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Reinnervation of the Tibialis Anterior Following Sciatic Nerve Crush Injury: A Confocal Microscopic Study in Transgenic Mice

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Abstract

Transgenic mice whose axons and Schwann cells express fluorescent chromophores enable new imaging techniques and augment concepts in developmental neurobiology. The utility of these tools in the study of traumatic nerve injury depends on employing nerve models that are amenable to microsurgical manipulation and gauging functional recovery. Motor recovery from sciatic nerve crush injury is studied here by evaluating motor endplates of the tibialis anterior muscle, which is innervated by the deep peroneal branch of the sciatic nerve. Following sciatic nerve crush, the deep surface of the tibialis anterior muscle is examined using whole mount confocal microscopy, and reinnervation is characterized by imaging fluorescent axons or Schwann cells (SCs). One week following sciatic crush injury, 100% of motor endplates are denervated with partial reinnervation at two weeks, hyperinnervation at three and four weeks, and restoration of a 1:1 axon to motor endplate relationship six weeks after injury. Walking track analysis reveals progressive recovery of sciatic nerve function by six weeks. SCs reveal reduced S100 expression within two weeks of denervation, correlating with regression to a more immature phenotype. Reinnervation of SCs restores S100 expression and a fully differentiated phenotype. Following denervation, there is altered morphology of circumscribed terminal Schwann cells demonstrating extensive process formation between adjacent motor endplates. The thin, uniformly innervated tibialis anterior muscle is well suited for studying motor reinnervation following sciatic nerve injury. Confocal microscopy may be performed coincident with other techniques of assessing nerve regeneration and functional recovery.

Keywords

transgenic mice; chromophore; sciatic nerve crush; motor endplate

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Introduction

Sciatic nerve crush is the most commonly studied nerve injury paradigm and has been used to test numerous neuroregenerative therapeutic modalities (Akassoglou, et al., 2002, Contreras, et al., 1995, Le, et al., 2005, Le, et al., 2005, Myckatyn and MacKinnon, 2004, Schiaveto De Souza, et al., 2004, Steiner, et al., 1997). Outcome measures following injuries to the sciatic nerve or its branches include histology, histomorphometry, electron microscopy, immunohistochemistry, nerve conduction, single motor unit potentials, and assessments of functional recovery (Gillingwater, et al., 2004, Inserra, et al., 1998, Kanaya, et al., 1996, Le, et al., 2005, Le, et al., 2005, Munro, et al., 1998, Nichols, et al., 2005, Schiaveto De Souza, et al., 2004, Terris, et al., 1999). Indirect measures of reinnervation following nerve injury include gauging muscle force and power production and assessing functional recovery using walking track analysis (Aydin, et al., 2004, Cederna, et al., 2000, Kobayashi, et al., 1997, Urbanchek, et al., 1999, Yoshimura, et al., 1999, Yoshimura, et al., 2002). These indirect measures of functional recovery, however, have limited sensitivity and vary not only with motor endplate reinnervation, but with cortical control of limb movement, post-operative stiffness, and the influences of a learning curve (Dellon and Mackinnon, 1989, Nichols, et al., 2005). Previously described techniques for measuring muscle force and power require specialized equipment, depend on the quality of muscle being innervated, and take place in an *ex vivo* environment (Cederna, et al., 2000, Yoshimura, et al., 1999, Yoshimura, et al., 2002).

More direct *in vivo* evaluation of axonal regeneration and motor endplate reinnervation can be done using recently developed transgenic mice whose axons (Feng, et al., 2000), or Schwann cells (SCs) (Zuo, et al., 2004), constitutively express chromophores. Direct evaluation of axons and SCs *in vivo* has primarily been performed in small, thin muscles like the sternomastoid, or platysma, where labeled axons of the spinal accessory nerve, or cervical branch of the facial nerve, can be serially evaluated (Feng, et al., 2000, Kang H, 2000, Nguyen, et al., 2002, O'Malley JP, 1999, Zuo, et al., 2004). Additionally, sequential *in vivo* live imaging has been done on dissected muscle whole mounts (Walsh and Lichtman, 2003, Pan et al. 2003). Unfortunately, small caliber nerves, like the spinal accessory and delicate facial nerve branches, are not easy to surgically manipulate. A larger caliber nerve, such as the sciatic, is required to maximize the utility of these transgenic chromophore-expressing mice for the study of traumatic nerve injury.

