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Force recovery and axonal regeneration of the sternomastoid muscle reinnervated with the end-to-end nerve anastomosis

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Abstract

Background—End-to-end nerve anastomosis (EEA) is a commonly used nerve repair technique. However, this method generally results in poor functional recovery. This study was designed to determine the correlation of functional recovery to the extent of axonal reinnervation after EEA procedure in a rat model.

Materials and Methods—Seven adult rats were subjected to the immediate reinnervation of an experimentally paralyzed sternomastoid (SM) muscle. The SM nerve was transected and immediately repaired with EEA. The SM muscle at the opposite side, without nerve transection, served as a control. Three months after EEA nerve repair, the muscle force of the SM muscle was measured and the regenerated axons in the muscle were detected using neurofilament immunohistochemistry.

Results—Three months after surgery, the reinnervated SM muscle produced limited anatomical and functional recovery (calculated as the percentage of the control). Specifically, the wet weight of the operated SM muscle (a measure of muscle mass recovery) was 78.0% of the control. The maximal tetanic force (a measure of muscle functional recovery) was 56.7% of the control. The area fraction of the neurofilament stained intramuscular axons (a measure of axonal regeneration and muscle reinnervation) was measured to be only 13.4% of the control. A positive correlation was revealed between the extent of muscle reinnervation and maximal muscle force.

Conclusions—The EEA reinnervated SM muscle in the rat yielded unsatisfactory muscle force recovery as a result of mild to moderate nerve regeneration. Further work is needed to improve the surgical procedure, enhance axonal regeneration, and/or develop novel treatment strategies for better functional recovery.

Keywords

Muscle reinnervation; end-to-end nerve anastomosis; muscle contraction; tetanic force; neurofilament staining; sternomastoid muscle

INTRODUCTION

Major peripheral nerve injuries are often associated with devastating functional deficits [1]. Epidemiological studies on civilian populations report that fall and motor vehicle traffic are the most frequent (67%) mechanisms of injury and peripheral nerve injury occurs in 2.8% of

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multitrauma patients admitted to regional trauma facilities in North America each year, translating to >17,500 cases annually in the United States [2].

Current management techniques fail to achieve adequate functional neural regeneration, and the development of adjunct therapies is necessary to improve outcomes. Recent efforts at enhancing the regeneration rate of peripheral nerves and developing axonal guidance channels or conduits have had limited success. At best, these efforts only partially restore motor functions and outcomes are inconsistent. Till now there is no effective treatment option for functional neural regeneration. Therefore new reinnervation treatments are needed to improve outcomes.

The sternocleidomastoid (SCM) muscle lies on the lateral side of the neck. Anatomically, it is composed of two bellies, a medially and superficially localized sternomastoid (SM) and a laterally and deeply positioned cleidomastoid (CM). As the SM belly is located superficially and has a relatively larger muscle mass when compared with the CM belly, it has been widely used as a muscle or myocutaneous flap for the reconstruction of oral cavity and facial defects [3,4]. More recently, we have chosen the SM as a target muscle to investigate muscle reinnervation in a rat model.

The present study is focused on determining relations between different characteristics of the rat's reinnervated SM muscle. Tetanic force was used as a measure of muscle functional recovery, number and area fraction of axons with neurofilament staining was used as measure of neuronal reinnervation, and wet muscle weight was used as a measure of muscle mass recovery. The muscle was first denervated by transecting the SM nerve and immediately reinnervated using the EEA procedure. Three months after reinnervation, each rat had muscle force measured at operated and control sides. Both muscles were removed, weighed and the level of reinnervation was assessed through immunohistochemical analysis (neurofilament staining). The characteristics of the SM muscle, measured after a 3-month recovery period, provided normative data, which could be used as a reference to evaluate the extent of recovery after muscle reinnervation with novel, more effective procedures.

