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The Topographic Specificity of Muscle Reinnervation Predicts Function

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Abstract

Functional testing has assumed a progressively dominant role in validating the success of experimental nerve repair. Results obtained in one model, however, cannot predict the results in others because they reflect the coordinated interaction of several muscles across multiple joints. As a result, many combinations of topographically correct and incorrect muscle reinnervation could produce the same result. We have developed a binary model in which elbow flexors and extensors are reinnervated, and elbow flexion and extension are the functions tested. The musculocutaneous and radial nerves of Lister-Hooded rats were subjected to axonotmetic injuries that produced increasing degrees of axonal misdirection at the site of injury ranging from simple crush to transection and rotational offset of proximal and distal stumps. Elbow function was tested with a device that requires coordinated elbow extension to reach sugar pellets and flexion to return them to the mouth. After 12 weeks of regeneration, motoneurons projecting to the distal musculocutaneous nerve were retrogradely labeled with WGA-Ruby and scored as to their location within musculocutaneous or radial motoneuron pools. The severity of axonal misdirection resulting from the initial surgery was mirrored by progressive degrees of inappropriate reinnervation of the musculocutaneous nerve by radial nerve axons. The specificity of reinnervation predicted elbow function ($r = 0.72$), whereas the number of motoneurons regenerating did not. This model is thus well-suited to study the interaction of regeneration specificity and function across a single joint, and to produce data that can be generalized more broadly than that obtained from more complex models.

Keywords

Lister-Hooded Rat; nerve regeneration; retrograde labeling; Montoya staircase

INTRODUCTION

Defining the functional consequences of nerve repair and reconstruction has assumed increasing importance since deMedinaceli introduced rat walking track analysis as a test of function after rat sciatic nerve repair (deMedinaceli *et al.*, 1982). It soon became apparent,

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however, that deMedinaceli's sciatic functional index (SFI) did not correlate with other measures of outcome (Kanaya *et al.*, 1996; Munro *et al.*, 1998). Recently, outcome measures were grouped by the aspects of regeneration they quantified in order to assess their predictive value (Brushart, 2011). Category IV measures demonstrate that regeneration has occurred; Category III measures show that end organs have been reinnervated; Category II measures are indicators of regeneration specificity; and Category I measures are tests of voluntary function. The majority of measures that predict Category I function are specificity-related measures in Category II (eg Lago & Navarro, 2006; Galtrey & Fawcett, 2007).

To fully understand the impact of nerve repair and regeneration on function, it is necessary to examine the neural circuitry that drives reinnervated muscle. The first Category II study to examine both the specificity of muscle reinnervation by retrograde labeling and function by SFI suggested a correlation between the two (Wikholm *et al.*, 1988). Recently, neuroma-in continuity and transection injuries were studied with retrograde labeling and the ladder rung slip test, and demonstrated an excellent correlation between regeneration specificity and complex function (Alant *et al.*, 2013).

Tests that examine the interaction of reinnervated muscles to produce function all involve the activity of multiple extremity muscles working across multiple joints, and all initially denervate the contact surface of the paw. Because of this complexity, the same result could be produced by multiple combinations of correct and incorrect reinnervation of each muscle involved, as well as by varying degrees of sensory recovery. In effect, the extremity functions as a black box; we can measure the input and the output, but have no idea what goes on inside. As a result, we cannot use the results of any Category I test to predict the results of any other. To overcome this limitation we have developed a simple, binary model to facilitate correlation of regeneration specificity and function. Surgical manipulations involve varying degrees of injury and misdirection of motor axons in the musculocutaneous (MCN) and radial (RN) nerves. The former is the only nerve that controls elbow flexion, and the latter is the only one to provide elbow extension; neither supplies sensibility to the paw. The motoneuron pools that serve these nerves have very little longitudinal overlap within the spinal cord, facilitating the use of robust retrograde labeling techniques to evaluate regeneration specificity. Function is evaluated with the Montoya staircase test (Montoya *et al.* 1991), a device that requires elbow extension to reach a sugar pellet and elbow flexion to eat it. By combining a binary measure of function (flexion-extension) with a binary measure of regeneration specificity (MCN pool/RN pool) we have derived a fixed relationship between the two that should predict the behavior of other single-joint models.