As the sciatic nerve branches proximally into the tibial nerve and common peroneal nerve, several corresponding muscle groups are available for assessing recovery following nerve injury. The tibial nerve innervates the ankle plantar flexors, including the gastrocnemius and soleus muscles, but these muscles have limited utility in studying motor reinnervation following sciatic or tibial nerve crush injury. One issue is that these plantar flexors are innervated along their deep surface, and require eversion of their thick muscle tissue to visualize motor endplates. Unfortunately, this eversion requires dissection and separation of various posterior compartment muscles (Myckatyn, et al., 2004). Even when meticulously performed, this manipulation leads to scar tissue that impairs imaging of the deep muscle surface. Separating muscle compartments also adversely affects normal functional recovery, which often serves as an outcome measure following the treatment of nerve injury. Lastly, the dense innervation of the soleus and gastrocnemius leads to overlap of motor endplates, terminal axons, and SCs during visualization, thus precluding independent characterization of these discrete structures during both vital and whole mount imaging. In this study, we have transitioned to using the long, thin, tibialis anterior muscle, which is innervated by the deep peroneal branch of the sciatic nerve. The tibialis anterior affords reproducible identification and imaging secondary to a band of motor endplates along its transverse axis.

To facilitate whole mount imaging, our study utilizes two distinct transgenic murine lines. We utilize the *thy1-YFP(16)* line that constitutively expresses yellow fluorescent protein (YFP) in nearly all axons in order to evaluate temporal patterns of motor endplate reinnervation and axonal branching following nerve injury (Feng, et al., 2000). We have also bred *thy1-CFP(23)* mice whose axons constitutively express cyan fluorescent protein (CFP) to *S100-GFP* mice whose mature, differentiated Schwann cells (SCs) robustly express green fluorescent protein (GFP), and whose denervated and immature SCs have diminished or absent fluorescence (Jessen and Mirsky, 2005, Zuo, et al., 2004). This double transgenic line enables visualization of the dynamic relationship between SCs and regenerating axons.

The purpose of this study is to directly visualize the course of muscle reinnervation after crush injury with respect to both axons and SCs, and to establish baseline data for the commonly used sciatic crush traumatic nerve injury paradigm. Direct visualization enables us to study: (1) the rate of motor endplate reinnervation over time, (2) the number of terminal branches contacting a given motor endplate, (3) the number of terminal SCs (TSCs) expressing S100, and (4) the intensity of S100 expression before and after injury. We propose that adaptation of imaging techniques designed to study the development of the peripheral nervous system will also have significant utility for testing any new neuroregenerative therapies after traumatic nerve injury.

Methods

Transgenic Mice

Homozygous *thy1-YFP(16)* mice (n=15), commercially available through Jackson Laboratories (Jackson Laboratories, Bar Harbor, Maine), express YFP in almost all peripheral sensory and motor fibers (including nerve terminals), and allow for direct visualization of axons following nerve injury. Additionally, the distribution and morphology of mature, differentiated SCs is assessed in *S100-GFP* mice, (generous gift from Dr. W. Thompson, University of Texas, Austin, now available through Jackson Laboratories) which utilize the S100B promoter to drive GFP expression (Allore RJ, 1990, Castets F, 1997, Jiang H, 1993, Ludwin SK, 1976, Stefansson K, 1982, Zuo, et al., 2004). *S100-GFP* mice (n=15), are also bred to *thy1-CFP(23)* mice (Jackson Laboratories) to produce *thy1-CFP(23)/S100-GFP* offspring that express cyan fluorescent protein (CFP) in their axons and GFP in their SCs (Feng, et al., 2000) (n=10). The presence of the GFP transgene is confirmed by observing green fluorescent protein expression in the retina under fluorescent light. The presence of the CFP transgene in heterozygous *thy1-CFP(23)* mice is determined by polymerase chain reaction (PCR) using genomic DNA extracted from mouse tail specimens. The primers used for PCR are recommended by the distributor. The mice are maintained in a central animal housing facility, and all described procedures are performed according to protocols approved by the Division of Comparative Medicine at Washington University School of Medicine.

Surgical Procedure

Adult animals are anesthetized for surgery with subcutaneous injections of ketamine (75 mg/kg) and medetomidine (100 mg/kg). The right hindquarter is then carefully shaved and depilation completed with generic Nair hair removal cream (Church and Dwight, Co., Inc.) prior to skin cleansing with Betadine swabs. The skin is incised 1 mm posterior and parallel to the femur, and the biceps femoris is bluntly split under 16x magnification to expose the sciatic nerve. The sciatic nerve is then crushed 5 mm proximal to its trifurcation with No. 5 Jeweler's forceps for 30 seconds (Bridge, et al., 1994), followed by wound irrigation and closure of the muscle with 6-0 vicryl, and closure of the skin with interrupted 6-0 nylon sutures. Animals recover on a heated surface and anesthesia is reversed with atipamezole hydrochloride (1mg/kg). Upon evaluation, animals are anesthetized, reshaved, and transcardially perfused

with 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS, pH 7.4). The tibialis anterior and distal 5 mm of the deep peroneal nerve are mobilized with careful dissection, placed on a Sylgard resin coated dish (Dow Corning), washed with PBS, and stained with alpha-bungarotoxin Alexa Fluor-594 (10 µg/ml; Invitrogen) for 30 minutes at room temperature. After washing with PBS, the whole muscle is mounted under a coverslip in Vectashield (Vector Laboratories) for confocal imaging.