MATERIALS AND METHODS

Animals

The experiments were performed on 7 adult (3.5 months old) Sprague-Dawley female rats (Charles River Laboratories, MA), weighing 350–450 grams. Sprague-Dawley rats were used as this species is a common experimental model for denervation and reinnervation related studies. Female rats were used because their body weights become stable in adulthood (starting at about 3 months) [5]. Studies by Cantillon and Bradford [6] showed that there is no difference between the male and female rat in the contractile properties of upper airway muscles, both in young and old animals. The rats were provided with *ad libitum* access to food and water and housed in standard cages in a 22 °C environment with a 12:12-h light-dark cycle. All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee prior to the onset of our experiments. The experiments were also performed 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). All efforts were made to minimize the number of animals and their suffering during the experiments.

SM muscle denervation

All animals had an experimentally paralyzed SM muscle on one side (right) through a transection of the SM nerve. The SM muscle on the opposite side, which was not paralyzed, served as a control.

SM muscles on both sides were exposed and their nerves were identified on the lateral edge of the middle portion of each muscle (Fig. 1). The right SM muscle was denervated by transecting its nerve 2 mm distal to the motor point.

EEA procedure

All animals underwent surgical repair of the experimentally paralyzed SM muscle immediately after SM nerve transection using the EEA procedure.

Details regarding the EEA procedure have been given in one of our recent publications [7]. Under an Olympus SZX12 Stereo zoom surgical microscope (Olympus America Inc., Center Valley, Pa), a midline cervical incision was made extending from the hyoid bone to the sternum to expose SM muscles and their innervating nerves. The right SM nerve was sharply transected approximately 3 mm proximal to the SM muscle. The proximal and distal ends of the transected SM nerve were immediately anastomosed with a classic EEA epineurial technique using two interrupted 10-0 nylon micro-sutures under a surgical microscope. After surgery, the wound was closed in layers with interrupted simple sutures of 4-0 prolene.

Muscle preparation for electrical stimulation and force measurement

At the end of the 3-month recovery period the functional and anatomical characteristics of reinnervated muscles (muscle force, muscle weight and the extent of axonal regeneration) were compared to those of control muscles from the opposite side. Details regarding the force measurement of the rat SM muscle have been given in our recent publications [7–10]. Briefly, the studied SM muscle was freed from surrounding tissue without interrupting its innervation. The distal tendon of the muscle was severed, tied with 5-0 silk suture and connected to a force transducer attached to a servomotor lever arm. The electrical stimulation was provided directly to the SM muscle at the level of the nerve entrance (Fig. 1). The same measurement procedures as in the operated muscle were repeated in the control muscle. Throughout testing, the SM muscle and nerve were regularly bathed with warm mineral oil to maintain a temperature between 35°C and 36°C. Body temperature was maintained at the same level with a closed circuit homeothermic blanket system for rodents (No. 50300; Stoelting Co, Wood Dale, IL).

Stimulation and recording system

We used a stimulation and recording system based on a multifunction National Instruments Acquisition Board, which was used in our previous studies [7–10]. Briefly, the system consisted of a NI USB board (6251, 16 bit 1.25 Ms/s, National Instruments, Austin, TX) connected to a DELL laptop with a custom written program using National Instruments' labVIEW 8.2 software. The system generated stimulation pulses, which after isolation from the ground through an optical isolation unit (Analog Stimulus Isolator Model 2200, A-M Systems, Inc, Carlsborg, WA) were used for the current controlled stimulation. The system also provided a position signal, which was used by the servomotor of the Dual-Mode Lever System (305B-LR; Aurora Scientific Inc., Aurora, Canada) to control muscle length. The Acquisition System collected stimulation data and a muscle force signal from the Dual-Mode Lever System. A Grass Amplifier System Model 15LT was available for signal conditioning and recording. Collected data were analyzed offline with DIAdem 11.0 software (National Instruments).

