UNIVERSITY OF CALIFORNIA SAN DIEGO

Peripheral Nerve Lengthening as a Novel Strategy for Treating Acute and Chronic Nerve Injury

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Bioengineering

by

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The Dissertation of Holly Marie Howarth is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chapter 2, in full, is a reprint of the material as published in Neural Regeneration Research in 2019, titled “Redistribution of nerve strain enables end-to-end repair under tension without inhibiting nerve regeneration.” My co-authors are Turki Alaziz, Shawn O’Connor, and Sameer Shah. The dissertation author is the primary author of the article.

Chapter 3, in full, is a reprint of the material as published in Tissue Engineering and Regenerative Medicine in 2019, titled “Nerve lengthening and subsequent end-to-end repair yields more favorable outcomes compared to autograft repair of rat sciatic nerve defects.” My co-authors are Adarsh Kadoor, Rayeheh Salem, Brogan Nicolds, Stephanie Adachi, Achilles Kanaris, Richard Lovering, Justin Brown, and Sameer Shah. The dissertation author is the primary author of the article.

Chapter 4, in part, is currently being prepared for submission for submission for publication. My co-authors are Elisabeth Orozco, Richard Lovering, and Sameer Shah. The dissertation author is the primary author of the paper.

Chapter 5, in part, is currently being prepared for submission for publication. My co-author is Sameer Shah. The dissertation author is the primary author of the paper.

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ABSTRACT OF THE DISSERTATION

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Peripheral nerve injuries, particularly transection injuries, are difficult to repair and treatment strategy is decided on a case-by-case basis. When there is a large gap between the proximal and distal stumps of the nerve, the use of a graft to bridge the distance between the stumps is the current clinical gold standard of treatment. However, grafts are often either expensive (allografts) or necessitate loss of function (autografts). Axons must also grow from the proximal stump, through the graft, to the distal stump, and so outcomes are often poor. Correspondingly, substantial clinical and pre-clinical evidence demonstrates that graft-free end-to-end repair of nerve endings would be preferable when possible. In

this dissertation, I examine lengthening of injured peripheral nerves followed by end-to-end repair as a new method to bridge large nerve gaps, using a novel device to directionally stretch the proximal nerve stump. Though tension is often considered to be damaging to the nerve, studies have shown that axons can in fact grow more quickly when under modest tension. We first showed that for moderate gaps, an end-to-end nerve repair under tension had equivalent functional and growth outcomes when compared to tension-free repairs. Following an acute large-gap injury, nerves that were lengthened had equal or better outcomes when compared to a graft. Following a chronic large-gap injury, nerves that were lengthened had equivalent outcomes when compared to a graft, but, consistent with prior literature, outcomes as a whole were inferior to those following acute injury. Finally, we used proteomic profiling to probe biological pathways upregulated during tension-based regeneration. Cumulatively, we found that nerve lengthening followed by end-to-end repair is a new and potentially efficacious approach for nerve regeneration. Future work will examine the possibility of lengthening the nerve over very large gaps as well as new strategies for regeneration following chronic injury.

Chapter 1: Introduction

1.1 Injuries of the peripheral nervous system

The peripheral nervous system consists of very long post-mitotic cells called neurons. Neurons are made up of a cell body, located in the spinal cord or dorsal root ganglion, and an axon. The axon travels from the cell body to the sensory and motor targets in the muscle, skin, and other organs (Debanne et al., 2011). Depending on the animal, an axon can be more than a meter long, and it is very important that this connection between the cell body and the functional targets remains intact. If the axon is transected, this connection is lost, and the axon must grow to the target. The farther proximally an injury is made, the farther the axon must regrow and more time will be required for regeneration. Equally important is the direction the axon is growing. If the neuron can find no pathway to grow through, neuron growth will be disorganized and may occur in a completely different direction from where the target actually is. Fortunately, a naturally occurring pathway for the axon does exist: the distal nerve stump (Lee and Wolfe, 2000).

Within 48 hours of a transection injury, the distal stump of the nerve begins to undergo Wallerian degeneration. The distal axons degenerate and macrophages begin to clear debris. Left behind are the Schwann cells which begin to dedifferentiate, becoming ready to nurture axon growth, and basal lamina, providing the path for axons from the proximal stump to grow. At early timepoints, this environment is very favorable for axonal regeneration, but within a couple months, the Schwann cells begin to deteriorate as well. Eventually, if the axon has not found its way to the distal stump, it will degrade completely (Gaudet et al., 2011; Stoll and Müller, 1999).

Peripheral nerve injuries are serious injuries causing pain and loss of function. In the United States, twenty million people suffer from the injuries (Grinsell and Keating, 2004). Unfavorable outcomes, even months after the initial injury, are common, particularly in cases of a complete transection of the nerve (Ruijs et al., 2005). In order to be certain that the proximal axons will find their way to the

distal stump after a transection injury, surgical intervention is required with a variety of treatments available.

1.2 Current clinical treatments for peripheral nerve injuries

The preferred treatment of surgeons varies on the size of the nerve gap. If there is a short gap between the proximal and distal stumps of the nerve, an end-to-end repair is performed, and the surgeon simply sutures the stumps together. The precise length of the gap for which an end-to-end repair can be used is debated, and some surgeons prefer not to perform an end-to-end repair if there is any risk of tension (Matsuyama et al., 2000; Sunderland et al., 2004). In the case of long gaps, surgeons will instead use a graft, commonly an autograft harvested from the sural nerve, though too long of a graft can lead to unfavorable outcomes (Meek et al., 2005). Other options include allografts and nerve conduits, which do not require nerve harvest but also will not provide the same regenerative environment from a nerve and should not be used for gaps any longer than 3 cm (Grinsell and Keating, 2004). Though end-to-end repairs have been shown to have superior outcomes, these other surgical strategies are used to avoid putting tension on the nerve, especially at the site of repair (Bhatia et al., 2017). A repair breaking following surgery can be catastrophic as the proximal axons will lose the connection to the distal stump pathway. Large amounts of tension have been shown to be detrimental to the health of the nerve (Terzis et al., 1975). However, smaller amounts of tension do not seem to be detrimental to an injured nerve if the repair remains intact (Sunderland et al., 2004; Scherman et al., 2004).

1.3 Peripheral nervous system response to mechanical loading

Besides end-to-end repair, other clinical situations exist which place the nerve under tension. One such case is limb lengthening, where nerve must stretch and grow to accommodate the changing length of the limb. While nerve conduction may be affected intermittently during this process, full return to function is often observed at the completion of the procedure (Simpson et al., 2013; Makarov et al., 1996). In another study, a nerve was slowly stretched over the course of the surgery until the proximal

and distal stumps were close enough for an end-to-end repair to be performed. The patient receiving this repair had good functional recovery following the surgery (McDonald and Bell, 2010).

Supporting these clinical results are studies of the growth of axons under tension. When an axon is towed along slowly in vitro, the rate of axon growth can increase dramatically (Bray, 1984). This has been observed in both sensory and motor neurons (Pfister et al., 2004; Katiyar et al., 2019). Similarly, mTOR and S6 proteins have been shown to be upregulated following sustained stretch of an intact nerve (Love et al., 2017). These results suggest that tension has a positive impact on peripheral nerve regeneration.

1.4 The effect of timing on peripheral nerve regeneration

As mentioned previously, the favorable growth environment of the distal stump of the nerve begins to deteriorate in the months that follow the initial injury (Dahlin, 2013). Besides the distal nerve, the dennervated muscle atrophies over time, leading to the deterioration of some of the target endpoints of the motor axons (neuromuscular junctions) (Scheib and Höke, 2013) . While these occur, the growth of proximal axons also eventually slows, leading to unfavorable conditions for peripheral nerve regeneration at chronic timepoints (Gordon and Tetzlaff, 2015).

The effect of this is seen clinically. Patients who receive a delayed repair (generally greater than two months post injury) have much poorer functional outcomes following recovery than those who receive a repair more quickly following the injury (Jivan et al., 2009; Kim et al., 2003). While immediate repair of peripheral nerves would be ideal, the severity of the injury or misdiagnosis can prevent patients from receiving surgery sooner. As such, methods to improve peripheral nerve regeneration following delayed repair are of interest to clinicians and researchers.

1.5 Lengthening peripheral nerves

Peripheral nerve regeneration presents many problems, but the behavior of axons also encourages possible solutions. A proximal axon that has been transected needs to regain its connection to its target

endpoints. This is especially difficult when there is a very large gap between the proximal and distal stumps of the nerve. A graft can be used to bridge this gap, but this graft must be harvested from elsewhere. It either must be bought, potentially incurring additional costs for the already high cost of neurosurgery, or it must be harvested from elsewhere in the body, causing loss of function of the donor site. Not only this, but the axons must then grow through the graft to reach the distal stump of the nerve. To circumvent the need for this, I suggest lengthening of the proximal nerve instead.

Tension increases the rate of axon growth, so lengthening of the entire nerve is simply scaling up this growth strategy. By stretching the nerve slowly over several days, the nerve will be given an opportunity to grow following intervals of tension. The proximal nerve can then be brought directly to the distal stump for an end-to-end repair, and recovery can follow.

In this dissertation, I use peripheral nerve lengthening in both acute and chronic models of nerve injury to repair nerve transections, as well as profile the sustained effect of nerve tension on nerve regeneration.

1.6 Dissertation Scope

Chapter 2, published in Neural Regeneration Research, is a study to determine the affect of tension on repair as well as the viability of a device designed to redistribute tension from the repair site. The hypothesis of this study was that redistributing tension away form the repair site would enable an end-to-end repair. This was tested by comparing repairs under moderate tension with the tension redistributing device implanted to repairs under no tension and repairs under moderate tension but with no device implanted. Interestingly, we found that there were no differences in recovery between any group, so the moderate tension on the repairs made no significant difference if the repair site did not fail.

Chapter 3, published in Tissue Engineering and Regenerative Medicine, is a study in which we use a novel device to lengthen the nerve following the creation of a nerve gap. Our hypothesis was that lengthening of the nerve followed by end-to-end repair would be superior to using a graft to bridge the nerve gap, and we found that regenerative outcomes were equal or superior in the nerves which were

lengthened than those using the graft. We concluded that nerve lengthening is a viable method of nerve repair.

Chapter 4 is a study using the same concepts which were used in Chapter 3 but applying them to a delayed-repair nerve injury model. We hypothesized that we would similarly see superior outcomes in the lengthening group. However, we found that all outcomes were equal in this group, though lengthening of the nerve did avoid the use of a graft. We then examined how this data correlated with the data which was found in Chapter 3 and noticed interesting relationships between outcomes variables.

Chapter 5 is a study which aimed to profile the proteomic changes that occur in a lengthened nerve following injury. We compared nerves which were lengthened after injury to nerves which had instead been repaired with a graft and nerves that had not been injured. We found that there were many similarities between both groups of injured nerves when compared to the control nerves, but also some interesting differences, particularly in transcription related proteins.

Chapter 6 is a study on a new method to evaluate and quantify elbow function, and in the case, being used to evaluate recovery following nerve transfer surgery. We found that this new test was sensitive to changes in function over time and useful in comparing the recovered function to that of a control subject. With this new method and other standard tests, we were able to comprehensively evaluate the recovery of two patients.

Chapter 7 summarizes the research which is presented in this dissertation and discusses future directions for further research in these topics as well as the clinical significance of the results of this research. Emphasized are the goals to use nerve lengthening in clinical setting as an alternative strategy for treating peripheral nerve injuries.

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Chapter 2: Redistribution of nerve strain enables end-to-end repair under tension without inhibiting nerve regeneration

2.1 Abstract

End-to-end repair under no or low tension leads to improved outcomes for transected nerves with short gaps, compared to repairs with a graft. However, grafts are typically used to enable a tension-free repair for moderate to large gaps, as excessive tension can cause repairs to fail and catastrophically impede recovery. In this study, we tested the hypothesis that unloading the repair interface by redistributing tension away from the site of repair is a safe and feasible strategy for end-to-end repair of larger nerve gaps. Further, we tested the hypothesis that such an approach does not adversely affect structural and functional regeneration. In this study, we used a rat sciatic nerve injury model to compare the integrity of repair and several regenerative outcomes following end-to-end repairs of nerve gaps of increasing size. In addition, we proposed the use of a novel implantable device to safely repair end-to-end repair of larger nerve gaps by redistributing tension away from the repair interface. Our data suggest that redistriubution of tension away from the site of repair enables safe end-to-end repair of larger gap sizes. In addition, structural and functional measures of regeneration were equal or enhanced in nerves repaired under tension – with or without a tension redistribution device – compared to tension-free repairs. Provided that repair integrity is maintained, end-to-end repairs under tension should be considered as a reasonable surgical strategy.

2.2 Introduction

Peripheral nerve injuries can be devastating to an individual's sensorimotor capabilities and quality of life. Though nerves are capable of regeneration, structural and functional recovery is hindered by the fact that growth tends to be highly disorganized (Meek et al., 2005). For complete nerve transections, functional recovery is especially poor, as axons must successfully and accurately regrow from the proximal stump into and through the distal stump, and reconnect at motor or sensory termini. As

positive outcomes are not typically observed for weeks to months following surgery, surgical decision-making at the time of repair is of the utmost importance (Pfister et al., 2011).

End-to-end repair is preferred for transected nerves with short gaps (Meek et al., 2005; Bhatia et al., 2017). However, if such a repair places the nerve or the site of repair under substantial tension, outcomes are likely to be poor and repair failure emerges as a significant concern (Terzis et al., 1975; Maeda et al., 1999; Sunderland et al., 2004); so, an intervening graft or conduit is deployed. Hollow conduits are acceptable for relatively short gaps, but for modest to long gaps, autografts remain the gold-standard (Matsuyama et al., 2000; Siemionow and Sonmez, 2010; Grinsell and Keating, 2014). Despite their utility, autografts have many disadvantages, including additional patient exposure to anesthesia, donor site morbidity, geometry mismatches between injured and donor nerves, and the presence of multiple interfaces across which axons must grow before even reaching the distal nerve (Schmidt and Leach, 2003). On the other hand, despite the prevalence of graft-based repairs, there is increasing evidence that low to moderate tension may be beneficial to nerve regeneration. In vivo and ex vivo models suggest accelerated axonal or nerve outgrowth under tension (Bray, 1984; Pfister et al., 2004; Franz and Guck, 2010; Simpson et al., 2014), and direct end-to-end nerve repairs under slight tension in fact outperform tension-free graft repairs for modest nerve gaps (Hentz et al., 1993; Sunderland et al., 2004).

In this study, we tested the hypothesis that redistributing tension away from the site of repair will enable the end-to-end repair of larger nerve gaps. Further, we hypothesize that such a strategy will not adversely affect structural and functional regeneration. In this study, we compare the integrity of repair and several regenerative outcomes in end-to-end repairs of varying nerve gaps (i.e., varying tension). In addition, we propose the use of a novel implantable device to safely repair end-to-end repair of larger nerve gaps by redistributing tension away from the repair interface.

2.3 Materials and Methods

Nerve-interfacing device

A nerve-interfacing device (NID; patent pending) was designed to redistribute tension away from the repair site (Figure 2.1). The device consists of two hemi-cylindrical clamps – one for each stump – which grip the nerve. A stainless steel guide rod connects the two clamps, and allows alignment and approximation of nerve ends to a desired strain during or after repair. Several additional design features enable the nerve to be secured without excessive compression: i) Epineurial barbs oriented against the direction of tension, to secure the nerve during repair and subsequent loading; ii) overlying sutures, to ensure that the nerve does not slip; iii) a hemicylindrical interface with the nerve, with a diameter slightly larger than that of the nerve, to ensure that the nerve is not excessively compressed by the sutures; iv) small footprint, to enable device to be seated within the nerve bed without modifying the nerve trajectory (i.e., no appreciable raising of the nerve from its bed); v) clamp biocompatibility and customization; clamps were printed on a Projet 3510 HD 3D Printer (3D Systems, Rock Hill, SC, USA) using Visijet M3 Crystal (USP class VI approved).

Nerve transection and repair

All animal experiments were performed under the approval of the Institutional Animal Care and Use Committee of University of California, San Diego (Protocol S11274). Surgeries were performed on 10–12 week old male (250–300 g; n = 38) Lewis rats (Envigo, Placentia, CA, USA) by an orthopedic surgeon after multiple successful practice repairs in terminal procedures that were inspected for quality by a senior attending hand surgeon. Anesthesia was induced with 5% isoflurane/95% oxygen, and maintained at 2% isoflurane/98% oxygen for the duration of the surgery. For survival surgery, anesthetized rats were injected with 0.03 mg/mL buprenorphine (Par Pharmaceutical, Chestnut Ridge, NY, USA) and Baytril (Bayer Healthcare, Shawnee Mission, KS, USA). Sciatic nerves of the right hindlimb were exposed by splitting the femoral biceps, and decompressed prior to transection and repair.

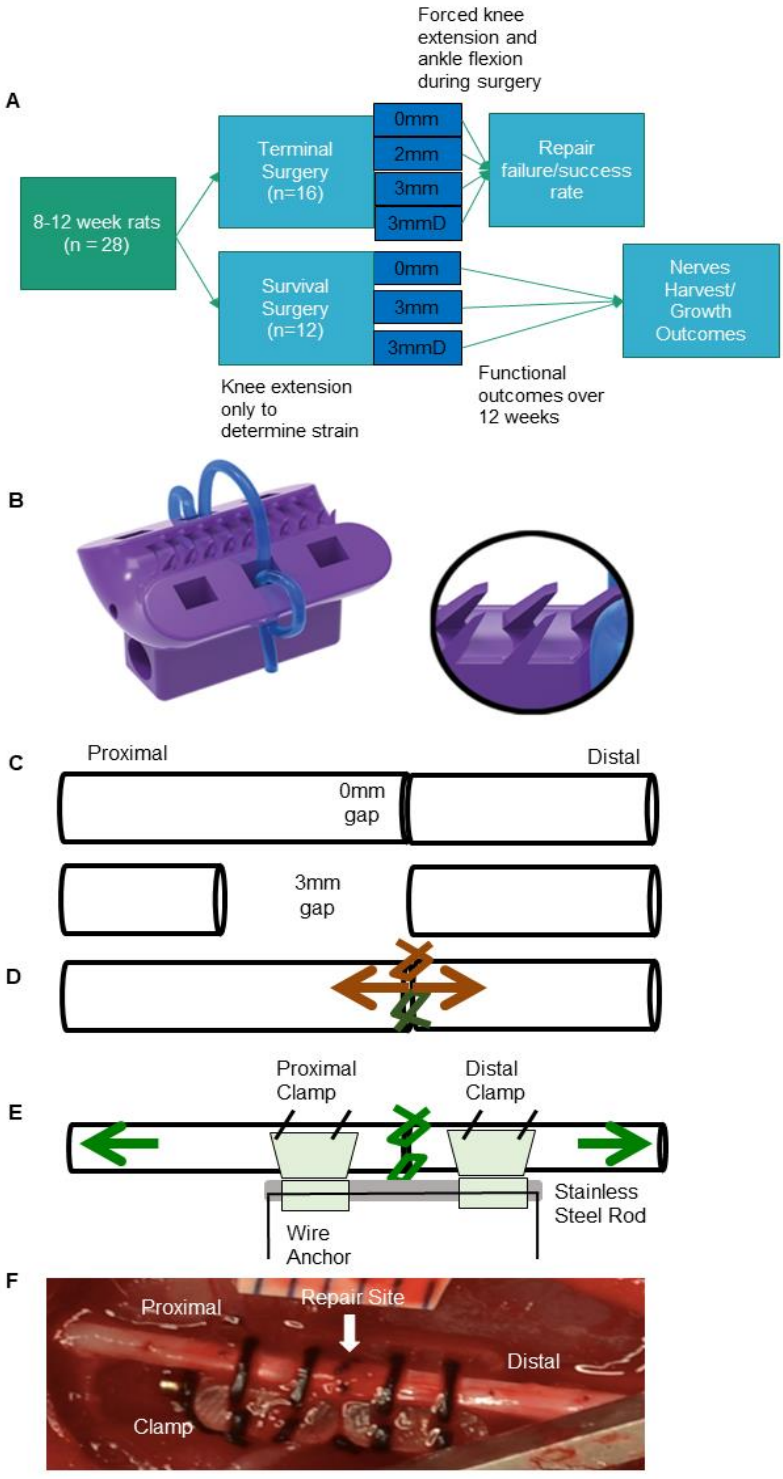

Figure 2.1: Summary of experimental design and implementation. (A) Experimental flow and outcomes collected from terminal and survival surgery cohorts. (B) Design of the 3D printed clamp used in the device, with inset showing epineurial barbs. (C–E) Surgical procedure for survival surgeries and design of nerve device. (C) Initial injury of the sciatic nerve for 0 mm group (top) and 3 mm/3 mmD (nerve-interfacing device implantation) groups (bottom). (D) End-to-end repair of the sciatic nerve. (E) Device implantation for 3 mmD group and image of a completed implantation (F). Arrows in D and E show site and direction of tension.

Two cohorts were tested. The first cohort (terminal surgery, n = 16) was divided into four groups. In the first three groups, 0, 2, or 3 mm of nerve (corresponding to no, moderate, or high tension) were removed following transection, to test the integrity of end-to-end repair during hindlimb joint manipulation in a terminal surgery (Figure 2.1A). In the 4th group, a 3 mm gap was created, and an NID deployed to redistribute tension away from the repair site. The nerve was placed within the clamp and sutured using 4-0 Nurolon (Ethicon, Guaynabo, Puerto Rico) to the device (Figure 2.1B). Clamps were brought together along the guide rod such that the repair site was tension free, and strain was redistributed proximally and distally. The proximal cuff was secured to underlying muscle with a single 4-0 suture, such that any device movement did not tether or excessively load the intact proximal stump. Nerves were repaired end-to-end with three epineurial stitches (8-0 Ethilon suture, Ethicon), with the knee and ankle in a neutral configuration. After this initial repair, maximum physiological strain was imposed on the nerve: with hips abducted, knees were fully extended (180 degrees) and ankles maximally dorsi-flexed. This loading was repeated three times. Typically, repair failure occurred immediately; however, joints were held in position for 2 minutes to allow for the possibility of slower failure. The integrity of repairs was then visually assessed using digital images, and additional epineurial sutures used to reinforce failed or failing repairs.

The second cohort (survival surgery, n = 18) was subdivided into three groups (Figure 2.1A, B, C, D). In group 1 (0 mm gap), the nerve was transected without any additional gap. In groups 2 (3 mm) and 3 (3 mm-NID), a 3 mm length of nerve was removed following transection. Nerves in all groups were repaired end-to-end with exactly three stitches around the nerve circumference. In group 3, the NID was implanted following repair as above (Figure 2.1E and Figure 2.1F). Contralateral nerves were decompressed, and served as sham controls. After surgery, in all groups, overlying muscle was sutured using 4-0 polyglycolide suture (Oasis, Mettawa, IL, USA), then skin was stapled together using AutoClips (MikRon Precision, Gardena, CA, USA). Rats were allowed to recover on a heating pad before being returned to their cages. Buprenorphine was provided twice a day for 3 days, for analgesia.

After 12 weeks, rats were anesthetized and the sciatic nerve was exposed as in the first surgery. Devices were removed, sciatic nerves from both legs were harvested, pinned to cork, frozen in liquid nitrogen cooled isopentane, and stored at –80°C until processing.