Motor Endplate Evaluation

The mobilized tibialis anterior muscle is stained with alpha-bungarotoxin conjugated to Alexa 594 (Molecular Probes, OR) to label motor endplates. Vectashield mounting medium with the nuclear stain DAPI is used to identify the presence of any nucleated cells *and ensures that GFP expression by S100-GFP mice is not related to background fluorescence or debris*. The entire muscle is then placed on a glass slide with a coverslip to facilitate whole mount imaging. The deep surface of the muscle is imaged using standard fluorescent filters under low magnification to survey the muscle surface before performing confocal microscopy of nerve terminals and endplates.

The confocal microscope used in the study is equipped with a multi-line argon laser (458nm, 488nm, and 515nm), diode lasers (405nm and 561nm), and a helium-neon laser (633nm) (Olympus FV1000). Images of YFP-labeled axons are obtained by defining the spectral detection window at 515 nm. To further evaluate chromophores of interest, spectral detection is set to image CFP-labeled axons (458 nm), GFP expressing SCs (488 nm), motor endplates (568 nm), and DAPI-labeled nuclei (405 nm). To discern CFP-labeled axons from GFP-labeled axons, two methods are employed. In the first, the slit after the diffraction grating in front of the photomultiplier tube is modulated (narrowed through the Olympus FV 1000 software) to capture emissions exclusively from a narrow 20 nm bandwidth at the peak of the two emission profiles. In addition, the emission profiles of the CFP and GFP chromophores are independently plotted and a spectral unmixing algorithm is employed to differentiate them using Olympus FV 1000 software.

These images are then compiled to fashion motor endplate reinnervation maps for uninjured control animals (t=0), and at one, two, three, four and six weeks after crush injury. To evaluate the irregular surface of the muscle, a z-series of images taken 5 µm apart is flattened into a two-dimensional image. At each time point, motor endplates captured within 25 randomly sampled areas of 100 µm² are evaluated. Calibrated regions of interest are then established to quantify: (1) the number of completely denervated motor endplates, (2) the number of synaptic terminal axons associated with a single motor endplate (with proximal axonal projection intact throughout the entire field of view), (3) the number of TSCs expressing S100 as determined by the GFP reporter at motor endplates, and (4) GFP intensity in these SCs.

GFP reporter intensity is also measured in peripheral SCs, which are defined as any SC not physically contacting a motor endplate, as opposed to TSCs which are associated with a motor endplate. To quantify GFP intensity, standardized regions of interest are created within SC boundaries, and intensity measurements are recorded for twenty SCs per image. Intensity measurements are reflective of gray scale units, with a spectral range of 0 (minimum) to 4095 (maximum). To subtract background signal, regions of interest outside of imaged structures are selected and averaged using the software (Olympus Fluoview, version 1.5) and then subtracted from Schwann cell intensity values. This ensures that measured intensity is attributable to SCs rather than the baseline detector readout, scattered fluorescence, or autofluorescence. Background fluorescence can be affected by different whole mount thicknesses and surface topographies.

To ascertain whether the absence of S100-labeled terminal SCs at the motor endplate results from migration, demise, or dedifferentiation, the persistence of DAPI labeled nuclei at motor endplates is assessed. Persistence of DAPI-labeled nuclei with the eventual renewal of S100 co-expression upon reinnervation suggests restoration of a mature SC phenotype (Son and Thompson, 1995, Son, et al., 1996). S100 expression is robust in mature SCs, but can be reduced or absent in immature SCs (Fornaro, et al., 2001, Jessen and Mirsky, 2005).

Walking Track Analysis

Prior to perfusion and sacrifice, the hind paws are dipped in developer solution and the mice walk down a 20x3 cm corridor lined with undeveloped x-ray paper. Complete footprints are used to measure print length on the uninjured or normal side (NPL) and the crushed or experimental side (EPL). Print length is measured from the distal most toe print to the proximal aspect of the heel print on a calibrated SummaSketch II Plus digitizing tablet (Summagraphics, Cal Comp Input Technologies). Toe spread, measured as the distance between the first and fifth toes, is similarly recorded from the uninjured (NTS) and experimental (ETS) prints. Sciatic functional indices are then calculated using the formula $SFI = 118.9 [(ETS-NTS)/NTS] - 51.2 [(EPL-NPL)/NPL] - 7.5$ (Insera, et al. 1998).