Adjustment of muscle length

Muscle length is an important variable affecting active muscle force generated in response to electrical stimulation. However, establishing the optimal length for a muscle to produce

maximal force requires lengthy investigation at many different lengths each time a new muscle is studied. In vivo study also requires length adjustment with each animal body movement. Placing the muscle at optimal length can be achieved by adjusting the tension with which the muscle is stretched. Different muscle groups require different passive tensions in order to generate maximal isometric tetanic force. Johns et al. [11] compared optimal passive muscle tension in the feline thyroarytenoid and digastric muscles. They showed that the optimal tension for the thyroarytenoid muscle is 0.14N whereas it is much lower (0.028N) in the digastric muscle. It was speculated that this difference might result from the physiological role played by the muscle. For example, the thyroarytenoid muscle must function as a fine tensor in the vocal fold in a high strain environment. Our preliminary study on the rat's intact SM muscle showed the largest tetanic force when the muscle was stretched with a tension of 0.08N before stimulation [8]. The force-tension data collected in our EEA study showed the same force-tension relation in the operated muscles as in intact muscles [7]. Therefore the same muscle tension of 0.08N was used in the present study.

Assessment of muscle contractile function

Muscle force measurement is a useful approach to assess the mechanical function and contractile properties of a muscle [12–18]. Our studies [7,8] and others [19–23] have demonstrated that force measurement is a reliable technique in determining the degree of functional recovery of a reinnervated muscle. During muscle force testing the SM muscle was stretched by the servomotor lever arm to the optimal length for the development of maximum force.

Isometric contractions of the SM were 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. The stimulation current was gradually increased from 0 mA to 1 mA. At least one minute breaks separated subsequent tetanic contractions to permit muscle recovery. At the moment of force measurement, the lever arm was stationary.

At each stimulation intensity the maximal force produced by a muscle was measured using DIAdem software, (Fig. 2A, at 0.1 mA stimulation level the maximal force was marked with the red arrow) and from those measurements the intensity–force plot was made. Maximal force from all stimulation currents was identified (marked with the red arrow in Fig. 2B). Maximal muscle force from the operated side was calculated as a percentage of maximal muscle force recorded from the control side.

Measurement of muscle weight

The denervated muscle is characterized by reduced muscle mass, decreased fiber size, increased connective tissue, and reduced metabolic capacity [24,25] which can be reversed by reinnervation [10,26]. Muscle wet weight has been widely used to evaluate muscle reinnervation [7,10,27–29]. At the end of the experiment, after the 3-month recovery period following EEA procedure, immediately after muscle force measurement, the operated and control SM muscles were removed (Fig. 3) and weighed. In each rat, the muscle weight on the operated side was calculated as the percentage of the control muscle weight on the contralateral side.

Neurofilament staining

Muscle sections obtained with a Cryocut 1800 (Reichert-Jung, Germany) were immunostained with neurofilament antibody to label regenerating axons as described previously with some modifications [30]. 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 phosphate-buffered saline (PBS); (3) treated with 0.1 mol/L glycine in PBS at room

temperature for 30 minutes; (4) dipped in 100% methanol at -20°C for 1 hour; (5) incubated in PBS containing 0.3% Triton and 3% bovine serum albumin at room temperature for 1 hour to inhibit nonspecific protein binding; (6) incubated with primary antibody SMI-31 (dilution 1:800; Covance Research Products, Berkeley, California) in PBS containing 0.1% Triton and 2% bovine serum albumin 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% bovine serum albumin at room temperature for 2 hours; and (9) rinsed in PBS several times. The stained sections were mounted with Permount, examined under an Axioplan Zeiss Photomicroscope (Germany) equipped with epifluorescence optics, scanned with an attached Sony color digital camera model DXC-5500 (Japan) and saved in electronic form with Adobe Photoshop 6.0 for Windows, running on a HP computer.