Biomechanical testing

During survival surgery (second cohort), 10-0 Ethilon (Ethicon) sutures were stitched into the epineurium, two proximal and two distal to the injury site, as markers to measure proximal and distal strain, respectively, using methods previously published (Mahan et al., 2015). Suture spacing was measured in two configurations – tension-free (1, knee and ankle neutral) and nerve tensioned (2, full knee extension and neutral ankle; ankles were not dorsiflexed to avoid damage observed in terminal studies (first cohort) with maximum physiological nerve strain). The length between the sutures was recorded before the injury (Lpre1, Lpre2), immediately after the injury and repair (Lpost1, Lpost2), and at 12 weeks just before tissue harvest (Lrecovery1, Lrecovery2). Strain was measured before injury (Lpre2–Lpre1)/Lpre2, immediately after injury and repair (Lpost2–Lpost1)/Lpost2, and twelve weeks after repair (i.e., just before sacrifice) proximal and distal to the injury site (Lrecovery2–Lrecovery1)/Lrecovery2, based on the change in marker spacing with nerves under tension compared to untensioned. In addition, as an indirect measure of growth, the spacing between markers in a tension free configuration was compared at 12 weeks after recovery vs. just after repair (Lrecovery1–Lpost1)/Lpost1, the rationale being that an increase in spacing reflects material addition between the sutures.

Electromyography testing

To test the effect of clamping on nerve conduction in an uninjured nerve (i.e., to test whether the device interface has the potential to damage the nerve by compression), sciatic nerves were exposed during a terminal surgery in a third cohort (n = 10). After decompression, clamps were secured onto intact nerves, as above. Nerve conduction velocity was assessed using previously published methods (Restaino et al., 2014; Love et al., 2017), 10–15 minutes following device implantation. Briefly, nerves were

stimulated with a miniature bipolar hook electrode (Item ID: 501650; Harvard Apparatus, Holliston, MA, USA) positioned at either of two locations proximal to the clamp, separated by 5 mm. Two monopolar needle recording electrodes (Grass F-E2) were positioned adjacent to the endplate zone (Westerga and Gramsbergen, 1993), and a ground needle electrode was placed in the contralateral limb. Stimulation pulses (SD9, Grass Instruments, Astro-Med Inc., West Warwick, RI, USA) were chosen to minimize the applied voltage, while maintaining a recordable and consistent electromyography response (6 monophasic 50 μs duration square pulses at 5 Hz, at an input voltage of 7 V (< 10 mA)). Five recordings were made at each location to ensure consistency of stimulation and recording, and averaged together to determine the latency between stimulus and recording. Velocity was measured based on the 5 mm spacing divided by the difference in latency between the recorded signals for each stimulation location.

Functional testing

To test hindlimb function, rats were run and recorded on a Treadscan treadmill (CleverSys Inc., Reston, VA, USA) for 20 seconds at a rate of 10 cm/s, given a brief period to rest, then run a second time. Data points were collected pre-operatively, then at 2 and 12 weeks post-operatively. The sciatic functional index (SFI) was measured by two lab members, one blinded to experimental groups. Video stills were taken at points in time where the rat fully placed its paw on the track during normal gait. The length and toe spreading were measured using ImageJ (NIH, Bethesda, MD, USA; Schneider et al., 2012), and the SFI was calculated using the formula from Bain et al. (1989). Results were averaged from 3 images to find a single SFI value for each rat at each time point.

Histology and imaging

Tissue was blocked in OCT (4583, TissueTek/Sakura Finetek USA, Torrance, CA, USA), and subsequently sectioned using a cryostat into 10 μm sections, sliced axially. Sections were collected approximately 5 mm proximal and 5 mm distal to the site of the injury. Slides were stored at –80°C until stained. Slides were washed in Millipore water and fixed in 10% formalin. Following fixation, slides were

permeated using 0.2% Triton-X. Slides were then blocked in goat blocking buffer (10% goat serum (v/v) and 3% BSA (w/v) in PBS, and then incubated with the primary antibodies, mouse anti-β3-tubulin (Sigma-Aldrich, St. Louis, MO, USA; Cat# T5076, 1:500 dilution) and rabbit anti-laminin (Sigma-Aldrich; Cat# L9393, 1:500 dilution), for 1 hour at room temperature. Slides were then incubated with AlexaFluor 488 goat-anti-rabbit and AlexaFluor 594 goat-anti-mouse secondary antibodies (LifeTechnologies; Cat# A-11008 and A-11005, respectively, 1:200 dilution) while being protected from direct light for 45 minutes at room temperature. Finally, slides were coverslipped with Vectashield (Vector Labs, Burlingame, CA, USA). Slides were then stored at 4°C and imaged within 1 week of staining.

Images were taken using a Leica SP-5 confocal microscope (Leica, Buffalo Grove, IL, USA) and 10×/0.4NA and 63×/1.3NA objectives, using filter sets appropriate for the above secondary antibodies. At 63×, images were taken confocally using z-stacks, from the bottom to the top of the sample. 63× images were taken randomly across the nerve section, covering approximately 50% of the nerve cross-sectional area, to representatively characterize the entire sample. Axons were counted using the z-projection of these 63× images by two individuals, using ImageJ; one member, blinded to the experiments, counted axons manually within each image. The other counted axons by image thresholding, using the "analyze particles" function in ImageJ, excluding very small particles (< 1 μm2 in area). Total axon counts reflected summed counts from each image. Counts showed no significant difference when compared between lab member, with averaged counts within 5% of each other, and the final count used was an average between lab members. The average axon density was found by dividing the sum of axon counts by the sum of analyzed image areas. Non-nerve regions and large blood vessels, identified morphologically, were not included in area calculations. Total intra-epineurial area (i.e., area of nerve within the inner epineurial border) was found by tracing the corresponding contour (ImageJ) in 10× images. Total axon counts were calculated by multiplying the average axon density by the inter-perineurial area. From the "analyze particles" function, average axon size (or the area stained at each

point) was collected from each image. Fraction axon coverage was calculated as the total area within the nerve cross-section covered by axons divided by the total area of the image.

Statistical analysis

Significance for strain measurements and SFI was tested using a mixed two-way analysis of variance (ANOVA) (across: group, within: time), and Tukey's honestly significant difference (HSD) post hoc test was used to compare means between individual groups. Axon growth was tested using one-way ANOVA, comparing proximal and distal ends separately, again using Tukey's HSD post hoc test to compare means. All analysis was done in GraphPad Prism 7.04 (GraphPad Software, San Diego, CA, USA). Effect sizes were calculated using G*Power. For sample sizes used in our study, we were able to detect an effect size of 0.81 with a power of 0.80 ($\alpha = 0.05$). Measurements are reported as the mean ± standard error of the mean (SEM).

2.4 Results

Integrity of varying nerve gaps repaired end to end

We first repaired rat sciatic nerve gaps of increasing length (corresponding to increasing tension) in terminal surgeries, and tested whether repairs could successfully tolerate joint movement corresponding to maximum nerve strain (knee extension and ankle dorsiflexion). All repairs were successfully completed in a neutral joint configuration. 1 and 2 mm gaps were successfully repaired with three epineurial sutures (n = 4 each), and did not fail following knee extension and ankle dorsiflexion. In contrast, though 3mm gaps were successfully repaired with three epineurial sutures (n = 4), all (100% of) repairs showed signs of failure after knee extension and ankle dorsiflexion. Modes of failure included suture pullout, tearing of the epineurium surrounding the suture, or changes in nerve geometry in the vicinity of sutures. Even after reinforcing repairs with up to 7–8 epineurial sutures, repairs failed 25% of the time following joint manipulation (Figure 2.2).

Based on these qualitative assessments, we established 3 mm as the maximum allowable nerve gap amenable to end-to-end repair in our model, but whose repair is susceptible to damage in extreme physiological joint configurations. We then tested the feasibility of reinforcing the repair site with our

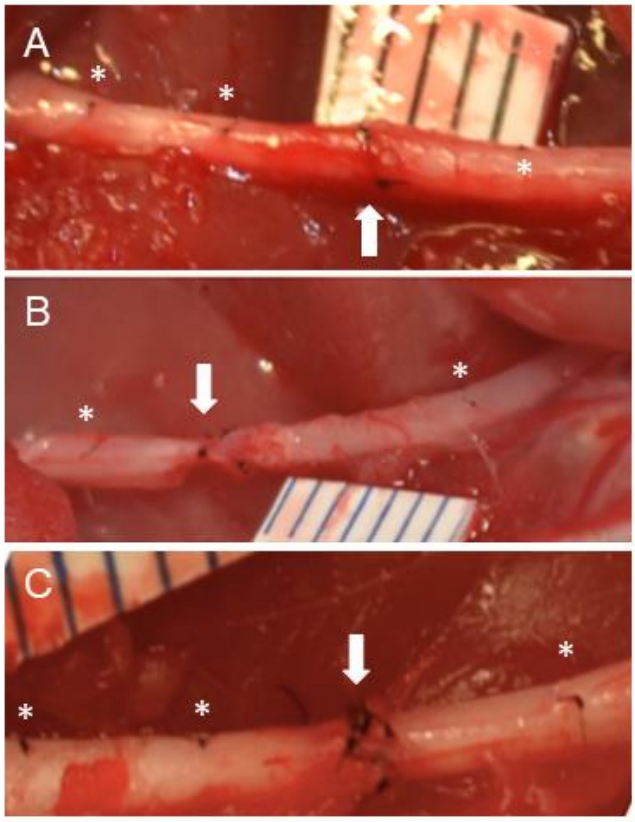

Figure 2.2: Successful and failed end-to-end repairs following sciatic nerve transection. (A) Successful end-to-end repair. (B) Failed end-to-end repair. (C) Failed end-to-end repair that had been anchored with additional sutures. The repair site is indicated with arrows. Epineurial sutures used to measure changes in nerve length are indicated with asterisks.

NID. First, we tested whether the NID itself was likely to cause compressive damage to the nerve, by deploying it upon an uninjured nerve. Nerve conduction velocity was not significantly changed after deploying the NID on untransected nerves (31.28 ± 2.89 vs. 26.02 ± 4.72, $P > 0.36$), suggesting that the NID itself, as envisioned by its design, did not cause appreciable damage to neural elements (Figure 2.3). We then repaired 3 mm gaps in the presence of the NID. In contrast to repairs without the NID, all repairs were executed with the usual three sutures, and withstood knee extension and ankle dorsiflexion with no

signs of repair failure or nerve damage. As an extreme example of NID functionality, we also repaired a 5-mm gap in the presence of an NID, with no signs of repair failure.

Regenerative outcomes following end-to-end repair of varying gap size

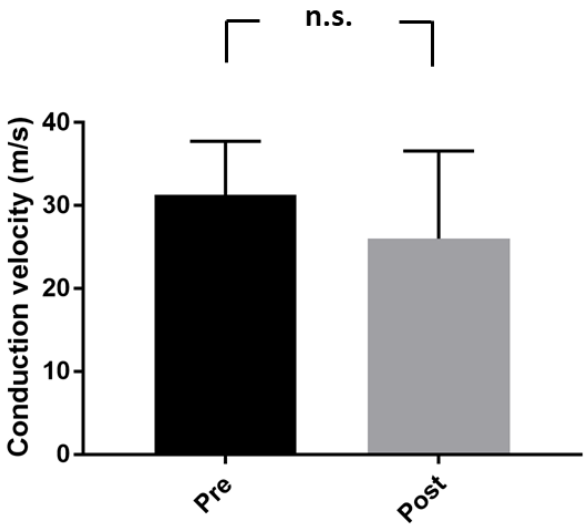

Figure 2.3: Conduction velocity pre- and post-device implementation on uninjured sciatic nerves. No significant changes occurred in velocity before and after device implantation in uninjured nerves. Slight difference in conduction velocity average between groups is due to a single outlier. Bars represent standard error (n = 10). n.s.: Not significant.

Based on the above observations in terminal surgeries, we performed survival surgeries to probe regenerative outcomes in three end-to-end repair groups: 0 mm gap (repair under minimal tension), 3 mm gap (repair under high tension), and 3 mm-NID group (repair under high tension, with tension redistributed away from repair site). For survival surgeries, based on the potential catastrophic repair failure of 3 mm gaps with combined knee and ankle movement, we ranged only the knee joint following repair, to confirm the integrity of repair. All repairs remained intact at the time of repair.

To probe local deformation following end-to-end repair in each group, we measured strain just proximal and just distal to the repair site. Immediately after repair, consistent with a larger gap, strain was significantly higher (P = 0.0002) in the distal region of the 3 mm gap group compared with the 0 mm gap. However, in the group with a device, this strain was partially relieved (Figure 2.4B). After 12 weeks, any differences in strain between groups were no longer significant (Figure 2.4C). In addition, after 12 weeks we observed significantly increased spacing between sutures with knee and ankle in a neutral

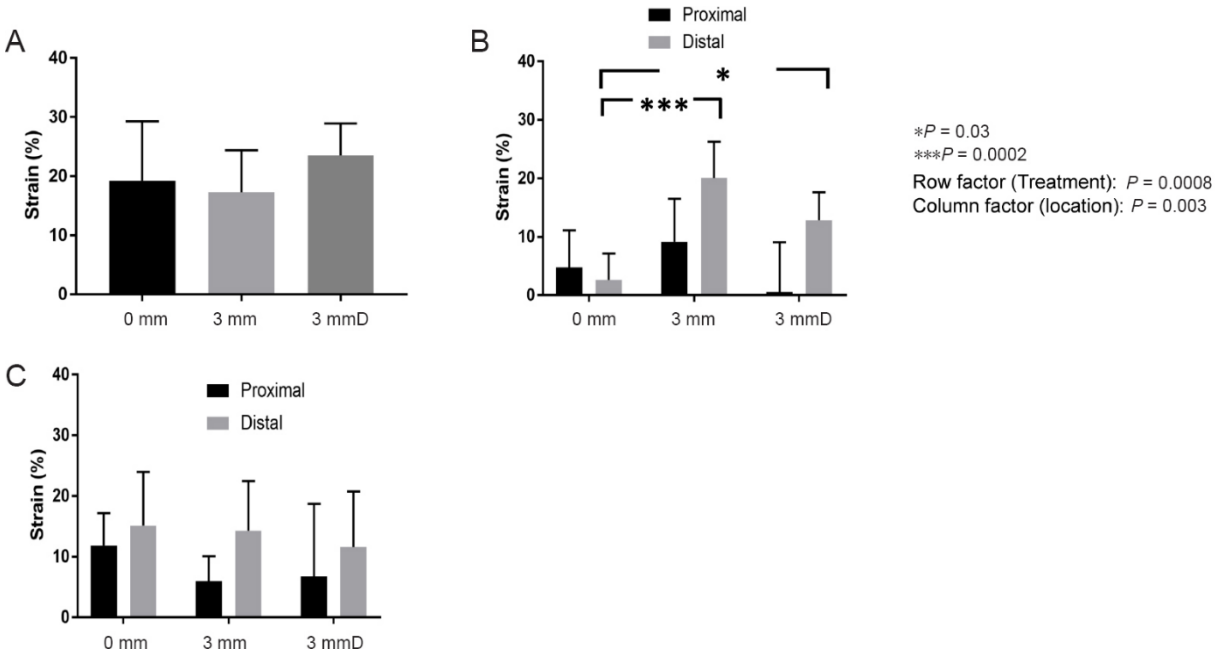

Figure 2.4: Strain in injured and uninjured sciatic nerves. (A) Strain in the uninjured contralateral nerve with knee extension. (B) Strain that occurred with knee extension after nerve injury. (C) Strain that occurred with knee extension after 12 weeks recovery. 3 mmD: nerve-interfacing device implantation. Bars represent standard error (n = 18). *P < 0.05, ***P < 0.001 (two-way analysis of variance followed by Tukey's honestly significant difference (HSD) post hoc test).

configuration, indicative of material addition ("growth") between the sutures over 12 weeks (Figure 2.5B). Such growth in both 3 mm and 3 mm-NID groups appeared to exceed growth in the contralateral limb.

Functionally, SFI decreased after injury, consistent with the loss of sciatic nerve function (Figure 2.6). During recovery, ANOVA revealed a significant effect of time, but no difference in SFI across experimental groups, suggesting consistent, but incomplete recovery after 12 weeks in all groups. At 12 weeks, upon exposure of the nerve, repairs appeared intact in all groups. Enhanced fibrotic deposition was observed in device groups; however, devices were readily removed from the attached nerve prior to tissue harvest (Figure 2.7). Consistent with the similarity of functional outcomes across groups, axon growth also appeared similar among groups, with no significant differences in axon counts between groups (Figure 2.8), (Figure 2.9A,B). Interestingly, the axon size was significantly higher in the 3 mm (no device) group compared with the other groups, consistent with increased gross indicators of growth (Figure 2.9C,D).

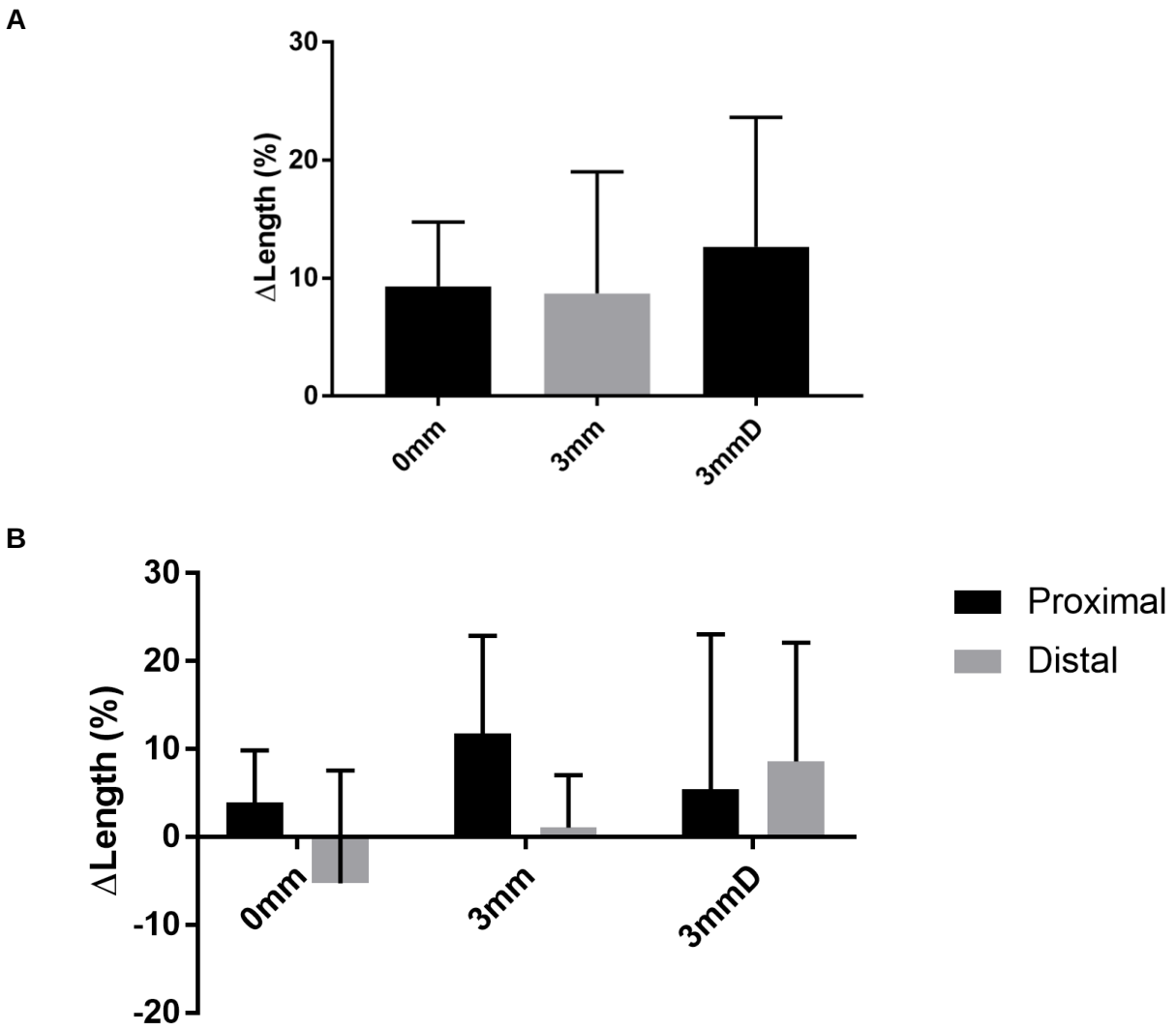

Figure 2.5: Nerve "growth" over 12 weeks after sciatic nerve injury and repair. Growth was assessed based on distance between two sutures in the contralateral nerve (A) and the injured nerve (B). Bars represent standard error (n = 18). 3 mmD: Nerve-interfacing device implantation.

