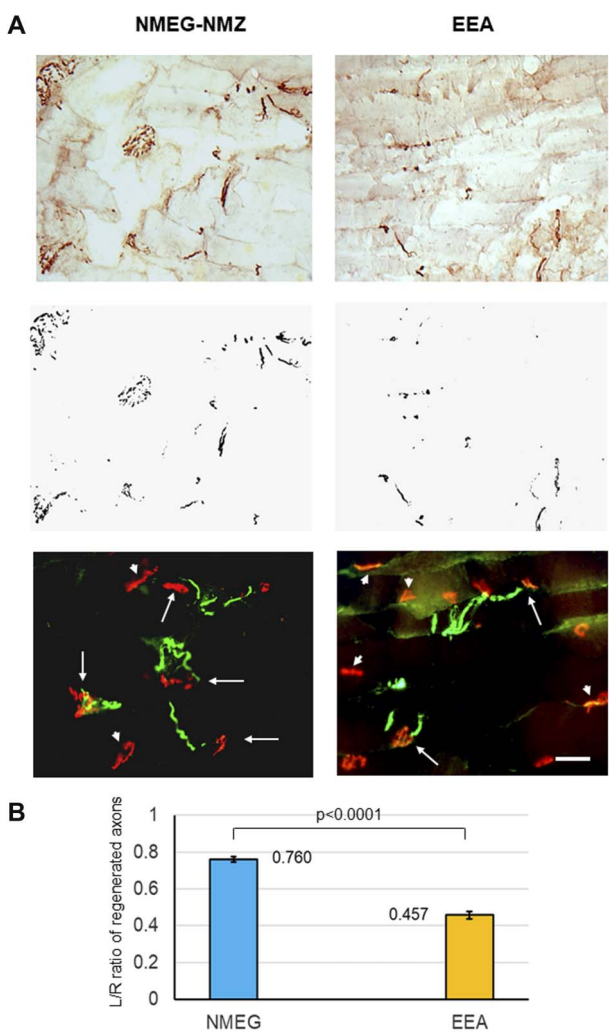


**FIGURE 5.** **A**, *Immunostained horizontal sections of the rat TA muscles re-innervated with NMEG-NMZ and EEA, showing intramuscular regenerated axons (top and middle graphs) and innervated (arrows in bottom graphs) and noninnervated (arrowheads in bottom graphs) MEPs. Regenerated axons (darkly stained threads and dots in top graphs and green in bottom graphs) were detected with SMI-31 monoclonal against neurofilaments, while MEPs (red) were labeled with α-bungarotoxin. The stained sections in top graphs were opened using ImageJ software and converted to binary images (middle graphs). The density of the regenerated axons was determined by estimating the number and area fraction of the positively stained axons within a section area (1.0 mm$^2$). For the rat with NMEG-NMZ surgery in top graphs (rat # 10), the operated TA had a very good muscle reinnervation as indicated by the mean axon count (813; 80.1% of the control) and the mean area (0.758; 76.3% of the control). For the rat with EEA nerve repair in middle graphs (rat # 2), the treated TA had less regenerated axons (421; 51% of the control) and area fraction (0.298; 47.8% of the control). Also note that the NMEG-NMZ–treated TA (middle graphs) had more reinnervated MEPs (red; arrows) as compared with EEA-treated muscle. Arrowheads indicate non–reinnervated MEPs. Bar = 100 μm.* **B**, *The bar graph shows that the mean ratio of axon counts in the NMEG-NMZ–treated TA (76% of the control) was significantly higher than that in the EEA-treated muscle (45.7%; P < .0001, unpaired t-test). EEA, end-to-end anastomosis; MEPs, motor endplates; NMEG, nerve-muscle-endplate grafting; NMZ, native motor zone; TA, tibialis anterior.*

MEPs in the NMZ of the target muscle. Finally, the muscle fibers at site of the NMZ have unique property that favors muscle re-innervation. This concept gains support from studies reported by others[32-38] and this research team.[11,14] Our previous study showed that implantation of the NMEG pedicle to a MEP-free area outside of the NMZ in the recipient muscle resulted in less favorable functional recovery (67% of the control).[11] This suboptimal result was related, at least in part, to the fact that the regenerating axons from the NMEG pedicle failed to reinnervate the denervated MEPs in the target muscle.[11,13] Because MEP reinnervation is critical for motor recovery,[9,10] we implanted the NMEG to the NMZ of the target muscle and obtained better results.[14]

One of the major causes leading to poor functional recovery after EEA is that a few regenerated axons could pass through the coaptation site to reinnervate the denervated muscle.[7,8] Our study showed that the mean number of the intramuscular axons in the EEA-treated TA (46% of the control) was much lower than that in the NMEG-NMZ–reinnervated TA (76%). In addition, we found that the mean number of reinnervated MEPs in the EEA-treated muscles (59%) was significantly lower than that in the NMEG-NMZ–reinnervated muscles (83%). We believe that both the decreased regenerated axons and the innervated MEPs attributed to the reduced muscle mass, fiber size, and muscle force generation.

## Limitations

This study also has some limitations. Although NMEG-NMZ resulted in encouraging outcomes, complete functional recovery of the treated muscles was not achieved. This may be due at least in part to nonsynergistic NMEG transplantation. After NMEG-NMZ transfer, the denervated TA muscle fibers were reinnervated by GM motor neurons with different physiological properties. Studies showed that after nonsynergistic or even antagonistic nerve transfers, the outcome may be less favorable.[39] After crossing of nerves to antagonistic muscles, motor readjustment of the spinal network organization takes place.[40] Successful neuromuscular remodeling at the spinal cord[41,42] and the target muscle motor unit composition[43,44] would be an important factor for optimal functional recovery of the reinnervated muscle. In addition, further work is needed to evaluate the effectiveness of NMEG-NMZ for delayed muscle reinnervation.

Our results indicate that NMEG-NMZ is effective for immediate limb muscle reinnervation. However, more work is needed to seek novel therapies for further improving the outcomes of the NMEG-NMZ. In this regard, focal application of exogenous neurotrophic factors[45-48] and intraoperative brief (1 hour) low-frequency (20 Hz) continuous electrical nerve stimulation[49-52] would be useful for promoting axonal regeneration and functional recovery. Our previous animal studies demonstrated that the focal application of NGF/FGF-2[53] and brief electrical stimulation[31] enhanced outcomes of NMEG-NMZ surgery in a rat neck muscle model. These findings encourage us to further investigate the beneficial effects of these nerve growth–stimulating methods on limb reinnervation with NMEG-NMZ.

## CONCLUSION

The denervated TA muscle in the rat was successfully reinnervated with NMEG-NMZ technique as demonstrated by electrophysiological, gross, and histological evidence of reinnervation at 3-month postoperatively. One of the critical aspects for successful reinnervation with the NMEG-NMZ technique is to know the anatomic locations of the NMZs within the recipient and donor muscles. These can be easily outlined by using whole-mount nerve staining and AChE stain as described in our recent publications.[11,14,25] Our findings give strong support to the NMEG-NMZ technique as an alternative surgical procedure to reinnervate paralyzed limb muscles such as TA caused by peroneal nerve injuries or lesions.

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### Disclosures

The authors have no personal, financial, or institutional interest in any of the drugs, materials, or devices described in this article.

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## COMMENT

The authors report on the use of a nerve-muscle-endplate grafting (NMEG) technique for limb muscle reinnervation. At 3 months after tibialis anterior muscle reinnervation by transfer of a pedicle from the lateral gastrocnemius muscle, they report increased average muscle mass, number of regenerated axons, and proportion of reinnervated motor endplates as compared with end-to-end nerve anastomosis controls. Comparative muscle force recovery and static toe spread are also presented as evidence of enhanced functional improvement. Taken together, the data are intriguing and may prove to be clinically relevant. I eagerly await follow-up comparative analyses of validated quantitative gait and kinematic variables, generally considered to be more reliable markers of functional recovery.

