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Nerve-Muscle-Endplate Band Grafting: A New Technique for Muscle Reinnervation

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Abstract

Background—As currently existing reinnervation methods result in poor functional recovery, there is a great need to develop new treatment strategies.

Objectives—To investigate the efficacy of our recently developed nerve-muscle-endplate band grafting (NMEG) technique for muscle reinnervation.

Methods—Twenty-five adult rats were used in this study. Sternohyoid (SH) and sternomastoid (SM) muscles served as a donor and a recipient muscle, respectively. Neural organization of the SH and SM muscles and surgical feasibility of the NMEG technique were determined. A NMEG contained a muscle block, a nerve branch with nerve terminals, and a motor endplate (MEP) band with numerous neuromuscular junctions. After a 3-month recovery period, the degree of functional recovery was evaluated using maximal tetanic force measurement. Retrograde horseradish peroxidase (HRP) tracing was used to track the origin of the motor innervation of the reinnervated muscles. The reinnervated muscles were examined morphohistologically and immunohistochemically to assess the extent of axonal regeneration.

Results—Nerve supply patterns and locations of the MEP bands in the SH and SM muscles were documented. The results demonstrated that the reinnervated SM muscles gained motor control from the SH motoneurons. NMEG technique yielded extensive axonal regeneration and significant recovery of SM muscle force generating capacity (67% of the control). The mean wet weight of the NMEG reinnervated muscles (87% of the control) was greater than that of the denervated SM muscles (36% of the control).

Conclusion—NMEG resulted in successful muscle reinnervation and functional recovery. This technique holds promise in the treatment of muscle paralysis.

Keywords

nerve-muscle-endplate band grafting; muscle reinnervation; peripheral nerve injury; muscle paralysis; sternomastoid muscle; sternohyoid muscle; tetanic force; retrograde horseradish peroxidase (HRP) neuronal tracing; histology; neurobiology; immunocytochemistry

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Introduction

Muscle paralysis can result from peripheral nerve injuries which are common, with 200,000 and 300,000 cases occurring in the United States and Europe per year, respectively.^{1,2} Peripheral nerve injuries are caused primarily by trauma,¹ especially motor vehicle crashes.³ In addition, iatrogenic surgical injuries also account for a large proportion of the peripheral nerve injuries.⁴ Peripheral nerve injury is a major source of chronic disabilities, which limit the opportunities to work and diminish the quality of life, and represents a challenging problem in microsurgery.

Muscle reinnervation continues to be a highly researched topic. Peripheral nerve injuries and paralyzed larynx,⁵⁻¹⁰ face,¹¹⁻¹⁶ and upper and lower extremities¹⁷⁻²⁵ have been extensively investigated and managed with various reinnervation procedures in animals and humans. To date, research efforts in rehabilitation of paralyzed muscle have focused primarily on reconstruction of the injured nerves as indicated by various modifications of nerve repair techniques reported in the literature. In general, nerve repair is accomplished by conventional end-to-end anastomosis (EEA) when the two stumps can be approximated without tension.²⁶⁻²⁹ If EEA nerve repair is not an option, end-to-side neurorrhaphy may be employed.³⁰⁻³⁶ In the presence of a nerve defect, autologous nerve graft^{37,38} or artificial nerve guide tube (tubulization)^{2,39-43} is often performed to bridge the nerve defect for axonal regeneration and nerve reconnection. If it is not possible to reinnervate muscle by using aforementioned methods, direct nerve implantation^{10,44,45} or nerve-muscle pedicle (NMP) transfer^{5,6,9,46,47} is an option for muscle reinnervation. Among these methods, end-to-end nerve repair has gained popularity in use for restoring paralyzed muscles.²⁷

Despite advances in microsurgery and extensive studies on nerve repair, the presently used reinnervation methods result in poor functional recovery. As reported, the results of nerve repair to date have been no better than fair, with only about 50% of patients regaining useful function.^{48,49} As current treatment options are far from ideal and an entirely satisfactory solution to dynamic rehabilitation of the paralyzed muscles has not yet been found, there exists a great need to seek novel approaches for the treatment of paralyzed muscles.

In an effort to achieve satisfactory reinnervation and functional recovery, we developed a novel technique called NMEG to reinnervate paralyzed muscles. This technique is based on the concept that a paralyzed muscle could be better reinnervated by transplanting a NMEG from a neighboring donor muscle. The NMEG is characterized by containing numerous motor endplates (MEPs) and nerve terminals which provide a rich source of axonal regeneration.

The purpose of this report is to provide detailed description about the NMEG technique and surgical outcomes in a rat model. Specifically, this research included two parts: 1) Neuroanatomical and surgical feasibility studies; and 2) NMEG reinnervation experiments, including surgical procedures, postoperative assessments, and functional and morphohistological outcomes.

Materials and Methods

Animals

In this study, twenty-five adult female Sprague-Dawley rats (Charles River Laboratories, MA) were used. The body weight of the animals ranged from 250 to 300 g at the time of initial operation. Ten rats were used to determine the neural organization of the SH donor and SM recipient muscles and surgical feasibility of the NMEG technique. Ten rats underwent SM nerve transection and immediate NMEG reinnervation. The remaining five

rats served as denervation control. 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). The animals were provided with food and water *ad libitum* in a 22°C environment with a 12:12-h light-dark cycle and housed in standard cages in the state of the art animal housing facilities of Hackensack University Medical Center. All efforts were made to minimize the number of animals and their suffering in the experiments.

Neuroanatomical and Surgical Feasibility Studies

As the NMEG technique involves in MEP band and its innervating nerve, the knowledge on the distribution of the nerves and MEPs in the donor and recipient muscles is a basic prerequisite for performing the NMEG. Therefore, ten rats were used for anatomical and surgical feasibility studies. The SH (donor) and SM (recipient) muscles on the left side were used to study the nerve supply patterns and the locations of the MEP bands in the muscles using Sihler's stain and combined acetylcholinesterase and silver (AChE-Ag) staining. The muscles on the right side were used to simulate NMEG implantation. After surgery, the right operated SM muscles were removed and stained to see if a NMEG could be successfully implanted. These studies were designed to provide useful data for formal NMEG reinnervation experiment.