2.5 Discussion

Redistribution of tension away from the repair site enabled end to end repair of larger gaps

In this study, we repaired nerves end-to-end under varying tension, with and without a new device that redistributed tension away from the site of repair. Repairs of large nerve gaps failed in physiological joint configurations resulting in maximum nerve strain. However, using a nerve-interfacing device we effectively redistributed tension at the repair site, and the novel clamp design held the nerves in

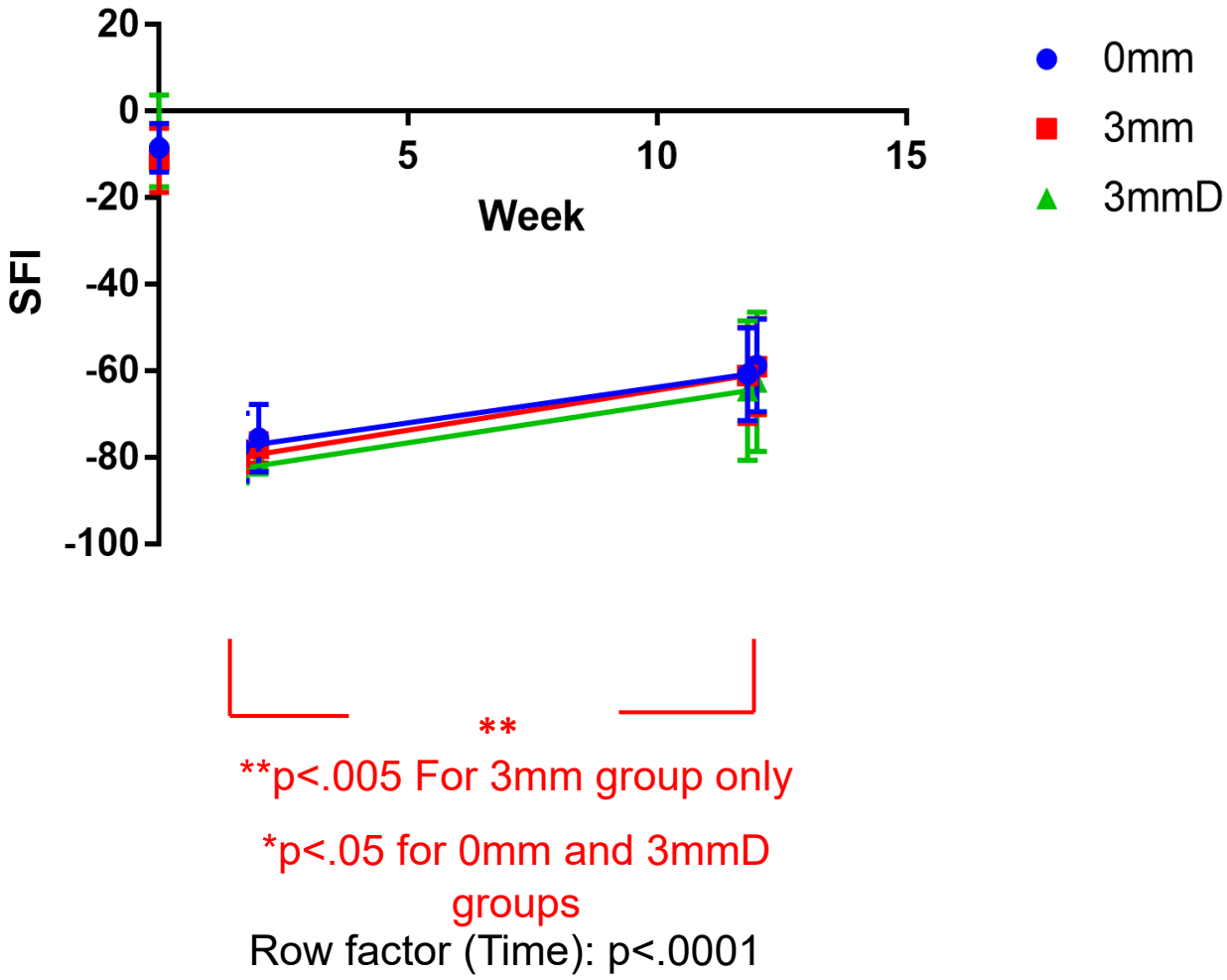

Figure 2.6: Change in SFI over time after sciatic nerve injury. SFI improves significantly ($P < 0.0001$) 12 weeks after injury (two-way analysis of variance followed by Tukey's honestly significant difference (HSD) post hoc test). Bars represent standard error (n = 18). 3 mmD: Nerve-interfacing device implantation group. SFI: Sciatic functional index.

place without damage. There was also no repair site failure when using the device, suggesting its potential utility in graft-free repairs, without detrimental effects. The device was also superior to simply adding additional epineurial sutures to the nerve to reinforce the repair, as loads could be distributed across the larger nerve-clamp interface, rather than at individual sutures. The strategy of redistributing tension away from the site of repair has been successfully tested surgically by Kechele et al. (2011), using a mesh, and Scherman et al. (2004), using anchoring sutures. Anecdotal evidence suggests that surgeons may also reinforce or protect repair sites by wrapping them with nerve conduits or other materials. In contrast, the use of a nerve interfacing device provides a simpler, more clinically reproducible and reliable approach.

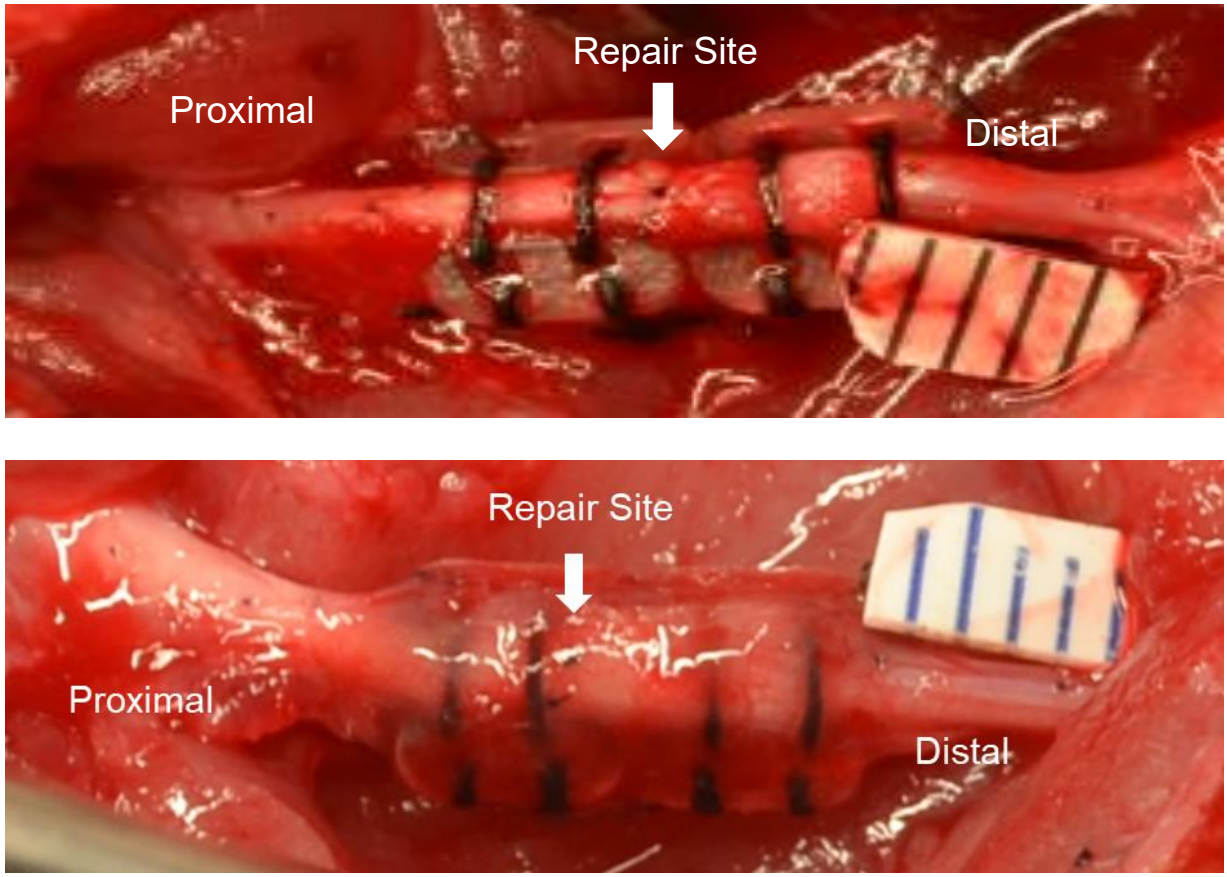

Figure 2.7: Injury appearance before and after recovery with device implantation. Nerve device immediately following implantation (top) and 12 weeks post implantation (bottom), showing a fibrotic layer that has formed over the clamps. Device was cleanly excised from the sciatic nerve upon removal of this layer.

Advantages include the ability to readily align and approximate nerves to a desired strain during the repair, in a manner analogous to non-implantable tissue approximators used intra-operatively (Forootan et al., 2014). Gripping the nerve via epineurial barbs also represents a new approach to securing the nerve without excessive compression, while preserving conductive elements.

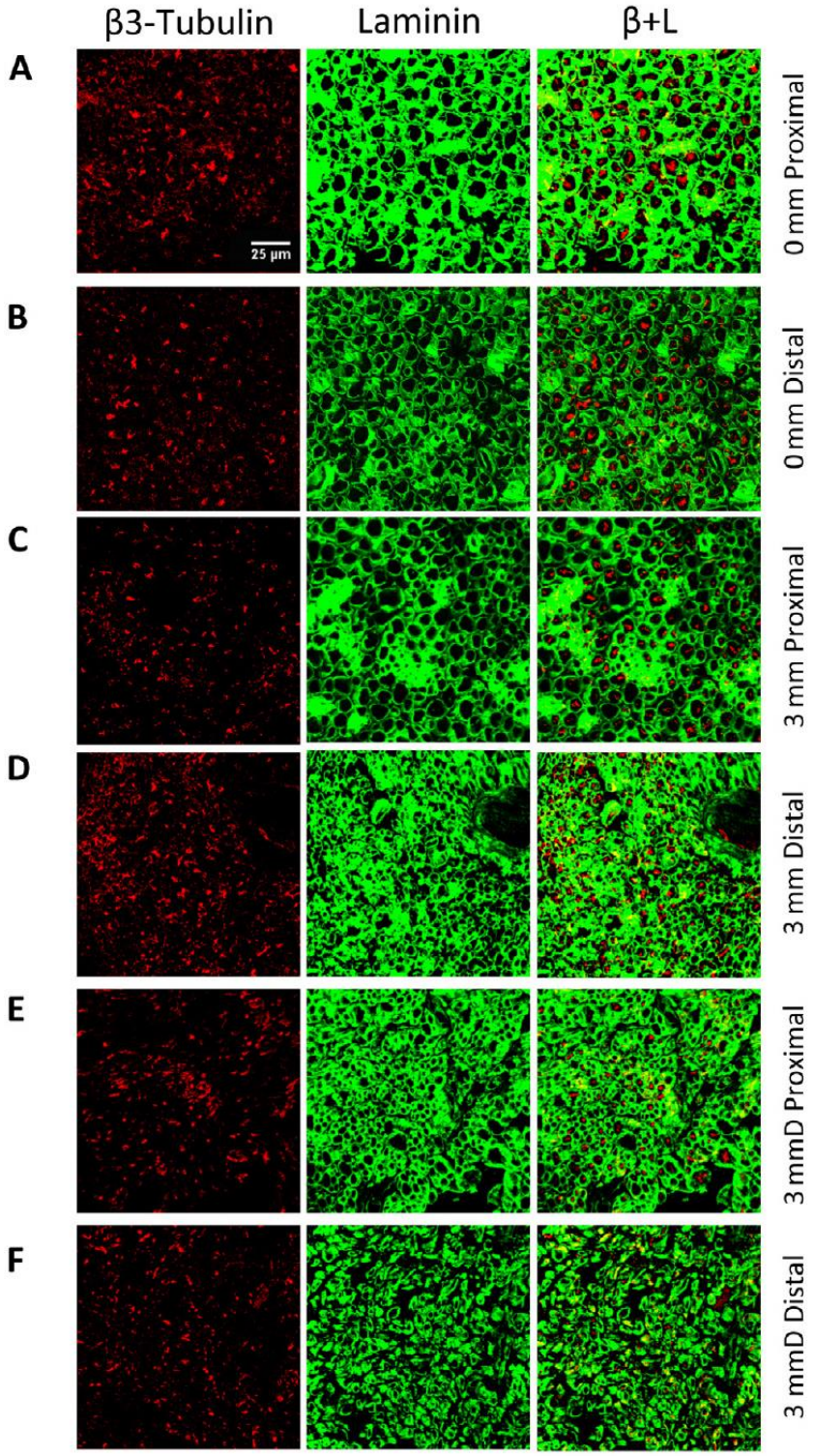

Figure 2.8: Staining with laminin and β3-tubulin antibodies of injured nerves 12 weeks after sciatic nerve repair. 0 mm group proximal (A) and distal (B) to the injury. 3 mm group proximal (C) and distal (D) to the injury. 3 mmD (nerve-interfacing device implantation) group proximal (E) and distal (F) to the injury. Blood vessels were excluded from the analysis, and all images are at the same scale. (G) 10× images of distal nerves from each group provide examples of images used to make total cross-sectional area measurements. Red is β3-tubulin, green is laminin.

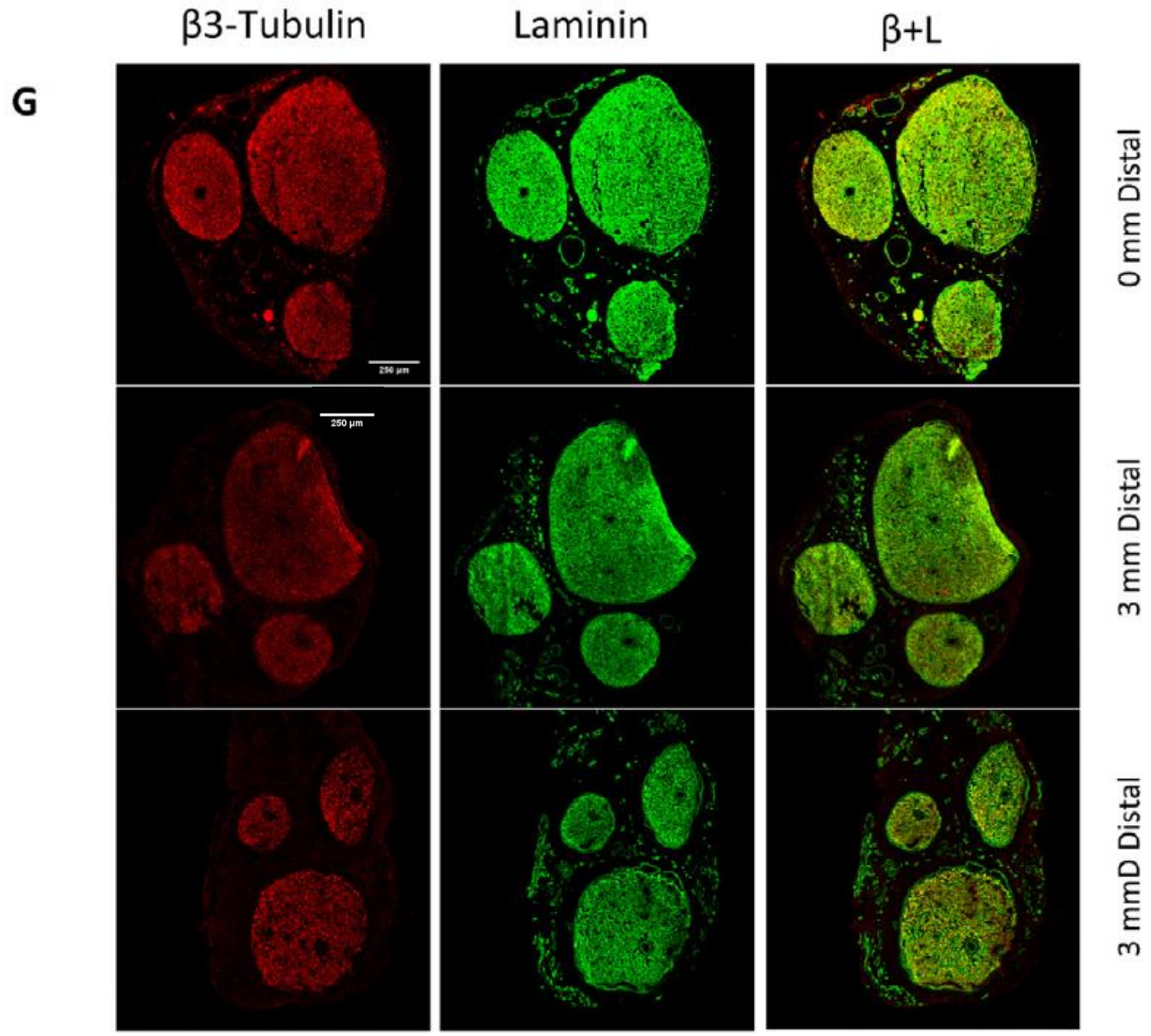

Figure 2.8 con't: Staining with laminin and β3-tubulin antibodies of injured nerves 12 weeks after sciatic nerve repair.

Cumulatively, our data suggested that the repairs made under tension were no worse than those made without tension. With respect to strain, functional, and histological outcomes, repairs under tension – with or without a device -- did not adversely affect nerve regeneration. The magnitudes of measured strains are consistent with previous studies in the rat sciatic nerve (Foran et al., 2017), and magnitudes of SFI and axon counts are consistent with several previous reports, which range from an average of 7000 to 12,000 axons distal to the repair (MacKinnon et al., 1991; Jonsson et al., 2013; Ganguly et al., 2017).

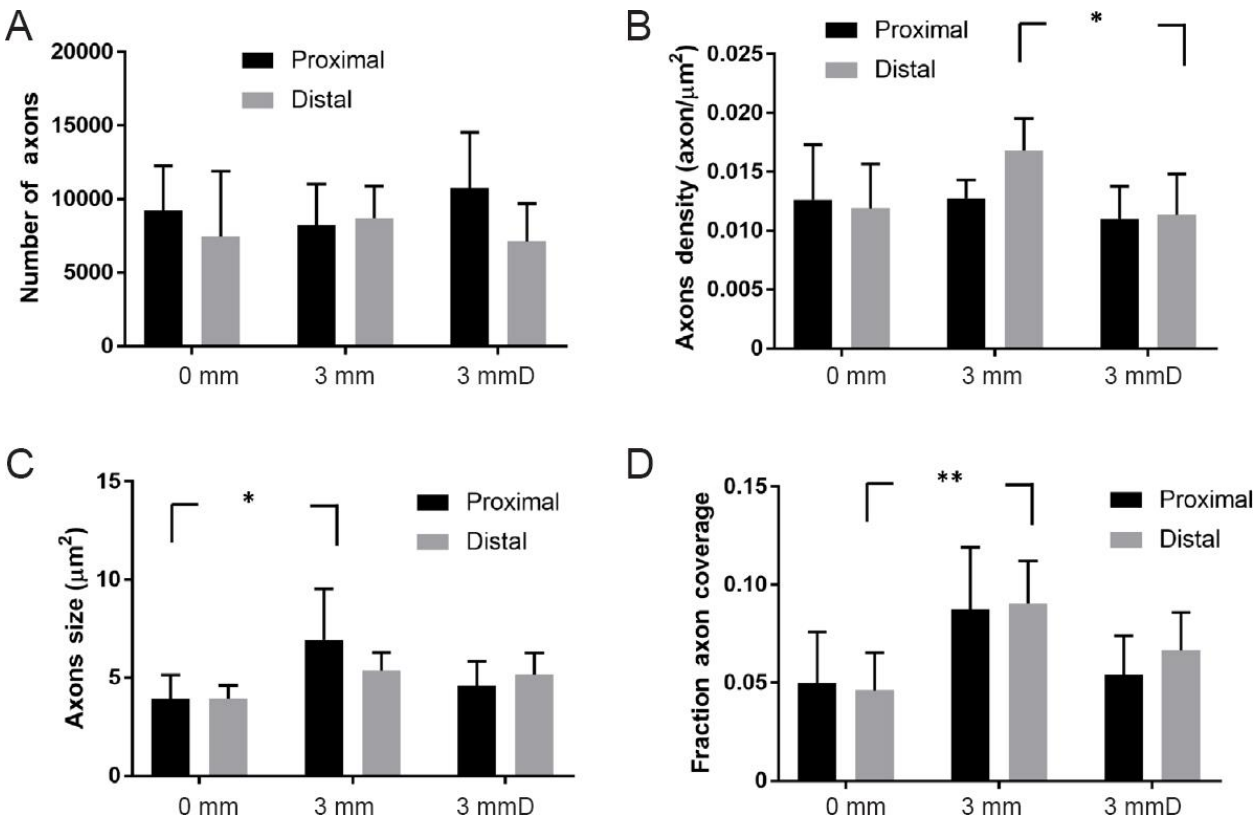

Figure 2.9: Quantitative analysis of confocal images at 12 weeks after sciatic nerve repair. (A) Number of axons; (B) axon density, or the number of axons per area; (C) axon size; (D) fraction axon coverage, or the area covered by axons divided by the total area inside the perineurium. Significant differences were found using multiple comparison tests between 3 mm and 3 mmD (nerve-interfacing device implantation) axon density, distally (*P < 0.05); between 0 mm and 3 mm axon size, proximally (*P < 0.05), and between 0 and 3 mm axon coverage, distally (**P < 0.01) (one-way analysis of variance followed by Tukey's honestly significant difference (HSD) post hoc test). Bars in all graphs represent the standard error (n = 17).

Our observed outcomes also reflect the key considerations that enter surgical decision-making. On one hand, repairs of the 3 mm device-free group failed catastrophically when nerves were maximally strained (knee extension and ankle dorsiflexion). However, no failures were seen with knee extension alone, or after 12 weeks with joint movement during recovery, and functional and structural outcomes trended more favorably compared to 0 or 3 mm-NID groups. We speculate that repair integrity may reflect the fact that after sciatic nerve injury, no active ankle dorsi- or plantar flexion can occur, thus protecting the nerve from any ankle-related tension. In addition, typical rat hindlimb postures do not maximally strain the sciatic nerve. Thus, if the repair holds, a tensioned repair of larger gaps is not unfavorable, and in fact, as demonstrated to be the case for smaller gaps, may even be favorable

(Hentz et al., 1993; Sunderland et al, 2004). On the other hand, the fact that 3mm gaps repaired without a device fail in extreme, albeit still physiological, joint configurations demonstrates the high risk associated with tensioned repairs despite their possible benefit. Protection of the repair site, as we have shown with our NID, may offer the benefits of a tensioned repair while mitigating the risk. We recommend protection of the repair site over a timeframe during which the repair is structurally stabilized. Biomechanical properties of transected rat nerves significantly improve within seven days post-repair, and approach a plateau within 3 weeks post-repair, suggesting robust and rapid strengthening of the repair sites (Abrams et al., 1998); future versions of a resorbable device could reflect such timelines.

Role of tension in growth

An intriguing observation was that comparatively high (though not super-physiological) nerve strain observed immediately after repair in the 3 mm groups was markedly reduced after 12 weeks of recovery. Further, in the 3mm group, growth appeared to be, surprisingly, even more robust based on larger axon diameter and increased axon coverage. These findings support a hypothesis that tension may stimulate growth. There is considerable precedent for a positive role for nerve tension on growth. Previous studies have shown that the rate of axon growth increases when axons are under tension, even to the extent of multiplying the rate of growth by eight (Bray, 1984; Pfister et al., 2004). Other studies show that tension activates certain translational pathways, such as mTOR-based pathways, promoting further axon growth and structural changes (Suter and Miller, 2011; Love et al., 2017). Nerve lengthening also occurs in limb lengthening procedures, and if done slowly enough, without detriment to the affected nerves (Simpson et al., 2013). Similarly, decompressed nerves redistribute their strain to reflect their dynamic environment (Foran et al., 2018), suggesting a remarkable capacity for remodeling in response to mechanical cues. This concept has increasingly been incorporated into neuroregenerative strategies by multiple research groups, with positive results (Saijilafu et al., 2008; Chuang et al., 2013; Vaz et al., 2014; Yousef et al., 2015). Finally, a case report suggests that dynamic tensioning can have favorable

outcomes as well (McDonald and Bell, 2010), providing early clinical support for tensioned repairs, under appropriate circumstances.

Limitations

Despite promising data in support of tensioned repairs, our study did have several limitations. First, because tissue was sampled at a single time point and location, it was not possible to determine how axon growth rate was ultimately affected by tension. However, we were able to determine that functional recovery and axon growth distal to the injury occurred, revealing that the repairs under tension were, at minimum, as effective as tension-free repairs. Further study, by allowing longer recovery times and sampling structural regeneration at additional intermediate data points, could reveal the effect of tension on axon growth with increased resolution. Another limitation was our small sample sizes, compared to other studies. However, power analysis suggests that we were able to detect effect sizes of 0.81 with a power of 0.80, and based on our data, establishing significant differences between groups would require at minimum 2–3× the sample size in these experiments. Thus our study is sufficiently powered to confirm a lack of major differences from group to group. A third limitation was our decision to use strain as a surrogate for tension, not measure tension itself. This was in part due to significant nerve-to-nerve differences in material properties, and in part due to the ability to readily measure regional differences in deformation. Another limitation was our selected approach for functional testing. We had two goals; the first was to measure conduction velocity in uninjured nerves, to demonstrate device implantation did not appreciably affect neural elements. This was achieved using electromyography-based conduction testing. Our second goal was to use simple functional outcomes to demonstrate that device implantation and repairs under tension did not adversely affect functional regeneration (i.e., that such approaches are safe). Towards this goal, we elected to use the SFI, a measure that is not considered as reliable for earlier time points, but shows good correlation with recovery at later time points, and so remains a simple and useful tool for longitudinally measuring recovery (Monte-Raso et al., 2008). Future studies would benefit from the use of additional functional tests, including conduction velocity testing, muscle contractility, and/or

joint torque testing. Finally, with respect to translation of our NID, while Visijet M3 Crystal is USP Class VI approved, this material is non-resorbable, and thus an additional surgery would be required for device excision. However, the use of resorbable materials (e.g., PLGA), which are widely used in a variety of biomedical devices, offer a solution to this translational challenge.