**Francis Farhadi**
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# Focal Application of Neurotrophic Factors Augments Outcomes of Nerve–Muscle–Endplate Grafting Technique for Limb Muscle Reinnervation

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## Abstract

**Background** We have developed a novel muscle reinnervation technique called "nerve–muscle–endplate grafting (NMEG) in the native motor zone (NMZ)." This study aimed to augment the outcomes of the NMEG-NMZ (NN) by focal application of exogenous neurotrophic factors (ENFs) for limb reinnervation.

**Methods** Adult rats were used to conduct NN plus ENF (NN/ENF) and autologous nerve grafting (ANG, technique control). The nerve innervating the left tibialis anterior (TA) muscle was resected and the denervated TA was immediately treated with NN/ENF or ANG. For NN procedure, an NMEG pedicle was taken from the lateral gastrocnemius muscle and transferred to the NMZ of the denervated TA. For ANG, the nerve gap was bridged with sural nerve. Three months after treatment, the extent of functional and neuromuscular recovery was assessed by measuring static toe spread, maximal muscle force, wet muscle weight, regenerated axons, and innervated motor endplates (MEPs).

**Results** NN/ENF resulted in 90% muscle force recovery of the treated TA, which is far superior to ANG (46%) and NN alone (79%) as reported elsewhere. Toe spread recovered up to 89 and 49% of the control for the NN/ENF and ANG groups, respectively. The average wet muscle weight was 87 and 52% of the control for muscles treated with NN/ENF and ANG, respectively. The mean number of the regenerated axons was 88% of the control for the muscles treated with NN/ENF, which was significantly larger than that for the ANG-repaired muscles (39%). The average percentage of the innervated MEPs in the NN/ENF-treated TA (89%) was higher compared with that in the ANG-repaired TA (48%).

**Conclusion** ENF enhances nerve regeneration and MEP reinnervation that further augment outcomes of NN. The NN technique could be an alternative option to treat denervated or paralyzed limb muscles caused by traumatic nerve injuries or lesions.

**Keywords**

- nerve–muscle–endplate grafting
- exogenous neurotrophic factors
- peripheral nerve injury
- tibialis anterior
- gastrocnemius

Peripheral nerve injuries (PNIs) to the extremities represent a significant cause of morbidity and disability in both military and civilian populations.[1] Among soldiers with extremity injuries, 37% had PNIs.[2] More than one-third of soldiers with extremity injuries were not able to return to active duty because of disability.[3] In the civilian population, PNIs are mostly caused by vehicle accidents[4] and surgical intervention.[5] It has been reported that 20 million Americans suffer from PNIs,[6] with a health care burden of $150 billion annually.[7] Severe PNI has a potentially devastating impact on patients' quality of life.

Multiple surgical methods have been used to manage PNIs for restoring motor function, including nerve end-to-end anastomosis (EEA), end-to-side neurorrhaphy, direct nerve

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implantation, autologous nerve grafting (ANG), nerve transfer, synthetic nerve conduits, and many others.[8] Unfortunately, only about half of the patients treated will regain useful function with variability in other nerve repair paradigms.[9] Our animal studies showed that muscle force of the EEA-treated tibialis anterior (TA) recovered only 51 to 57% and average number of the intramuscular regenerated axons was reduced to 31 to 46% of the control in rats.[10,11] The unfavorable outcome of the EEA is associated with insufficient regenerating axons that pass through the suture site to reach the target muscle as a result of scar tissue formation.[10–12]

Nerve gap defects (segmental nerve injuries) are generally repaired with ANG, nerve conduits, or nerve transfer. However, the recovery rate of motor function for ANG, the gold-standard treatment for nerve gap injuries, is only about 40%.[13] The outcomes are even poorer with nerve conduits, resulting in functional reinnervation in only 13% of cases with larger nerve defects.[14,15] If the proximal nerve stump of an injured nerve is unavailable, nerve transfer is often used to repair the injured nerves, with mixed results.[16] The variable outcomes may be associated with a number of other factors, including the type of injury, the size of the nerve defect, the distance between nerve repair site and the target muscle, the time interval between nerve injury and nerve surgery, the age and general health condition of the patient, and the experience of the surgeon. Despite intensive research and plentiful published procedures, few methods have achieved optimal functional recovery.

After PNIs and nerve repair, unsatisfied functional recovery is due mainly to insufficient nerve regeneration and reinnervation of denervated motor endplates (MEPs) in the target muscle.[17] Recognizing the need for a better treatment approach, we developed a new reinnervation technique called "nerve–muscle–endplate grafting (NMEG)."[18] The idea is that a functionally important denervated or paralyzed muscle following PNI can be reinnervated by transferring an NMEG pedicle from a neighboring expendable muscle. In our studies, an NMEG pedicle was taken from the native motor zone (NMZ) of the donor muscle and implanted it into the NMZ in the recipient muscle. We have demonstrated that NMEG-NMZ (NN) results in promising motor recovery of the reinnervated neck (82%)[19] and limb (79%)[11] muscles in the rat.

This study aimed to further augment the outcomes of NN by focal administration of exogenous neurotrophic factors (ENFs) to promote nerve regeneration and functional recovery.[20–23]

## Methods

### Animals

Thirty 3-month-old female Sprague-Dawley rats (Charles River Laboratories, MA) were used in this study as female rats, unlike males, retain a constant body weight throughout adulthood. Furthermore, we were able to compare the results with those obtained in our previous studies of the NN reinnervation in neck[18,19] and limb[11] muscles, which also utilized female rats. Experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee and by the USAMRMC Animal Care and Use Review Office. All animals were housed and handled in accordance with the NIH *Guide for the Care and Use of Laboratory Animals*.

### Animal Groups, Target Muscles, and Reinnervation Methods

Thirty adult rats were randomly assigned into two groups (15 rats per group): NN plus ENF (NN/ENF) group and ANG (technique control) group. The left TA was denervated by resecting a 10-mm segment of its nerve and immediately reinnervated by NN/ENF or ANG as described in the following.

### Procedures for NN, ANG, and Focal Application of ENFs

Under general anesthesia with an intraperitoneal injection of a mixture of ketamine (80 mg/kg) and xylazine (5 mg/kg), surgical procedures were performed under aseptic conditions on the rat left legs. The left TA and gastrocnemius muscle (GM) and the three major branches of the sciatic nerve were exposed by an incision made in the posterior thigh and calf of the hind limb (►**Fig. 1**).

Animals in the NN/ENF group were subjected to NN procedure and focal administration of ENFs (a mixture of nerve growth factor [NGF] and basic fibroblast growth factor [FGF-2]) as described.[11,24] Briefly, a 10-mm segment of the TA nerve was resected to denervate the muscle. The denervated TA was treated with NN/ENF. For NN procedure, the NMZs in the left TA and GM were outlined according to the locations of their MEP bands as visualized by whole-mount acetylcholinesterase staining and nerve supply patterns as revealed by Sihler's stain, a whole-mount nerve staining (►**Fig. 1**) as described.[11,24] An NMEG pedicle is composed of a block of muscle ($\sim 8 \times 6 \times 4$ mm) in continuity with its innervating nerve branch, axon terminals, and an MEP band with numerous neuromuscular junctions. The pedicle was taken from the NMZ of the lateral GM muscle (GM-l). Then, a muscular defect (recipient bed), with the same dimensions as the NMEG pedicle, was made on the superficial layer of the muscle fibers in the NMZ of the TA. Finally, the NMEG pedicle was embedded into the muscular defect and sutured with 10–0 nylon microsutures (►**Fig. 1**).