Sihler's Stain—Five removed normal SH and SM muscles on the left side and five operated SM muscles on the right side were processed with Sihler's stain as described in our previous publications.⁵⁰⁻⁵⁴ Briefly, the muscles were: (1) fixed in 10% unneutralized formalin for 3 weeks; (2) macerated in 3% aqueous potassium hydroxide solution for 2 weeks; (3) decalcified in Sihler's solution I for 1 week; (4) stained in Sihler's solution II for 3 weeks; (5) destained in Sihler's solution I for 3 hr; (6) rinsed in 0.05% lithium carbonate solution for 1 hr; (7) cleared in 50% v/v aqueous glycerin for 3 days; and (8) preserved in 100% glycerin with a few thymol crystals for 4 weeks for transparency. After transillumination by a xenon light source (model 610; Karl Storz, Endoscopy-America, Culver City, CA), the stained muscles were dissected and photographed.

Wholemount AChE-Ag Staining—Five removed normal SH and SM muscles on the left side and five operated SM muscles on the right side were treated with AChE-Ag staining as described in our previous publications.^{50,54-57} Briefly, the muscles were: (1) fixed in 10% phosphate-buffered formalin for 2 hr; (2) washed in 0.1 M phosphate buffer at pH 7.4 and pH 6.0 for 15 min in each; (3) incubated in stock solution at pH 6.0 and 37°C for 2 hr; (4) rinsed in 40% sodium sulfate (anhydrous) for 15 min; (5) washed in distil water (DW) for 15 min; (6) immersed in 20% potassium ferricyanide for 15 min; (7) washed in DW for 1 hr; and (8) transferred into 50% glycerin for preservation. The stained specimens were photographed after transillumination by a xenon light source. The AChE-stained muscles were then impregnated with 10% silver nitrate at 37°C for 1 hr, rinsed in DW for 90 min, reduced in Bodian's developer for 5 min, and rinsed in DW for 2 hr.⁵⁰ The AChE-Ag stained operated SM could show whether the implanted NMEG contains MEP band and nerve terminals.

NMEG Reinnervation Experiment

Ten rats were used to perform formal NMEG reinnervation experiment as described below. Additional five animals served as denervation controls (DEN). In each of the DEN animals, a segment of the right SM nerve was resected to prevent nerve regeneration.

Surgical Procedures—Microsurgical procedures were performed under aseptic conditions. Animals underwent general anesthesia with a mixture of ketamine (80 mg/kg body wt) and xylazine (5 mg/kg body wt) administered intraperitoneally.

First, a midline cervical incision was made extending from the hyoid bone to the sternum to expose the right SM and SH muscles and their innervating nerves (Figure 1) under an Olympus SZX12 Stereo zoom surgical microscope (Olympus America Inc., Center Valley, PA).

Second, the right SM muscle was denervated by resecting a 5-mm segment of its innervating nerve. The nerve cut ends were then coagulated using a bipolar cautery to prevent nerve regeneration (Figure 2).

Third, a NMEG was harvested from the right SH muscle. The SH nerve branch was identified on the lateral margin of the middle portion of the muscle and traced from motor point (the entry point of the motor branch into the muscle) to the motor zone where axon terminals and a MEP band are located. A NMEG containing a block of muscle (~6×6×3 mm), axon terminals, and a MEP band was outlined and harvested from the SH muscle in continuity with its pedicle of motor nerve branch and feeding vessels (Figures 3 and 4). The presence of functioning NMEG was confirmed by observing its twitch contractions on nerve stimulation.

Fourth, a muscular defect of the same dimensions as the NMEG was made in an endplate-free region in the caudal portion of the right denervated SM muscle (~5 mm rostral to the native endplate zone) (Figure 4).

Finally, the well-prepared NMEG was embedded in the SM muscle defect and sutured with four to six 10-0 nylon microsutures. The fibers of the NMEG were lined up with those of the SM muscle (Figure 4).

After surgery, the wound was closed in layers with interrupted simple sutures of 4-0 prolene. The animals were awakened before being returned to the animal colony.

Postoperative Assessments—At the end of the 3-month recovery period, the efficacy of the NMEG technique was evaluated using electrophysiological, histological and immunohistochemical techniques as described below.

Maximal Tetanic Force Measurement: The details regarding the force measurement of the rat SM muscle have been given in our recent publication.⁵⁸ Briefly, the SM muscle examined was dissected free from the surrounding tissue. The rostral tendon of the muscle was cut close to the insertion, tied together with a 2-0 suture, and connected to a servomotor lever arm (Model 305B Dual-Mode Lever Arm System, Aurora Scientific Inc., Aurora, Ontario, Canada). After the implanted NMEG in the right SM was outlined by localizing the surgical microsutures, the SH nerve branch supplying the NMEG was identified, isolated, and placed on a bipolar stimulating electrode for nerve stimulation. On the control side, muscle force of the left normal SM was measured by stimulating the intact SM nerve. Care was taken to avoid damage to the nerve branch and blood vessels. In addition to nerve stimulation, direct muscle stimulation was also used in some cases. The SM was stimulated with a pair of needle electrodes placed in a region ~5 mm caudal to the implanted NMEG.

A stimulation and recording system (National Instruments Corporation, Austin, TX) controlled by user written LabVIEW software (National Instruments Corporation, Austin, TX) was used to deliver biphasic rectangular pulses to the nerve stimulated (Figure 5). Isometric contraction of the SM muscle was obtained with 200 ms trains of biphasic

rectangular pulses (the duration of each phase of stimulation pulse was set at 0.2 ms and train frequency was set at 200 pulses/s). A break of at least 1 min was used before trying next measurement. The maximum value of muscle force during 200 ms contraction was identified as well as initial passive tension before stimulation. The difference between the two values, maximal active force and preloaded passive force, was used as the muscle force measurement. The force generated by the contraction of the SM muscle was transduced with the servomotor of a 305B lever system and displayed on a computer screen. At the moment of force measurement, the lever arm was stationary and the muscle was stretched to the optimal length for the development of maximum force as described.⁵⁸ During experiment, the rat was placed supine on a heating pad (homoeothermic blanket system, Stoelting, Wood Dale, IL) and the core body temperature was monitored with a rectal thermistor and maintained at 36°C. The muscle and nerve examined were regularly bathed with warmed mineral oil throughout the testing to maintain muscle temperature between 35-36°C.

The physiological data were obtained and processed by an Acquisition System built from a multifunction I/O National Instruments Acquisition Board (NI USB 6251, 16 bit 1.25Ms/s, National Instruments, Austin, TX) connected to a DELL laptop with a custom written program using LabVIEW 8.2 software. The system produced stimulation pulses, which after isolation from the ground through an optical isolation unit (Analog Stimulus Isolator Model 2200, AM Systems, Inc, Carlsborg, WA) were used for the current controlled nerve stimulation. The Acquisition System was also used to control muscle length and collect a muscle force signal from the 305B Dual-Mode Lever System. Collected data were analyzed offline with DIAdem 11.0 software (National Instruments).