Conclusions and clinical implications

Currently, clinicians are rightfully reluctant to make nerve repairs under tension. However, this study as well as other previous literature suggests that tension could be a valuable alternative to the use of grafts in cases of small to moderately sized nerve gaps. With the caveat that the repair is stable – a concern addressed by our novel NID – tension is not detrimental to the repair and may even promote faster growth. This concept may be pushed further in future research, in which dynamic tensioning via modified versions of the NID may be used to bridge much larger nerve gaps prior to performing end-to-end repairs (Vaz et al., 2014).

2.6 Acknowledgements

Chapter 2, in full, is a reprint of the material as published in Neural Regeneration Research in 2019, titled "Redistribution of nerve strain enables end-to-end repair under tension without inhibiting nerve regeneration." My co-authors are Turki Alaziz, Shawn O'Connor, and Sameer Shah. The dissertation author is the primary author of the article. I also would like to acknowledge the generous support of the Department of Veterans Affairs (VA MERIT IRX001471A).

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Chapter 3: Nerve lengthening and subsequent end-to-end repair yields more favorable outcomes compared to autograft repair of rat sciatic nerve defects

3.1 Abstract

Outcomes of end-to-end nerve repairs are more successful compared to outcomes of repairs bridged by nerve grafts. However, end-to-end repairs are not always possible for large nerve gaps, as excessive tension may cause catastrophic failure. In this study, we built on previous nerve lengthening studies to test the hypotheses that gradual lengthening of the proximal stump across a large nerve gap enables an end-to-end repair and such a repair results in more favorable regenerative outcomes than autografts, which represent the gold-standard in bridging nerve gaps. To test these, we compared structural and functional outcomes in Lewis rats after repair of sciatic nerve gaps using either autografts or a novel compact internal fixator device, which was used to lengthen proximal nerve stumps towards the distal stump over two weeks, prior to end-to-end repair. Twelve weeks after the initial injury, outcomes following nerve lengthening/end-to-end repair were either comparable or superior in every measure compared to repair by autografting. The sciatic functional index was not significantly different between groups at 12 weeks. However, we observed a reduced rate of contracture and corresponding significant increase in paw length in the lengthening group. This functional improvement was consistent with structural regeneration; axonal growth distal to the injury was denser and more evenly distributed compared to the autograft group, suggesting substantial regeneration into both tibial and peroneal branches of the sciatic nerve. Our findings show that end-to-end repairs following nerve lengthening are possible for large gaps, and that this strategy may be superior to graft-based repairs.

3.2 Introduction

Peripheral nerve injuries have long presented a difficulty clinical challenge, in terms of treatment strategy and degree of recovery. Transected nerves in particular result in poor recovery, because original connections from the proximal to distal portions of the nerve are lost completely and new axon growth

emerging from the severed proximal stump is disorganized (Meek, Coert, & Robinson, 2005). For proper nerve regeneration to occur, the axons in the proximal stump must grow back into and through the distal stump, which provides a path for organized axon growth. For transected nerves, such recovery is facilitated through surgical repair, with the particular repair strategy chosen driven by the size of the gap between the proximal and distal ends. End-to-end repairs are preferred for small gaps and lead to superior clinical outcomes compared with graft-based repairs (Bhatia et al., 2017; Deumens et al., 2010; Siemionow & Brzezicki, 2009). However, current clinical practice does not recommend end-to-end repairs for certain transection injuries, due to the possibility of excess tension along the nerve and at the repair site; attempting the repair can result in tissue damage and catastrophic repair failure (Maeda, Hori, Sasaki, & Maruo, 1999; Sunderland et al., 2004). Because of this rationale, surgeons typically use a graft to repair any nerve gap larger than 3 cm in length (Grinsell & Keating, 2014; Siemionow & Sonmez, 2010). Autologous grafts are the current gold standard for repairing nerve gaps, but grafts are not an ideal solution because they introduce an additional interface and length through which axons must grow before finally reaching the distal stump of the nerve (Bhatia et al., 2017; Brown, Shah, & Mackinnon, 2009). Other drawbacks of nerve grafts include donor site morbidity, geometry mismatch, and limitations on length and viability of grafts that can be harvested (Schmidt and Leach, 2003; Meek et al., 2005).

On the other hand, there is compelling evidence in small animal and primate models that nerves can be repaired under modest tension, resulting in improved outcomes compared with autograft or conduit repair (Hentz, Rosen, Xiao, McGill, & Abraham, 1993; Sunderland et al., 2004). In addition, modest tension (<20% strain) distributed along axons in vitro not only does no harm to neurons but also dramatically increases the rate of axonal growth (Bray, 1984; Pfister, Iwata, Meaney, & Smith, 2004; Franze and Guck, 2010; Simpson et al., 2013). Based on these observations, nerve lengthening should correspondingly have a positive effect on nerve regeneration, by increasing the rate of growth along the entire nerve. These concepts have been borne out in models of limb lengthening (Simpson et al., 2013) and nerve lengthening (Saijilafu, Hara, Yoshii, & Ochiai, 2008; Yousef et al., 2015) with generally

positive outcomes. Limb-lengthening studies show that nerves can indeed be lengthened over time in short intervals, with only transient functional deficits (Simpson et al., 2013). In addition, end-to-end repairs have been successfully completed following nerve lengthening across a nerve gap, with variable outcomes (Saijilafu et al., 2008; Yousef et al., 2015). Despite these successes, nerve-lengthening strategies have not yet been translated, possibly due to the invasiveness of lengthening strategies and/or inconclusive outcomes. In this study, we implemented a new and compact technology for directional nerve lengthening, to repair transected nerves in an acute nerve injury model. We deployed a comprehensive set of structural and functional outcomes to test the hypotheses that (a) gradual lengthening of the proximal nerve end towards the distal stump will enable end-to-end repair of large nerve gaps and (b) such a repair strategy will result in improved outcomes compared with graft-based repair. This study provides a new strategy for repair of long nerve gaps, which, by combining the positive benefits of nerve lengthening with the potential advantages of end-to-end nerve repair, could offer a superior alternative to autografts.

3.3 Methods

Surgeries

Lewis inbred rats weighing 300–350 g were arbitrarily separated into two different groups: autograft and lengthening/end-to-end repair (LETER) group. Rats were anaesthetized in an incubation chamber using 5% isoflurane. Surgery was performed on a heating pad with continuous isoflurane at 1.5–2% provided to rats via a nose cone. The rats were injected subcutaneously with 0.03 mg/ml of buprenorphine (Par Pharmaceutical, Chestnut Ridge, NY) and Baytril (Bayer Healthcare, Shawnee Mission, Kansas). A dorsal incision was made in the hindlimb, and the femoral biceps was split to expose the sciatic nerve. The sciatic nerve was then decompressed.

A 10-mm gap represents a moderate-to-large defect in rat models and requires a graft to ensure repair integrity (Terzis, Faibisoff, & Williams, 1975; Wong & Scott, 1991). Therefore, for the autograft

group, 10 mm of the sciatic nerve was cut cleanly using a no. 11 blade, between the hip and knee and above the bifurcation. This nerve piece was then flipped and resutured to the proximal and distal ends using three epineurial sutures (8-0 Ethilon, Ethicon, Guaynabo, Puerto Rico; Grinsell & Keating, 2014). Muscle was then sutured together using 4-0 polyglycolic acid suture (Oasis, Ottawa, IL), and the skin was stapled using AutoClip staples (MikRon Precision, Gardena, CA). The rats were then allowed to recover on a heating pad prior to rehousing.

For lengthening surgeries, the proximal stumps of transected nerves were lengthened by 12 mm to span a final gap of 10 mm at the time of repair. Either a 10- or 3-mm segment of the nerve was removed prior to lengthening. For the former, an additional 2 mm of nerve stumps was trimmed prior to end-to-end anastomosis, and so, the repair was under slight tension (as is also typical for flipped autografts). For the latter, additional nerve was trimmed back at the time of surgery, such that 10-mm total nerve was excised before a tension free repair. Both groups were analysed separately, and ultimately, data were pooled for comparison with autograft, as there were no significant differences in any outcomes ($p < .05$; Table 3.1).

Proximal and distal nerve ends were secured to the nerve-lengthening device, using our published methods (Howarth, Alaziz, Nicolds, O'Connor, & Shah, 2019; Figure 3.1a). Briefly, proximal and distal stumps were firmly secured to the nerve clamps present on the device using 4-0 Nurolon suture (Ethicon, Guaynabo, Puerto Rico) with directional barbs penetrating the epineurium to help prevent slippage of the nerve during lengthening (Figure 3.1b). The proximal clamp was mounted on a stainless steel rod, for alignment and guidance during subsequent lengthening. In order to avoid interference, the distal clamp was positioned perpendicular to the proximal clamp; its purpose was to hold the distal clamp in place for the duration of lengthening. Two 10-0 Ethilon sutures (Ethicon) were made in the epineurium to approximate change in length of the proximal stump. Muscle and skin incisions were closed as for autografts. Nerves were lengthened approximately 1 mm/day and 12 mm total over the next 2 weeks by means of a manually actuated guidewire that exited the rat but was secured to the proximal clamp

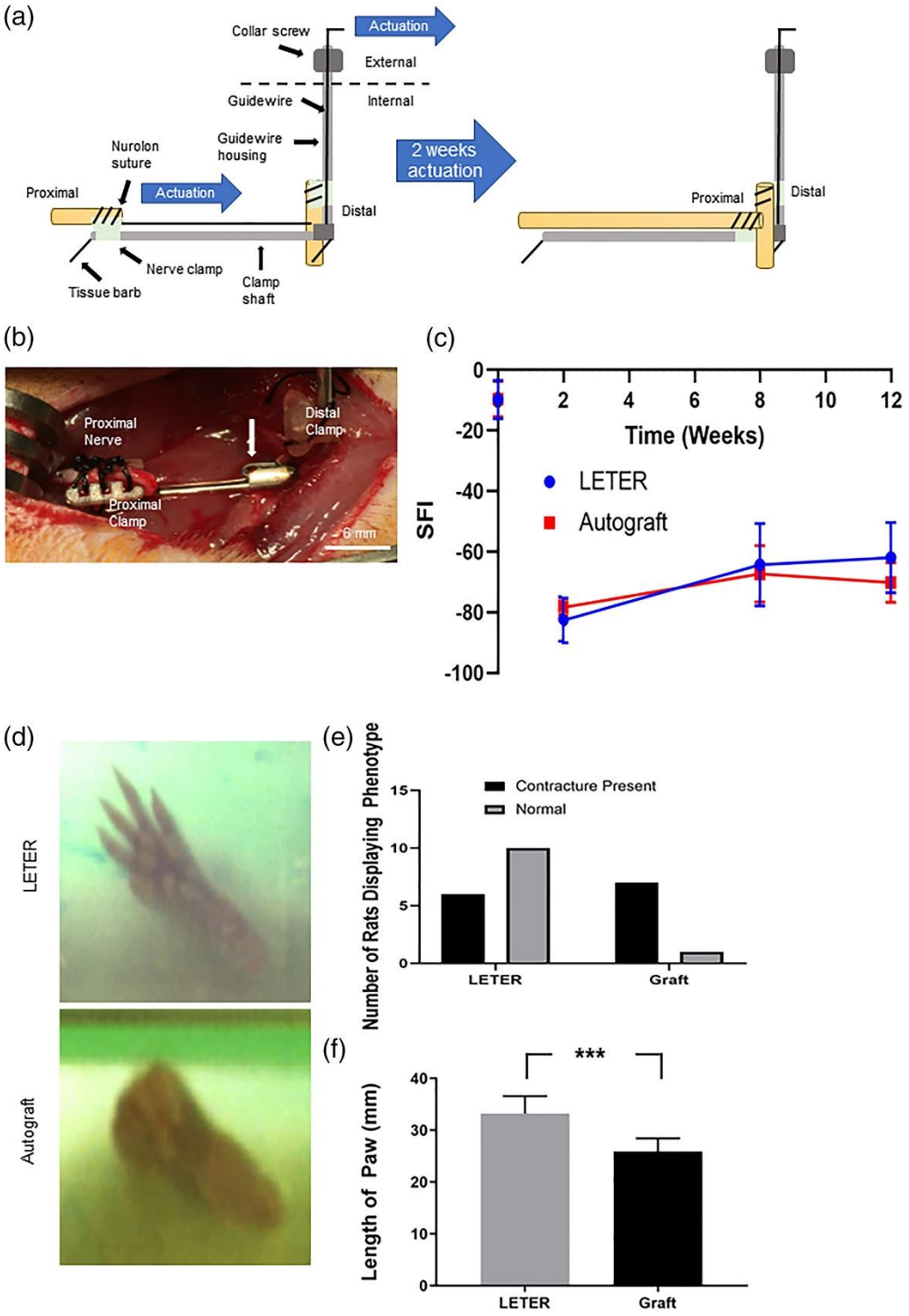


Figure 3.1: (A) Schematic of nerve lengthening device, before and after actuation. Important components are labeled in figure. (B) Image of implanted nerve device, showing proximal nerve resting in clamp. (C) Change in Sciatic Functional Index (SFI) over time Row factor (time): $p<0.0001$ Column factor (group): $p=0.4419$. Statistical analysis was done only with post-op measurements, so pre-op measurement (0 weeks) was not connected to post-op time points. (D) Example of differences in paw appearance due to nerve palsy. (E) Number of rats from each group that displayed contracture compared to the number that did not. (F) Differences in paw length between graft and lengthening groups due to contracture ($p = 0.0002$) (n=24).

internally (Figure 3.1a). Rats were gently restrained, but not anaesthetized, during each lengthening. Fourteen days after the initial surgery, a second surgery was performed. Rats were prepared as they were in the previous surgeries, the device was carefully extracted from the surrounding fibrotic tissue and from the nerve itself, and clamped nerve ends were trimmed off using a no. 11 blade in order to create a clean interface for subsequent end-to-end repair. Nerves were repaired end to end using three epineurial sutures (8-0 Ethilon, Ethicon, Guaynabo, Puerto Rico), and incisions were closed as in the previous surgeries.

Functional Testing

Rats were placed on a TreadScan treadmill (CleverSys Inc, Reston, VA) for paw print analysis. Tests were taken presurgery and 2, 8, and 12 weeks post-surgery. Rats were run on the treadmill at a rate of 10 cm/s for 20-s intervals. Image stills from the recorded video were analysed using ImageJ to measure paw length, toe spread, and intermediate toe spread, parameters that comprise the sciatic functional index (SFI, Bain, Mackinnon, & Hunter, 1989). Because toe curling (i.e., contracture, such as that occurring during a nerve palsy, Weekley et al., 2012) precluded flat placement of the paw during gait, thus potentially affecting the accuracy of the SFI calculation, calculations were only made from steps where both the heel and some portion of the toes rested on the track. As paw length was calculated as the distance between the forward-most and rearmost toe and heel contact points, respectively, curled toes resulted in a shorter paw length. Two researchers, one blinded to experiments, calculated the SFI from image stills, averaging the results between them. The presence (yes/no) of toe curling/contracture was also assessed at the 12-week time point.

Tissue Harvest

Sciatic nerve harvest took place 12 weeks after nerve repair in both autograft and LETER groups. Rats were anaesthetized as in the previous surgeries, and surgical approach was the same as above. Any fibrotic tissue that surrounded the nerve was gently removed (with adherent tissue left in place), and the sciatic nerve was removed, pinned to cork, and placed in 10% formalin for 24 hr. This was followed by submersion in 15% and 30% sucrose overnight, to provide cryoprotection. Nerves were then frozen in

liquid nitrogen-cooled isopentane and stored at −80°C until processing. Rats were euthanized via carbon dioxide inhalation, and muscles were innervated by the sciatic nerve (tibialis anterior [TA], extensor digitorum longus, and gastrocnemius) were harvested, weighed, then pinned to cork, and preserved using the same methods.

Histology

Nerve samples were taken from approximately 5 mm proximal and 5 mm distal to the nerve injury. In the autograft group, a sample was also taken halfway between the proximal and distal ends of the graft. Nerve samples were then blocked in OCT (4583, TissueTek/Sakura Finetek USA, Torrance, CA) and then sectioned into 10-μm axial sections. The intact contralateral nerve was also sectioned for comparison. Slides were stored at −80°C until staining.

Prior to staining, slides were allowed to thaw to room temperature. At room temperature, sections were permeabilized in 0.2% Triton X-100, washed with phosphate buffered saline (PBS) and blocked (10% goat serum and 3% bovine serum albumin in PBS), incubated in SMI-31 and anti-laminin primary antibodies (anti-mouse NF-H, 1:500, Sigma-Aldrich NE1022 and anti-rabbit laminin, 1:500, Sigma-Aldrich L9393) for 1 hr, rinsed 3× in PBS, and incubated in fluorescent AF488 goat anti-mouse and AF594 goat anti-rabbit secondary antibodies for 1 hr. Finally, slides were washed in PBS once more and coverslipped using VectaShield Hardset (Vector Laboratories H-1400). Slides were stored at 4°C and imaged within 1 week of staining. CGRP 1:500 (Abcam ab81887) antibody-labelling and isolectin B4 1:200 (IB4 Farmingdale, NY, USA) costaining were also performed. For this combination, primary antibody was applied overnight at 4°C, and PBS washes were replaced by washes with TBST. IB4 was conjugated with 488 and added during the same step as the secondary antibody.

Muscle

The TA and gastrocnemius muscles were split longitudinally before sectioning (100-μm-thick sections). Labelling was initially as previously described, but α-bungarotoxin, conjugated to AF488, was incubated overnight at 4°C.

Imaging and Analysis

Slides were imaged at 10×/0.4 numerical aperture and 63×/1.3 numerical aperture using a Leica SP5 confocal microscope. At 10×, an image of the entire nerve cross section was taken. At 63×, several images were taken at randomly selected locations throughout the sample for each nerve, to ensure approximately 50% coverage of the entire nerve. A z stack of the nerve was taken at each location.

Images taken at 63× were analysed using ImageJ to count the number of axons. Axons were analysed by image thresholding (Otsu's method) and then counted using the Analyze Particles function, which also determined the average particle size. Images were randomly selected to have axons counted manually, in order to affirm accuracy of measurements. Total axon counts were calculated by summing the number of axons found in all regions, dividing by the total area represented by these axons, and then multiplying this number by the total intraepineurial area found from images taken at 10×. CGRP was imaged similarly, and max z projections were quantified by determining the area the antibody covered in comparison with the entire area of the image. Fractional area covered by the CGRP antibody for each image was averaged for each individual and then averaged between groups.

Approximate distances between axons were measured using the Delaunay–Voronoi plugin in ImageJ and axon locations found using the Analyze Particles function in order to calculate the Delaunay triangulation, which represents an objective and more comprehensive assessment of the proximity of objects (cf. nearest neighbour spacing, Liebling & Pournin, 2012). Histograms showing the distributions of these distances were generated and compiled in Matlab R2018b, overlaying analyses from LETER and graft groups from distal sections and a separate overlaid histogram from proximal sections in order to

compare visually. Also measured were the mean and median from each dataset. Each image analysed was averaged for the individual, and then individuals were averaged by groups.

Neuromuscular junctions (NMJs) were imaged using a modification of published methods (Pratt, Iyer, Shah, & Lovering, 2018). Max z projections were created to project 3D structure and arboriaton into a 2D plane. NMJs were quantified by manual counting within an area sampled within the endplate-enriched region comprising the proximal one third of each muscle. Counts (per unit area) are approximate, as a 100-μm section only represents a fraction of total muscle volume. The dispersion index, defined as the total stained area/the total area × 100, of individual NMJs was calculated from the morphology using the methods of Pratt et al. (2018).

Statistical Tests

Differences between means for SFI were assessed using mixed two-way analysis of variance (within factor: time; between factor: treatment). Differences between means for histological outcomes were measured using a Student's t test. Tukey's honestly significant difference post hoc test was used to compare individual means. For our primary outcome, SFI, with $\beta = .2$, $\alpha = .05$, and a moderate effect size of 0.45, a sample size of $n = 8$ was required for each group. The 10- and 3-mm LETER groups were combined for the purpose of statistical analysis following comparison by t test, as justified above; for $n > 8$ in each of these groups, our effect size for SFI was 0.043. Functional and structural outcomes for these groups are in Table 3.1. All comparisons were performed in GraphPad Prism 7.0.4. Power calculations were performed in G*Power 3.1.

3.4 Results

Device Implantation and Lengthening

Lengthening devices were successfully implanted and were actuated over 14 days free of anaesthesia. Rats were active and showed no behavioural signs of pain (e.g., excessive vocalization, grooming deficiencies, excessively sedentary behaviour, or autotomy), even 72 hr postsurgery, when

analgesics were discontinued. At 14 days, lengthened nerves could be repaired end-to-end tension free, suggesting that nerves were lengthened. To assess the region in which nerves were lengthened, we measured the spacing between two sutures proximal to the proximal nerve clamp, before and after actuation. There was a significant increase in suture spacing in the untensioned nerve following actuation, suggesting some growth in this region. However, the difference in means (1.25 ± 1.49 mm) was far less than the total change in lengthening (10 mm), suggesting that, as expected based on clamp location, most of the lengthening occurred more proximally, where we were unable to measure changes in length.

Functional Recovery

To test the hypotheses that repair of rat sciatic nerve gaps with nerve LETER results in improved functional recovery compared with autograft-repaired groups, we compared several functional outcomes. SFI improved over time in all groups, and no difference was found between groups throughout the recovery (Figure 3.1c). In contrast, rats showed phenotypic differences between groups, with rats displaying increased incidence of paw contracture (clawing) during gait at 12 weeks in the autograft group when compared with the lengthening groups (Figure 3.1d,e). Quantitatively, contracture was indicated by significant differences in average paw length between groups (Figure 3.1f).