MATERIALS AND METHODS

Experiments were performed on 250 gm male Lister Hooded rats (Harlan Laboratories, U.K.). This strain was chosen because of superior performance on behavioral tests in comparison with other rat strains (Galtrey & Fawcett, 2007). Rats were housed 2 per cage and maintained on a natural light-dark cycle. Food and water were normally available *ad libitum*, but food was withheld on the days that preceded behavioral testing. Procedures

were approved by the Animal Care and Use Committee of the Johns Hopkins Medical Institutions.

Surgical Procedures

Rats were anesthetized by intraperitoneal injection of a mixture of ketamine (100 mg/Kg) and xylazine (6 mg/Kg) in normal saline. The brachial plexus was exposed unilaterally through a pectoralis-splitting incision (Figure 1-A). The elbow was paralyzed by interrupting axons controlling elbow flexion through the musculocutaneous nerve (MCN) and elbow extension through the radial nerve (RN). This was achieved by generating lesions that imposed increasing degrees of misdirection upon regenerating axons (Figure 1-B). The least disruptive axotomy was produced by individually crushing the MCN and RN for 10 seconds with a number 4 jewelers forceps so that the crushed area became transparent (n=5). Intraneural disruption without inter-neural cross-over was achieved by transection and individual repair of MCN and RN (n= 4) using 10-0 suture (Ethicon). Repairs were confined within 2 mm segments of silicon tubing (0.035 inches ID; Dow Corning) to prevent escape of axons from one repair to the other. Both intra- and interneural disruption were facilitated by placing both MCN and RN within a single silicon tube (0.04 inches ID). Progressive disruption was imposed by correctly aligning the MCN and RN (n=8), by creating 90 degree rotational offset of proximal and distal stumps (n=4), and by creating a full 180 degree malalignment (n=6) so that the proximal MCN was aligned with the distal MCN, and vice versa (Figure 1-C).

Behavioral Testing

Post-operative elbow function was assessed with the Montoya staircase test (Montoya *et al.*, 1991) using the commercially-available Motility Staircase Test device (Campden Instruments). This device confines the rat to a platform flanked on both sides by a staircase that descends from rostral to caudal and is accessible by a narrow slit that is wide enough to admit the paw and arm but excludes the snout from all but the top level (Figure 2-A). A shallow cup has been cut into each step of the staircase; each cup contains two 45 mg sugar pellets. To successfully retrieve a pellet, the rat must extend the elbow, grasp the pellet, and flex the elbow to bring the pellet to its mouth. The more coordinated elbow function is compromised, the more difficult it will be to reach and retrieve pellets from the more distant levels. In a standard trial, the animal is left in the device for 15 minutes, after which the number of pellets retrieved is recorded.

Initially, prospective experimental subjects underwent a 2-week training period to familiarize them with the device and to identify individuals that showed the greatest engagement in the testing procedure. Two 15 minute training sessions were performed daily for two weeks, during which food intake was restricted to 15 gms of rat chow per day. By the sixth day all rats were participating in the testing, and by the tenth day they were retrieving a maximum of 10 to 14 pellets. The last three days were used to obtain baseline values. At the end of the training period, rats that weighed at least 250 gms, retrieved 10 or more pellets in baseline trials, and could reach to at least the fifth level were chosen to be experimental subjects and were operated upon within the following week. Behavioral testing was resumed 7 days after surgery and was performed twice weekly for three months

thereafter. Each week's performance was characterized as the mean number of pellets retrieved in the two trials. Data from the 12 week time period was used to compare with motoneuron counts obtained on the same day.