Assessment of Axonal Regeneration

The extent of axonal regeneration in the reinnervated SM was assessed by use of the neurofilament stained cross sections from the region where the SM nerve entered the SM muscle. The density of the regenerating axons was evaluated by estimating the number of the neurofilament-positive axons and the area fraction (a standard stereological measure, which estimates the total amount of a partition as a fraction of total sample volume) of the neurofilament-labeled axons within a section area (1.0 mm^2). For each rat, between 6 and 10 sections were randomly selected with at least 3 sections from the operated muscle and at least 3 sections from the control muscle. Neurofilament staining data in each slide were measured and averaged results for each muscle side were calculated. The assessment procedure is illustrated in Fig. 4. Under two neurofilament stained cross sections from the operated and control muscles, two corresponding pictures converted to binary black and white are shown. Areas with neurofilament staining were outlined, measured with the public domain ImageJ software (version 1.45s, National Institutes of Health) and the relation between the operated and control muscles were calculated both for the number and the area fraction of labeled axons.

Statistical analysis

Four parameters of the muscle: (1) Maximal muscle force observed during muscle stimulation with increasing current up to 1 mA, (2) muscle weight, (3) number and (4) area fraction of axonal neurofilament staining were collected. The percentage of the operated to control side was calculated for each of the 4 parameters in each of the 7 rats. The group mean and standard error were computed for each parameter. Those 4 parameters were compared with a Kruskal–Wallis test, followed with post-hoc analysis with Bonferroni corrected multiple comparisons on ranks. Pearson correlation coefficient was calculated to evaluate correlation between muscle force and area of axonal staining. Statistical computations were performed using the Statistical Analysis System 9.2 (SAS, Inc., Cary, NC). Statistical significance was set at $p < 0.05$.

RESULTS

Axonal Regeneration

Our data showed that EEA procedures produce limited axonal regeneration after a 3-month period of recovery following SM nerve transection and immediate anastomosis. The density of the regenerating axons was evaluated by calculating the number and cumulative cross-section area of the NF-positive axons within a 1.0-mm^2 . Representative examples of neurofilament (NF) stained cross sections from the operated and control muscles are shown in Fig. 4. Both the number of stained axons and total cross-section area of them were substantially smaller in the reinnervated muscle than in control not-denervated muscle. In

the reinnervated muscle, the average (across of all 7 animals) number of stained axons was reduced to 31.0% of the control, whereas the total area of stained axons was reduced to only 13.4% of the control. These findings suggest that new reinnervation approaches, which might accelerate axon growth, are needed to achieve optimal reinnervation of the muscle.

Muscle Force

Tetanic force results in SM muscles reinnervated by EEA were extensively described in our previous paper [7]. Muscle force data in all 7 rats as well as group average and standard error are shown in Fig. 5. The rates shown in this figure represent maximal muscle force observed at the operated side divided by maximal muscle force from the control muscle at the opposite side. The average percentage rate of maximal tetanic force at the operated side was only 56.7% of that observed at the contralateral side.

Muscle Weight

Muscle reinnervation with the EEA procedure does not lead to the full recovery of muscle weight. A comparison of SM muscles at an operated and a control side in a representative rat is illustrated in Fig. 3. On average, muscle weight at the operated side reached 78.0% of the control non-operated muscle (see Fig. 5). This weight decrease in the operated muscle was statistically significant ($p < 0.0001$).

Comparison between 4 outcome measures of reinnervation

Reductions at the operated side of muscle force, muscle weight, number and cross-section area of stained axons are shown in Fig. 5. These 4 measures of muscle reinnervation with immediate EEA were compared with Kruskal-Wallis test. The test showed statistically significant differences between these measures ($p < 0.0001$, Chi-Square 22.0, $df = 3$). Post-hoc analysis with Bonferroni corrected multiple comparisons on ranks showed that all these measures were significantly different from each other (at least $p < 0.05$). The most significant differences ($p < 0.0001$) were between cross-section area and muscle weight (rank difference 19.4) as well as between cross section area and muscle force (rank difference 13.3). The least significant differences ($p < 0.05$) were between muscle force and weight (rank difference 6.1) and between number and cumulative area of stained cross-sections (rank difference 6.4). Combined area of neurofilament staining in different operated animals (calculated as percent of stained area at operated side to control side) was significantly correlated with maximal muscle force produced by the SM muscle to direct muscle stimulation $p < 0.05$, $r = 0.77$, $df = 6$, see Fig. 6).