Structural Regeneration

In order to probe a structural basis for differences in functional outcomes, we examined axonal growth and morphology of the nerves. Regrowth was quantified based on the number and density of the axons, as well as their size and total cross-sectional coverage. Regrowth patterns differed between groups as well as proximally and distally within the nerve (Fig. 3.2) as indicated by SMI31 labeling (red). Distal to the injury, axon count (Fig. 3.3a) coverage (3.3b), size (3.3c), showed significant increases in the LETER group, and axon count trended towards higher density (3.3d). No significant differences were found between these groups proximally and in the contralateral nerves (Table 3.1). Potential changes in

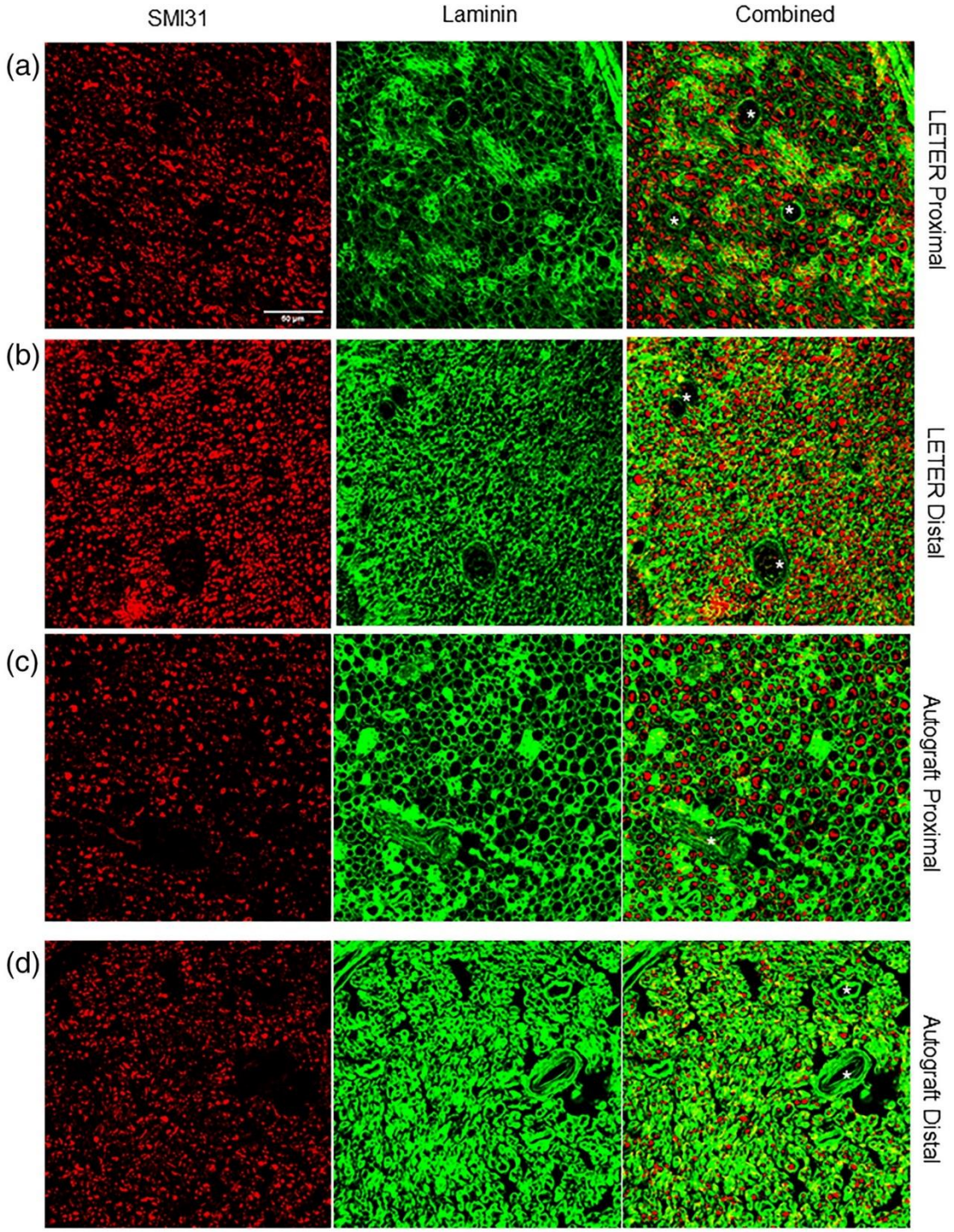


Figure 3.2: Representative image of nerve cross section showing axons (red) and laminin (green) at 63x for each group, both proximal and distal to the injury (A-D). Axon growth distally appears to be more clustered in the LETER tissue and more connective tissue appears in the graft group. Representative image taken at 10x of entire nerve section distal to LETER repair (E), in autograft (F), and section distal to autograft (G). (H) Representative image of CGRP (red) and IB4 (green) at 63x in the distal nerve of the LETER group (left) and autograft group (right). A portion of the image is enlarged to show detail. Asterisks in all images at 63x show blood vessel which are present throughout the nerve.

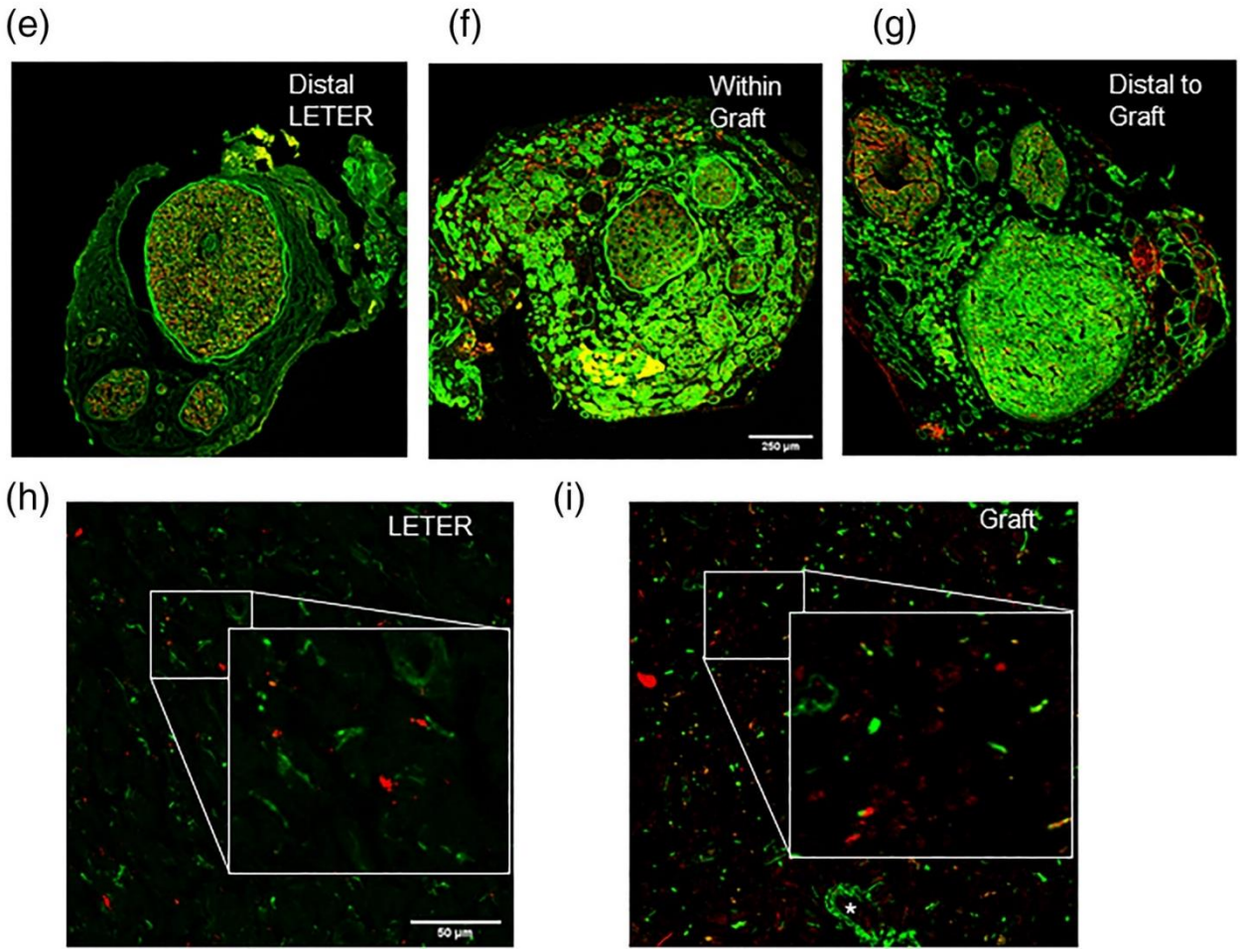
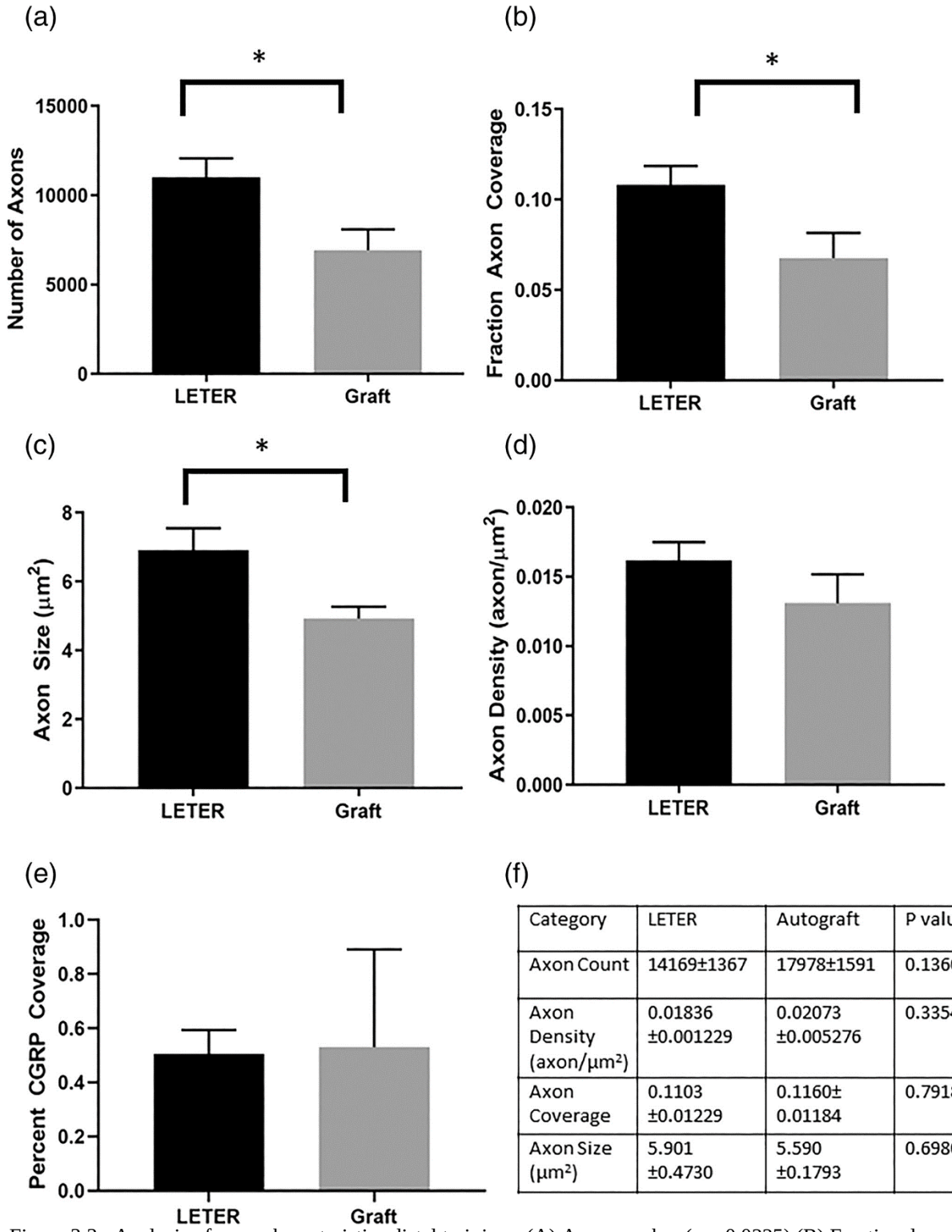


Figure 3.2 con't: Representative image of nerve cross section.

sensory axon distribution were examined by staining for CGRP and IB4. CGRP and IB4 were observed to be present in nerve from both groups. No significant difference between groups was found (Fig. 3.3e). Typical images are also shown (Fig. 3.2h and i).

To more closely examine the differences in axon density and clustering between groups, we used Delaunay triangulation to visualize the distribution of axonal proximity to neighboring axons. This approach was selected to identify heterogeneity in axonal regrowth that would not be discernible by only examining axon density, due to the possibility of spatial clustering. We determined that the mean and median distance from axons were significantly greater in the graft group than in the lengthening group (Fig. 3.4a). The mean is always larger than the median, indicating that the data is skewed right. These



(f)

Category	LETER	Autograft	P value
Axon Count	14169±1367	17978±1591	0.1360
Axon Density (axon/μm²)	0.01836 ±0.001229	0.02073 ±0.005276	0.3354
Axon Coverage	0.1103 ±0.01229	0.1160± 0.01184	0.7918
Axon Size (μm²)	5.901 ±0.4730	5.590 ±0.1793	0.6980

Figure 3.3: Analysis of axon characteristics distal to injury. (A) Axon number (p = 0.0225) (B) Fractional axon coverage (p = 0.0295) (C) Axon size (p = 0.0384) (D) Axon density (p = 0.2019) (n=22). (E) Percent CGRP coverage in the nerve (p=0.14). Comparisons between groups in the proximal end of the nerve is given in Table 1. Error bars represent the standard error. (F) A table showing the results from the same analysis but for tissue proximal to the injury. No significant differences were found between groups.

Table 3.1: Comparison between 10mm and 3mm group, showing p-values from the t-test. Displayed as mean +/- standard error.

Category	3 mm	10 mm	P value
SFI	-52.45±4.530	-53.08±5.334	0.9300
Axon Count	9815±829	13167±2414	0.1321
Axon Density (axon/μm^2)	0.01519±0.001627	0.01796±0.002222	0.3316
Axon Coverage	0.1127±0.01054	0.09999±0.02354	0.5786
Axon Size (μm^2)	7.763±0.8401	5.368±0.5161	0.0704

results indicate that axons grew more tightly packed in the lengthening group (Fig. 3.4b) and the distribution of distances between axons approached that seen in contralateral and proximal nerves (Fig. 3.4c). An example Delaunay triangulation diagram generated by ImageJ is included as an inset in Figure 3.4b.

Neuromuscular Morphology

We examined muscle to determine if an axonal path to the muscle and NMJ were present after sciatic nerve injury. NMJs were observed in both healthy and affected muscles. Examples of NMJs observed in injured and contralateral muscle are shown in Figure 3.5a-c. Also shown are the NMJs observed distributed throughout the atrophied muscle (Fig. 3.5d-e) in the proximal 1/3 of the TA muscle. Endplate distribution appeared qualitatively different; however, no significant difference between the number of NMJs nor the dispersion index (DI) between groups was found (Fig. 3.5f-g). There was also no significant difference in muscle mass between the TA, EDL, or gastrocnemius muscles when compared as fractions of the contralateral limb muscles (Fig. 3.5h).

3.5 Discussion

In this study, we combined and built upon two concepts suggested by prior research, that tension can enhance axonal growth and that end-to-end repairs are favorable to graft-based repairs. We implemented a new device to test our hypotheses that lengthening proximal stumps of severed nerves is feasible, and when followed by end-to-end repair (LETER), may be superior to grafting. Our results showed comparable or better functional recovery and enhanced growth outcomes, including greater axon coverage, increased density, and more homogeneous regrowth of axons in all nerve fascicles in the LETER group compared to autografts. We observed NMJs in muscles (tibialis anterior and gastrocnemius) of both groups at the time of harvest, indicating successful reinnervation of motor endplates. Together, our results show that LETER offers a potentially superior alternative to graft-based repair of long gaps.

Efficacy of nerve lengthening/end-to-end repair

Our hypotheses are predicated upon two principles of nerve plasticity and repair: the efficacy of end-to-end repair and a positive role for tension in nerve regeneration.

End-to-end repairs: If an end-to-end repair fails, the results are catastrophic. Because functional outcomes cannot be evaluated until weeks to months after the repair, failure may often not be detected until a very late time point. Therefore, clinical practice has tended to steer more conservatively, avoiding tension as much as possible during nerve repairs (Millesi, Berger, & Meissl, 1972). However, if failure does not occur, outcomes are better for tensioned end-to-end repairs than grafts, even at moderate strains (Hentz et al., 1993; Sunderland et al., 2004). These findings are consistent with clinical evidence of the superiority of end-to-end repairs over grafts (Meek et al., 2005; Terzis and Williams, 1975), especially in clinical situations that require very long grafts (Koller et al., 1997). Thus, there is a significant benefit to developing strategies that facilitate safe end-to-end repair of severed nerves. In a previous study, we demonstrated the efficacy of redistributing tension away from the repair site, to perform end-to-end repairs on moderate gaps (Howarth et al., 2019). In this study, we proposed a strategy for

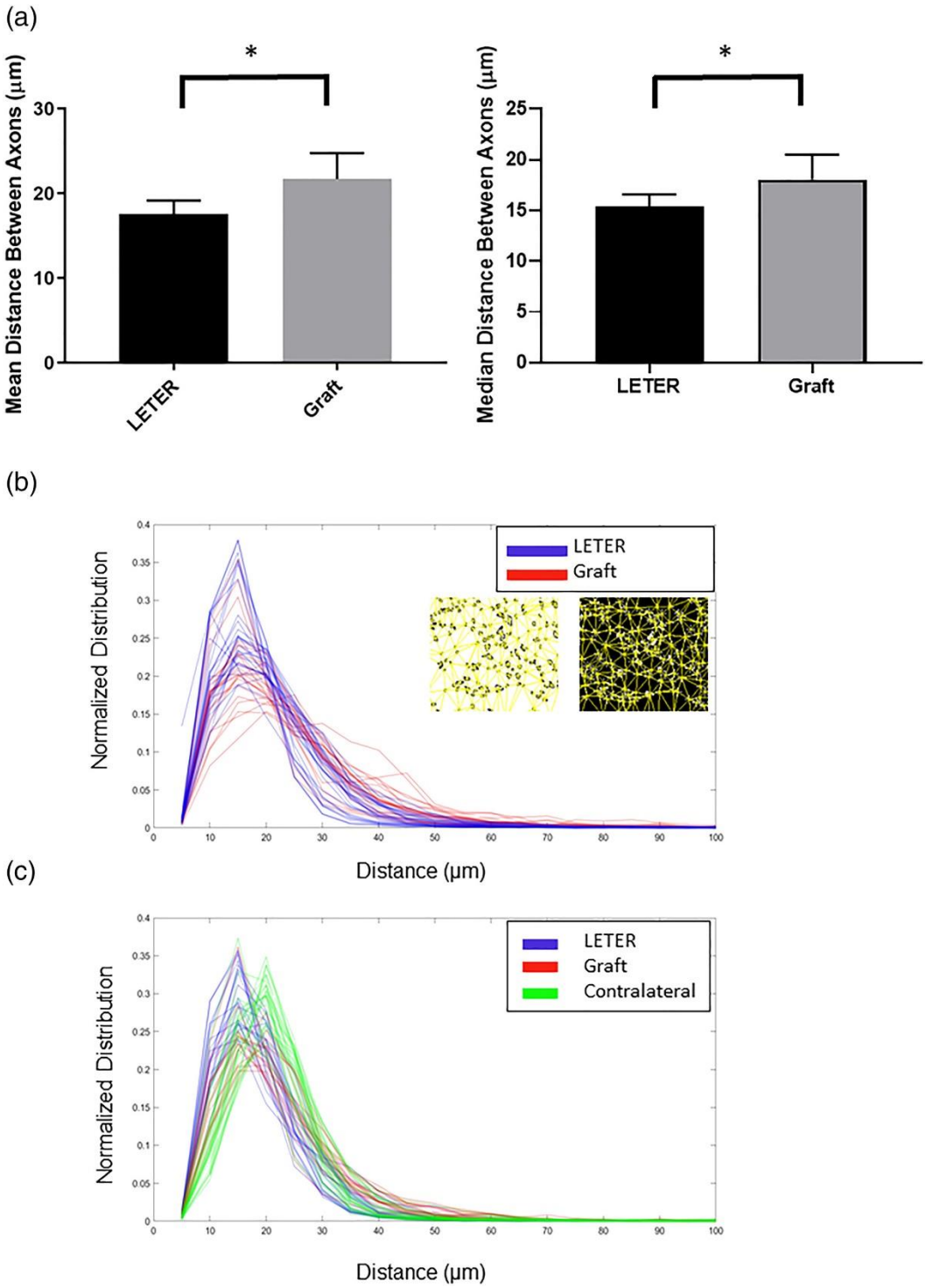


Figure 3.4: (A) Mean (p = 0.0116) and median (p = 0.0289) distances between axons from Delaunay analysis. (B) Overlaid histograms from Delaunay analysis showing distances distal to the injuries in the LETER group (blue) and graft group (red). Inset: example of Delaunay triangulation performed in ImageJ. The black outlines are axons as processed by ImageJ, and the yellow lines between axons are the distances generated by the triangulation (left). The image on the right shows the axons in white and the background in black to better visualize the lines. (C) Histograms showing distances proximal to the injuries in the LETER group (blue) and graft group (red), as well as in the contralateral nerve (green). Error bars represent the standard error.

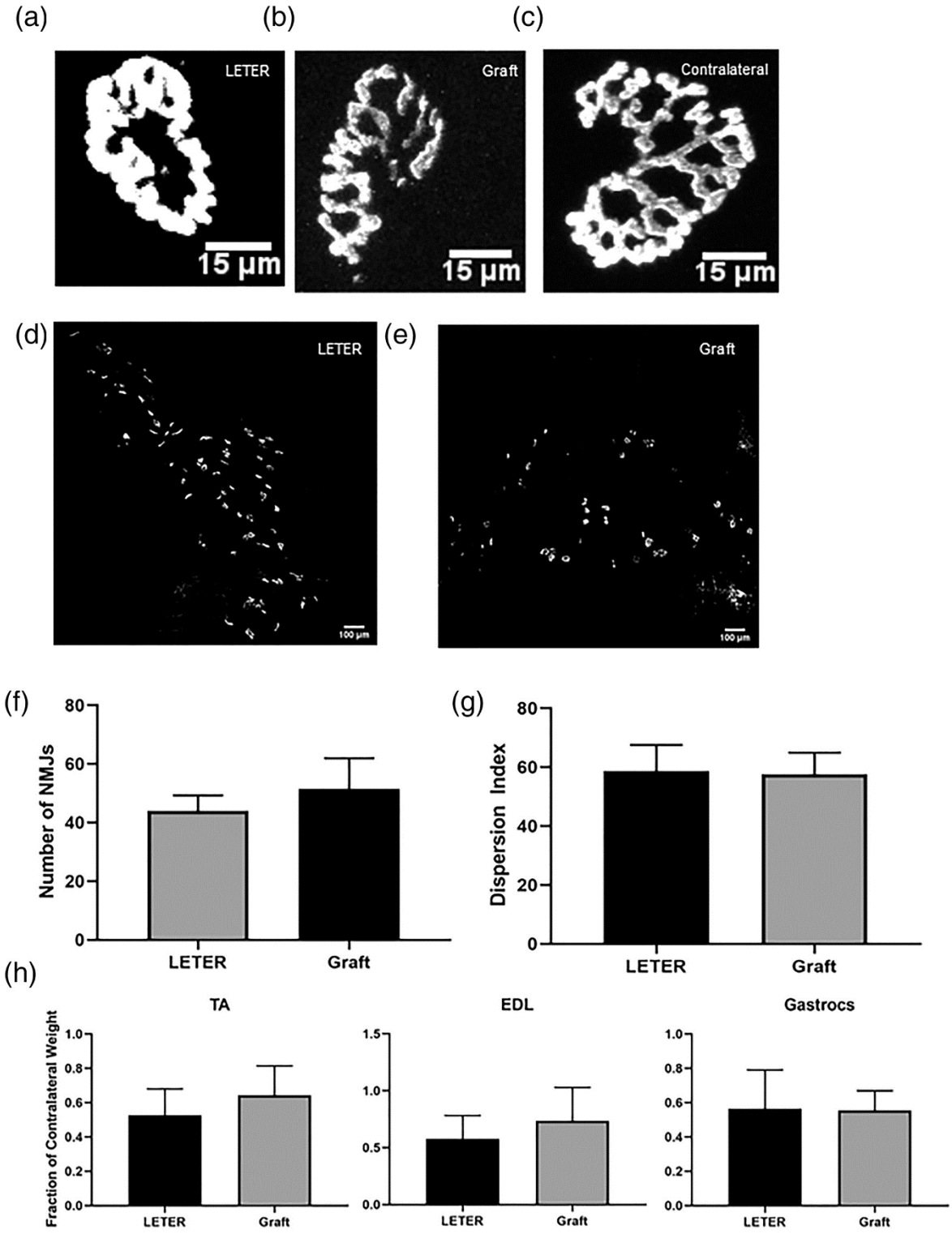


Figure 3.5: NMJs in the affected and contralateral tibialis anterior (TA) muscle: (A) LETER, (B) graft, (C) contralateral. NMJs in muscle, imaged at 10x LETER TA muscle (D), TA graft muscle (E). Location is approximately 1/3 down from where it was attached to the knee. (F) Number of NMJs in a 100 µm thick TA muscle sample ($p = 0.3140$) (n=7). (G) Dispersion index of NMJs in each group ($p = 0.087$). Average fraction muscle weights when compared to contralateral for TA ($p = 0.0918$), EDL ($p = 0.1057$), and gastrocnemius ($p = 0.8981$) muscles (H) (n=22).

tension-free end-to-end repair of longer gaps, following gradual lengthening of the proximal nerve stump via tension.