►**Fig. 2** illustrates steps for NN procedure and focal application of NGF/FGF-2. During NN procedure, the muscular defect created on the TA was filled with 0.5 mL of fibrin sealant (TISSEEL Kit; fibrinogen 90 mg/mL, thrombin 500 IU/mL, 4 mmol/L $CaCl_2$; Baxter Healthcare Corp., Westlake Village, CA), containing recombinant rat NGF (100 ng/mL) and FGF-2 (100 µg/mL) (R&D Systems, Minneapolis, MN). As reported, focal application of NGF and FGF-2 at these concentrations resulted in better functional recovery.[25,26] Then, the NMEG pedicle was placed on the fibrin sealant and sutured with four 10–0 microsutures. Finally, the wound was closed in layers.

In this study, ANG was conducted as a technique control. Clinically, ANG is often used to manage nerve gap defects (segmental nerve injuries). This type of nerve injury is also an indication for NN. Immediately after muscle denervation by resecting a 10-mm segment of TA nerve 5 mm proximal to

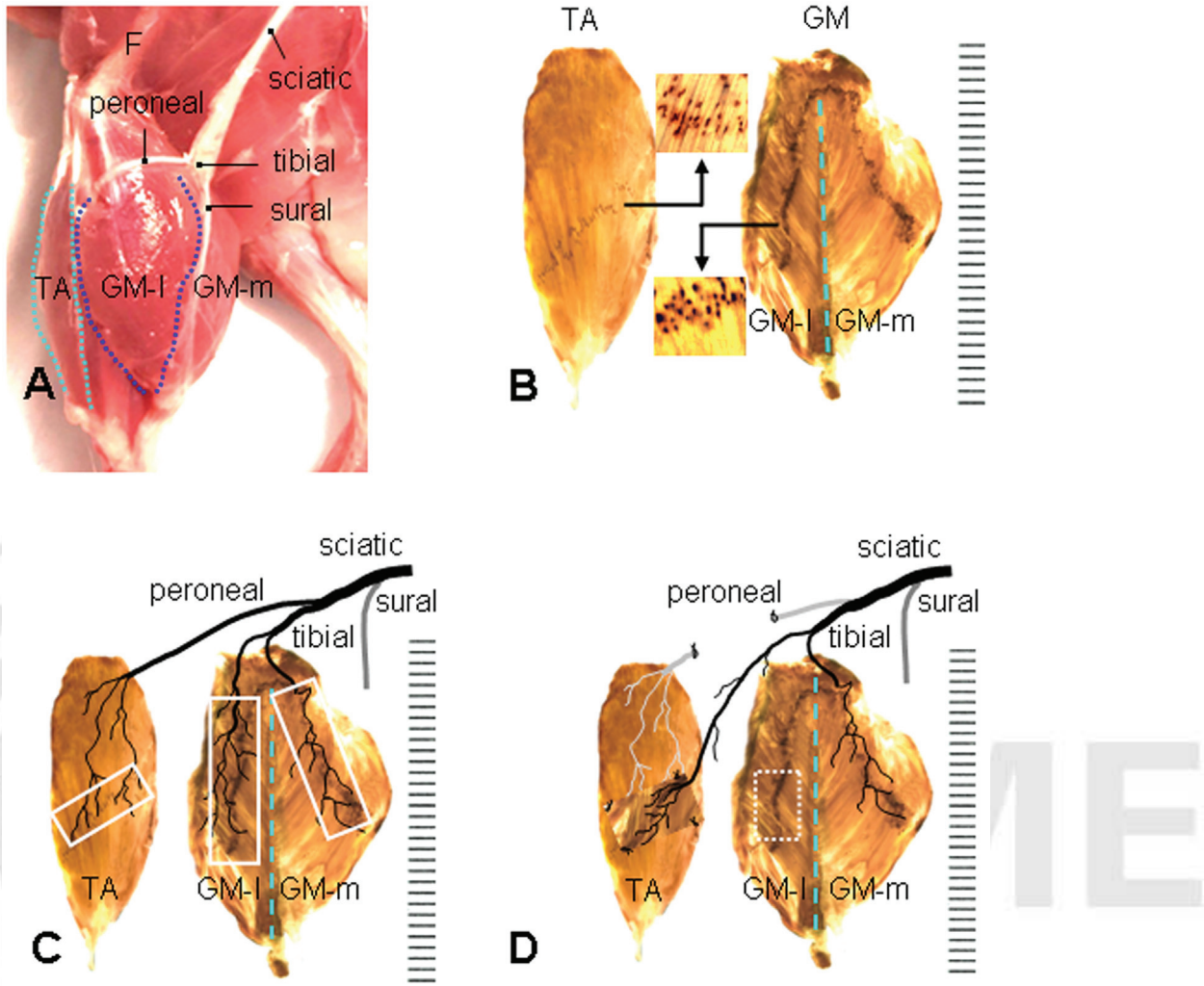


**Fig. 1** Neuromuscular organization of the rat tibialis anterior (TA) and gastrocnemius (GM) muscles and implantation of NN. (**A**) Lateral view of a rat left fresh hind limb, showing the anatomical relationship between the TA and GM as well as sciatic nerve and its major branches (i.e., peroneal, tibial, and sural nerves). Note that the GM is composed of two neuromuscular compartments, lateral (GM-l) and medial (GM-m). The tibial nerve enters the GM on the top between GM-l and GM-m. F, femur. (**B**) Locations of motor endplate (MEP) bands with numerous MEPs (*black dots*) within the rat TA, GM-l, and GM-m as demonstrated by wholemount acetylcholinesterase (AChE) staining. *Vertical dashed line* in the GM indicates midline. (**C**) Native motor zones (NMZs) containing MEPs and their innervating axons (*boxed regions*) within the TA, GM-l, and GM-m. Nerve branching and supply patterns are determined by Sihler's stain, a wholemount nerve staining technique. (**D**) Images showing that the denervated TA muscle by resecting a segment of its nerve is reinnervated with NN. An NMEG pedicle with a nerve branch is taken from the NMZ (*boxed region*) of the GM-l and transplanted to the NMZ of the denervated TA muscle.

the motor point, the nerve gap defect was bridged with the same length of sural nerve (sensory) using 10–0 nylon microsutures.

Three months after surgery, postoperative evaluations were performed on both sides. A given parameter examined was recorded and reported as a ratio of the operated to the contralateral unoperated side.

## Evaluating Functional Recovery

### Static Toe Spread Analysis

Static toe spread (TS) analysis proposed by Bervar[27] has been demonstrated to be a useful method for assessing limb motor function.[28,29] In this study, static TS analysis was performed just before muscle force measurement as described.[11] In brief, the rat was placed in an acrylic $40 \times 20 \times 20$ cm container on a transparent base plate. The plantar surface of the rat hind limb paws was photographed with a camera that was positioned underneath the base plate of the container. The images from a given rat were used to measure the distance (mm) between the 1–5 TS and between the 2–4 intermediate TS (ITS) on the operated (O) and nonoperated (N) sides. The mean values of TS and ITS in each group were used to determine the TS and ITS factors (TSF and ITSF): $TSF = (OTS - NTS)/NTS$ and $ITSF = (OITS - NITS)/NITS$. Bervar[27] proposed a static sciatic index (SSI) for TS analysis. The SSI is calculated using the static TSF and ITSF in the equation: $SSI = (108.44 \times TSF) + (31.85 \times ITSF) - 5.49$. An index score of 0 is defined as normal and an index score of −100 indicates a complete functional loss.