DISCUSSION

EEA resulted in limited axonal regeneration and reduced muscle force

Outcome results, expressed as the ratio of NF labeled axons at the experimental side to the control side [10,31]), showed that the EEA reinnervated muscle had very limited axonal regeneration (stained area, 13%) as compared to control, the non-operated muscle. There was also general atrophy observed in the operated muscle (78%), although muscle weight was not so deeply affected as axonal regeneration. Poor anatomical recovery of the EEA reinnervated muscle (indicated by reduction of muscle weight) and very limited functional connections between nerve and muscle (indicated by very small area fraction of axonal staining) resulted in weak maximal tetanic muscle force (57%).

Limited effectiveness of reinnervations with EEA observed in other studies

Limited outcomes from presently available reinnervation methods (including EEA) used in the treatment of human limb injury were recently reviewed [1]. The authors concluded that

currently used management techniques fail to achieve adequate functional neuronal regeneration. At best, EEA leads to only partial functional recovery, preinjury strength is never fully recovered, and outcomes are inconsistent. Also denervation atrophy of target muscles is one of the most significant issues related to nerve repair.

A meta-analysis of published studies on facial nerve function after hypoglossal-facial nerve anastomosis in humans showed that transection of the hypoglossal nerve inevitably results in ipsilateral tongue paralysis and atrophy [32]. Many of the 214 analyzed patients had some degree of difficulty with eating and swallowing, ranging from 20% to 45% of cases, some patients also had problems with speech, ranging between 10% and 60% of cases, but ipsilateral tongue atrophy was the most common problem, ranging from 50% to 75% of cases. Synkinesis from miss-directed regenerating nerve fibers seems to hamper functional recovery.

Swanson et al. 2008 [18] compared the outcome of EEA with neurotization performed after a period of chronic denervation of rat's tibialis anterior. At three months after immediate EEA repair, maximal muscle force was 94% (with a large individual variability of 30%) of that observed in a non-operated muscle at the contralateral side. Muscle weight was 75% of weight at control side. With neurotization corresponding muscle force was 50%, whereas muscle weight 65%. EEA was superior to neurotization if the repair was done immediately after nerve damage. However, as the duration of denervation increased, the difference between outcomes of EEA and neurotization narrowed, suggesting that neurotization may offer a viable surgical alternative in the setting of prolonged denervation.

Reduction of maximal muscle force after EEA repair was reported previously in other rat muscles. Meyer et al. [33] analyzed the force produced by the soleus muscle following transection and traditional end-to-end epineurial repair of the sciatic nerve in the rat. By 32 weeks, the maximum isometric muscle force recovered to about 70% of the force of an intact muscle. Interestingly, recovery was much better (90% of normal) when the nerve went through a crash injury instead of transection. The authors concluded that poor recovery after nerve transection could be caused the innervation of a muscle by inappropriate axons. Master et al. [34] studied the force produced by the gastrocnemius muscle in rats that had their tibial nerve transected (4–5 mm proximal to the neuromuscular junction) followed by immediate neurorrhaphy. At 3 months postoperatively, the mean force of the operated gastrocnemius returned to 68% of the force produced by the non-operated one.

The mechanisms responsible for the reduced force produced by denervated and reinnervated muscles are not completely understood but may include muscle atrophy, reduced axonal numbers, altered axonal spatial organization, diminished muscle oxidative capacity, motor unit remodeling and alternations in muscle fibers [12].