Our results demonstrate improved functional recovery with nerve LETER compared to autografts. Most prominently, we see a significant reduction in paw contractures. As plantarflexing/toe flexing nerve-muscle units represents the dominant contributors to contracture, our data indicate sufficient reinnervation of dorsiflexing/toe extending muscles (most notably the TA and EDL) via the peroneal nerve, in lengthened nerves repaired end-to-end. This functional outcome is consistent with improved structural regeneration of axons distal to the repair site. In addition to overall increases in axon number, density, and size, axons are also more evenly and less variably distributed across the entire nerve cross-section, including branches of both the tibial and peroneal nerve. Confidence in our assessment of our findings is bolstered by a new quantitative outcome measurement. We applied Delaunay triangulation analysis, which provides a robust and comprehensive assessment of axonal proximity to describe axonal organization; LETER groups demonstrated more dense and homogenous regrowth compared to autografts. This enhancement and homogeneity in axon growth in the distal nerve after lengthening may be a benefit for an end-to-end repair. Because axons were not required to grow through connective tissue that may emerge within the graft, and also avoided an additional interface (i.e., entry into and exit from the graft, as opposed to entry into the distal stump following end-to-end repair), we posit that proximal axons are more likely to reach the distal end and less likely to change direction during growth. Specific magnitudes of structural and functional outcomes in this study are similar to those in nerves repaired end-to-end, both tension-free and under tension (Howarth et al., 2019), indicating the consistency and suitable quality of end to end repair.

Tension in nerve regeneration. Our outcomes are also consistent with a positive role for strain in nerve plasticity. Models of lengthening have been well-described in uninjured nerves, most extensively during limb lengthening, when nerves must stretch and grow in order to accommodate the increasing limb length. Studies examining the effects of this lengthening process on the nerves show some temporary

decrease in function, but almost always a complete recovery over time (Galardi et al., 1990; Ippolito et al., 1994). Limb lengthening also represents an important translational model for facilitating tissue remodeling in clinical settings, as patients are capable of successfully accommodating a variety of invasive traction devices (Simpson et al., 2013). In addition to limb lengthening, several other studies indicate a positive role for tension in nerve remodeling. Axons, the most delicate cellular component within nerves, grow more quickly when stretched (Bray, 1984; Pfister et al., 2004), and both animal and human studies demonstrate the capacity of nerves to respond actively and favorably to an altered tensile biomechanical environment (Foran et al., 2016; McDonald and Bell, 2010; Howarth et al., 2019). These observational studies are supported by several more mechanistic studies by our group and others, which provide a biological basis for nerve response to lengthening. Love et al. showed that nerves respond to strain by eliciting activation of mTOR pathways, which underlie upregulated translation of structural and myelin-associated proteins to facilitate growth (Love et al., 2017). Such growth has also been noted in limb-lengthening models, which show increased internode spacing in lengthened nerves (Simpson et al., 2013). Finally, a recent study shows the importance of mechanically-gated ion channels on nerve regeneration, as the knockout of the Piezo ion channel markedly curtails nerve regeneration (Song et al., 2019). Future studies will examine the response of such players in tension-based regenerative strategies.

Technologies for nerve lengthening

A few previous studies have used lengthening as a strategy to repair the peripheral nerve, with some success. Ochiai and colleagues used an extracorporeal device, anchored into the bone, which lengthened just the proximal stump in one iteration, and both the proximal and distal stumps of the nerve in another iteration (Saijilafu et al., 2006; Saijilafu et al., 2008; Sharula, Nishiura, Saijilafu, & Ochiai, 2010). Their studies also showed increased axon counts distally following the repair, compared with autograft. Another group (Yousef et al., 2015) designed a device that used a screw-driven spring to control nerve lengthening. Their results also showed positive functional outcomes for the lengthened nerves. We used SFI as opposed to sciatic stationary index but with similar toe-spreading following

recovery. Their calculated axon density was similar in magnitude to ours; though no significant differences were found, this parameter appeared to trend in the opposite direction (i.e., decreased density) compared with our study.

Our nerve-lengthening technology has undergone several rounds of improvement, and the current technology compares favourably to earlier efforts. Previously, our group showed proof of concept of a related design, including outgrowth of axons into an engineered conduit following nerve lengthening (Chuang, Wilson, Love, Fisher, & Shah, 2013); however, neuroma formation at the distal conduit–nerve interface precluded optimal regeneration. More recently, we demonstrated the successful implementation of the current device to enable the repair of moderate-length gaps end to end under tension (Howarth et al., 2019). Unlike the current study, these repairs represented a one-time lengthening at the time of end-to-end repair. This work confirmed the feasibility of a new nerve-cuff design and positive nerve response to tension. In the current study, we demonstrated the ability to continually lengthen nerves over a 2-week period, through the use of a compact, internal fixator device. Key features of the modular design include the comfortable localization of the device within the nerve bed, successful and reliable clamping and lengthening of nerves without tearing or compression-induced damage to more proximal regions, a simple manually actuated guidewire that may be readily converted to an internalized actuation strategy, animal-specific design features to prevent guidewire migration intracorporeally, and prevention of rats chewing on the device guidewire by means of a locking collar screw on the guidewire housing. Implicit in device usage, mirroring surgical freshening of nerves prior to repair, is the ability to more aggressively trim back poor quality nerve at both stumps prior to anastomosis, both at the time of device implantation and at the time of device excision and end-to-end repair. In comparison with previous strategies for nerve lengthening, advantages of our device include comparatively low impact on surrounding tissue, minimal extracorporeal components (with an intuitive path to full internalization), and low distress of animals. The surgery required to implant the device is minimally invasive, as the device rests in the sciatic nerve bed and is anchored by two intramuscular barbs. Device anchorage enables lengthening against a fixed frame

of reference, despite the possibility of some loads during animal movement. Such loading did not adversely impact lengthening in any animal and, as noted above, may even be helpful (Foran et al., 2016; Howarth et al., 2019; McDonald & Bell, 2010).

Limitations

Among the possible functional outcomes that could be tested (Wood, Kemp, Weber, Borschel, & Gordon, 2011), our primary focus was on those associated with gait and contracture, as these reflect the composite structural and functional regeneration of antagonistic nerve–muscle units. SFI is a less reliable functional metric and may be confounded by stepping differences with toe curling, but our results reflect consistent measurements between two researchers and consistence with previous findings (Ganguly et al., 2017; Jonsson et al., 2013; Mackinnon, Dellon, & Obrien, 1991). Further, by inspecting specific elements of the SFI calculation (e.g., paw length), we were able to extract meaningful conclusions regarding the reinnervation of antagonistic muscle groups.

We also acknowledge caveats associated with our sampling approach to calculate morphological outcomes (Cai, Cash, Thompson, & Blumbergs, 2002; Kaplan, Geuna, Ronchi, Ulkay, & von Bartheld, 2010; Saxod, Torch, Vila, Laurent, & Stoebner, 1985). We sampled approximately 50% of each nerve section (cf. Tang & Ebbesson, 1972), which enabled us to confidently capture outcomes for nerves with minor artefactual defects in small regions of a given section that may have confounded whole-section sampling. This approach was suitable to detect statistically significant differences between groups, despite any inaccuracy associated with stereological sampling. Our use of a contralateral control was also a limitation, as natural variation occurs not only between rats but also between both nerves with an individual (Christensen & Tresco, 2015; Muglia, Vita, Laura, Mammola, & Germana, 1997).

Another limitation was the maximum nerve gap achievable in a rat model. Although future studies in larger animals (e.g., rabbits or primates, Sharula et al., 2010) will be required to demonstrate efficacy of nerve lengthening for larger gaps, this study demonstrates the viability of this device and

potential efficacy as a strategy for nerve regeneration. The use of manual actuation is an additional limitation of our work, as it limits control of strain rate and accuracy of lengthening. We opted to actuate daily for a low strain (<10%) at a relatively low strain rate, to avoid potential complications of overlengthening and viscoelastic impacts at higher strain rates. Incorporation of motorized actuation will enable more accurate testing of varying strains and strain rates and represents a future direction that will also be enabled within a larger animal model.

Conclusions and future perspectives

Our data suggest that the combined effect of initial nerve lengthening and subsequent end-to-end repair supports improved nerve regrowth, though additional work will be required to fully decouple what benefits came from each factor, towards further enhancement of functional recovery. Clinically, there are a number of potential applications. A lengthening strategy would be most useful for regeneration of very large gaps, which are too large for a graft or reinforced end-to-end repair to otherwise be feasible or effective. Another application is for use in nerve transfers. The selection of a donor nerve is in part limited by its proximity to a recipient stump, but our research raises the possibility of identifying new proximal donors among lengthened nerves.

En route to translation, there are several intuitive future directions, including i) implementation of nerve lengthening in a larger animal model (e.g., rabbits, pigs, or non-human primates); ii) implementation of nerve lengthening in a chronic injury model, in which larger swaths of tissue at nerve ends may need to be excised and in which clinical decision-making is more time-sensitive (Pfister et al., 2011) ; iii) internalization and motorization of nerve lengthening technology, to enable improved accuracy and comparison of strain-rate; iv) examination of proteomic and genomic pathways related to regeneration and mechanical transduction. These represent important and reasonable future directions for our work.

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Chapter 4: A comparative assessment of lengthening followed by end-to-end repair and isograft repair of chronically injured peripheral nerves

4.1 Abstract

In order to repair chronic nerve injuries (injuries repaired after a long delay), the damaged nerve segments are resected and stumps are bridged by grafts. Autografts remain the gold-standard, but outcomes are typically poor, even after long periods of recovery. In a recent study, we described the use of a nerve lengthening device to gradually elongate the proximal stump of a transected nerve towards the distal stump, enabling a tension-free end-to-end repair. This approach showed significantly improved outcomes in comparison to autografts in repairing acutely injured nerves. In this study, we compared the use of nerve lengthening/end-to-end repair (LETER) to isograft repair of chronically transected nerves in a rat model. Structural and functional regenerative outcomes following LETER were comparable to isograft-based repair, with no significant differences found in outcomes involving functional recovery or axon growth. These data demonstrate the feasibility of nerve lengthening as a viable graft-free strategy for repairing chronically injured nerves. Not unexpectedly, outcomes for chronic nerve injuries were less favorable in both groups compared to repair of acutely injured nerves. Nonetheless, the findings provide insight into barriers to restoring function after chronic nerve injury through novel comprehensive characterization of a diverse set of neuromuscular outcomes. This analysis revealed key parameters predicting functional recovery.

4.2 Introduction

Nerve injuries can severely limit an individual's motor and sensory capabilities. Transection injuries (i.e. neurotmesis), especially resulting in large gaps, can be particularly devastating, as regenerating axons lose guidance to their targets (Pfister et al., 2011). The size of the gap between proximal and distal stumps after nerve transection is a major consideration for surgical options (Grinsell and Keating, 2014). When possible, the preferred and most efficacious method of repairing a transected

nerve is an end-to-end repair, providing similar stump geometry at the interface and obviating the need for a graft (Bhatia et al., 2017). In cases where the gap is too large, repair stumps must be bridged by a conduit or graft to reduce tension at the site. Conduits and allografts have been used with success for smaller gaps (Siemionow and Sonmez, 2007; Meek and Coert, 2002), but autologous grafts remain the gold standard. However, autografts are limited in viable length, and must be harvested from another site, leading to donor site morbidity (Meek et al., 2005; Koller et al., 1997). As a consequence, in addition to providing functional benefits of an end-to-end repair, strategies that bypass the need for a graft are of high significance.

The timing of repair following injury strongly influences outcomes. For the best possible outcomes, nerves ideally should be repaired as quickly as possible (Jivan et al., 2009). However, weeks or even months may pass before surgical repair can be initiated. This can be for a variety of reasons, including severity of damage to the surrounding tissue, misdiagnosis, or revision a failed repair (Secer et al., 2007). In these cases of the likelihood of functional recovery is low, as shown by several studies that have examined outcomes at time points when repair occurs more than 2-3 months following initial injury (Jivan et al., 2009; Jonsson et al, 2013; Gordon et al., 2015). Several factors have been proposed to underlie the poor functional outcomes following a delayed nerve repair: 1) From a surgical standpoint, chronic injury often requires resection of large swaths of poor quality tissue from the remaining nerve stumps (i.e., refreshing) prior to repair, thus precluding end-to-end or conduit-based repair (Dahlin, 2013). 2) Biologically, axon growth slows over time, especially in motor axons (Gordon et al., 2015). 3) The degenerating distal stump offers an increasingly unfavorable environment over time, due to changes in both trophic support from Schwann cells as well as structural changes in the basal lamina and extracellular matrix (Gaudet et al., 2011; Belin et al., 2015; Gordon and Tetzlaff, 2015). 4) Denervation results in severe muscle atrophy and endplate remodeling or even synapse withdrawal, providing additional barriers to functional reinnervation (Scheib and Höke, 2013).

To address the challenges associated with nerve grafting, we developed a device-based regenerative strategy to repair large nerve gaps, in which degenerated stump margins are excised and the proximal stump is gradually lengthened towards the distal stump before performing a graft-free end-to-end repair. Our approach is motivated by prior research showing the benefits of tension on axon growth, nerve protein synthesis pathways (Bray, 1984; Pfister et al., 2004; Love et al., 2017), and on neuro-regenerative outcomes (Sunderland et al., 2004; Sharula et al., 2004). Most recently, our device yielded superior structural and functional outcomes compared to autografts in an acute nerve injury model (Howarth et al., 2019). In this study, we extended this work to test the hypothesis that nerve lengthening followed by end-to-end repair will result in favorable functional and regenerative outcomes compared to isograft repair of chronically injured nerves. As a secondary analysis, we additionally examined the relative efficacy of repair strategies for chronic versus acute nerve injury, and identified key potential outcomes predicting functional recovery.recovery.

4.3 Methods

Surgery and Functional Testing

All procedures were approved by the VA San Diego institutional animal use committee (IACUC). Nerve transection surgery was performed as published, on 6-7 week-old Lewis inbred rats, weighing 150-200 grams. Briefly, rats were placed on a heating pad with continuous isoflurane (1.5-2.5%) inhalation anesthesia for the duration of the surgery. To expose the sciatic nerve, an incision was made in the posterior thigh of the hindlimb and the heads of the biceps femoris were split. The sciatic nerve was transected with a blade, to create a 3 mm gap. This gap size enabled excision of 3-4 mm of proximal and distal stumps (sufficient to expose better quality nerve), to create a final gap size of 10 mm at the time of repair. Spontaneous outgrowth of the proximal stump was blocked with a PDMS (Polydimethylsiloxane) cap, secured to the end of the nerve with 4-0 Nurolon suture. Following the procedure, the muscle was sutured using 4-0 PGA suture (Oasis, Ottawa, IL) and skin was stapled using AutoClips (MikRon Precision, Gardena, CA). Rats were allowed to recover on a heating pad. Twelve weeks following the

injury, a second procedure was performed where the PDMS cap was removed and adherent tissue was cleared from the nerve stumps.

Rats were then separated into two groups. In the isograft group (Graft), a 10 mm isograft from the sciatic nerve of another Lewis inbred rat was sutured to the proximal and distal stumps using 2 epineurial stitches (8-0 Ethilon, Ethicon, Guaynabo, Puerto Rico), allowing for a tension free repair. A syngeneic isograft is considered equivalent to an autograft (Hudson et al., 2004), and precludes any detrimental impacts of using a contralateral autograft (i.e., creating a bilateral injury). In the second group, nerve stumps were lengthened followed by end-to-end repair (LETER). A nerve lengthening device was secured to the proximal stump as described previously by means of secure nerve cuffs (Howarth et al., 2019). Surgical approach and closing of the wound were performed as in the first surgery. For the LETER group, the nerve was lengthened daily by 1 mm for 12 days by means of an external guidewire. Finally, a third surgery was performed on the LETER group to remove the device and repair the nerve using an end-to-end repair, after nerve refreshing. A summary of the surgical steps and timing for both experimental groups is given in Figure 4.1.

For both Graft and LETER groups, rats were run on a treadmill (DigiGait, Framingham, MA) at 10 cm/s at four different timepoints. Once before the second surgery (i.e., just before repair of the chronic nerve injury), and four, eight, and twelve weeks thereafter. Image stills of each foot during normal gait were taken from videos in order to calculate a modified version of the sciatic functional index (SFI) using the equation of Bain et al. 1989.

At the terminal 12 week time point, rats from both groups were anesthetized for muscle functional testing. Briefly, the foot was strapped to a footplate attached to a dual-mode force transducer (Model 6650, Cambridge Technologies, Cambridge, MA, USA). The fibula nerve was exposed and placed in a nerve cuff (subminiature electrode, Harvard Apparatus, Holliston, MA, USA) and stimulated (Model S48, Grass Instruments, Quincy, MA, USA) as described previously (Peters et al., 2003; Minamoto et al., 2007). The fibula nerve was stimulated with a single pulse, and the corresponding

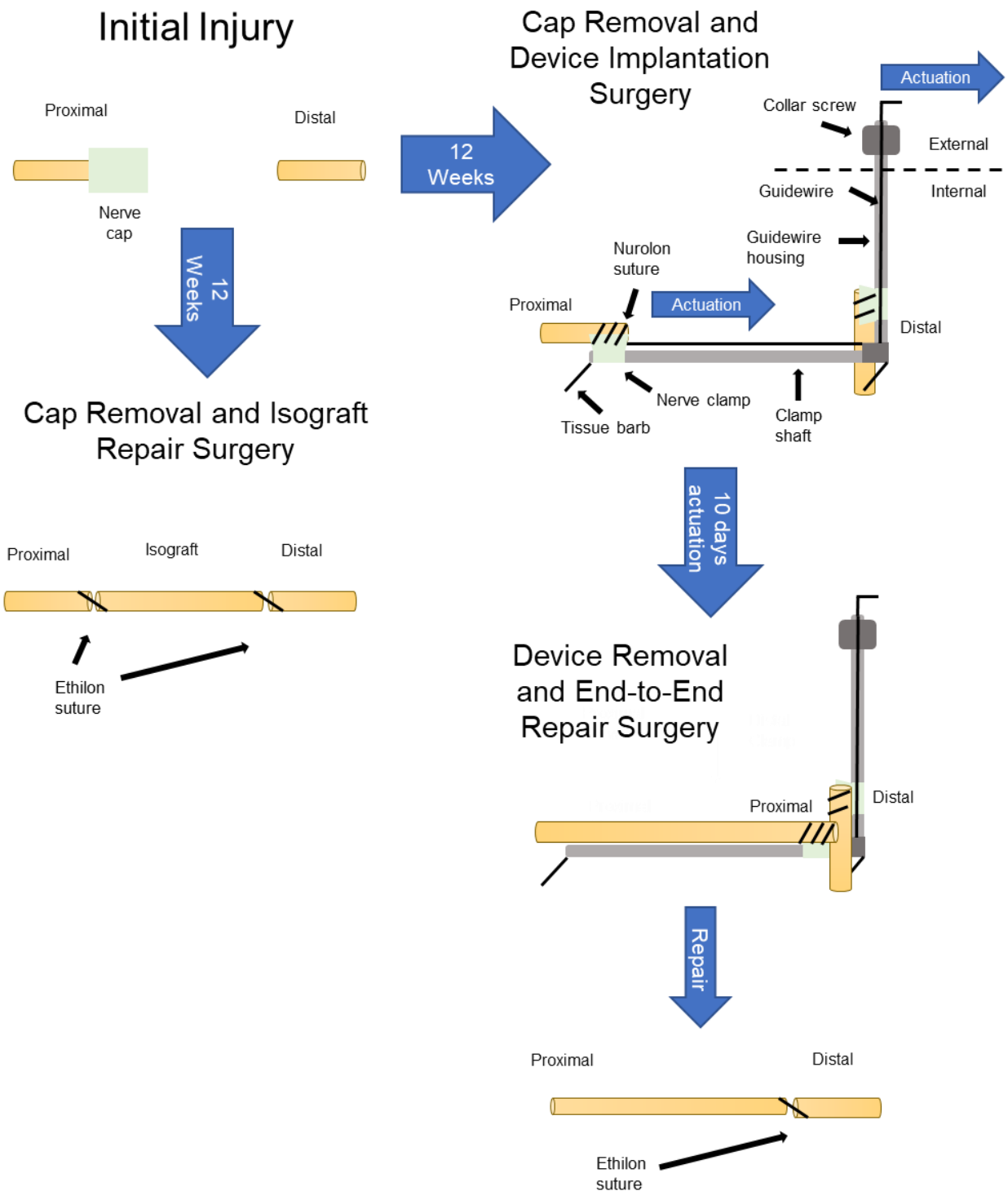

Figure 4.1: Diagram of the experimental flow after nerve transection for isograft rats (left) and experimental flow for LETER rats as well as a diagram of the lengthening device implanted into rats with important components labeled (right).

dorsiflexion maximal twitch force was measured. Following testing, rats were promptly euthanized and the sciatic nerve, L5 dorsal root ganglion (DRG), and target muscles innervated by the sciatic nerve (tibialis anterior (TA), extensor digitorum longus (EDL), and gastrocnemius muscles) were harvested bilaterally. Muscles were fixed in 10% formalin for 24 hours and then washed in 15% and 30% sucrose overnight. Tissue was then frozen in liquid nitrogen cooled isopentane and stored at -80^{o}C until processing.