 

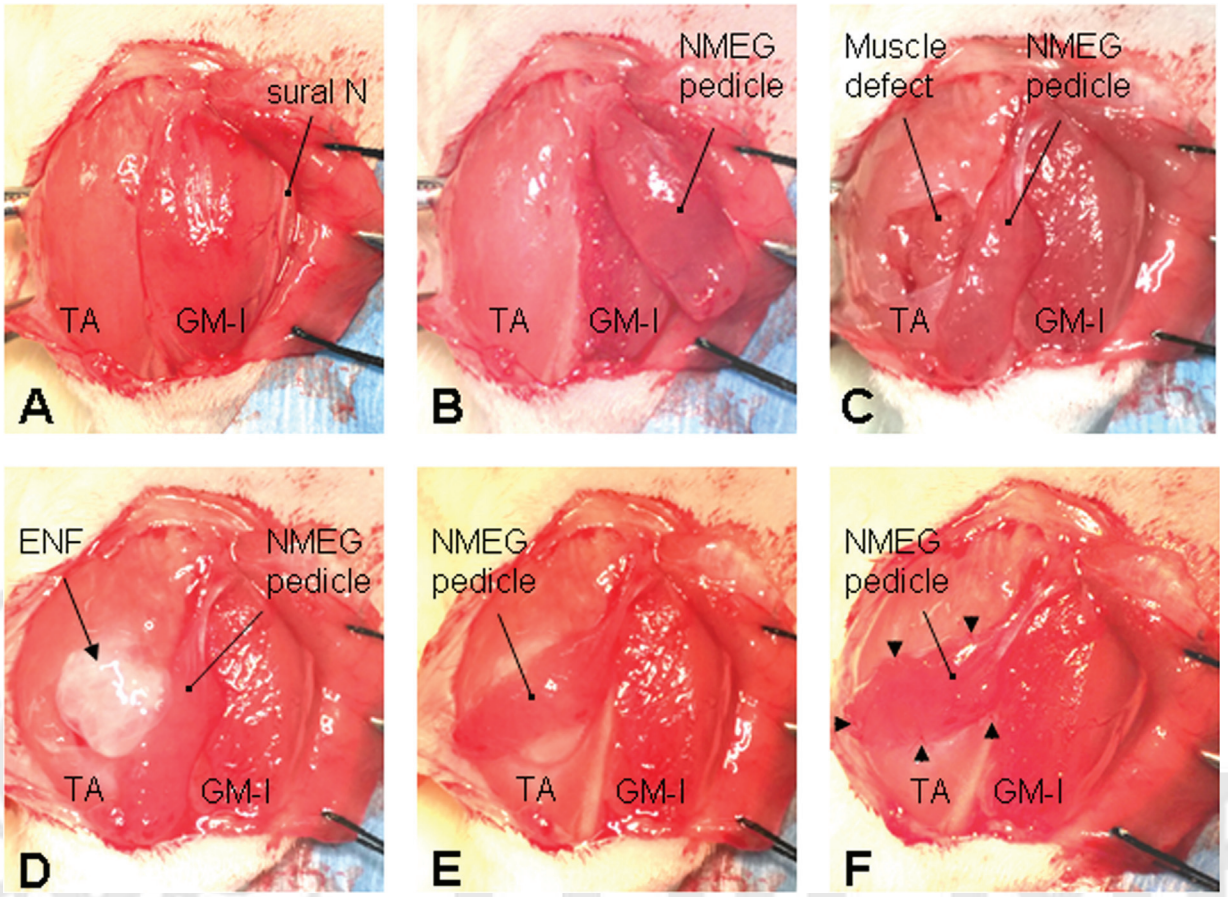


**Fig. 2** Images showing NN/ENF procedures. (**A**) Lateral view of the recipient TA and donor GM-I in the rat left hind limb. (**B**) An NMEG pedicle is taken from the GM-I. (**C**) A muscle defect is created on the surface of the NMZ of the TA. (**D**) A fibrin sealant, containing exogenous neurotrophic factors (ENF) NGF and FGF-2, is applied locally to the TA muscle defect. (**E**) The fibrin sealant is covered by the prepared NMEG pedicle. (**F**) The transferred NMEG is sutured with 10–0 nylon microsutures as indicated by *arrowheads*.

### Maximal Muscle Force Measurement

Three months after treatment, animals underwent maximal muscle force measurement under general anesthesia to quantify the extent of motor recovery of the left TA as described.[11] Briefly, the transplanted tibial nerve branch supplying the NMEG (NN/ENF group) or the repaired peroneal nerve (ANG group) was isolated for electrical stimulation.

The TA's distal tendon was cut, and the muscle was separated from surrounding tissues proximally to the level of its origin. The transected TA tendon was tied with a 4–0 suture and attached to force transducer connected to a servomotor lever arm of the Dual-Mode Lever System (305B-LR; Aurora Scientific Inc, Aurora, Ontario, Canada). Biphasic rectangular pulses were delivered to the nerve examined through a stimulation and recording system (National Instruments Corp, Austin, TX) controlled by user-written LabVIEW 8.2 software (National Instruments Corporation). The nerve was placed on hook-shaped stimulating electrodes and stimulated with 200-ms trains of biphasic rectangular pulses at the optimal muscle length. The duration of each phase of stimulation pulse was set at 0.2 ms and train frequency was set at 200 pulses/s. The stimulation current was gradually increased from 0 to 5 mA. A break of at least 1 minute was taken between two stimulations to permit muscle recovery.

DIAdem 11.0 software (National Instruments) was used to analyze the collected force data. Maximal muscle force from the treated TA was reported as a percentage of the muscle force from the contralateral healthy control muscle. During force measurement, the muscle and nerve examined were bathed regularly with warmed mineral oil to keep muscle temperature between 35°C and 36°C and the core body temperature was maintained at the same level with a closed circuit homeothermic blanket system for rodents.

## Examining Muscle Weight, Fiber Morphology, Denervated Myofibers, and Fiber Type Grouping

Immediately after muscle force measurement, both TA muscles were removed, weighed using a microscale, photographed, and prepared for tissue studies. Each of the removed TA muscles was divided into two segments: superior one-third and inferior two-thirds, which were frozen and sectioned on a cryostat (Reichert-Jung 1800; Mannheim, Germany) at −25°C. The superior segments were sectioned transversely (10-µm thick) and stained with hematoxylin and eosin (H&E) and monoclonal antibodies to assess muscle fiber morphology, fiber type grouping, and denervated and

 

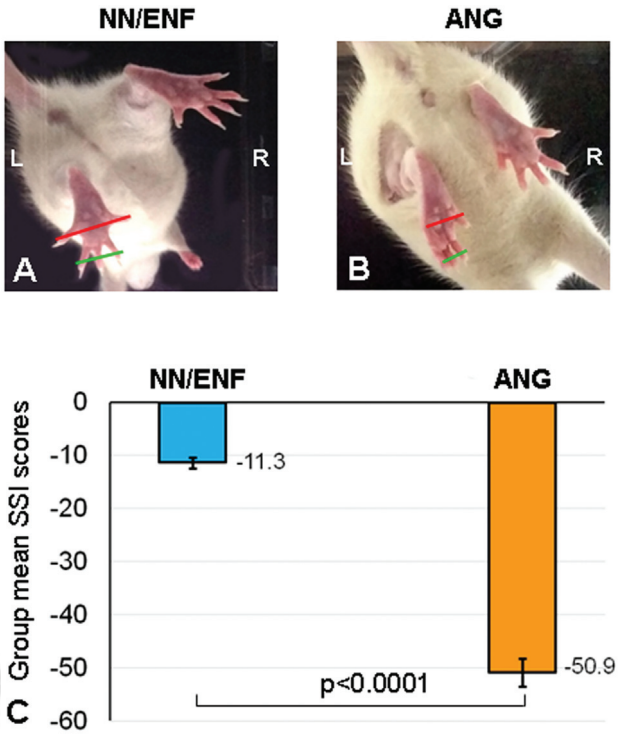


**Fig. 3** Toe spread recovery. Comparison of hind limb footprints between left (L) operated and right (R) nonoperated sides in the rats treated with NN/ENF (**A**) and ANG (**B**). Note that NN/ENF resulted in better toe spread recovery compared with ANG. (**C**) Average group data, showing that the differences in calculated static sciatic index (SSI) are statistically significant between both groups ($p < 0.0001$).

atrophied myofibers. The inferior segments were cut horizontally (50-µm thick) and immunostained to quantify regenerated axons and reinnervated MEPs.