Retrograde Horseradish Peroxidase (HRP) Tracing: Immediate after muscle force measurement, four rats in the NMEG group underwent retrograde HRP labeling to track the origin of the motor innervation of the NMEG reinnervated SM muscles. The details regarding HRP labeling have been given in our recent publication.⁵⁹ Briefly, 30 % HRP (type VI A, Sigma Chemical, St. Louis, MO) solution was injected at two points (5µL/point) into the MEP band in the right NMEG reinnervated SM muscle and the left SH muscle (control) with Hamilton microsyringe. After a survival time of 48 hours, the animals were deeply anesthetized with sodium pentobarbital (60 mg/kg i.p.) and perfused transcardially with 200 ml of a fixative solution containing 1% paraformaldehyde, 1.25% glutaraldehyde, and 3% Dextrose in 0.1 M phosphate buffer at pH 7.4 and 4°C. After perfusion, the fixed lower medulla oblongata (MO) and cervical spinal cord (C1-C4) were dissected out, post-fixed 4 hours at 4°C in the same fixative, and then stored in graded concentrations (10% and 25%) of phosphate-buffered sucrose solution at pH 7.4 and 4°C for 2 hours and overnight, respectively.

The fixed MO and upper cervical spinal cord were frozen and sectioned at 40 µm on a cryostat (Reichert-Jung 1800; Mannheim, Germany) in the transverse plane. The sections were reacted for 20 min in the dark with 3,3',5,5'-tetramethylbenzidine (TMB) as the chromogen to develop the HRP reaction product.⁶⁰ The processed sections were mounted on gelatin-coated slides and counterstained with 1% aqueous neutral red (PH 4.8).

The stained serial sections were examined under a Zeiss photomicroscope (Axiophot-2; Carl Zeiss, Goettingen, Germany) and photographed using a digital camera (Spot-32; Diagnostic Instruments, Keene, NH) attached to the photomicroscope and connected to a personal computer with image analysis software. HRP-labeled motoneurons in each of the anatomical locations were counted. The total number and distribution of the SH motoneurons on the operated and contralateral sides in the MO and spinal cord were recorded.

Documenting the NMEG-Established Motor Zone: Some of the operated muscles were prepared for Sihler's stain and AChE-Ag staining to identify the implanted NMEG and newly established motor zone.

Detecting the Evidence for NMEG-Induced Nerve Regeneration: At the end of the experiments, the operated and contralateral SM muscles in each rat were removed, weighed, and examined using histological and immunohistochemical techniques to detect NMEG-induced direct and indirect evidence for nerve regeneration.

Muscle Weight: Wet weights of the operated and contralateral SM muscles in each rat were measured. The muscle weight was expressed as the percentage of values between the SM muscles on both sides.

Fiber-Type Grouping and Regenerating Axons: Some muscle samples were frozen in melting isopentane cooled with dry ice. Serial cross sections were cut (10- μ m thick) in a cryostat at -25°C and stored at -80°C. The sections were used for routine hematoxylin and eosin (H&E) staining to examine muscle structure and for immunohistochemical reaction to detect muscle fiber-type grouping and axonal regeneration as described below.

Immunoperoxidase: Type-specific anti-myosin heavy chain (MHC) monoclonal antibody (mAb) NOQ754D (Sigma; St Louis, MO) was used to identify type I MHC-containing fibers for identifying fiber-type grouping. The avidin-biotin complex (ABC) method was used as described in our previous publications.^{50,61-63} Briefly, the frozen sections were fixed in 4% paraformaldehyde for 10 min, blocked in 2% bovine serum albumin (BSA) with 0.1% Triton X-100 for 20 min, and incubated with primary mAb NOQ7-5-4D (dilution 1:1,000) for 1 h at room temperature (RT). Then the sections were incubated with a Vectastain anti-mouse IgG (ATCC; Rockville, MD) for 1 h, reacted in ABC reagent for 1 h at RT, and reacted for 10 min at RT with a solution containing 3,3'-diaminobenzidine (DAB) as chromogen to localize peroxidase for primary antibody according to a DAB substrate kit (SK-4100; Vector Laboratories, Burlingame, CA). The sections were then dehydrated in graded concentrations of ethanol, cleared in xylene, and mounted with Permount. Control sections were stained as described above except that the incubation with the primary antibody was omitted.

Neurofilament staining: Some muscle sections were immunostained using neurofilament antibody to label regenerating axons as described previously with some modifications.⁶⁴ Briefly, the sections were: 1) dried on gelatin-coated slides and fixed in Zamboni fixative with 5% sucrose overnight at 4°C; 2) rinsed several times in PBS; 3) treated with 0.1 mol/L of glycine in PBS at room temperature (RT) for 30 min; 4) dipped in 100% methanol at -20°C for 1h; 5) incubated in PBS containing 0.3% Triton and 3% BSA at RT for 1h to inhibit nonspecific protein binding; 6) incubated with primary antibody SMI-31 (dilution 1:800; Covance Research Products, Berkeley, CA) in PBS containing 0.1% Triton and 2% BSA at 4°C overnight for detecting neurofilaments; 7) rinsed several times in PBS; 8) incubated with a secondary antibody (donkey anti-mouse antibody conjugated to Alexa 488; dilution 1:1000) in PBS containing 0.05% Triton and 2% BSA at RT for 2 h; and 9) rinsed in PBS several times. The stained sections were mounted with Permount and examined under a Zeiss photomicroscope equipped with epifluorescence optics and photographed.

Assessment of Axonal Regeneration: The extent of axonal regeneration in the reinnervated SM was assessed using the neurofilament stained cross-sections from the region where the implanted NMEG was located. A SM muscle was divided into two halves, ventral (close to the implant) and dorsal (distal to the implant). The density of the regenerating axons was evaluated by estimating the number of the neurofilament-positive axons within a 1.0 mm²

area in a given half. The density of the neurofilament-labeled axons was divided into three degrees: 1) Degree 1: <10 axons per square mm, 2) Degree 2: 10-20 axons per square mm, and 3) Degree 3: >20 axons per square mm.

Data Analysis

Anatomical data on the distribution of the nerves and MEPs in the donor SH and recipient SM muscles, topographic locations of the HRP labeled motoneurons, and distribution of the regenerating axons were reported in detail through photographs and written descriptions. The number of the HRP labeled motoneurons in the cervical spinal cord was calculated. One-way analysis of variance (ANOVA) was used to make statistical comparison between the mean numbers of labeled motoneurons at different levels.