Retrograde Labeling

Retrograde labeling was used to define the location of the motoneuron pools that normally serve the MCN and RN, and to identify motoneurons that reinnervated the MCN after the various nerve lesions (Figure 2-B). Five normal MCN pools and 9 normal RN pools were labeled to serve as controls. Fluorescent label was injected into a newly-crushed segment of nerve to facilitate local uptake by axotomized motor axons. In control preparations, the labeling crush was made 1 cm distal to the nerve's exit from the brachial plexus. In animals that had undergone nerve transection and repair, the crush was made 1 cm distal to the tube, which was left in place. We have previously shown that passive diffusion of the tracer used here extends a maximum of 2mm proximal to the site of introduction, so only axonally-transported tracer will reach the site of nerve repair in these experiments (Brushart et al., 2002). A glass micropipette containing a 5% solution of a conjugate of Alexa Fluor 555 and wheat germ agglutinin (Invitrogen, Carlsbad Calif., catalog #W32464) was introduced into the nerve distal to the crush and advanced to the edge of the crushed area. A Picospritzer (Parker Hannafin, Fairfield NJ) set at a pressure of 20 mm Hg was then used to fill the crushed area with tracer, restoring the rounded contour of the nerve. Immediately after removal of the pipette the nerve was sealed with fibrin glue to prevent leakage of the tracer. Forty-eight hours were allowed for proximal transport of the tracer, after which rats were deeply anaesthetized and perfused through the left ventricle. A warm saline flush (200 ml) was followed by 500 ml of 4% paraformaldehyde in 0.1 M sodium acetate buffer (pH 7.4) over 30 minutes, after which the cervical spinal cords were removed, post-fixed in the perfusate solution for one hour, and stored in 20% sucrose in 0.1 M Sorensen's phosphate buffer (pH 7.4). A sliding microtome was then used to cut the cords into 40 micron sections that were serially mounted on gelatin-coated glass slides, dried, and overlain with coverslips using DPX (Aldrich, Milwaukee, WI) to minimize background fluorescence.

Each tissue section was viewed with fluorescent light (555 nm excitation, 580 nm emission) by an observer who was unaware of the nerve labeled (MCN or RN) in the case of controls, or of the type of nerve lesion received by experimental animals. Labeled motoneurons in which the nucleus could be defined were counted and potential double-counting corrected for by using the formula of Abercrombie (Abercrombie, 1946). In both control and experimental preparations a normal MCN pool was labeled on one side of the spinal cord. In controls, either the MCN pool or the RN pool was labeled on the contralateral side. In experimental animals, the motoneurons that reinnervated the MCN distal to the crush or repair were labeled on the contralateral, experimental side. In each spinal cord series, sections were numbered from rostral to caudal, beginning at the rostral extent of the normal control MCN pool. In experimental animals, the boundary between MCN and RN motoneuron pools on the experimental side was defined as the caudal extent of the normal, unoperated MCN pool on the contralateral side of the cord.

Analysis

Of the motoneurons reinnervating the MC nerve, the percentages that originated in the correct MC motoneuron pool (% correct reinnervation) in each experimental group were compared by logistic regression analysis. ANOVA was used to compare the total numbers of motoneurons regenerating in each group, while Poisson regression analysis was used to compare the number of pellets retrieved by animals in these groups. To evaluate a possible correlation between the topographic specificity of regeneration and pellet retrieval, a Poisson regression model was used to compare fitted vs observed values to establish the correlation between functional outcome and the topographic specificity of motoneuron regeneration.

RESULTS

Normal Motoneuron Pools

The normal MCN motoneuron pool was confined to the fifth and sixth cervical levels, with occasional extensions to C4. A total of 27 normal MCN pools were labeled, 5 in control animals and 22 contralateral to the experimental limb in operated animals. These 27 MCN pools contained a mean of 240 (SE 7.8) motoneurons. In controls in which both MCN pools were labeled there was remarkable symmetry, with rostral and caudal boundaries never differing from side to side by more than 3 spinal cord sections (Figure 3-A). The normal RN pool extended from C5 to C8, with the majority of labeled motoneurons confined to the C7 and C8 segments (Figure 3-B). In nine control preparations, the RN pool contained a mean of 489 (SE 26.3) motoneurons.