Statistical Analysis

In the analysis of confocal images, investigators have previously counted the number of terminal axons per motor endplate and the ratio of innervated vs. denervated endplates (Nguyen, et al., 1998). In our study, two independent investigators analyze whole mount confocal z-series images of the tibialis anterior muscle in control animals, and following sciatic nerve crush at one, two, three, four, and six week time points to record all previously described quantifiable parameters. For counting, images are taken using a 20x objective (N.A. 0.75) and with the higher power 40x (N.A. 0.90) and 60x (N.A. 1.42, oil) objectives to inspect synapses at greater magnification. Images are then analyzed via the Olympus FV100 software (Olympus Fluoview, version 1.5) using regions of interest and digital zoom for ultrastructure visualization (Olympus FV1000). Multiply innervated synapses were distinguished from sprouted synapses by scanning through the z-series of muscle whole mounts, and following axons to their most distal point in a specimen, in addition to obtaining images using higher power objectives. Data obtained from each investigator is compiled and interrater reliability established using Statistica version 6.0 (Statsoft Inc., Tulsa, Oklahoma), with only two of thirty parameters yielding significant ($p < 0.05$) differences between raters. Counts from each investigator are then averaged, and time points following crush injury are compared to one another to evaluate for significant differences using Student's t-test. In the analysis of the walking track data, a mean SFI with standard deviation is calculated for each experimental time point and significant differences confirmed following performance of a one-way analysis of variance.

Results

Axon Counts

Adult *thy1-YFP(16)* mice are characterized by 1:1 relationships between terminal axons and motor endplates. Following sciatic nerve crush injury, this relationship becomes dynamic, as imaged nerve terminals are completely denervated after one week following injury, partially reinnervated at two weeks, hyperinnervated at three and four weeks, and return to a 1:1 terminal axon to motor endplate ratio by six weeks, as noted in Table 1. Shown in Figures 1A and 2A, 100% of endplates are singly innervated prior to injury ($t=0$), while 100% are denervated one week after nerve crush (Figs 1B&2A). Two weeks after crush injury, $27.7 \pm 3.7\%$ are singly innervated, $3.3 \pm 0.5\%$ are dually innervated and the rest remain denervated (Figs 1C&2A). By three weeks (Figs 1D&2A), all are innervated ($54.9 \pm 3.7\%$ singly, $45.1 \pm 3.1\%$ dually), while at four weeks $66.2 \pm 3.3\%$ are singly innervated and $31.3 \pm 2.9\%$ are dually innervated (Figs

1E&2A). By six weeks, 100% are singly innervated and resemble controls (Figs 1F&2A). In no case are three or more terminal axons associated with a single motor endplate in the crush injury paradigm. There are no statistically significant differences in the degree of dual innervation between three and four weeks post crush. The number of singly innervated motor endplates seen at three and four weeks is significantly less than uninjured controls ($p < 0.001$ and $p < 0.005$, respectively) and animals that are six weeks post-injury ($p < 0.001$ and $p < 0.05$, respectively).

Schwann Cell Counts and Intensity

In uninjured control specimens of the mouse tibialis anterior muscle, the number of TSCs per endplate with detectable S100 expression approximates a 3:1 ratio (2.9 ± 1.0 TSCs per endplate, Table 1). One week following nerve crush, this number decreases to 1.7 ± 1.2 TSCs per endplate ($p < 0.001$ compared to control). From two to four weeks following crush injury, the number of visualized TSCs expressing S100 per endplate increases, and peaks at four weeks with 3.8 ± 1.4 TSCs per endplate ($p < 0.001$ compared to control). By six weeks after crush injury, the TSC/endplate ratio (2.8 ± 1.0) is not significantly different than control numbers.

GFP signal intensity is proportional to S100B promoter activity in *S100-GFP* mice (Zuo, et al., 2004), and signal changes are noted in all mice following sciatic nerve crush injury. In uninjured control animals, TSCs express GFP with significantly ($p < 0.001$) more intensity (852.2 ± 401.5 gray scale units) than SCs in the periphery (252.1 ± 102.3 gray scale units). Following injury, the intensity measurements of TSCs (Table 1) demonstrate a decrease until three weeks post crush, at which time a robust signal increase is noted. Thereafter, there is a downward trend in signal intensity from three to four weeks, whereupon signal intensity approximates control levels. Compared to control TSC intensity measurements, values at one and two weeks post nerve crush are both significantly different ($p < 0.003$ and $p < 0.0001$, respectively). There are no significant differences between intensity levels at four and six weeks post crush compared with the uninjured control. Of note, there is a significant difference between independent investigator measurements of TSC intensity at three weeks, with mean values reported as 1121.1 ± 376.7 gray scale units by one evaluator, and 1387.6 ± 466.9 gray scale units by the other.

Peripheral SC S100 expression also changes in response to sciatic nerve crush injury (Fig 3A-F). One week after crush injury, GFP intensity increases ($p < 0.001$ compared to controls), followed by a decrease at two weeks ($p < 0.05$) and peak at three weeks. After three weeks, GFP intensity decreases at four and six weeks (Table 1). The SC intensity in the periphery six weeks after sciatic crush injury is not significantly different than control values. Of note, there is a significant difference between independent investigator measurements of peripheral SC intensity one week after nerve crush, with mean values reported by each as 822.7 ± 293.0 gray scale units and 536.0 ± 303.3 gray scale units, respectively.