Wet weights and force values of the operated and unoperated SM muscles in each rat were computed. Both variables of the reinnervated SM muscles were expressed as the percentages of the values of the contralateral control ones. All data were reported as mean $\pm$ SD. Student *t* test was used to compare differences in the mean muscle force and mean muscle weight between operated and unoperated SM muscles. A difference was considered statistically significant at $P < 0.05$.

Results

Neuromuscular Organization of the SM and SH Muscles and Surgical Feasibility Studies

SM and SH muscles in the rat are located in the ventral aspect of the neck. Anatomically, both muscles are located at the same level with short distance (Figure 1) which facilitates NMEG procedure. Sternocleidomastoid (SCM) is a paired muscle which originates from the anterior surface of the episternum and the medial portion of the clavicle and inserts, with a strong tendon, into the lateral surface of the mastoid process. It is composed of two bellies, a medially and superficially located SM belly and a laterally and deeply positioned cleidomastoid (CM) belly (Figure 2). Under a surgical microscope, a single branch from the spinal accessory nerve (XI) was identified between both bellies to enter the SM at the lateral margin of the middle portion of the muscle (Figures 2 and 6A). Wholemount AChE staining showed that the SM had a single MEP band (Figure 6A). Sihler's stain showed that the SM nerve gave off numerous nerve terminals (Figure 6B).

SH muscle was found to be composed of two bellies or neuromuscular compartments (NMCs), superior (s-SH) and inferior (i-SH). The s-SH, which is larger than the i-SH (4:1 in length), was used to harvest a NMEG in this study (Figure 6C). Each of the NMCs had a MEP band (data not show) in its middle portion and was supplied by a separate nerve branch derived from ansa cervicalis that is formed by the hypoglossal (XII) nerve and C1 to C3 spinal nerve (Figure 6B,C). For the s-SH, a nerve branch entered the muscle at the lateral margin of the middle portion of the muscle. After entering the muscle, the nerve branch together with blood vessels traveled rostrally and lateromedially on the deep (dorsal) surface of the muscle (Figures 3 and 6B,C).

The nerve supply pattern and the location of the MEP band in the SH donor muscle are useful for precisely harvesting a NMEG, whereas those in the SM recipient muscle are helpful for choosing an endplate-free area to implant the NMEG. Surgically outlined motor zone in the SM recipient muscle or the SH donor muscle was confirmed to contain nerve terminals and a MEP band (Figure 6A,C). Successful preparation and implantation of the NMEG were documented by several ways. First, a functional NMEG from the SH muscle was confirmed by the observation of contractions of the NMEG on stimulation of its innervating nerve immediately after harvesting the NMEG. Second, NMEG implants were precisely outlined in the motor zone within the SH donor muscles during surgery. Finally,

surgical feasibility studies showed that the harvested NMEGs from the SH muscles were accurately and successfully implanted into an endplate-free areas in the caudal portion of the SM recipient muscles with a success rate of 100% of time. The above findings suggest that the NMEG can be reliably performed without technical problems.

Outcomes of NMEG Reinnervation

Three months after NMEG reinnervation, postoperative assessments demonstrated that all the NMEG implants were healed so well that they could not be distinguished microscopically from the recipient muscles without microsutures (Figure 7A). AChE-Ag stained specimens demonstrated that the implanted NMEG was located in an endplate-free area of the caudal SM and contained a SH nerve branch, nerve terminals, and a MEP band with numerous neuromuscular junctions (Figure 7B).

Maximal Tetanic Muscle Force Measurement—After a period of 3 months, maximum tetanic tension in the NMEG reinnervated SM muscle was evaluated. The magnitude of functional recovery of the reinnervated SM muscle was determined as compared with that of the contralateral control muscle in each rat. To study the SM muscle at optimal length, the muscle was stretched with moderate tension of 0.08 N. This tension was established in our preliminary studies on 10 control rats as described.⁵⁸ Electrical stimulation of the nerve branch innervating the NMEG at low intensity (0.02-0.04 mA) produced visible muscle contraction. Similar stimulation threshold was obtained when the nerve innervating the left intact SM muscle was stimulated. Increase of stimulation current resulted in increase of muscle force until it reached horizontal asymptote. Increase of stimulation current resulted in increase of muscle force until it reached horizontal asymptote. During nerve stimulation on the operated side this saturation level was reached with 0.2 mA. The saturation level was reached at smaller current (0.1 mA) on the control side. Direct muscle stimulation in the motor region required above 0.5 mA current to produce maximal muscle force (Figure 8). The reinnervated SM showed an average maximum tetanic force of 0.75 N (± 0.10) (control 1.12 N (± 0.15 ; $P < 0.05$)). The NMEG reinnervated SM muscles produced 67% of the maximal tetanic tension of the contralateral control muscles. Maximal muscle force evoked by nerve stimulation was similar to that generated by direct muscle stimulation.

HRP Labeled Motoneurons—The distribution of the HRP labeled SH motoneurons was examined and compared between both sides at a given level in the upper cervical spinal cord. In each animal, HRP labeling showed tracer uptake only in the motor nucleus of the SH nerve, which is located in the ventral horn cells in the C1-C2. In the C1, the HRP-labeled SH motoneurons were located in the ventromedial (VM) region of the ventral horn (Figure 9A), whereas in the C2 the labeled SH motoneurons were organized into two columns, VM and ventrolateral (VL) (Figure 9B). The locations and distribution patterns of the HRP-labeled motoneurons supplying the right NMEG reinnervated SM in the C1 and C2 were consistent with those innervating the intact SH on the left side. These findings indicate that the SM muscles reinnervated by NMEG gained motor innervation from the SH nerve. A direct comparison of the arrangement of SH and SM motoneurons was made in a rat where the intact SM muscle on one side and the intact SH muscle on the contralateral side received intramuscular HRP injections in the same animal (Figure 9C). As reported, the SM motoneurons were typically located in the lower MO and spinal ventral grey horn in the C1-C2.⁵⁹ For example, the SM motoneurons in C1 were concentrated predominantly in the dorsomedial (DM) region, whereas the SH motoneurons at this level were located mainly in the VM region of the ventral horn (Figure 9C).

On average, there were 96 HRP-labeled motoneurons innervating the SH in the rat. In each animal, labeled SH motoneurons were more abundant in the C1 (61/96, 64%) than in the C2 (35/96, 36%). The mean number of HRP-labeled motoneurons on the operated side was 89 ± 14 , whereas that on the unoperated side was 102 ± 10 . There were no significant difference in the number of the labeled motoneurons between the NMEG reinnervated SM and the normal SH muscles ($P > 0.05$).