Representative Individual Cases

Twenty-two of the original 27 surgical preparations yielded complete datasets. Five animals were excluded because of infection, anaesthesia-related death during the labeling procedure, or inadequate perfusion. Twelve weeks after surgery, the MCN nerves on both normal control and contralateral operated sides of the rat were exposed to WGA-Ruby: to label the normal MCN motoneuron pool in the former, and to label motoneurons that had projected axons across the injury site to the distal MCN in the latter. The rostro-caudal location of motoneurons that reinnervated the MCN was highly dependent upon the severity of malalignment imposed by the surgical lesion. After crush, the symmetry of operated and control motoneuron pools was restored to normal (Figure 4-A). Similar accuracy was seen after individual repair of the MCN and RN within separate tubes (Figure 4-B). When transected MCN and RN were confined within a single tube, a mixture of correct reinnervation from the MCN and incorrect reinnervation from the RN was observed with both correct alignment and 90 degree rotational offset (Figures 4-C, 4-D). After 180 degree offset of proximal and distal nerve stumps, in contrast, the distal MCN was reinnervated almost exclusively by motoneurons that originally served the radial nerve (Figure 4-E).

Post-operative grasping function is exemplified by the response to crush and 180° rotation. In both preparations the number of pellets retrieved plummeted in the immediate postoperative period (Figure 5). After nerve crush, the ability to control elbow flexion and extension then returned rapidly, with most animals returning to baseline in 2–3 weeks

(Figure 5-A). After 180° rotation of proximal and distal stumps, in contrast, functional recovery remained poor throughout the 12 week period (Figure 5-B).

Numerical Data

The labeling in each animal was scored as percent correct- the percentage of the regenerated motoneurons that lay within the confines of the normal MCN pool as defined by comparison with the opposite, unoperated side of the rat. Box plots of the labeling results grouped by lesion severity revealed the consequences of crush and individual repair to be similar, as were those of tube repair with 0 degrees and tube repair with 90 degrees offset (Figure 6-A). Using logistic regression on percent correct with group as a categorical variable, there was no significant difference ($p > 0.5$) between the crush and individual repair groups, so these were combined to form the Mild Malalignment group (97% correct reinnervation; SE 1%; $n=9$) (Figure 6-B). Similarly, there was no significant difference between the tube repair with 0 degrees and tube repair with 90 degrees offset ($p > 0.5$), so these were combined to form the Moderate Malalignment group (75% correct reinnervation; SE 6%; $n=7$). The tubes with 180 degrees rotation remained as the Severe Malalignment group (36% correct reinnervation; SE 7%; $n=6$). Logistic regression analysis of these three groups revealed them to differ significantly from one another (all comparisons $p < .001$). The degree of malalignment imposed by the surgical lesion thus predicts the accuracy with which motoneurons reinnervate the periphery.

The actual numbers of motoneurons regenerating in each of the three groups were then compared by ANOVA to determine if surgical alignment influenced the number of motoneurons that reinnervated the MCN without reference to specificity (Mild: mean 248 motoneurons, SE=25; Moderate: mean 237 motoneurons, SE=23; Severe: 304 motoneurons, SE=24). This analysis revealed that the degree of malalignment did not significantly influence the number of motoneurons that reinnervated the MCN distal to the nerve repair (Figure 6-C; p values for comparisons between .2035 and .2769). There is actually a non-significant increase in motoneuron regeneration when the MC and RN are aligned with one another in the Severe Malalignment group, potentially because the RN motoneuron pool is substantially larger than the MCN pool (Figure 3-B).

Once the Mild, Moderate, and Severe Malalignment groups had been defined on the basis of % correct reinnervation of the distal MCN, pellet retrieval was scored using the same grouping. Rats in the Mild group recovered a mean of 11 pellets (SE=0.6), while those in the Moderate and Severe groups both recovered a mean of 4 pellets (SE for Moderate group= 1.02, for Severe group 1.16). Poisson regression analysis comparing the number of pellets retrieved by animals in these groups revealed that the Moderate and Severe groups both differed significantly from the Mild group ($p < .01$), but not from each other. A Poisson regression model was used to compare fitted vs observed values to establish the correlation between functional outcome and the topographic specificity of motoneuron regeneration (Figure 6-D). This revealed a positive correlation ($r=0.72$), and demonstrated that a 10% increase in regeneration accuracy resulted in a 20% improvement in pellet retrieval.