Although the fluorescence of SCs at the nerve terminal is dynamic following nerve injury, DAPI labeling confirms the presence of any nucleated cells in proximity to motor endplates (Fig 4B-F). One week following nerve crush injury, there is a paucity of DAPI-labeled cells noted at the motor endplates (Fig 4C), coinciding with a reduction in GFP-labeled TSCs overall (Table 1). Two weeks after injury, the number of DAPI- and GFP-labeled cells increases, but the intensity of GFP expression is still reduced (Fig 4D, Table 1). Based on colocalization of GFP expression with DAPI staining, peripheral SC hyperproliferation is noted 1-2 mm proximal to the motor endplates two weeks following crush injury (Figure 4E) before dropping back to baseline levels four weeks after injury (Figure 4F). By four weeks, the number of TSCs per endplate is increased beyond pre-injury levels while the intensity of GFP expression approaches pre-injury levels (Fig 4F, Table 1).

Concurrent observation of the location and distribution of TSCs and axons is possible via confocal imaging of *thy1-CFP(23)/S100-GFP* mice (Fig 4A). Quantitative assessment, however, is exclusively performed on single transgenic *thy1-YFP* mice in this study, as YFP-labeled axons are brighter and more easily counted than CFP axons. Also, GFP-labeled SCs obscure CFP-labeled axons in some cases, even with the implementation of spectral unmixing algorithms (as described in motor endplate evaluation).

Walking Track Analysis

There is no validated test that specifically measures functional recovery following denervation of the tibialis anterior muscle. Functional recovery following sciatic nerve injury, however, can be monitored by calculating the sciatic functional index (SFI), which includes weighted contributions from print length and toe spread. The SFI is scaled such that -100 represents a complete nerve injury and 0 represents normal function (George, et al., 2003, Inserra, 1998, Yao, 1998). The walking track data demonstrates no difference between experimental and control SFI prior to injury, followed by a substantial decrement in sciatic function following crush injury with statistically significant improvements ($p < 0.05$) relative to the preceding time point noted at 2 and 4 weeks (Fig 2B). This correlates with the time to reinnervation of the tibialis anterior observed in imaging studies with early evidence of reinnervation of some endplates first noted at 2 weeks and ~99% with at least single innervation by 4 weeks. Plantar flexion, as demonstrated by restoration of print length, demonstrates statistically significant improvements over preceding time points 3, 4, and 6 weeks after injury.

Discussion

We aim to establish a model for studying motor endplate reinnervation following traumatic nerve injury, and to report baseline data relevant to the timing and extent of axonal reinnervation, functional recovery, SC distribution, and S100 expression during regeneration. Transgenic mice, whose axons constitutively express fluorescent chromophores, provide a remarkably useful tool for the study of developmental neurobiology in the peripheral nervous system (Feng, et al., 2000, Nguyen, et al., 2002, Pan, et al., 2003, Walsh and Lichtman, 2003). Previous investigations have expanded the utility of these mice to study the rate of nerve regeneration via imaging of both sensory and motor reinnervation. The saphenous sensory nerve has been transcutaneously imaged, and patterns of motor endplate reinnervation have been evaluated by crushing or transecting the small motor branches of the sternomastoid or platysma muscles (Nguyen, et al., 2002, Pan, et al., 2003). In the study of traumatic nerve injuries, larger caliber nerves are more amenable to technically sound and reproducible surgical manipulation, and include the sciatic nerve and its branches (Aguayo, et al., 1977, Genden, et al., 2002, Gillingwater and Ribchester, 2003, Gillingwater, et al., 2004, Inserra, et al., 2000, Le, et al., 2005, Myckatyn and MacKinnon, 2004, Naveilhan, et al., 1997), the femoral nerve (Eberhardt, et al., 2006, Martini, et al., 1994, Mears, et al., 2003) and the primary branches of the facial nerve (Ferri, et al., 1998, Sendtner, et al., 1997). Although we have previously imaged the facial mimetics to study the effects of facial nerve injury, and the soleus, gastrocnemius, and tibialis posterior muscles to study the effects of sciatic crush injury (data not shown), the long, thin tibialis anterior muscle has become our muscle of choice since it is innervated by the deep peroneal branch of the sciatic nerve and allows for whole mount confocal microscopy. Additionally, its evaluation does not preclude other modalities of evaluating nerve regeneration. Following sciatic nerve injury, histomorphometry, nerve conduction studies, muscle physiology, and functional assessment can still be performed on the other regenerating branches of the sciatic nerve.

Following sciatic nerve crush in *thy1-YFP16* mice, we report that at one week following injury, 100% of endplates are denervated, as shown by areas of clear discontinuity between the axon

and its endplate in neuromuscular whole mounts. Denervation is also demonstrated at this time point by some axons making distal contact with endplates, but lacking proximal continuity. The presence of incongruent distally-intact terminal axons contacting their endplates likely illustrates the “compartmental” theory of (Fig 1B-C) neurodegeneration, in that the neuronal soma, projecting axon, and synaptic terminal axon all degenerate via separate and distinct processes (Araki, et al., 2004, Gillingwater and Ribchester, 2001, Gillingwater and Ribchester, 2003). By two weeks, some continuous axons are seen, suggesting that concomitant regeneration and degeneration take place at this time point. Amorphous fluorescing YFP labeled-material is seen at both one and two weeks following sciatic nerve crush and likely represents the incomplete phagocytosis of neural elements (Pan, et al., 2003).