### Immunohistochemistry for Detecting Fiber Type Grouping

Monoclonal antibody NOQ7-5-4D was used to label slow type I muscle fibers for detecting fiber type grouping as described.[30] Some cross-sections were fixed in 4% paraformaldehyde for 10 minutes; blocked in 2% bovine serum albumin (BSA) and 0.1% Triton X-100 for 20 minutes; incubated with monoclonal antibody NOQ7-5-4D (dilution: 1:1,000; Sigma, St. Louis, MO) for 1 hour; treated with a Vectastain antimouse immunoglobulin G (IgG; ATCC, Rockville, MD) for 1 hour; treated in ABC[Q3] reagent for 1 hour; and processed with DAB substrate kit (SK-4100; Vector Labs, Burlingame, CA) for 10 minutes. Control sections were stained without primary antibody.

The stained muscle sections were viewed on a Zeiss photomicroscope (Axiophot-1; Carl Zeiss, Goettingen, Germany) and photographed with a USB 3.0 digital microscope camera (Infinity 3-3URC; Lumenera Corp., Ottawa, Ontario, Canada). Three stained cross-sections of a given muscle were randomly selected at different spatial levels through the muscle to identify fiber type grouping that is indicative of partial denervation and reinnervation.

### Neural Cell Adhesion Molecule Immunohistochemistry for Detecting Denervated Myofibers

Neural cell adhesion molecule (N-CAM) is rich on the surface of early embryonic myotubes, decreases in level as development proceeds, almost disappears in the adult muscles, reappears in adult denervated muscles, and is lost in reinnervated muscles.[31] Therefore, N-CAM is a molecular marker of muscle fiber denervation. In this study, some cross-sections were immunostained to detect denervated myofibers as described.[30] In brief, the sections were fixed with methanol at −20°C for 20 minutes; blocked with 5% goat serum (Sigma, St.

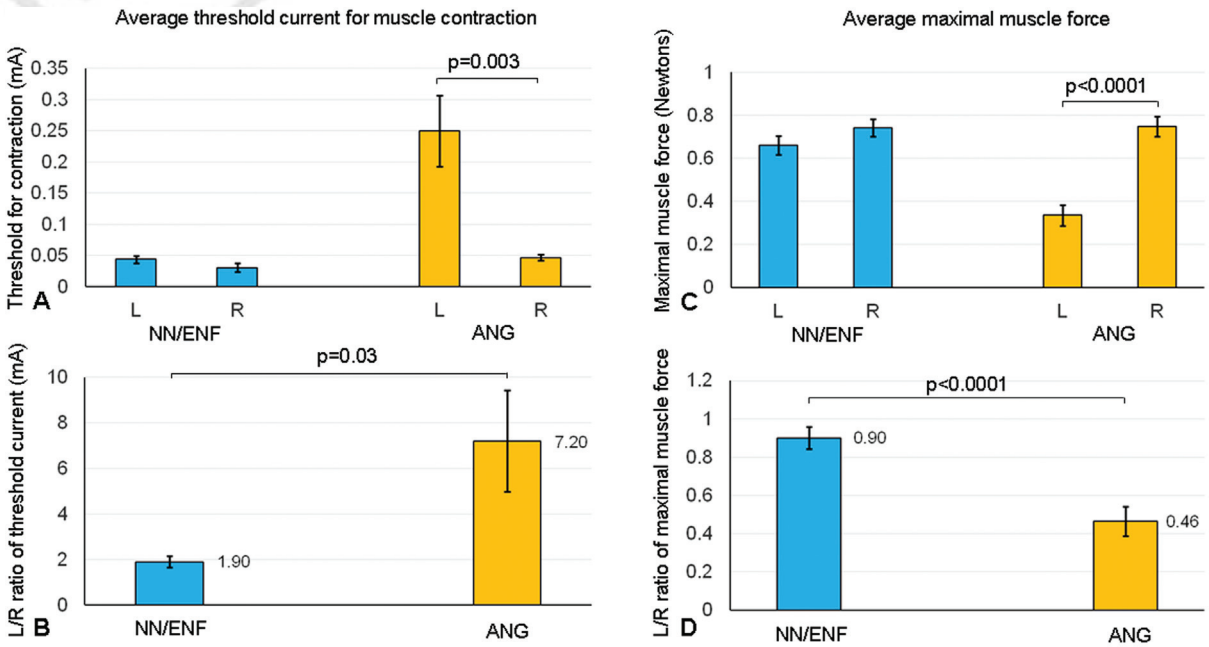


**Fig. 4** Comparison of muscle force from rat TA muscles treated with NN/ENF and ANG. (**A**) Average threshold current for muscle contraction. (**B**) Left (L) to right (R) ratio of threshold current. (**C**) Average maximal muscle force. (**D**) L/R ratio of maximal muscle force.

 

Louis, MO) in phosphate-buffered saline (PBS) for 30 minutes; incubated with a primary monoclonal rabbit antirat N-CAM antibody (Chemicon, Temecula, CA) for 2 hours; and incubated with a secondary CY3-conjugated goat antirabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 hour. Control sections were stained without primary antibody. The stained sections were mounted with Vectashield mounting medium (Vector) and photographed.

Three stained sections of a given muscle were randomly selected at different spatial levels through the muscle to identify N-CAM positive fibers. The mean proportion of the denervated fibers for each group was computed.

## Quantifying Regenerated Axons and Reinnervated MEPs

### Neurofilament (NF) Staining to Label Regenerated Axons

Monoclonal antibody SMI-31 (Covance Research Products, Berkeley, CA), a marker for all axons, was used to stain horizontal muscle sections as described.[11,19] In brief, the sections were blocked in 2% BSA for 30 minutes; incubated with SMI-31 (1:800) in PBS containing 0.03% Triton at 4°C overnight and antimouse biotinylated secondary antibody (1:1,000; Vector) for 2 hours; treated with a VectaStain ABC kit (1:1,000; ABC Elite; Vector); and reacted with diaminobenzidine-nickel as chromogen to visualize peroxidase labeling. Control sections were stained without primary antibody.

The stained tissue sections were photographed. The density of the intramuscular axons was determined by computing the NF immunoreactive (NF-ir) axons and the area fraction of the axons within a section area (1.0 $mm^2$) as described.[11] Three stained sections at different spatial levels through a muscle were randomly selected to compute NF-ir axons with ImageJ (v1.45s; National Institutes of Health, Bethesda).

### Double-Fluorescence Staining to Identify MEPs

Double-fluorescence staining was used to label MEPs as described.[11,19] In brief, some horizontal sections were fixed in Zamboni fixative at 4°C for 20 minutes; blocked in 4% normal goat serum for 30 minutes; incubated overnight at 4°C with primary antibody SMI-31 to label axons (1:1,000, Covance Research Products Inc); and incubated for 2 hours both with Alexa Fluor 488 goat antimouse IgG secondary antibody (1:500; Invitrogen Corp., Carlsbad, CA) to label axons and with Alexa Fluor 596–conjugated α-bungarotoxin (1:500; Invitrogen) to visualize postsynaptic acetylcholine receptors (AChRs) at MEPs. Control sections were stained without primary antibody.

The stained muscle sections were examined under a photomicroscope equipped with epifluorescence optics and photographed. Intramuscular axons (*green*) were labeled with SMI-31, while postsynaptic AChR site in the MEPs (*red*) was labeled with α-bungarotoxin. At least 100 stained MEPs in each muscle were randomly selected to determine the percentages of the innervated (visible axon attachment) and noninnervated (no visible axon attachment) MEPs.