DISCUSSION

The goal of this study was to develop and validate a rat model of peripheral nerve injury and regeneration that would 1) facilitate the correlation of regeneration specificity with the function resulting from this regeneration, and 2) provide binary measures of both reinnervation specificity and function that could be correlated to provide data with predictive value. The MCN and RN are well-suited to such a model. The MCN is the only nerve that provides elbow flexion, and the RN is the only one that controls elbow extension. From a surgical perspective, the nerves are adjacent to one another and thus amenable to surgical manipulation (Figure 1-A). Furthermore, assessing the specificity of regeneration is facilitated by the minimal overlap of the RN and MCN motoneuron pools along the longitudinal axis of the cervical spinal cord (Figure 3-B). Motoneuron pools are highly symmetrical from side-to-side (Brushart & Mesulam, 1980), so it is possible to retrogradely label motoneurons that reinnervate the MCN and determine their pool of origin (RN or MCN) by comparison with the normal MCN pool, also retrogradely labeled, on the opposite side of the spinal cord.

This model identifies the pool of origin of regenerated motoneurons with certainty when they are caudal to the normal MCN pool, as there is no overlap of RN and MCN pools at this level; all labeled motoneurons must be from the RN. Absolute certainty is not possible, however, for regenerated motoneurons that are contralateral to the normal MCN pool, as it is overlapped by a small rostral tongue of RN motoneurons (Figure 3-B). As a result, some motoneurons scored as correct MCN projections may actually originate in the RN pool, leading to underestimation of axonal misdirection. We attempted to identify the RN motoneurons that normally overlap the MCN pool by their location on transverse spinal cord sections, but the two pools intermingle to a degree that precluded this distinction. Accuracy could potentially be increased by sequential double-labeling of motoneurons projecting through the distal MCN before surgery and after regeneration. Unfortunately, no technique capable of doing this reliably over a 12-week period has been described (discussed in Brushart, 2011).

Elbow function is assessed using the Montoya staircase device (Figure 2). This construct evaluates coordinated upper extremity function by requiring the restrained rat to reach and retrieve sugar pellets that are placed at increasing distances from the shoulder (Montoya et al., 1991). The pellets are contained within shallow recesses in the treads of a staircase that descends in the rostro-caudal direction, so that the lowest pellets are beyond the reach of any rat. Outcome is scored as the number of pellets retrieved. Although the test was designed to evaluate the effects of CNS lesions (Montoya et al., 1991), it was subsequently evaluated as a measure of function after median and ulnar nerve injuries (Galtrey & Fawcett, 2007). Both the severity of injury and the time elapsed since injury were found to have a significant effect on the number of pellets retrieved. Additionally, rat strain influenced the willingness of rats to participate in testing, with male Lister-Hooded rats proving to be the most readily engaged.

The Montoya test was chosen for the current experiments because its performance is highly dependent upon the ability to flex and extend the elbow, a binary function that is controlled

by the musculocutaneous and radial nerves. This differs from its initial application to the PNS (Galtrey and Fawcett, 2007), which tested the more complex grasping function of the paw provided by the median and ulnar nerves. The MCN is the only nerve that stimulates elbow flexion; it has no secondary effects on either the sensory or motor performance of the paw itself. Similarly, the RN is the only nerve that controls elbow extension; it contributes no sensibility to the paw. Radial nerve transection, however, also denervates the wrist and digital extensors. Multiple video observations of test performance confirmed that active control of wrist and digital extension is not needed for successful pellet retrieval in the staircase model. The rat need only place his paw on the stair, which can then be used to stabilize the wrist as the pellet is grasped, an observation consistent with previous measurements of normal grip strength in the absence of radial nerve function (Wang *et al.*, 2008). The utility of the Montoya test in providing a meaningful assessment of MCN/RN control of elbow function was confirmed when it accurately reflected the severity of axonal misdirection across the range of surgical manipulations (Figure 5-A, B).