Tissue Processing

Tissue processing was performed as described previously (Howarth et al., 2019). Briefly, nerve was sectioned proximal and distal to the injury/isograft. Nerve was stained with primary antibodies SMI31 (1:500, anti-mouse NF-H, Sigma-Aldrich NE1022) and anti-rabbit laminin (1:500, Sigma-Aldrich L9393) and secondary antibodies fluorescent AF488 goat anti-rabbit and AF594 goat anti-mouse. Tibialis anterior muscle was sectioned longitudinally into 100 μm sections at the center of the muscle and the motor endplate was stained with alpha-bungarotoxin conjugated to AF488. Slides were imaged on a Leica SP5 confocal microscope.

Tissue Analysis

Axons were counted and analyzed as described previously (Howarth et al., 2019). Briefly, we used the Analyze Particles function in ImageJ to find the count and average size of axons, based on binary thresholding of axonal immunolabeling. These outcomes were then used to calculate axon density and fractional coverage of the nerve cross-sectional area. The Delaunay analysis was done in ImageJ, using the Delaunay-Voronoi plug-in to calculate distance between particles.

Neuromuscular junction (NMJ) morphology was analyzed as described previously (Pratt et al., 2017) by calculating the Dispersion Index (DI), number of discontinuities, and amount of branching. The DI is defined as the total stained area/the total area X100. The number of discontinuities is defined as a point where a line that ends at a single point, disconnected from the rest of the NMJ. Branching is

defined as any point that is connected to more than two other points, meaning a place where lines connect/intersect each other.

Statistics

Statistical analyses were performed in GraphPad Prism 8. Student's t-tests were used to compare two groups. Main effects of treatment and time were evaluated using two-way ANOVA, with Tukey's post hoc comparisons of individual means. Correlation matrices were generated using Python 3.7 and using two different methods: Pearson's correlation coefficient and the Kendall Tau correlation coefficient. Multiple regression analysis and plots were done in MATLAB R2019b.

4.4 Results

Rat Well-Being and Functional Testing

No rats showed signs of pain after withdrawal of analgesics (three days post-surgery). LETER rats adapted well to the device and no rats in either group had to be euthanized for complications related to the experiments.

To examine the recovery of sciatic function with time, we tested the Sciatic Functional Index (SFI) throughout the twelve-week recovery period. Two-way ANOVA and post-hoc tests revealed no significant differences between groups, but consistent with slight improvements in function, did show a significant effect of time ($p<0.0055$, Figure 4.2A). As some reports suggest that SFI may not be a sensitive metric for functional recovery in chronic nerve injury models (Dellon and Mackinnon, 1989), we also examined muscle contractile function by testing dorsiflexion strength (maximal twitch force) immediately before euthanasia. No significant differences were found between groups ($p=0.55$), and though muscle response to stimulation was evident, the resultant force was much lower than that measured contralaterally (Figure 4.2B). Example twitch traces from data collection are also shown (Figure 4.2C).

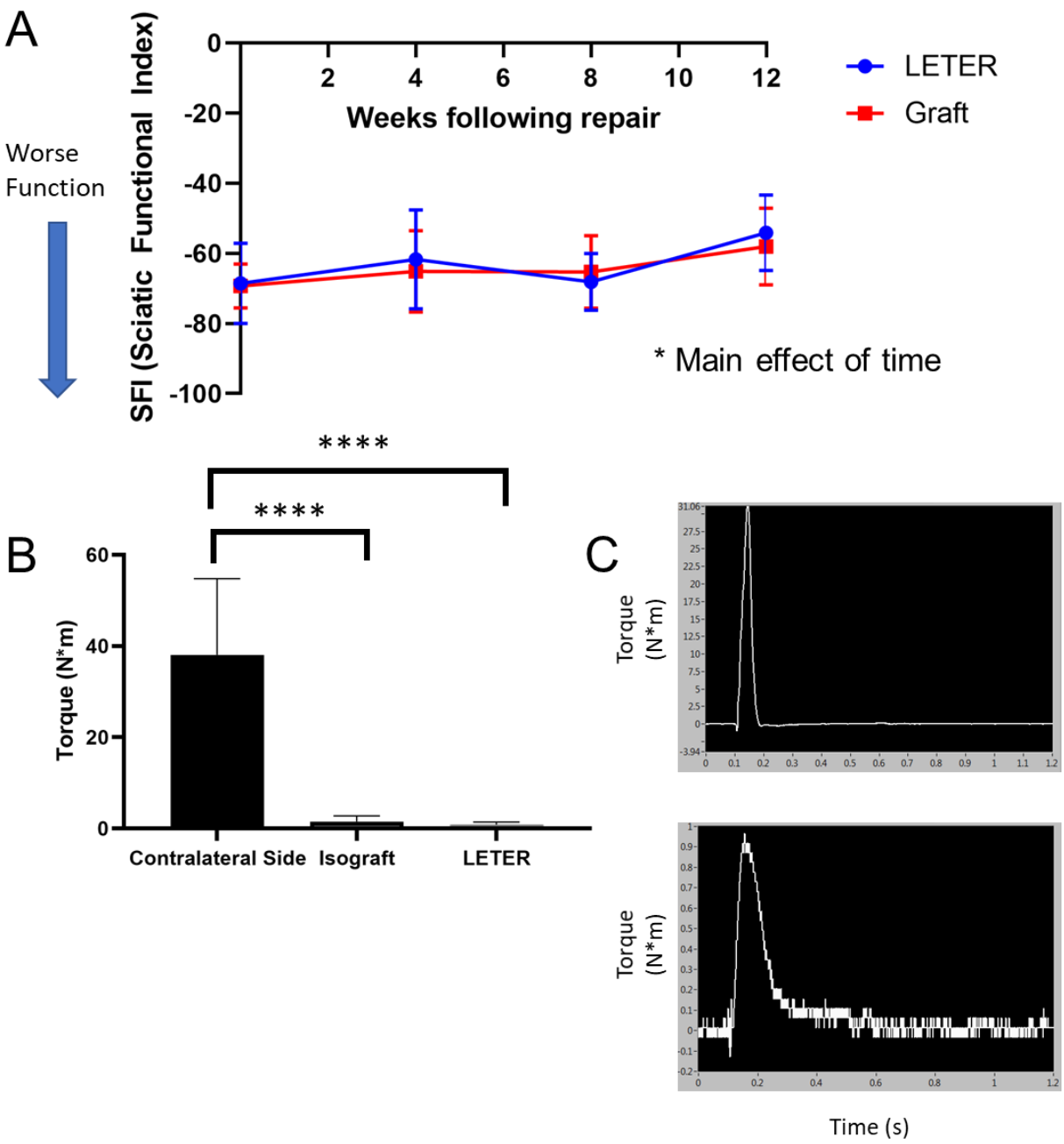

Figure 4.2: Measurement of the Sciatic Functional Index (SFI) throughout the recovery period of the rats (A). No significant difference was found between groups using multiple comparisons tests. However, a significant main effect of time using two-way ANOVA (p=0.0055). Maximum measurements of dorsiflexion strength of affected side of each group compared to the contralateral side twelve weeks after the initial treatment of the nerve (B). Bars represent the standard deviation. Examples of the traces from twitch measurements taken from the affected LETER side (top) and contralateral side (bottom) (C).

Axon Regeneration

To evaluate axonal regrowth, axons were counted and analyzed from portions of the nerve proximal and distal the injury. Representative images of nerve sections from each group are shown in Figure 4.3 A-G). Axon growth distal to the injury was evident in all nerves, though severe atrophy of the distal nerve was also observed. The presence of increased extracellular matrix and basal lamina deposition (deep purple in color) in the distal nerve was evident from the trichrome and laminin stains. No significant differences were found distal to the injury between groups in any of the following categories: number, density, fraction axon coverage, axon size, or nerve area (Figure 4.4 A-E).

Because there appeared to be variation in the distance between axons (some areas appeared sparse and others densely packed with axons), we assessed the distribution of axons. The distribution of axons in the LETER group was more similar to the contralateral (control) axons than those in the Graft group (Figure 4.4H). However, Delaunay-Voronoi analysis did not indicate a statistically significant difference between the average mean or average median spacing of each group (Figure 4.4F,G).

Motor Axons/NMJs

To characterize chronically denervated muscle after repair, we examined muscles of the distal hindlimb. All muscles were severely atrophied at 12 weeks and weighed only a fraction (on average 39% +/-22%) of the muscle from the unaffected contralateral side. To more accurately compare groups, we used the fraction weight of the affected muscle compared to the contralateral muscle. No significant differences were found between groups for any muscle (Figure 4.5A-C, TA, EDL, and gastrocnemius respectively).

In all groups, we observed a number of NMJs with normal, intact morphology (Fig. 4.5, green), and a corresponding motor axon (Fig. 4.5, red) in the same location (Figure 4.5D-F). NMJs were also present throughout the affected muscles (Figure 4.5G-H). However, despite the presence of NMJs with normal morphology, about half of the NMJs also appeared disrupted in both groups (Figure 4.5I-J).

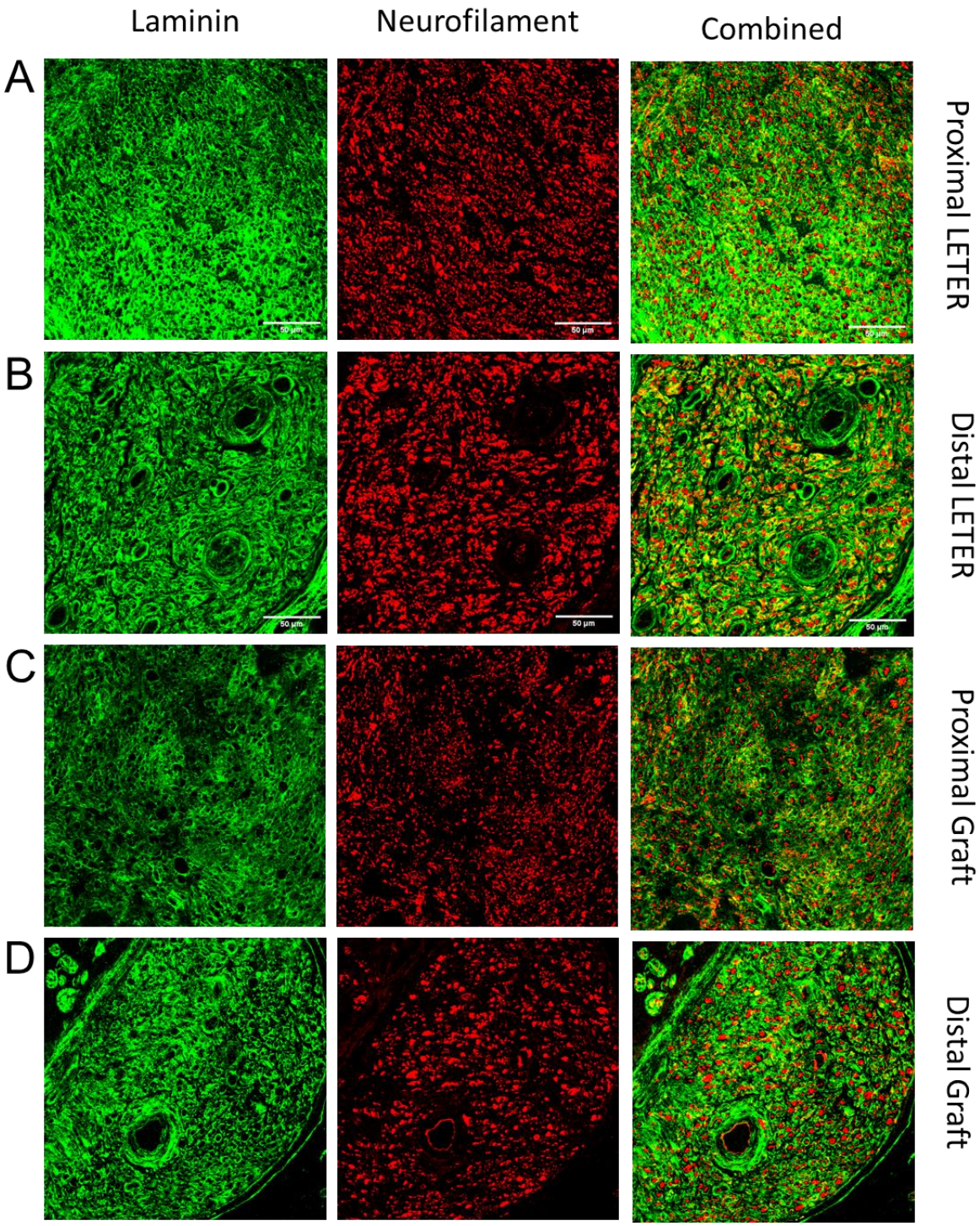

Figure 4.3: Images of the sciatic nerve taken using confocal microscopy at 63X (A-D). SMI31 (axons) are red, laminin is green. (A) Proximal and (B) distal to the injury of the LETER group. (C) Proximal and (D) distal to the injury of the graft group. Also included are images of the sciatic nerve taken at 10X (E-G). Labeling is the same. Distal to the LETER (E) and graft (F) groups, as well as an example of a contralateral (uninjured) nerve (G). Images from trichrome staining indicating differences in the extracellular matrix between Graft and LETER groups and proximally and distally to the injury, taken using a scan at 40X (H-K). (H) Proximal and (I) distal to the injury of the LETER group. (J) Proximal and (K) distal to the injury of the graft group.

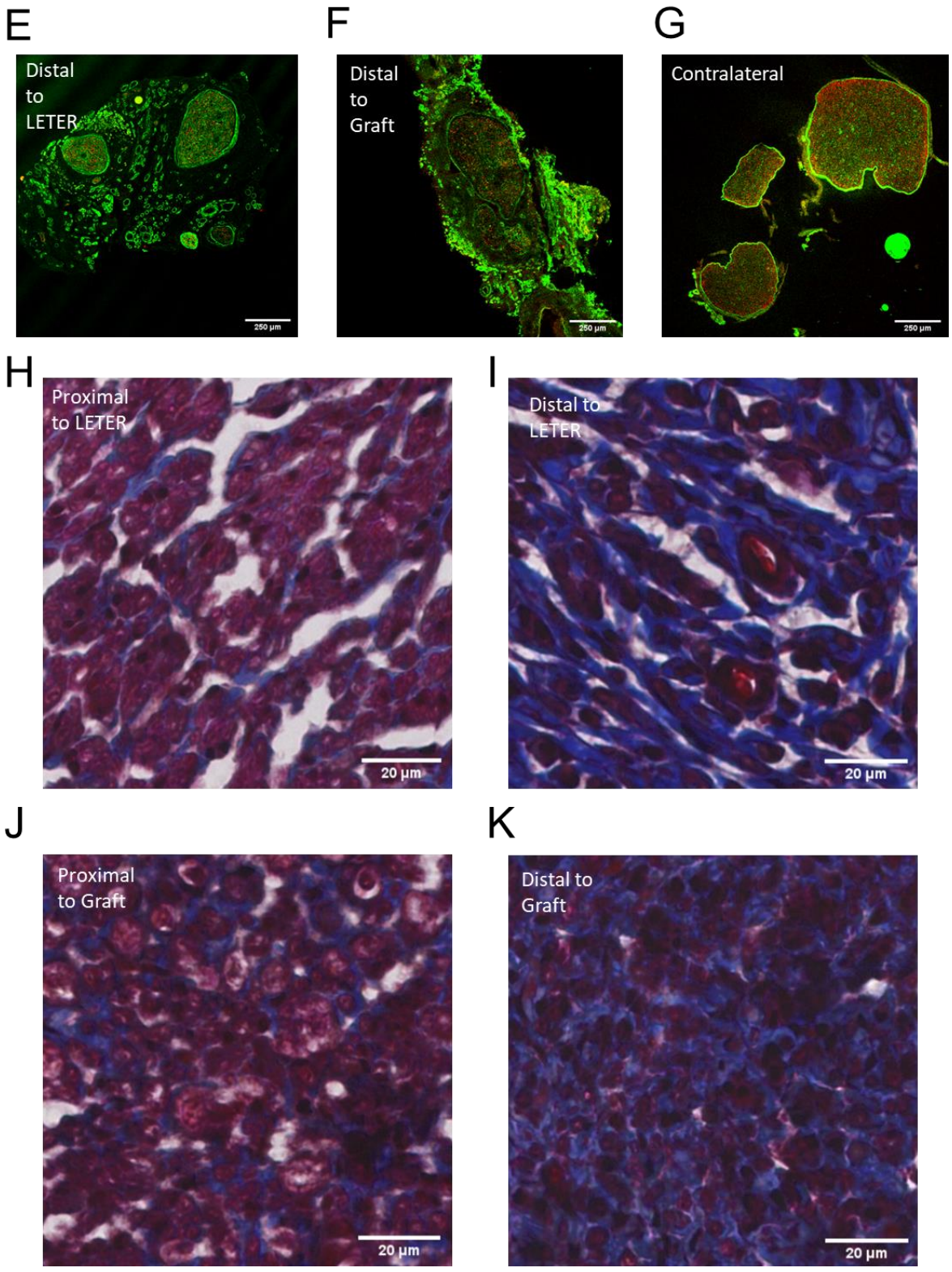

Figure 4.3 con't: Images of the sciatic nerve

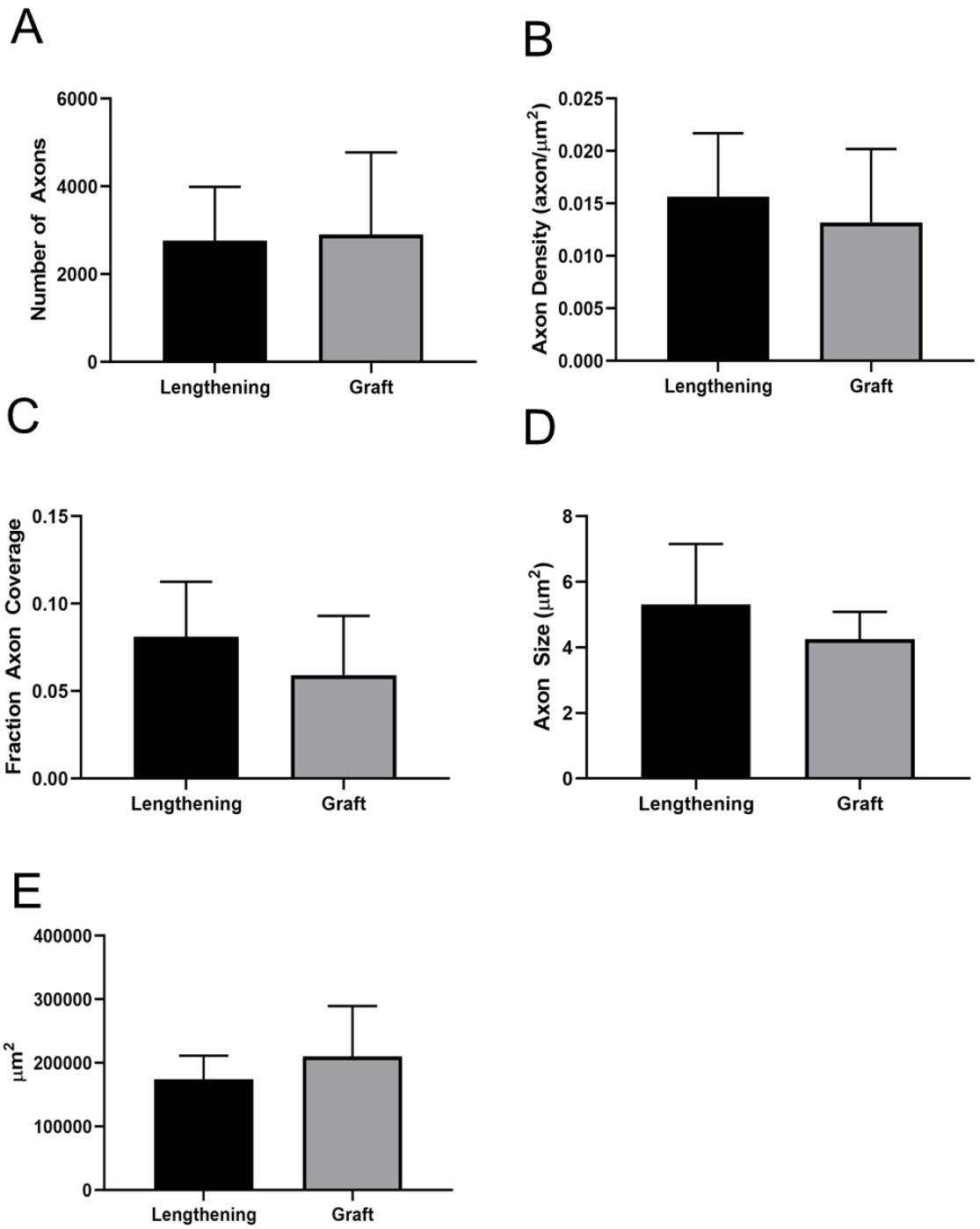

Figure 4.4: Quantification of analysis of axons distal to the injury: number of axons (A), axon density (B), fraction axon coverage (C), axon size (D). Also compared is the total area of the nerve distal and proximal to the injury (E). No significant difference was found between any group and growth appeared similar between all groups and regeneration appeared similar ($p = 0.89$, 0.54, 0.29, 0.20, and 0.37, respectively). Nerves appeared atrophied distal to the injury, especially when compared to the nerve area proximal to the stump. However, Delaunay mean (F) and median (G) showed no significant difference was found between groups ($p = 0.42$ and 0.35 respectively). The histograms generated from the Delaunay analysis from distal LETER axons (blue), graft axons (red) and control axons (green) (H) Inset shows example of Delaunay diagram from ImageJ.