### Statistical Analysis

Static TS measures, muscle force values, wet muscle weights, NF-ir axon counts, and innervated and noninnervated MEPs

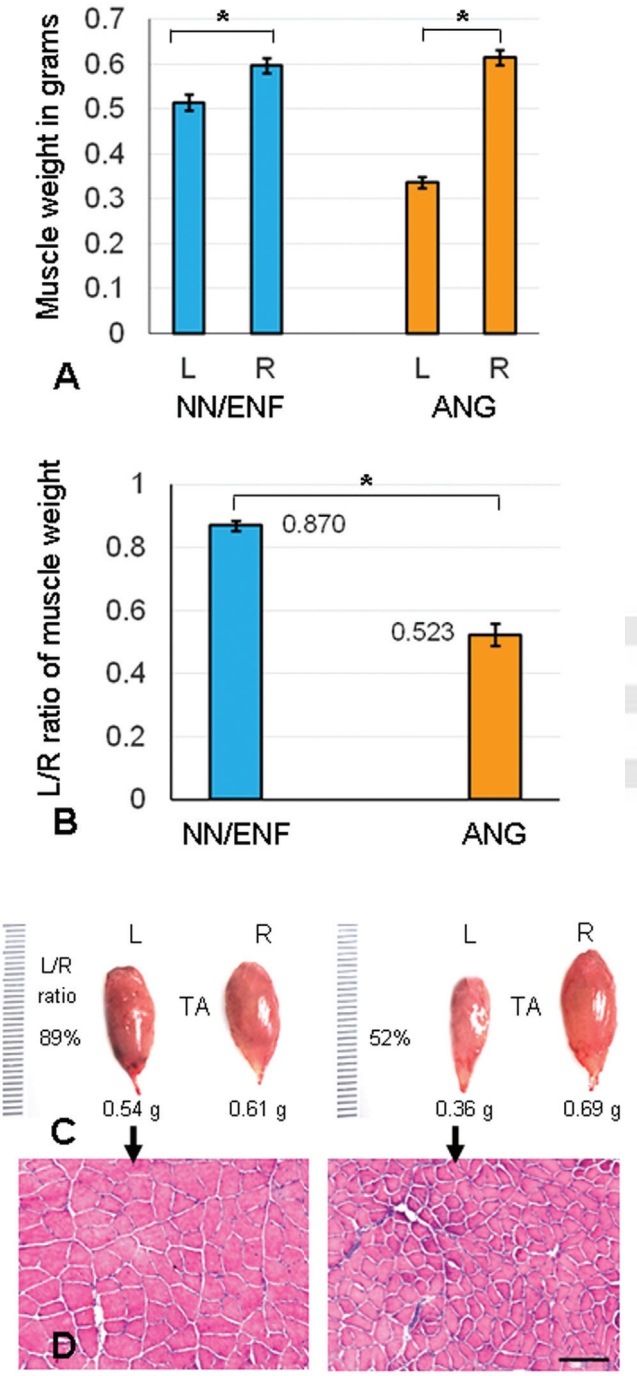


**Fig. 5** (**A**) Mean group data on muscle weights of the left (L) treated and right (R) control TA muscles. Note that the mean weight of the left TA treated with NN/ENF is higher than that of the ANG-repaired TA. (**B**) L/R ratio of the muscle weight in the NN/ENF and ANG groups (*$p < 0.0001$). *Bars* represent standard error. (**C**) Comparisons of the muscle masses of the left treated and right control TA muscles from individual rats in the NN/ENF (left pair, rat #5) and ANG (right pair, rat #3) groups. (**D**) H&E-stained muscle sections of TA treated with NN/ENF (left) and ANG (right), showing differences in fiber size and structure between the treatments. Note that NN/ENF-treated muscle exhibited very good preservation of muscle structure with less fiber atrophy compared with ANG-treated muscle. Scale bar = 150 µm.

 

of the TA muscles in each rat were computed. The Student *t*-test (paired or unpaired as appropriate) was used for comparison of normally distributed data. Mann–Whitney *U* test (for unpaired comparisons) or Wilcoxon signed rank test (for paired comparisons) was used for comparison of nonparametric data. Type I error at $p < 0.05$ (two-tailed *t*-test) was treated as statistically significant. All comparisons were made with SAS 9.4 software or with online statistics calculators.

## Results

### Static Toe Spread

The rats in the NN/ENF group (►**Fig. 3A**) had better TS recovery compared with those in the ANG group (►**Fig. 3B**). The mean SSI score was calculated to be −11.3 and −50.9 for the NN/ENF and ANG groups, respectively (►**Fig. 3C**). In other word, the TS recovered up to 89% of the control for the NN/ENF group and 49% for the ANG group ($p < 0.0001$).

### Maximal Tetanic Muscle Force

►**Fig. 4A** shows the average threshold current that produced visible muscle contraction of the left (L) treated and right (R) healthy control TA muscles. ►**Fig. 4B** shows the L/R ratio of threshold current for muscle contraction. The threshold currents from the treated muscles were significantly higher than those from the controls in the ANG group ($p = 0.003$), but not in the NN/ENF group ($p > 0.05$). On average, ANG-repaired TA had a higher L/R ratio of the threshold stimulation currents (7.20) compared with NN/ENF-treated TA (1.90, $p = 0.03$).

►**Fig. 4C** shows the average maximal muscle force of the left treated and right control TA muscles, and ►**Fig. 4D** shows the L/R ratio of the maximal muscle force for the NN/ENF and ANG groups. Increasing stimulation current was accompanied by increasing muscle force until muscle force reached horizontal asymptote at the current of about 0.1 mA. Maximal muscle force was calculated in response to larger stimulation currents from 0.5 to 0.75 mA. Maximal muscle force of the treated TA was significantly smaller than that of the control in ANG group ($p < 0.0001$), but the difference did not reach statistical significance in the NN/ENF group ($p > 0.05$). NN/ENF resulted in much better muscle force recovery (90%) compared with ANG (46%) and NN alone (79%) as reported elsewhere.[11] The rate of treated TA to control muscle force was significantly higher ($p < 0.0001$) in the NN/ENF group compared with ANG group.

### Muscle Weight and Muscle Fiber Morphology

Muscle weight, size, and fiber morphology of the left treated and right control TA muscles in each group are displayed in ►**Fig. 5**. Mean group data on weights of the left treated and right control TA muscles (►**Fig. 5A**) and left (L)/right (R) ratio (►**Fig. 5B**) showed that NN/ENF resulted in better muscle weight recovery than ANG ($p < 0.0001$). The TA muscle weights were recovered to 87 and 52% for the NN/ENF and ANG groups, respectively (►**Fig. 5B**).

►**Fig. 5C** shows paired TA muscles of some individual rats selected from NN/ENF (left) and ANG (right) groups. Note that the size of the TA treated with NN/ENF was close to that of the right control, whereas the ANG-treated TA had a significant loss of muscle mass. H&E-stained cross-sections (►**Fig. 5D**) showed that NN/ENF-treated muscle (left) exhibited less fiber atrophy compared with the ANG-treated TA (right).

### Fiber Type Grouping and Denervated Muscle Fibers

Type I fiber grouping was observed frequently in the ANG-repaired TA muscles (►**Fig. 6A**) and occasionally identified in the NN/ENF-treated muscles (data not shown).

Denervated and atrophied muscle fibers in the rat TA were labeled with N-CAM immunohistochemistry. N-CAM was expressed around muscle fibers and/or muscle fibers displayed sarcoplasmic expression. The N-CAM–positive fibers were identified more frequently in the ANG-treated TA muscles (►**Fig. 6B**) than in the NN/ENF-treated muscles. The N-CAM–positive fibers accounted for 32% of the total fiber population in the ANG-treated TA and 8% in the NN/ENF-treated TA.