Muscle Weight—Gross muscle examination at 3 months postoperatively revealed mild atrophy of the operated SM as evidenced by a slightly decrease in muscle mass compared to the contralateral muscle. Figure 10 illustrates a comparison of muscle masses among the normal, denervated, and NMEG reinnervated SM muscles. The NMEG reinnervated muscles were closer in size to the contralateral controls than denervated ones.

The mean value of the wet weight of the NMEG-reinnervated SM muscles (0.330 g) was slightly smaller when compared with that of the contralateral control muscles (0.378 g; $P < 0.005$), but much larger than that of the denervated SM muscles (0.199 g; $P < 0.025$). Specifically, NMEG reinnervated SM muscles weighed 87% of the weight of contralateral control muscles (Table 1). The mean percent of muscle wet weight (in relation to normal contralateral side) in the NMEG group (87%) was much higher than that in the denervation group (36%; $P < 0.001$). The muscle wet weight could be considered as one of the morphological indexes of muscle atrophy degree.

Histological and Immunohistochemical Findings—Histologically stained cross-sections showed that the NMEG reinnervated muscles exhibited better preservation of muscle structure with less fiber atrophy and connective tissue hyperplasia as compared with the denervated muscles. In the NMEG reinnervated SM, there were a majority of normal-looking fibers either outside or within the implanted NMEG although fiber size was slightly smaller than the normal control (Figure 11). In contrast, denervation resulted in significant atrophy of muscle, marked by a reduction in the fiber size and an increase in connective tissue. The myonuclei were often found in the center of cell (Figure 11).

Our experiments demonstrated that NMEG reinnervation technique resulted in successful muscle reinnervation (Figure 12) as revealed by fiber-type grouping (Figure 12B), neurofilament-labeled regenerating axons (Figure 12C,D), and AChE-Ag stained newly formed MEPs (Figure 12E) and axonal sprouts (Figure 12F). Fiber-type grouping is known to be a sign of nerve regeneration. The regenerating axons were derived from the implanted NMEG and grew into the recipient muscle. Some regenerating axons were found to reach the distal part of the reinnervated SM muscle (Figure 12).

The density of the regenerating axons in the reinnervated SM was different between the ventral and dorsal portions of the muscle. The ventral portion (close to the implant) of the muscle in the majority of cases (76%) had extensive axonal regeneration with degree 3 density, whereas others had moderate axonal regeneration with degree 2 (18%) or degree 1 (6%) density. In contrast, the dorsal portion (distal to the implant) of the muscle had moderate or mild axonal regeneration with degree 2 (62%) or degree 1 (38%) density.

Discussion

Overview of Key Findings

In this study, we described in detail about the NMEG reinnervation technique on the basis of anatomical and surgical feasibility studies. The extent of axonal regeneration and functional recovery of the NMEG reinnervated SM muscles was evaluated 3 months after surgery using electrophysiological, morphohistological and immunohistochemical methods.

Functionally, the NMEG technique yielded a satisfactory recovery of muscle force (67% of the control). Morphohistologically, injection of HRP neuronal tracer demonstrated uptake exclusively in the central motor nuclei of the donor nerve innervating the SH muscle. The mean weight of the NMEG reinnervated SM muscles (87% of the control) was greater than that of the untreated denervated SM muscles (36% of the control). The NMEG reinnervated SM muscles exhibited a better preservation of muscle structure with less fiber atrophy and connective tissue hyperplasia as compared with the denervated muscles. NMEG resulted in extensive axonal regeneration and successful reinnervation. Numerous newly formed MEPs, regenerating axons, and axonal sprouts derived from the NMEG were identified to supply the recipient SM muscle. The optimal reinnervation is most likely attributed to the fact that NMEG could provide sufficient nerve terminals and MEPs to reinnervate the denervated muscle. We believe that the NMEG technique would become a valuable tool in the treatment of muscle paralysis in certain clinical situations.

Correlation of the Functional Outcomes to Different Reinnervation Methods

When the nerve supplying a muscle is transected, the muscle fibers undergo profound denervation atrophy and degeneration, which result in loss of normal muscle structure and function. Early muscle reinnervation could reduce denervation-induced fibrosis and resultant loss of function.⁶⁵

In the past decades, multiple techniques have been developed for the reinnervation of denervated muscles. However, few of these methods have provided satisfactory outcomes. Although many factors such as the level and extent of injury, the duration of the muscle denervation, surgical procedures used, and the skill of the surgeons influence the success of reinnervation, functional recovery is dependent largely on the amount and accuracy of the regenerating axons that reach and innervate the denervated muscle fibers. The quantity of the regenerating axons could be related to the reinnervation methods used.

One technique that has demonstrated some experimental and clinical success in laryngeal and facial muscle reinnervation is the NMP transfer. The NMP reinnervation method is to transfer a donor nerve branch with a small piece of its muscle tissue to a recipient denervated muscle. It was originally developed in 1970's by Tucker and colleagues^{5,46,66-71} for laryngeal reinnervation in animals and human patients. In 1980's, Cummings and colleagues applied the NMP technique to facial reinnervation in animal experiments.⁷²⁻⁷⁴ Clearly, this method has been employed primarily to treat paralyzed larynx and face. Up to date, many studies on the NMP reinnervation procedure have been reported in the literature over the past 40 years. However, this procedure yields inconsistent results. For example, Tucker reported satisfactory functional recovery,^{5,6,46} whereas others failed to document NMP-induced reinnervation and functional recovery using histochemical and electrophysiological analyses.^{75,76} There are experimental data that argue for and against NMP.

It is difficult to objectively evaluate the contractile function of the NMP reinnervated laryngeal muscles because of their small size, inaccessibility, and technical problems. To further assess the technique's validity, some researchers investigated the NMP electrophysiologically and morphologically using cervical strap muscles in animal models. Broniatowski et al., (1989)⁴⁵ assessed the contractile function of the sternothyroid (ST) muscle reinnervated by transferring a NMP harvested from the contralateral ST muscle in rabbits. They reported that mean force curve reached 45.6% of the contralateral normal value 21.5 weeks after NMP reinnervation. Anonsen et al., (1985)⁷⁷ also evaluated the functional outcomes of the NMP reinnervated muscles in a rabbit model. In their experiments, the SH muscle on one side was denervated by resecting its motor nerve branches. A NMP was harvested from the contralateral ST muscle and transferred to the

denervated SH muscle. The authors reported that the average force for the NMP reinnervated SH muscles was 46% of the control 7 to 8 weeks after surgery. Evidently, our NMEG technique yielded a better recovery of muscle force (67% of the control) as compared with the conventional NMP transfer.