Parameters, which could best measure the outcome of the reinnervation process

Substantial individual differences in levels and relations between all 3 features of measured muscle in our rats (muscle wet weight, area fraction of connecting axons and maximal tetanic force) points to a large variability of outcomes from nerve repair with EEA. Statistically significant positive correlation was observed only between axonal recovery (measured by area fraction of the neurofilament-labeled axons) and maximal force produced by the muscle (which is a direct, sensitive and reliable method of assessing muscle function of individual muscles distal to a nerve injury [35]).

Kanaya et al 1996 [36] analyzed 14 different functional, electrophysiological and morphological parameters of the rat sciatic nerve and anterior tibial muscle, 3 months after immediate EEA. The authors' goal was to determine, which parameters were the best

measures of nerve regeneration. Interestingly there was surprisingly low correlation between commonly accepted markers of regeneration including muscle force, weight and axonal diameter. Therefore interpretation of experimental studies on nerve regeneration, in which only single parameter is measured should be limited to this particular technique. Extrapolation from one parameter to the other parameters can lead to inappropriate interpretation of success of reinnervation. Selection of the appropriate assessment parameters is critical for assessment of reinnervation success produced by a particular method.

Need for more effective treatment strategies to restore paralyzed muscles

In an acute transection injury, the primary method of treatment is the coaptation of the free ends of the injured nerve. If the lesion creates a segmental nerve defect and primary repair is not possible, the current standard of care involves reconnecting the free ends with a nerve auto-graft or through the use of axonal guidance channels from various biomaterials [1]. However, repair of a segmental nerve defect leads to the additional loss of muscle function. The limited anatomical and functional recovery in our rats after the EEA procedure reiterates the necessity to develop new, more promising techniques for muscle reinnervation.

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FIGURE 1. Force recording in a SM muscle

Picture of the SM muscle with attached pulling string shown with the black arrow. Muscle is attached to a lever, which extends the muscle to optimal length and sense active muscle force during electrical stimulation. The white arrow shows the SM nerve.

FIGURE 2. Calculation of maximal muscle force. Muscle force was recorded to 200 ms trains of biphasic pulses (width 0.2 ms) of increasing current (0–1 ms). Red arrows show presence of maximal force for this muscle

A. The sequence of force - time responses to increasing stimulation currents

B. The force – current characteristic created from maximal forces recorded at different stimulation currents.

control operated with EEA

FIGURE 3.

Pictures of removed SM muscles in one of the rats. The left side shows the control muscle, which has had intact SM nerve, whereas the right side shows the operated muscle, which has had the SM nerve transected and immediately repaired using the EEA procedure.

FIGURE 4.

A representative example of the rat SM muscle sections with axons stained with neurofilament. The operated muscle (at the right) was denervated and immediately reinnervated with EEA. The control muscle (at the left) is the not-operated muscle from the opposite side. Pictures demonstrating areas selected for the calculation of number and cumulative area of staining are shown below neurofilament stained sections. The cumulative area of neurofilament staining was measured and calculated as a fraction of the total section area [37,38]. Results for this particular example are shown at the bottom. The EEA reinnervated muscle showed a small amount of axons with neurofilament staining.

FIGURE 5.

A comparison between 4 parameters of the SM muscle reinnervated with EEA: force, weight, number of axons and their cumulative area. For each of the 4 parameters we show the percentage of operated to control side for all 7 rats. At the right side of each histogram representing a particular parameter are the group mean and standard error. The area of axonal staining had the lowest percentage rate (13.4%) whereas the muscle weight had the highest (78.0%).

FIGURE 6.

Comparison of axonal regeneration with muscle force recovery. Images of cross-section areas with neurofilament stained axons from three EEA reinnervated muscles (A, B and C) and from a control muscle (D). Maximal tetanic force rates (calculated as the percentage of operated to control side) are shown at the right to the neurofilament-stained sections from reinnervated muscles. Data from reinnervated muscles with large (A), intermediate (B) and small (C) maximal force are presented. Note that the extent of axonal regeneration positively correlates with the maximal force produced by the EEA reinnervated SM muscle.