Motor endplate recovery in the tibialis anterior is first noted two weeks following sciatic crush injury (0.4 ± 0.6 axon: endplate ratio) with hyperinnervation noted three (1.4 ± 0.6) and four (1.3 ± 0.5) weeks later. Restoration of a 1:1 axon to endplate relationship resembling uninjured controls is uniformly noted by six weeks, and is consistent with the regenerative specificity with which axons reach their target endplates when endoneurial tubes remain intact following nerve crush (Lichtman and Sanes, 2003, Nguyen, et al., 2002). Although other investigators have reported polyinnervation of motor endplates up to 6 weeks following *tibial nerve* crush (with a mean of 2.3 inputs per endplate), our data differs in that four and six weeks are evaluated as separate time points (with statistically significant differences between the two), instead of being grouped together (Gillingwater, et al., 2004). Our study reveals a clear selection of a dominant nerve terminal by six weeks. Additionally, the distance from the crush site to target motor endplates, and therefore the amount of time required for reinnervation may also contribute to variations in data obtained.

Improved functional recovery is the *sine qua non* for any neuroregenerative therapy accompanying reconstructive nerve surgery (Hadlock, et al., 2005, Hadlock, et al., 1999, Inserra, et al., 1998, Jabaley, et al., 1976, Lin, et al., 1996, Nichols, et al., 2005, Varejao, et al., 2003, Varejao, et al., 2001, Varejao, et al., 2004, Yu, et al., 2001). For the sciatic nerve or its branches, walking track analysis is a validated functional assay (Bain, et al., 1989, Beirowski, et al., 2004, Brown, et al., 1991, Brown, et al., 1989, de Medinaceli, et al., 1982, George, et al., 2003, Inserra, et al., 1998). The tibialis anterior muscle - an extrinsic dorsiflexor of the ankle - lacks a validated test to monitor functional recovery. However, the sciatic functional index (SFI) provides an accurate indicator of sciatic neuronal recovery following denervating injury. Obtaining imaging studies together with SFI throughout the course of sciatic nerve regeneration allows for correlation between endplate reinnervation and the recovery of muscle function. The morphologic data presented here demonstrate that 100% of motor endplates are denervated at one week following a sciatic nerve crush injury. At two weeks, 68% of endplates remain denervated, correlating with the finding of a statistically detectable but only modest improvement in the SFI at the two week time point. Hyperinnervation of motor endplates at three weeks, together with incomplete functional recovery, likely represents immaturity of endplates with ongoing synaptic competition. As expected based on the reinnervation data, the SFI improves significantly by four weeks, and approaches normal values by six weeks.

Incomplete functional recovery during a period of hyperinnervation is expected. Previous work on tibial nerve crush injury suggests that although motor endplates may be transiently hyperinnervated, they are not hyperfunctional (Gillingwater, et al., 2004). Synaptic activity is not a prerequisite for motor endplate reinnervation following sciatic nerve injury (Costanzo, et al., 2000). However, in the setting of hyperinnervation, synaptic activity has been shown to contribute to the outcome of competition between axons (Buffelli, et al., 2003). The vast majority of research on synapse activity, formation, elimination, and stabilization, however, relates to the recapitulation of development by adult mouse neuronal cells following nerve injury (Lichtman and Colman, 2000). Evidence regarding neuromuscular development

demonstrates that synaptic competition is likely dictated by trophic support from postsynaptic cells and activity between a terminal axon and its motor unit (Callaway, et al., 1987, Ridge and Betz, 1984), in addition to stabilization and maturation of the neuromuscular junction (Eusebio, et al., 2003, Jaworski and Burden, 2006, Lin, et al., 2001, Pun, et al., 2002, Sanes and Lichtman, 2001). Presumably, motor function is complete when the dominant synapse matures and the non-dominant synapse is eliminated.

The dynamics of neuromuscular recovery following nerve injury encompasses changes in SC behavior and morphology, as well. It is known that SCs optimize the extracellular environment around peripheral nerves by demonstrating a considerable degree of plasticity (Jessen and Mirsky, 2005). In the uninjured state, mature SCs exist in a myelinating or non-myelinating state, characterized by a specific complement of transcription factors (Le, et al., 2005, Le, et al., 2005), signaling proteins (Ogata, et al., 2004), and extracellular markers (Taveggia, et al., 2005). Additionally, the expression of S100B, among other proteins, is strongly expressed in mature SCs, but is significantly downregulated in immature SCs and their precursors (Fornaro, et al., 2001, Jessen and Mirsky, 2005). In the uninjured state, both peripheral (non-terminal) SCs and TSCs associated with the tibialis anterior strongly express S100, but the level of expression is at least three times as strong in TSCs (252.1 ± 102.3 versus 852.2 ± 401.5 gray scale units, respectively).