Some investigators^{45,72,73} performed animal experiments to compare direct nerve implantation (DNI) with NMP using strap muscles such as SH and ST in rabbits. They found that neither method had a clear advantage over the other, at least in terms of function. Studies showed that NMP transfer resulted in functional outcomes similar to the DNI when evaluated by electromyography (EMG) and muscle tension.^{72,74} NMP technique has been hampered by the fact that the muscle pedicle is harvested at the motor point (entry of the nerve into the muscle) where a nerve branch enters the muscle without considering the MEP band and nerve terminals. Some authors felt it probable that the small NMP might in fact contain cut ends of the nerve that acted in a fashion similar to that of a DNI.^{72,74} This seems likely because the nerves supplying a given muscle do not immediately terminate on entering the muscles, but run up or down for several millimeters and then give off numerous terminals innervating the MEPs (Figure 6). While removing a 2- to 3-mm cube of the donor muscle at the motor point,^{45,72-74} the donor nerve may be cut at the level of the main trunk or major branches. If this is so, the removed small muscle pedicle may contain only a single or multiple cut ends of the donor nerve. Thus, there may be little difference in practical terms between the NMP and DNI. This is most likely the major reason for the similar functional outcomes obtained from the muscles reinnervated with NMP and DNI.

EEA is a classical method for nerve repair. Partial reinnervation and poor functional recovery after microsuture repair of the injured peripheral nerves are most likely related to mechanical trauma, tension or infection of the anastomosis, devascularization of the nerves after dissection, neuroma formation, scarring, misdirected axonal growth at the repair site, and the loss of nerve fiber population that prevents axons reaching their target organs.⁷⁸ Usually, a small number of regenerating axons could pass through the repair site. Thus, few axons make functional connections which are not enough for activating a paralyzed muscle.

Concept and Characteristics of the NMEG Technique

The poor outcomes obtained from the currently existing reinnervation methods are closely related to inadequate axonal regeneration and reestablishment of the nerve-muscle connections. Development of the NMEG technique was based on the concept that neuromuscular junction is the physiological interface between the motor neuron and the muscle fiber. A NMEG containing a MEP band with numerous neuromuscular junctions and nerve terminals could provide an abundant source of axonal regeneration which is critical for successful reinnervation and satisfactory functional recovery.

Our NMEG is characterized structurally by containing three components: a block of muscle (~6×6×3 mm), an intact donor nerve branch with feeding vessels and numerous nerve terminals, and a motor endplate band with numerous neuromuscular junctions. There are several distinct morphological and neurobiological features of the NMEG that offer this technique to have the substantial advantages over the NMP and EEA.

First, the NMEG provides an abundant source of nerve terminals and MEPs. The relatively large sized NMEG preserves a MEP band and numerous intact nerve terminals. Therefore, a large number of the axons are available for muscle reinnervation. In contrast, the NMP most likely contains limited nerve terminals with a few or no MEPs. The almost normally innervated NMEG may support its attached nerve terminals for a sufficient period to allow them to establish fully functional connections.

Second, NMEG leads to minimal nerve fiber injury and degenerative reaction. As most of the nerve terminals and MEPs within the NMEG retain intact, thus preventing subsequent nerve degeneration before reinnervation. In addition, the transected axons in the cut margins and the denuded surface of the NMEG are much more distal, thereby retaining a greater proportion of the donor nerve fibers and developing minimal nerve degeneration. The transected axons in the NMEG are considerably separate from each other in three-dimensional space compared with the density of axons in a peripheral nerve. The anastomosed nerve trunk with EEA and the intramuscular nerve branches in a NMP are completely transected. The NMP may contain only the 2nd-order nerve branches or only a nerve stump, serving as direct nerve implantation. In this case, the implanted nerve branches will undergo progressive degeneration and eventually fail to regenerate and reinnervate the recipient muscle. Thus, the denervated muscle fibers may become severely atrophic before reinnervation.

Third, the NMEG provides sufficient space and short distance for reinnervation. It has relatively larger pedicle-recipient muscle interfaces which offer more space for axonal regeneration and a shorter distance between the NMEG and the distal portion of the denervated muscle as compared with the NMP and EEA. The axons could start regenerating at multiple points in the NMEG and grow across the pedicle-recipient muscle interfaces to easily reach and reinnervate the majority of the recipient muscle fibers. In contrast, NMP and EEA may need prolonged period of time for axon proliferation over large distances between the anastomotic site and the more distal parts of the target muscle.

Finally, the well-prepared NMEG, rich in fibronectin and laminin from the muscle fibers in the NMEG, provides an excellent medium for the growth of regenerating axons. Like the distal nerve stumps and targets following peripheral nerve injury, the cut ends of the nerve fibers on the surface of the NMEG could release neurotrophic and neurotropic factors, fibroblast growth factor, and extracellular stroma molecules such as laminin, fibronectin, and nerve cell adhesion molecules for axonal growth.^{79,80} The morphological and neurobiological features of the NMEG permit this technique to maximize nerve regeneration.

Future Research Directions

This study provided encouraging results even though functional recovery of the reinnervated muscle was incomplete. The findings suggest that NMEG technique holds great promise for muscle reinnervation. Our data provided an experimental basis for future clinical application. However, further work is needed to refine the NMEG technique and enhance axonal regeneration for full functional recovery.

First of all, the implantation site of the NMEG is considered to be a factor influencing surgical outcomes. Evidence from previous reports and our own observations led to our hypothesis that transferring a NMEG into original motor zone in the recipient muscle could favor functional connections between the donor regenerating axons and the recipient denervated neuromuscular junctions. Previous studies demonstrated that the MEP regions of mammalian muscle fibres are preferentially reinnervated.^{81,82} The reinnervation of the MEPs occurs as a consequence of some property of the muscle fibre at this site which is conducive to synapse formation.⁸¹ Some authors⁸³ performed reinnervation studies using DNI method and found that implanted nerve close to or far from native MEP zone influenced the morphology of reinnervation. Our further studies are underway to determine the effect of distance between NMEG implant and native MEP band on the functional recovery. We hypothesized that if the NMEG is implanted into the native MEP band in the recipient muscle, the regenerating axons from the NMEG could easily establish synaptic

contact with the denervated MEPs, thereby greatly increasing the degree of functional recovery.

Next issue would be to enhance axonal regeneration and sprouting. Many studies have documented that some of the neurotrophic factors such as nerve growth factor (NGF), basic and acidic fibroblast growth factors (bFGF and aFGF), brain-derived neurotrophic factor (BDNF), and others are beneficial for nerve regeneration.⁸⁴⁻⁸⁸ We will test whether focal administration of exogenous neurotrophic factors into NMEG could accelerate axonal regeneration and functional recovery to a greater degree than NMEG procedure alone.