Quantitative retrograde labeling techniques define both the number of motoneurons regenerating and the specificity of their peripheral connections (reviewed in Brushart, 2011). The current study found no correlation between the sheer number of motoneurons regenerating and function (Figure 6-C), consistent with previous evaluations of regeneration across lesions in which all the axons within a given experimental nerve were injured to the same degree (Valero-Cabre *et al.*, 2004; Galtrey *et al.*, 2007). Recently, however, the number of motoneurons regenerating and the functional restoration they provide have been correlated when axons within each nerve have been injured to varying degrees by creating a neuroma-in-continuity (Alant *et al.*, 2013).

These experiments provide three broad categories of mechanical axon guidance to explore the functional consequences across the entire spectrum of regeneration specificity: axonotmesis with the basal lamina intact (Crush), axonotmesis with regenerating axons confined to a single nerve (Individual Repair), and neurotmesis with varying degrees of axon intermingling from antagonistic nerves (0°, 90°, and 180° Rotation Tube Repairs). In the Crush group, the majority of axotomized axons regenerate within their original basal lamina tubes (Nguyen *et al.*, 2002), and both regeneration accuracy and functional restoration are excellent (Figures 5-A, 6-A). A similar outcome is obtained when regenerating motor axons are confined to their parent nerve branch in the Individual Repair group, allowing us to combine Crush and Individual Repair results into a single Mild Malalignment group (Figure 6-B). Although motor axons no longer reinnervate their original motor endplates, they contact endplates that serve the same function, and neither regeneration accuracy at the muscle level nor grasping behavior are significantly altered. However, when axons serving antagonistic functions are comingled (0°, 90°, and 180° Tube Repairs), individual muscles are reinnervated by both agonists and antagonists (Brushart and Mesulam, 1980) and their function is progressively degraded as mismatch increases (Wikholm *et al.*, 1988; Alant *et al.*, 2013). The degree of intermingling was similar in 0° Tube Repairs and 90° Tube Repairs (Figure 6-A), allowing us to combine them into a single Moderate Malalignment group (Figure 6-B). Regeneration accuracy was significantly worse in the 180° Tube Repairs (Figure 6-A), with predominant cross-reinnervation of MN and RN

(Figure 4-E). When entire populations of axons are reversed higher primates are able to compensate (Sperry, 1947; Brinkman et al., 1983), but rats are not (Sperry, 1941; Cohen, 1978). This raises the interesting possibility that, in humans, complete reversal of axon populations might be better tolerated than axonal intermingling.

The most significant finding of the present study is the positive correlation ($r=0.72$) between the accuracy of motoneuron regeneration as determined by retrograde labeling and the resulting function as measured with the Montoya grasping test. A possible correlation between the accuracy of motoneuron regeneration and sciatic function index (SFI) was first suggested by Wikholm *et al.* (1988). Subsequent work demonstrated that voluntary function could be predicted by indirect measures such as the distribution of motor axons within reinnervated nerve (Lago & Navarro, 2006) or purposeful manipulations of the alignment of nerve repair (Galtrey & Fawcett, 2007). Most recently, the specificity of motoneuron regeneration as determined with retrograde labeling was found to be strongly predictive of complex function as tested with the ladder rung slip test (Alant et al., 2013). In each instance, however, the results cannot be generalized to predict function in other models. Our findings, in contrast, demonstrate that the function of a single joint can be predicted by the mix of correct and incorrect reinnervation of its flexors and extensors. In this model, a 10% increase in regeneration accuracy resulted in a 20% improvement in function. This relationship highlights the vital role played by regeneration specificity in determining outcome. Whereas % correct varied continuously across the full range of surgical malalignment (Figure 6-B), function dropped off precipitously between mild and moderate malalignment (mean pellet retrieval: Mild: 11 pellets, Moderate: 4 pellets, Severe: 4 pellets). Even modest amounts of axonal misdirection will thus have a major impact on function. This relationship can now be tested in other single joint models and used as a baseline for experimental suppression of incorrect projections to improve function.

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Abbreviations

RN	radial nerve
MCN	musculocutaneous nerve

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Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.