Our study confirms the dynamic nature of SC populations at the nerve terminal following injury. At baseline, TSCs are intimately associated with motor endplates in a ~2-3:1 ratio (TSC number: endplate), providing a “glial cap” that buffers the extracellular milieu at the neuromuscular junction (NMJ) and modulates its function (Robitaille, 1998) (Reddy L., 2003) (Kang, et al., 2003). The number of TSCs at an endplate correlates to endplate size, age, and the muscle in question, with each cell projecting processes that cover the NMJ (Lubischer and Bebinger, 1999, Zuo, et al., 2004).

In response to denervation, the population and morphology of SCs changes, as SCs demonstrate the ability to revert from their mature myelinating or non-myelinating phenotypes, to assume promyelinating or migratory phenotypes (Jessen and Mirsky, 2005). At this time, TSCs surround and phagocytose degenerating fragments of terminal axons (Miledi, 1970, Miledi, 1968) and project long processes that can penetrate muscle (Reynolds and Woolf, 1992). One week following sciatic nerve crush, an abrupt decline in TSC numbers is noted, and may be caused by the migration of TSCs from denervated NMJs, a complete lack of S100 promoter activity, or cell death. It is known that GFP expression may persist for greater than one week in injured and degenerating axons that constitutively express fluorescent proteins, and may also remain after the SCs previously expressing this protein have died (Pan, et al., 2003). We surmise, then, that the lack of GFP expression and nuclear staining at motor endplates one week following injury is most consistent with migration of SCs away from the endplates and cell death (Weinberg and Spencer, 1978). Consistent with these findings, previous work has shown that SCs will abandon previous synaptic sites following denervation, and that over time, SC-deprived endplates result in a reduction in receptor area (Kang and Thompson, 2002). Restoration of TSC number at the NMJ is seen by two weeks (2.9 ± 1.0) and exceeds baseline numbers four weeks following sciatic nerve crush injury (3.8 TSCs/NMJ), before reverting to control values (2.8 ± 1.0) six weeks after crush injury.

Qualitatively, TSCs possess a relatively circumscribed morphology prior to injury that is restored three to six weeks after the crush (Fig 3A-F). Two weeks following crush injury, numerous interconnections are noted between TSCs, including the extension of GFP-labeled processes between distinct motor (Fig 3C2). These TSC processes can act as substrates for axonal extension (Son and Thompson, 1995, Son, et al., 1996), and previous work suggests that they form a network between adjacent endplates (Kang, et al., 2003, Tian and Thompson,

2002). Previously occupied endoneurial tubes may lead some nerve fibers back to motor endplates, while other axons continue to extend along the TSC process networks. These meandering axonal projections are termed “escaped fibers” and are believed to be strongly influenced by SC guidance during periods of denervation or shortly following reinnervation (Gutmann, 1944, Kang, et al., 2003, Ramon y Cajal, 1928). We note that this SC bridging (Tian and Thompson, 2002), is accompanied by the proliferation of peripheral SCs proximal to the motor endplates two weeks following crush injury (Fig 3C2 and Fig 4E).

Although imaging conditions may affect spectral intensity measurements of the GFP reporter in *S100-GFP* mice, (Lichtman and Conchello, 2005), S100 promoter activity can still be extrapolated. The diminished expression of GFP intensity in TSCs adjacent to motor endplates at one and two weeks following nerve injury may represent dedifferentiation into a more immature phenotype that does not robustly express S100. The dramatic upregulation of TSC GFP expression by three weeks following nerve crush (beyond baseline levels) coincides with replenished levels of S100 calcium binding protein in mature TSCs. The return of S100 expression to baseline levels by four weeks after the crush injury marks SC maturation.

Increases in peripheral SC signal intensity, particularly in the first week after crush injury may relate to migration of TSCs away from motor endplates and into the periphery and the persistence of GFP protein expression for at least a week after S100 promoter activity is downregulated. The reduction in peripheral SC signal intensity two weeks after crush likely corresponds to SC denervation and dedifferentiation into a promyelinating phenotype. This is supported by the fact that at one and two weeks following crush injury the majority of distal peripheral SCs are clearly denervated based on time-matched imaging of axonal regeneration.

Confocal microscopic evaluation of dynamic changes at the neuromuscular junction in murine models expressing neuronal and SC chromophores expands the armamentarium of outcome measures used to assess recovery following traumatic nerve injury. Following sciatic nerve crush, a 1:1 terminal axon to motor endplate ratio is established within six weeks, during which time accompanying SCs demonstrate a series of morphologic and phenotypic changes that ultimately stabilize within this time frame. Further studies using this technique will investigate the effects of various neuroregenerative therapies, reinnervation specificity, and the pattern of SC differentiation following disruptive nerve injuries requiring primary suture or nerve graft repair.