Further studies are also needed to determine the time when the regenerating axons reinnervate the majority of the denervated muscle fibers. Our data showed that 3-month recovery period following NMEG is not sufficient for full range of muscle reinnervation. As showed by Figure 12C and D, most of the regenerating axons from the NMEG did not reach the distal part of the recipient muscle. This appears to be closely associated with the degree of functional recovery. Therefore, a longer recovery period following surgery may be necessary for better axonal regeneration and functional outcomes.

We believe that once the advantages, limitations, and long-term benefits of the NMEG are documented by animal experiments, NMEG technique would have widespread applications to reinnervation of many denervated skeletal muscles.

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Abbreviations

ABC	avidin-biotin complex
AC	ansa cervicalis
AChE-Ag	acetylcholinesterase and silver staining
aFGF	acidic fibroblast growth factor
ANOVA	analysis of variance
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
CM	cleidomastoid muscle
DAB	3,3'-diaminobenzidine
DEN	denervation
DM	dorsomedial
DNI	direct nerve implantation
DW	distil water
EEA	end-to-end anastomosis

EMG	electromyography
H&E	hematoxylin and eosin
HRP	horseradish peroxidase
i-SH	inferior belly of the SH muscle
mAb	monoclonal antibody
MEP	motor endplate
MHC	myosin heavy chain
MO	medulla oblongata
NGF	nerve growth factor
NMC	neuromuscular compartment
NMEG	nerve-muscle-endplate band grafting
NMP	nerve-muscle pedicle
OH	omohyoid muscle
SCM	sternocleidomastoid muscle
SD	standard deviation
SH	sternohyoid muscle
SM	sternomastoid muscle
s-SH	superior belly of the SH muscle
ST	sternothyroid muscle
TMB	tetramethylbenzidine
VL	ventrolateral
VM	ventromedial
XI	accessory nerve
XII	hypoglossal nerve

Figure 1. Anatomical locations of the sternomastoid (SM) and sternohyoid (SH) muscles. The dashed line indicates the midline between both SH muscles. H, hyoid bone; L, left side; R, right side; S, sternum.

Figure 2.

A, The nerve (arrow) innervating the sternomastoid (SM) is located between the cleidomastoid (CM) and SM bellies. **B**, A segment of the SM nerve (white arrow) was removed to denervate the muscle. Both cut ends (black arrows) of the nerve were electrically coagulated. SH, sternohyoid muscle.

Figure 3.

Harvesting of a nerve-muscle-endplate band graft (NMEG) from the sternohyoid (SH) donor muscle. **A**, While the right SH muscle was being retracted laterally with fine forceps, the main trunk of the SH nerve (large arrow) and motor point (small arrow) are identified. The motor zone within the muscle is outlined (boxed region). Note that there is a distance between the motor point and the motor zone. The inset in A is a removed NMEG from the outlined motor zone. H, hyoid bone; OH, omohyoid muscle; S, sternum; SM, sternomastoid muscle; T, trachea. **B**, A NMEG harvested from the right SH muscle (boxed region in A) before implantation. Note that no nerve and blood vessels can be seen on the ventral surface of the NMEG (arrow). **C**, Dorsal view of the same NMEG as that in B. Note that a nerve

branch (white color; large arrow) and blood vessels (red color; small arrow) are visualized on the dorsal surface of the NMEG.

Figure 4.

Preparation for the harvested nerve-muscle-endplate band graft (NMEG) and recipient site.

A, An implantation site was outlined by shallow cuts (~3 mm in depth) on the surface of caudal sternomastoid (SM) muscle (arrows). S, sternum. **B,** A muscular defect (recipient bed) of the same dimensions as the NMEG was made in the caudal portion of the right denervated SM muscle. The superficial muscle fibers on the ventral aspect of the NMEG were also removed to create a denuded surface before implantation. **C,** Completed implantation of the NMEG. Note that NMEG harvested from the right sternohyoid (SH) muscle was well-embedded in the recipient bed and sutured with 10-0 nylon microsutures (white arrows). Also note that the implanted NMEG contained a nerve branch (large black arrow) and blood vessels (small black arrow). **D,** Schematic illustration of the implantation of the NMEG. Note that the motor zone containing nerve terminals and a motor endplate (MEP) band in the SM (left) or SH (right) is outlined by two dashed lines. The SM is denervated by transecting its nerve. A NMEG (middle) is harvested from the motor zone in the SH muscle and implanted into an endplate-free area in the caudal SM muscle.

Figure 5.

Schematic representation of the data acquisition system, which provides electrical stimulation and records muscle force. The experiment is controlled by a Dell laptop with user written software in LabVIEW 8.2. The SM muscle is detached from its rostral tendon and attached to the lever of servomotor, which controls muscle stretch and measures muscle force. Electrical stimulation with parameters controlled by LabVIEW software is generated by the Multifunctional board 6251 and delivered to the nerve examined. Data are analyzed off-line with DIAdem 11.0 software.

Figure 6.

Motor zones containing motor endplate (MEP) band and nerve terminals in the normal sternomastoid (SM) and sternohyoid (SH) muscles of the rat. **A**, A fresh (left) and an AChE-Ag stained (right) SM muscle. The left image shows the SM nerve (arrow head) and surgically outlined motor zone by cutting superficial muscle fibers (arrows). The right image shows the MEP band (arrow) which is confined to the motor zone marked in the left fresh muscle. **B**, Sihler's stained right SM and SH muscles, showing their nerve supply patterns. Note that the nerve terminal region (arrow) in the SM corresponds to the MEP zone as demonstrated in A. SH muscle is innervated by a nerve branch (arrow head) from ansa cervicalis (AC). Note that there is a distance between motor point (arrow head) and nerve terminals (arrow). CM, cleidomastoid belly; H, hyoid bone; S, sternum; XI, accessory nerve; XII, hypoglossal nerve. **C**, A fresh (left) and a Sihler's stained SH muscle. The left image shows the SH nerve (arrow head). According to nerve supply pattern as shown in B, a nerve-muscle-endplate band graft (NMEG) was outlined by cutting superficial muscle fibers (black arrows). This muscle was then processed with Sihler's stain (right image). Note that the surgically outlined motor zone (black arrows) contains nerve terminals (vertical arrow). Red arrow in either left or right image indicates the intersection between the inferior (i-SH) and superior (s-SH) compartments within the SH muscle. H, hyoid bone.

Figure 7.