### Regenerated Axons and Reinnervated MEPs

►**Fig. 7** summarizes regenerated axons and reinnervated MEPs in the treated TA muscles. The profiles of the

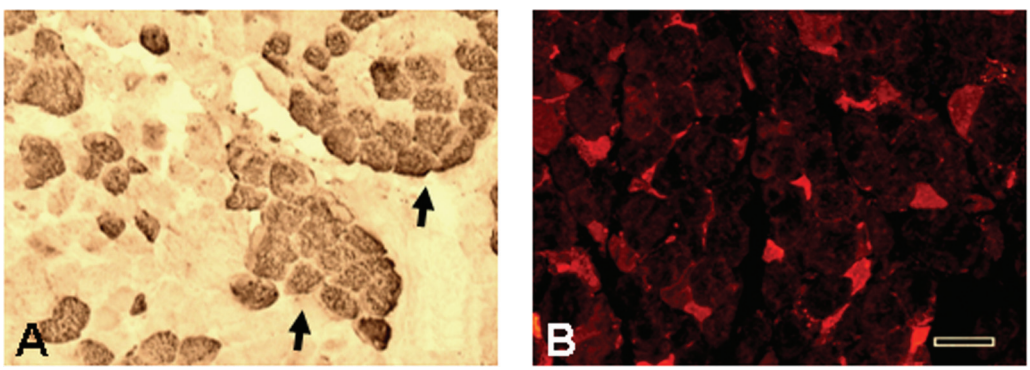


**Fig. 6** Cross-sections of the rat TA treated with ANG, showing fiber type grouping and denervated and atrophied fibers. (**A**) A section stained with monoclonal antibody NOQ7-5-4D specific for slow type I fibers (*dark staining*), showing type I fiber grouping (*arrows*). (**B**) A section immunostained for N-CAM, showing denervated and atrophied (*bright staining*) fibers. Scale bar = 50 µm for A and B.

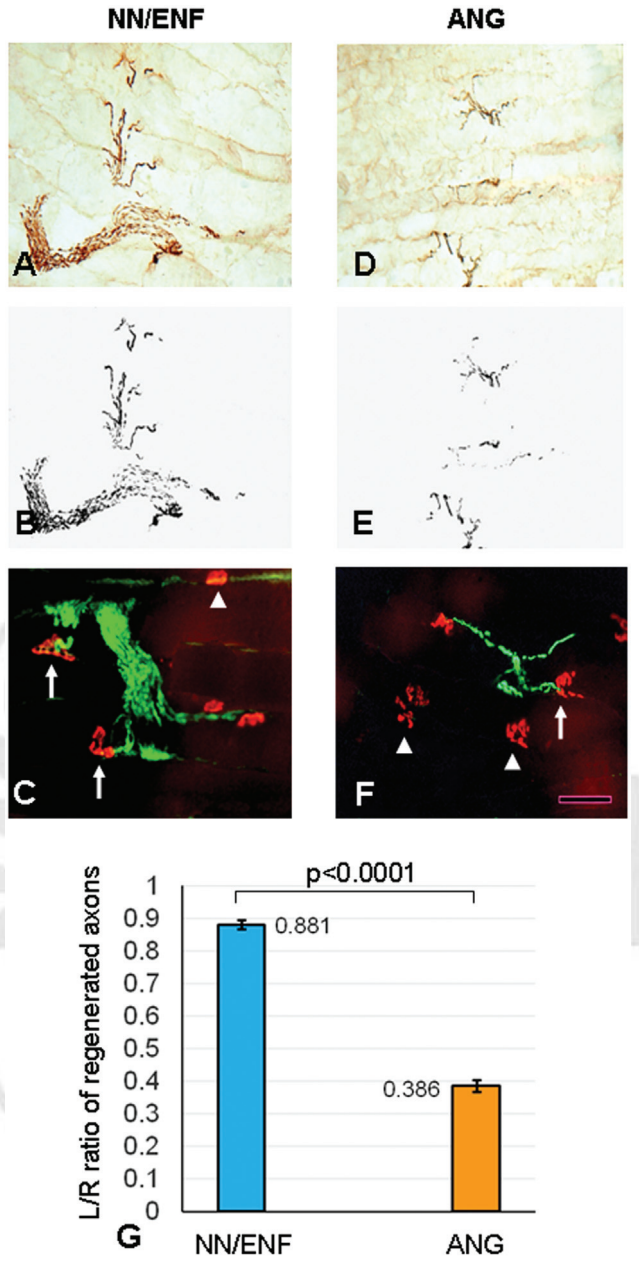


**Fig. 7** Immunostained horizontal sections showing regenerated axons and reinnervated MEPs in the TA muscles treated with NN/ENF (**A–C**) and ANG (**D–F**). Muscle sections stained with NF staining (**A, D**) showing intramuscular regenerated axons (*darkly stained threads and dots*). The NF-stained sections were converted to 8-bit (binary) images by using ImageJ software to calculate intramuscular axons (**B, E**). The density of the axons was evaluated by estimating the number of the NF-positive axons within a section area (1.0 $mm^2$). The sections stained with double fluorescence staining (**C, F**) showed MEPs (*red*) and their innervating axons (*green*). *Arrows* indicate innervated MEPs with visible axon attachments, whereas arrowheads indicate noninnervated MEPs without visible axon attachments. Note that the TA muscles treated with NN/ENF (**A–C**) exhibited much more regenerated axons and reinnervated MEPs compared with those treated with ANG (**D–F**). Scale bar = 50 µm for A through F. (**G**) Mean group data on the left/right (L/R) ratio of the regenerated axons. The mean number of the regenerated axons on the left treated TA was 88% of the right control for the NN/ENF, which was significantly higher than that for the ANG-treated muscle (39%) ($p < 0.0001$).

regenerated axons (►**Fig. 7A, D**) and converted images (►**Fig. 7B, E**) for computing the numbers of the regenerated axons are presented. The TA muscles treated with NN/ENF (►**Fig.7A–C**) exhibited more axonal regeneration compared with the ANG-treated muscles (►**Fig. 7D–F**). ►**Fig. 7G** shows group mean data on the left/right (L/R) ratio of the regenerated axons. The mean number of the regenerated axons in the NN/ENF-treated TA was 88% of the control, which was significantly higher than that in the ANG-repaired TA (39%) ($p < 0.0001$). The innervated and noninnervated MEPs were labeled with double-fluorescence staining (►**Fig. 7C, F**). The average percentage of the innervated MEPs in the NN/ENF group (89%) was higher compared with that in the ANG group (48%).

## Discussion

To the best of our knowledge, this is the first study to augment outcomes of NN in limb reinnervation by using fibrin-based neurotrophic factor delivery system. Our experiments documented that NGF/FGF-2 could promote motor restoration of the NN-reinnervated limb muscle. The extent of functional recovery is positively correlated to the extent of muscle atrophy, axonal regeneration, and MEP reinnervation.

Unlike current nerve repair procedures, NN potentially bypasses the zone of injury, obviates the need for an intercalary nerve graft, physically shortens nerve regeneration distances, decreases regeneration time by directly transferring the NMEG pedicle to the NMZ of the target muscle, provides an abundant source of axons that favor axonal regeneration, rapidly establishes axon–MEP connections, and results in favorable functional recovery. Therefore, NN can be used for treating denervated muscles in the legs, arms and shoulders, face, larynx, and many others caused by different types of PNIs, including segmental nerve injuries.