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Figure 1.

A. Confocal imaging of the tibialis anterior shows that motor endplates are singly innervated by terminal axons in uninjured adult mice. Axons are constitutively labeled with YFP in *thy1-YFP(16)* mice, while Alexa 594-bungarotoxin labeled acetylcholine receptors delineate motor endplates. **B** One week after sciatic nerve crush, all motor endplates are denervated, but YFP persists during the process of Wallerian degeneration. Therefore, some terminal axons contact motor endplates, but proximally there is a lack of axonal continuity. **C** Two weeks after injury, less YFP-labeled axonal debris remains. Although the majority of motor endplates remain denervated, a few rapidly regenerating terminal axonal sprouts are noted. **D** Three weeks after injury, all endplates are singly or dually innervated, but there are also a number of unmatched, disorganized terminal axons. **E** Four weeks after injury, the majority of endplates are singly innervated with very few unmatched terminal axons. **F** Six weeks after injury, all terminal axons are matched and all motor endplates are singly innervated.

Figure 2.

A. The percentage of endplates that are either denervated, or reinnervated with one or two axons is shown before crush injury and up to 6 weeks after injury. **B.** Functional recovery following sciatic crush injury is correlated to the sciatic functional index (SFI) and print length factor (PLF). Weekly statistically significant improvements ($p < 0.05$) are noted starting three weeks after crush injury and approximate pre-injury levels by 6 weeks following crush injury.

Figure 3.

A. Confocal image of uninjured tibialis anterior muscle demonstrates mature peripheral SCs and TSCs. **B.** One week after crush injury, there are qualitatively fewer TSCs expressing S100, and those that are present are less bright. Peripheral SCs possess less uniform morphology, and express GFP above baseline levels. **C1.** Two weeks later, TSCs are less bright. TSCs appear to extend processes that interconnect multiple motor endplates in a pattern not observed in the uninjured state or after complete recovery. **C2.** Magnification of the inset outlined in **C1.** demonstrates the proliferation of peripheral SCs proximal to the motor endplates at two weeks following crush injury (see arrows). **D.** Three weeks later, GFP expression by TSCs is drastically increased. **E.** Four weeks later, both peripheral and TSCs remain brightly labeled. **F.** Six weeks later, SC distribution, morphology, and GFP expression approximates the pre-injury state.

Figure 4.

A. *Thy1-CFP(23)/S100-GFP* mice combine the SC and terminal axon characteristics seen in single transgenic mice. Quantitative assessment, however, is exclusively performed on the single transgenic mice to reduce the interrater variability that occurs with double transgenic mice. Specifically, CFP-labeled axons are not as bright as YFP axons and hence more difficult to accurately count. **B.** To demonstrate cellularity at the motor endplate while GFP signals are changing, SC nuclei are also labeled with DAPI. Prior to injury, many of the cells associated with motor endplates are characterized by blue-green colabeling. **C.** One week after crush injury, there are very few DAPI-labeled cells that are colabeled with GFP. **D.** Two weeks later, motor endplates are densely populated with DAPI-labeled nuclei and colabeling with an attenuated GFP signal is noted by some TSCs. This suggests that TSCs are present and viable, but have regressed to a more immature phenotype that expresses S100 less intensely. **E.** Proximal to these TSCs, robust proliferation of peripheral SCs is noted along the paths of regenerating terminal axons two weeks following crush injury. **F.** Four weeks after injury, TSCs colocalizing GFP and DAPI show restoration of the mature S100-expressing SC phenotype.

Table 1 Schwann cell-axonal relationships of the tibialis anterior muscle following sciatic nerve crush injury.

	Time following sciatic nerve crush injury					
	Control	1wk	2 wks	3 wks	4wks	6wks
Number of axons per endplate	1 ± 0.0	0 ± 0	0.4 ± 0.6	1.4 ± 0.6	1.3 ± 0.5	1 ± 0.0
Number of SCs expressing S100 per endplate	2.9 ± 1.0	1.7 ± 1.2	2.9 ± 1.0	2.6 ± 1.0	3.8 ± 1.4	2.8 ± 1.0
Terminal Schwann cell fluorescent intensity**	852.2 ± 401.5	487.0 ± 168.7	96.2 ± 68.9	1254.3 ± 421.7	815.1 ± 188.0	901.0 ± 415.1
Peripheral Schwann cell fluorescent intensity**	252.1 ± 102.3	677.4 ± 298.2	105.8 ± 106.2	717.5 ± 398.8	675.0 ± 207.5	459.5 ± 193.1
Percentage of denervated endplate	0%	100%	68%	0%	0%	0%

* = p> 0.05 between investigator counts of same image
** = intensity measurements are reflective of gray scale units with a spectral range of 0 (minimum) to 4095 (maximum) for intensity range