Photographs from rat sternomastoid (SM) muscles reinnervated by nerve-muscle-endplate band grafting (NMEG) 3 months after surgery. **A**, A fresh right SM muscle reinnervated by NMEG. Note that the implanted NMEG was indicated by microsutures (small white arrows) after further microdissection. Within the NMEG, a main nerve branch (large white arrow) was identified. **B**, A wholemount AChE-Ag stained right SM muscle reinnervated by NMEG. Note that the NMEG was precisely harvested from the sternohyoid (SH) muscle and implanted with sutures (red arrows) to an endplate-free area in the caudal portion of the SM muscle. Also note that the NMEG was composed of a SH nerve branch, a muscle block as outlined by sutures, nerve terminals and a motor endplate (MEP) band (white arrow) with numerous neuromuscular junctions (black dots). The NMEG was implanted ~3 mm rostral to the SM native MEP band (arrow head).

Figure 8. Muscle force as a function of stimulation current in operated (NMEG) and control groups. These group averages were collected using nerve (upper panel) and muscle (lower panel) stimulation. Nerve was stimulated with two hooked wires placed under the nerve, whereas muscle was stimulated with two needle electrodes inserted into the muscle at the level of the nerve entrance. Vertical bars represent the standard error of the mean. The passive tension was set at a moderate level (0.08 N). Stimulation was made with 0.2s train of 0.2ms width biphasic pulses at frequency of 200 Hz.

Figure 9.

Retrograde horseradish peroxidase (HRP) labeled motoneurons in the rat. **A**, A transverse section from upper cervical spinal cord segment C1 following injections of HRP into the right (R) sternomastoid (SM) muscle reinnervated by nerve-muscle-endplate band grafting (NMEG) and the left (L) intact sternohyoid (SH) muscle in a rat. The right SM was denervated by transecting its nerve and immediately reinnervated with a NMEG harvested from ipsilateral SH muscle. Three months after muscle reinnervation, right SM and left SH (control) muscles received HRP injection. Note that the HRP labeled motoneurons supplying the right NMEG reinnervated SM were located in the same area as those innervating the SH on the left side. Both neuron populations in C1 were confined to the ventromedial (VM) region of the ventral horn of the spinal cord. The small arrow indicates the central canal, whereas the large arrow indicates the cut marked on the left spinal cord. $\times 80$. **B**, A transverse section from upper cervical spinal cord segment C2 from the same rat as that used in A, showing the arrangement of the HRP labeled SH motoneurons. Note that the SH motoneurons in C2 were organized into two columns, VM and ventrolateral (VL). Both motoneuron groups and their similar locations indicate that the right reinnervated SM muscle gained its motor nerve supply from the SH nerve. Arrow indicates the central canal. $\times 80$. **C**, A transverse section from upper cervical spinal cord segment C1 in a normal adult rat, showing different locations of the SM and SH motoneurons. HRP solution was injected into the right (R) SM and left (L) SH muscles. Note that in the C1 the SH motoneurons were confined to the VM region, whereas SM motoneurons were located in the dorsomedial (DM) region of the ventral horn. Arrow indicates the central canal. $\times 80$.

Figure 10.

Photographs from the removed rat sternomastoid (SM) muscles on the operated and unoperated sides 3 months after surgery. **A**, A pair of SM muscles from a rat in the nerve-muscle-endplate grafting (NMEG) group. Note that the mass of the NMEG reinnervated SM muscle on the right side (R) was close to that of the control on the left side (L). Also note that some sutures around the implanted NMEG (arrow) were visible. **B**, A pair of SM muscles from a rat in the denervation (DEN) group. The right SM was denervated by transecting its nerve. Note that the denervated SM showed a more significant loss of muscle mass as compared with contralateral control or the NMEG reinnervated SM in A.

Figure 11.

Cross-sections of the rat sternomastoid (SM) muscles stained with hematoxylin and eosin (H&E), showing fiber morphology after reinnervation. **A**, A contralateral SM muscle (normal control). **B**, A SM muscle 3 months after denervation (DEN) by transecting its nerve. Note that the majority of myofibers were atrophied (black arrows). The myonuclei were often identified in the center of cell (squared cells). **C**, A SM muscle 3 months after nerve-muscle-endplate band grafting (NMEG) reinnervation. The section was taken from the reinnervated SM muscle approximately 5 mm caudal to the implanted NMEG. **D**, The section was taken from the implanted NMEG in the same muscle as that in C. Initial magnification $\times 200$ for A-D.

Figure 12.

Photomicrographs of immunohistochemically stained cross-sections from the operated and unoperated sternomastoid (SM) muscles in the rat. **A**, A cross section from left intact SM (control) muscle immunostained with monoclonal antibody NOQ7-5-4D specific for type I myosin heavy chain (MHC) isoform. Note that normal SM muscle contained fast type II fibers (light staining). $\times 200$. **B**, A NOQ7-5-4D stained cross section from right NMEG reinnervated SM muscle. Arrows indicate the interface between the NMEG and SM. Note that NMEG reinnervation produced fast-to-slow (dark staining) fiber type transformation and fiber-type grouping (circled fibers) in the recipient SM muscle under the implanted NMEG, indicating axonal regeneration. $\times 100$. **C**, A cross section from right NMEG reinnervated SM muscle immunostained with antibody against neurofilament, showing that NMEG resulted in numerous regenerating axons (bright staining) which were concentrated in the recipient SM (vertical arrows) mainly under the implanted NMEG. Some regenerating axons were identified to reach the distal part of the SM muscle (horizontal arrows). Note that the locations of the regenerating axons were consistent with those of fiber-type grouping in **A**. $\times 100$. **D**, High-power view of **C**, clearly showing the regenerating axons. $\times 400$. **E**,

Low-power view of the AChE-Ag stained sagittal section from the NMEG reinnervated SM muscle. Note that there were numerous newly formed small motor endplates (MEPs) in the SM muscle (black dots). $\times 100$. **F**, High-power view of the AChE-Ag stained section in E, showing axonal sprouts (arrows). $\times 400$.

TABLE 1
Wet Muscle Weight Measurement for the Rat Sternomastoid Muscles Reinnervated by
Nerve-Muscle-Endplate Band Grafting (n=10)^a

Animal No.	Body Weight (g)	Right Reinnervated SM (g)	Left Intact SM (g)
1	311	0.321	0.327
2	346	0.294	0.416
3	371	0.335	0.426
4	354	0.372	0.433
5	300	0.276	0.348
6	276	0.248	0.316
7	323	0.297	0.302
8	454	0.477	0.485
9	402	0.319	0.360
10	349	0.356	0.371
<hr/>			
Total	3,486	3.295	3.784
Average	348.6	0.33	0.38
Ratio		(87%)	(100%)

^aSM, sternomastoid muscle