Previous studies have demonstrated that NGF improves axonal regeneration and functional recovery.[21,32] FGF-2 promotes nerve regeneration,[33] increases the numbers of functional Schwann cells and regenerating axons[33] and MEPs,[25] and improves motor recovery.[25] The delivery of biologically active molecules locally during regeneration has been extensively pursued in animal models for decades. Various ENFs have been used in nerve repair[21] and tubulization.[34] They have been administrated focally via direct injection into the target nerve[35] or muscle,[25] or a surgically placed osmotic pump.[36] More recently, PNI and nerve repair models are often managed using focal administration of a fibrin sealant containing ENFs. This approach permits slow continual release of ENFs directly to the damaged and/or repaired nerve in order to accelerate nerve regeneration for better functional recovery.[20,21,37,38] In vitro, ENFs in fibrin sealant can be released locally over periods of 2 to 4 weeks.[20,38] Studies have demonstrated that combined use of more ENFs results in better results than single factors alone.[36,39] Thus, the fibrin-based ENF delivery system is a promising method for slow continual release of ENFs that

 

favors accelerating axonal regeneration, thereby improving reinnervation outcomes. The findings from this study suggest that the combination of NGF and FGF-2 can improve outcomes of NN and may become a useful adjunct therapy for muscle reinnervation.

We have shown that focal application of ENFs has the potential to enhance axonal regeneration, MEP reinnervation, and muscle mass preservation, thereby augmenting outcomes of NN. However, further studies are needed to assess the efficacy of the NN and nerve growth stimulation methods for delayed muscle reinnervation. Clinically, some injured nerves cannot be repaired in the acute phase of PNI due to concomitant injuries. In this case, nerve reconstruction is often performed after 3 to 6 months' delay if there is no evidence of reinnervation. Unfortunately, by 3 months after PNI, the number of distal axons are reduced by about 80 to 90%,[40] and chronic denervation or delayed muscle reinnervation results in insufficient axonal regeneration and very poor functional recovery.[17] Our recent studies showed that denervation of the rat sternomastoid muscle for 3, 6, and 9 months resulted in a progressive decline in muscle weight (38, 31, and 19% of the control, respectively), fiber diameter (52, 40, and 28%), and number of MEPs (79, 65, and 43%).[41] Thus, muscle loss and reduced axons and MEPs would be major factors leading to poor functional recovery. At present, however, there is a lack of effective methods to prevent or delay muscle and MEP loss after denervation. There is a pressing need to develop specific therapeutic strategies to minimize these complications of delayed reinnervation.

This study also has some limitations. Rodents are known to have a super-regenerative capacity and the so-called "blow-through effect" with regenerating axons, whereas axonal regeneration in humans is relatively slow. In addition, our findings reported here come from rat immediate reinnervation model. Clinically, PNIs are often treated in a delayed fashion. Therefore, further work is needed to assess the efficacy of NN and focal administration of NGF/FGF-2 for delayed muscle reinnervation.

## Conclusions

The results generated from this study allow us to make the following conclusions:

- First, the outcomes of NN reinnervation technique can be augmented by focal application of ENFs.
- Second, the NN could be an alternative option to manage denervated limb muscles caused by PNIs, especially segmental nerve injuries.
- Finally, more work is warranted to determine the effectiveness of this combined approach for delayed limb reinnervation.

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### Conflict of Interest[Q2]

None declared.

### Acknowledgments

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# Transition Plan Questionnaire

**Directions: Please answer all questions that apply for each product under development. Please fill out one document per product.**
***This is not an application for funding; however, answers will help us understand the outcomes and products from your award.***

**1.** After the award closes, would you be willing to periodically provide voluntary information (via email) regarding the project status (i.e. where the research is headed)? **Yes** ☑ or **No** ☐
*These responses will help CDMRP demonstrate the return on its investments and will help demonstrate that the CDMRP is a responsible and successful steward of federal research funding.*

**2.** What **conclusion(s)** does your final data support?

Our data generated from this project allow us to make the following conclusions:

First, the nerve-muscle-endplate grafting (NMEG) in the native motor zone (NMZ) results in better functional recovery in both immediate and delayed reinnervation models as compared with commonly used end-to-end anastomosis (EEA) and autologous nerve graft (ANG). NMEG-NMZ technique could be an alternative option to manage denervated limb muscles caused by peripheral nerve injuries, especially segmental nerve injuries.

Second, the outcomes of the NMEG-NMZ technique for 3-month delayed reinnervation were less optimal compared to those of immediate NMEG-NMZ reinnervation. Delayed reinnervation causes muscle atrophy which contributes to suboptimal outcomes. More work is warranted to develop novel strategies to maintain muscle mass following nerve injuries for delayed limb reinnervation.

Third, outcomes of the NMEG-NMZ surgery can be augmented by focal application of exogenous neurotrophic factors (ENFs) such as NGF/FGF-2 for nerve regeneration and muscle reinnervation.

Finally, surgical-biological combination approach would be an ideal way to manage peripheral nerve injuries.

**3**. Will you/have you applied for/obtained follow-on-funding for this project? **If yes**, please list (a) funding organization, (b) total budget requested/obtained, and (c) title of the funded proposal. *This information will be recorded as an outcome to this award*.

I submitted a grant application (log number: PR220096) entitled "Combination strategies for limb reinnervation" in May 2022. This application was responsive to the FY22 PRMRP (IIRA) Topic Area – Neuroscience – Topic Areas "Peripheral Neuropathy" and "Trauma" and one of the Strategic Goals – Treatment – "Develop and test treatment strategies to manage symptoms and improve quality of life for those affected by associated neurological conditions." This proposal focused on improving outcomes of NMEG-NMZ for long-term delayed limb reinnervation. The total requested budget was $1.6M for 4 years. Unfortunately, this grant was not funded.

**4.** What will be **the next step(s)** for this project?

As mentioned above, delayed muscle reinnervation is a major clinical challenging issue. We will seek funding opportunities to continue this type of research. Our future studies will focus on improving outcomes of the NMEG-NMZ technique for long-term (≥ 6 months) delayed muscle reinnervation. We will determine the effectiveness of the combined strategy (i.e., NMEG-NMZ plus adjunct therapies) for long-term delayed limb reinnervation.

**5.** How would you classify your **lead candidate product**? f

(a) Therapeutic (Small Molecule, Biologic, Cell/Gene Therapy): Please choose, if applicable
(b) Diagnostic
(c) Device
(d) Research Tool to Address a Research Bottleneck
(e) Knowledge Product (Non-material product such as a compound library, database, something that improves clinical practice, education, etc.)
(f) Other - Please Specify: Novel surgical method for muscle reinnervation

**6.** How does your candidate product aid the Warfighter, Veteran, Beneficiary, and/or General Population?

Limb trauma and related peripheral nerve injuries (PNIs) are common in both military and civilian populations. Unfortunately, currently used nerve repair methods usually result in poor functional recovery. Our studies have demonstrated that NMEG-NMZ technique results in much better outcomes as compared with commonly used nerve repair methods. The data from animal experiments would be useful for future clinical application of the NMEG-NMZ that will benefit for the warfighter, veteran, beneficiary, and/or general population.

**7. Therapy / Product Development, Transition Strategies, and Intellectual Property**

Describe the steps and relevant strategies required to move the candidate product (knowledge or tangible) to the next phase of development and/or commercialization. Please address any issues with intellectual property.

*PIs are encouraged to explore the technical requirements and the current regulatory strategies involved in product development as well as to work with their organization's Technology Transfer Office (or equivalent regulatory/legal office), federal/international regulatory experts, to develop the transition plan and to explore developing relationships with industry, DoD advanced developers (e.g. USAMMDA), and/or other funding agencies to facilitate moving the product into the next phase.*

Our rat experiments showed that functional recovery of the muscle with 3-month delayed NMEG-NMZ is inferior to the muscle with immediate NMEG-NMZ reinnervation due to muscle atrophy and motor endplate (MEP) degradation. Our preliminary studies showed that neuroprotective therapies (i.e., focal NGF/FGF-2 injection into the MEP zone and sensory protection) have the potential for preserving muscle mass and MEPs in the denervated muscle prior NMEG-NMZ surgery. Further work is needed to determine the effectiveness of the neuroprotective therapies in augmenting outcomes of NMEG-NMZ for delayed muscle reinnervation. We believe that surgical-biological approach would be an ideal way for successful delayed muscle reinnervation.