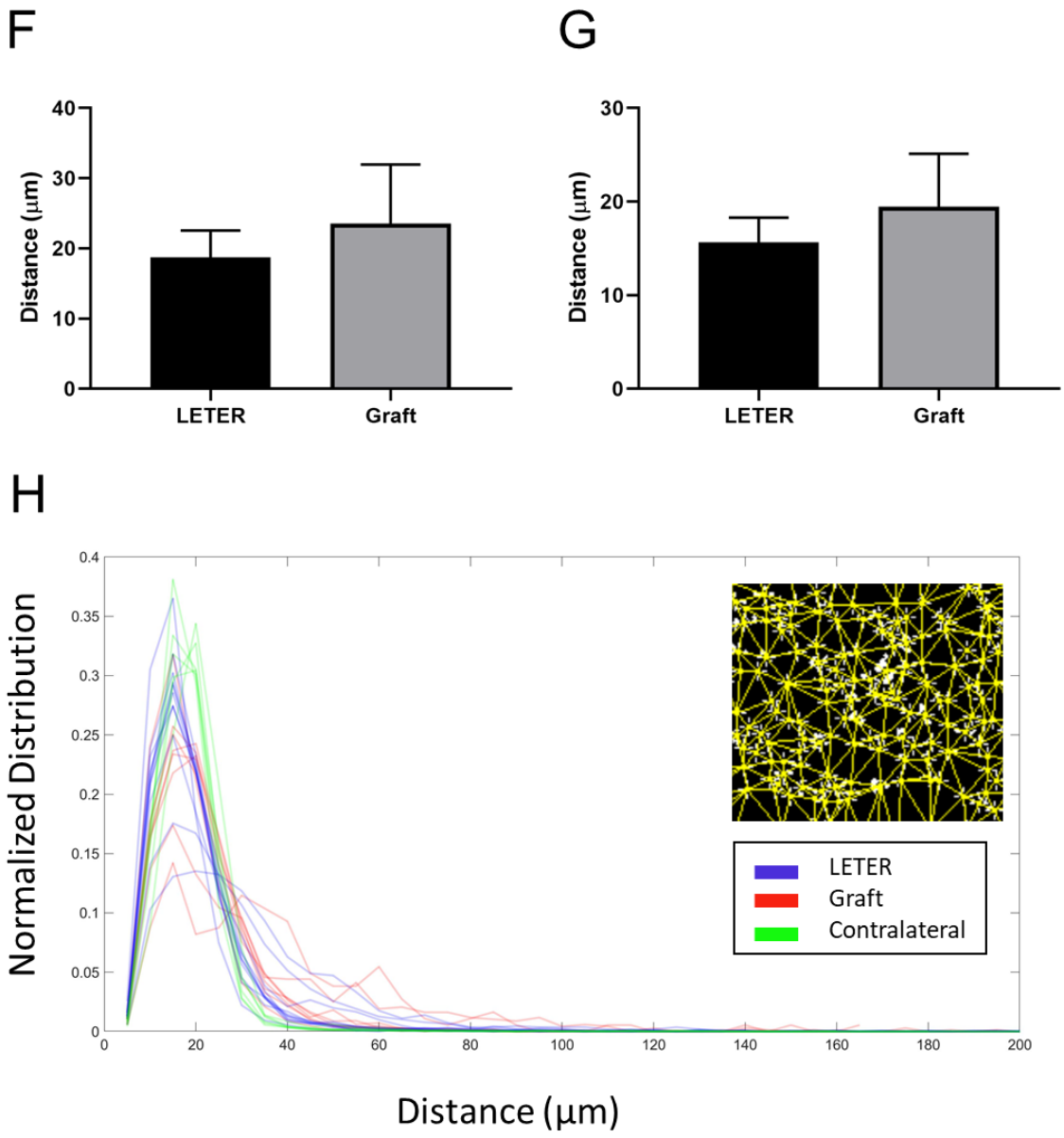

Figure 4.4 con't: Quantification of analysis of axons distal to the injury

Altogether, no significant differences between groups could be found in various measurements of NMJ morphology in the affected muscle (Figure 4K-M). Cumulatively, these data confirm the presence of viable neuromuscular connectivity between proximal stumps, distal stumps, and musculature. Despite the data showing neuromuscular connectivity and many NMJs with normal morphology, this did not result in recovery of sciatic function.

Correlation and Comparison to Acute Injuries

To identify key features that may have contributed to incomplete functional recovery, outcomes were compared between chronic (this study) and acute (Howarth et al., 2019) injury. There were several obvious differences between acute and chronic nerve injuries, especially distal to the injury. In the chronic group, the nerve was atrophied compared to the acute group (Figure 4.6A-C), with corresponding decreased number of average axons in the lengthening (2760 vs 11012) and graft group (3359 vs 6926). We also examined the correlation between variables measured in both groups (Supplemental Figures 4.1 and 4.2). Increased correlation is shown in darker blue (positive) or darker red (negative) in Supplemental Figure 1. In Supplemental Figure 4.2, the same dataset is instead displayed with individual data points are shown, colored by group (blue is acute, orange is chronic). Some of these plots are highlighted individually in Figure 4.6D, specifically axons vs perineurium area, SFI vs axons, and SFI vs TA muscle weight. Unsurprisingly, SFI had little correlation to the other variables in the chronic injury groups due to the little functional recovery and difficulty to measure. Interestingly, muscle weights showed a stronger correlation to axon growth in the chronic group compared to the acute group, possibly because more axons in the chronic group has a greater impact than it does in the acute group to muscle recovery. Both methods of correlation revealed the same patterns, though correlation typically seemed higher when using the Pearson's coefficient.

4.5 Discussion

In this study, we tested a new, recently described (Howarth et al., 2019) nerve-lengthening strategy to address poor outcomes associated with chronic nerve injury. The most important factors that limit recovery following chronic injury are unclear, but may involve the greatly slowed growth of motor axons at long timepoints following injury, poor regenerative environment of the distal stump, or muscle atrophy and endplate disruption. Via liberal excision of poor-quality nerve sections and subsequent nerve lengthening, our data validated the feasibility of nerve lengthening as a strategy for repairing chronic

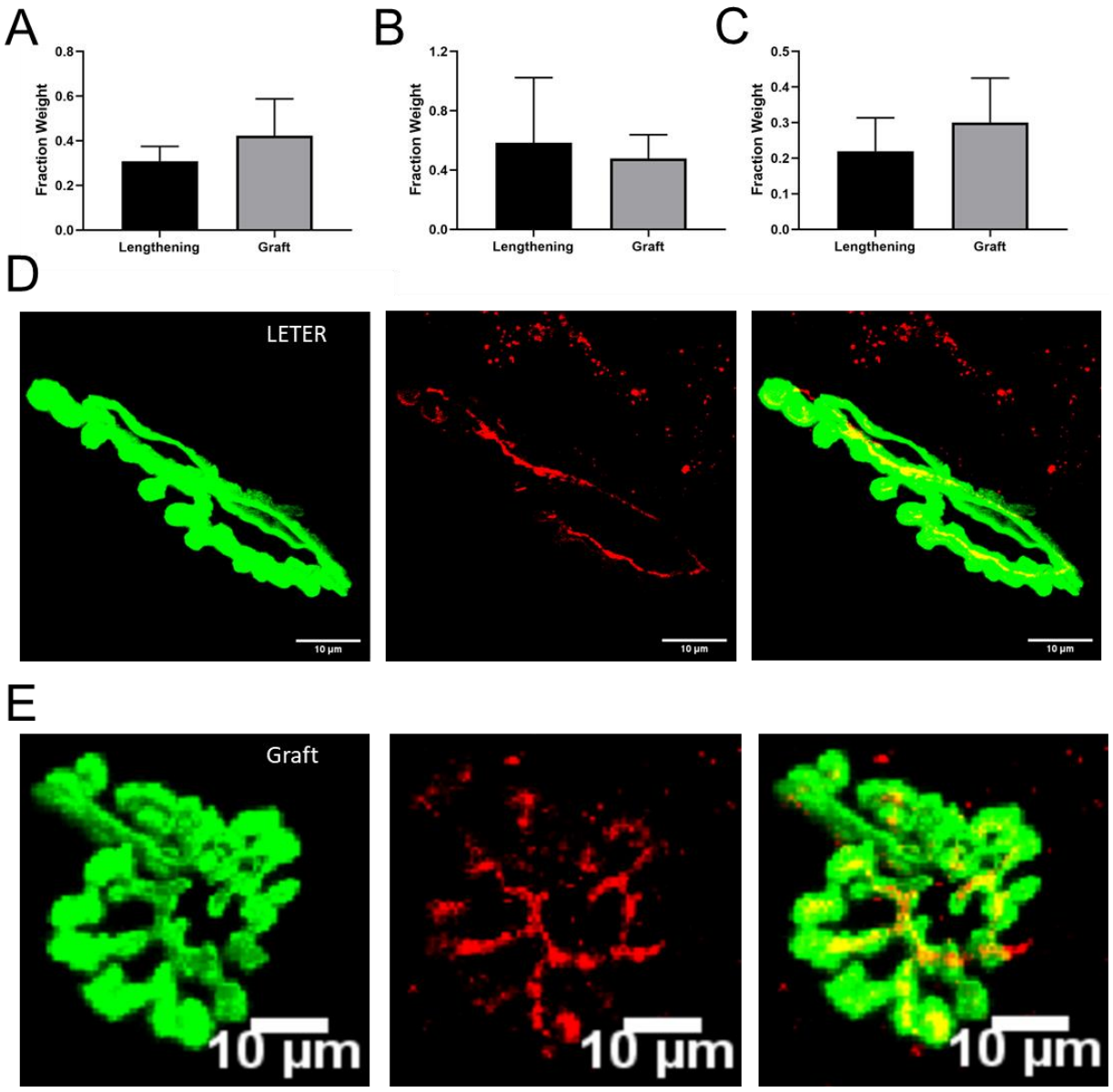

Figure 4.5: Mass of affected TA (A), EDL (B), and gastrocnemius (C) muscles as a fraction of the contralateral (unaffected) muscle. No significant differences in fraction muscle mass were found (p=0.13, 0.58, and 0.21, respectively). Examples of individual neuromuscular junctions (NMJs, green) and axons (red) found in the affected LETER TA muscles (D), graft muscles (E), and contralateral muscles (F). Lower power microscopy showing NMJs observed throughout the muscle for LETER (G) and graft (H) groups. Examples of disrupted NMJs observed in the LETER (I) and graft (J) TA muscles. Analysis of NMJ morphology (Dispersion Index, branching, and discontinuities) showing no significant difference between groups (K-M).

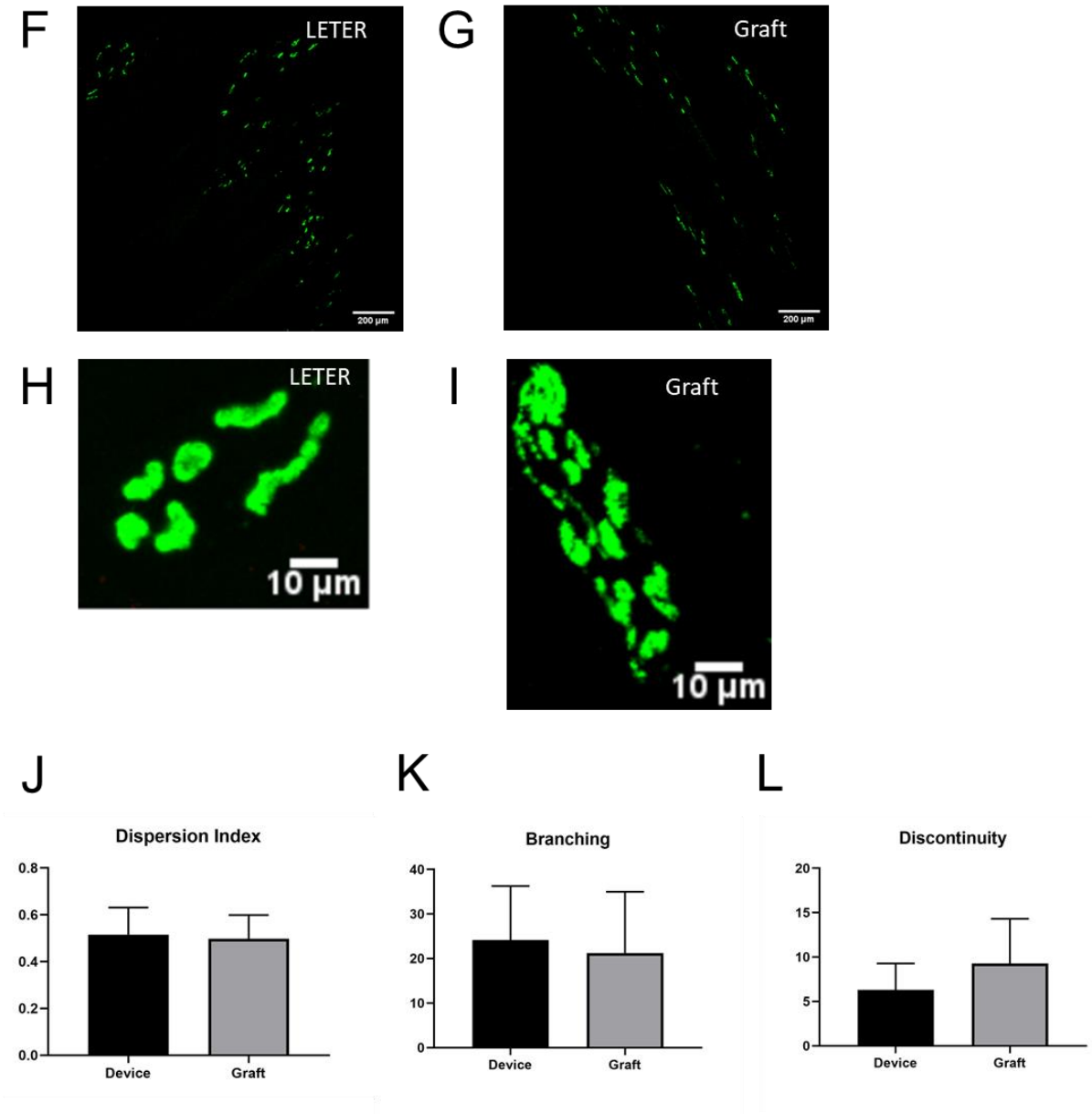

Figure 4.5 con't: Affected NMJs

nerve injuries. Nerve lengthening followed by end-to-end repair demonstrated equivalent structural and functional outcomes to isograft-repaired nerve gaps, but consistent with previous literature, regeneration in both experimental groups was inferior to that after an acute nerve injury, likely due to severe atrophy of both the distal nerve stump and muscle prior to repair. We performed a comparative and comprehensive

analysis of outcomes in acute (Howarth et al., 2019), and now chronic, nerve injury models, which have provided insight into barriers to, and predictors of, successful regeneration.

Nerve Lengthening as a Regenerative Strategy

Our nerve-lengthening device has previously been shown to effectively lengthen the proximal nerve stump, enabling an end-to-end repair (Howarth et al., 2019). When applied to an acute model, this strategy showed outcomes to be as good or better than a nerve repaired using an autograft (Howarth et al., 2019). This corroborates extensive previous research showing benefits of end-to-end repairs (Bhatia et al., 2017), that tension increases the rate of axon growth (Bray, 1984; Pfister et al, 2004), and positive outcomes from alternate peripheral nerve lengthening strategies in acute injury models (Saijilafu et al., 2006; Yousef et al., 2015; McDonald and Bell, 2010). In the acute model, long nerve gaps were readily bridged, without the structural and kinematic changes associated with chronic injury. Further, we found no significant differences in outcomes between lengthening and graft groups. As our device-based approach bypassed the need to harvest a graft from elsewhere, our data support an alternate strategy for nerve repair. The current study represents the initial application of nerve lengthening for a chronic injury model.

Differences Between Acute and Chronic Injury

Previous animal and human studies indicate a worse outcome for chronic nerve injuries compared to acute injuries (Jivan et al., 2009; Kim et al., 2003; Furey et al., 2007); our data showed that there were substantial deficits in the recovery of chronically injured nerves regardless of the repair method. Several studies have attempted to understand what the major barriers are to recovery in a chronic injury model, and we attempted to overcome several of these barriers.

After a nerve transection, axon growth in the proximal stump slows over time (Gordon et al., 2015), possibly due to the downregulation of many important proteins involved in axonal regeneration. For example, there is a twofold decrease in actin and tubulin expression as well as a threefold decrease in

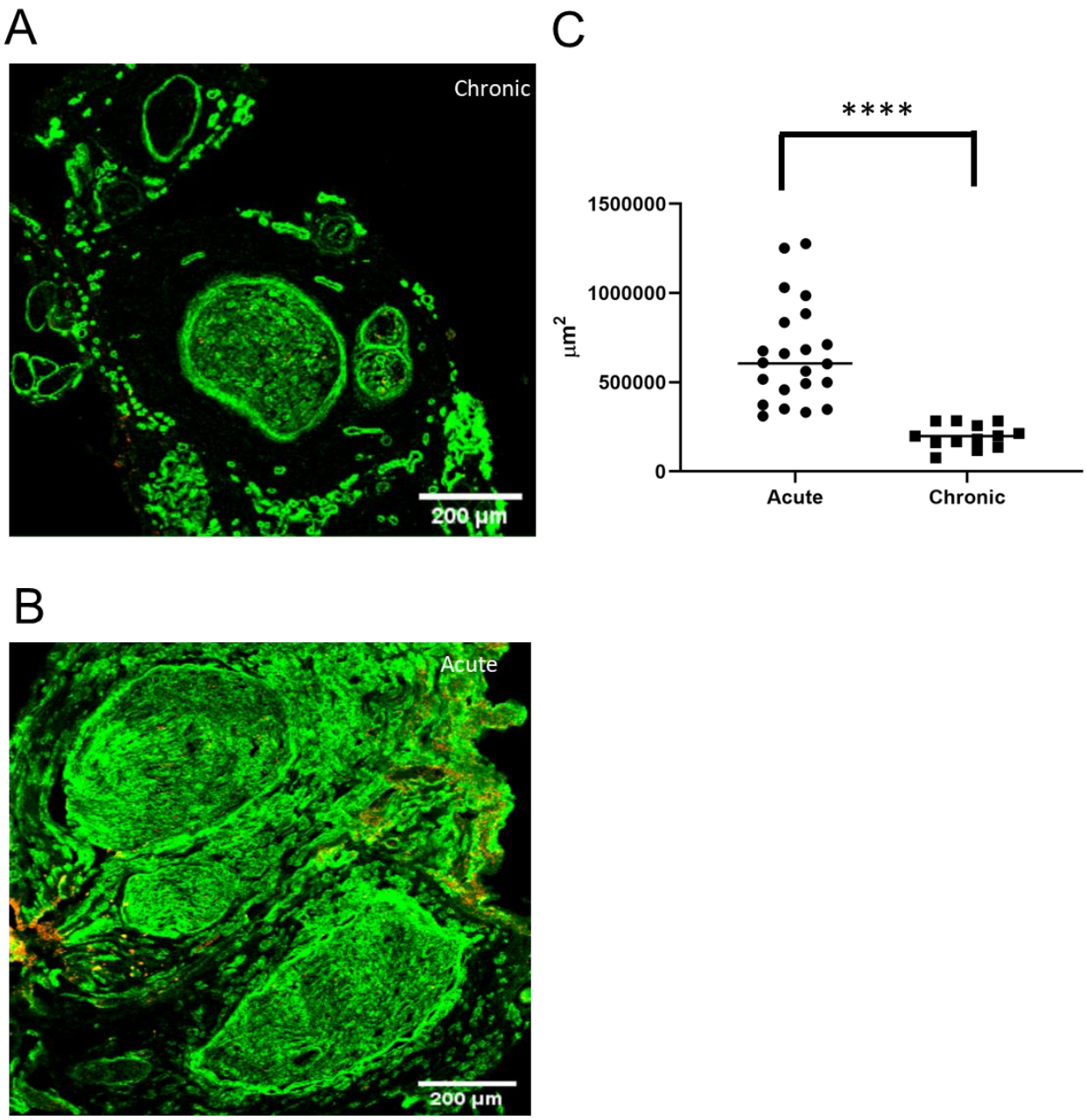

Figure 4.6: An atrophied nerve distal to the graft in the chronic group (A), compared to a nerve distal to the graft from our acute experiments (B), showing a large ($p<0.0001$) difference in nerve size following chronic injury. The actual differences in nerve area compared to acute and chronic nerves distal to the injury (C). Selected correlations between variables (D). Axon count vs. perineurium area (top), SFI vs. axon count (middle), and SFI vs tibialis anterior muscle weight (bottom) are all positively correlated with each other. Chronic datapoints are orange, and acute datapoints are blue.

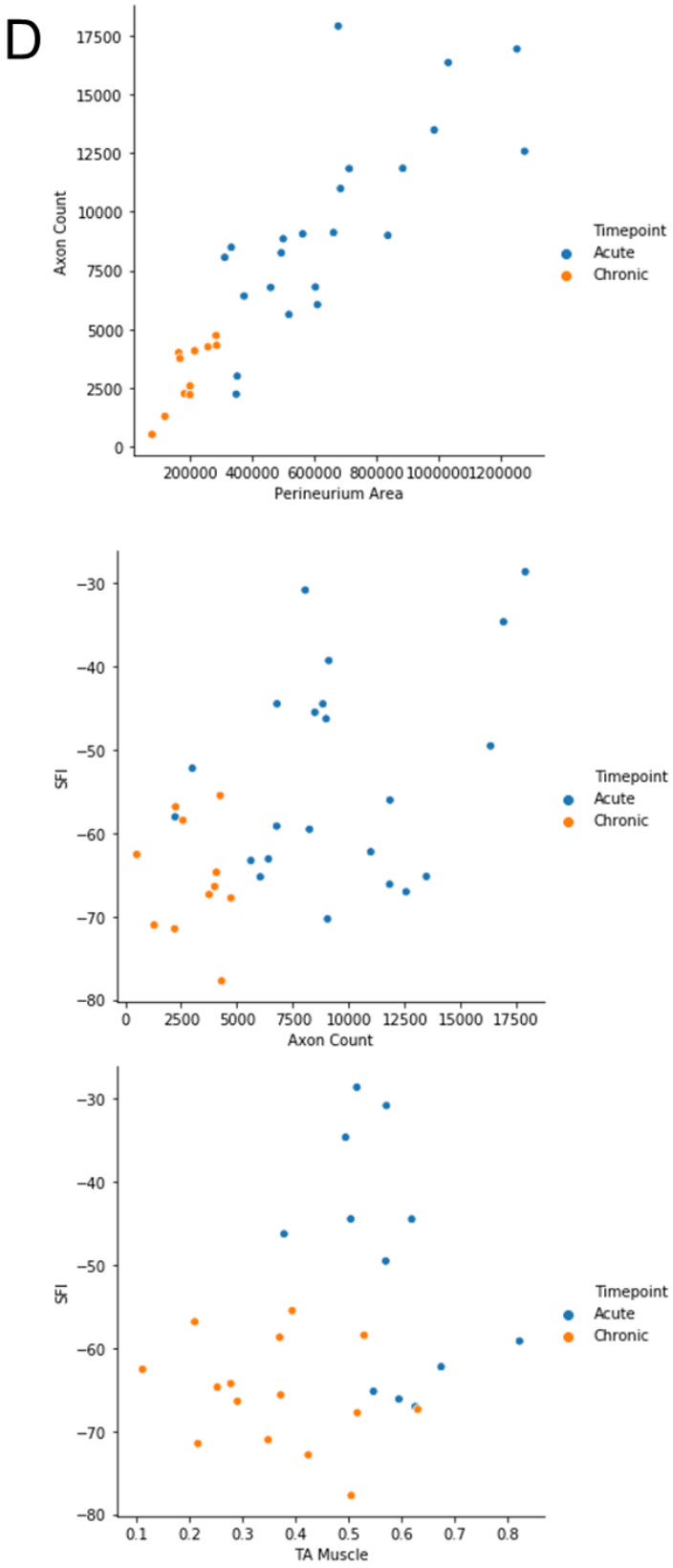

Figure 4.6 con't: Selected correlations

GAP43 expression at chronic timepoints (Gordon and Tetzlaff, 2015). In addition, prominent changes occur in the distal stump, where the extracellular matrix (ECM) produces proteins involved in glial scars and reduces expression of matrix-degrading matrix metalloproteinases (MMPs) (Heine et al., 2004; Bradley et al., 1998), with tissue quality particularly poor close to the injury site. Schwann cells also reduce their expression of chemoattractive growth factors and their receptors (Hoke et al., 2002; Boyd and Gordon, 2003). Compellingly, while skeletal muscle health influences the efficacy of repair following chronic denervation, implantation of GDNF-releasing polymers or stem cells at the nerve repair site showed some promise in coaxing functional recovery after chronic denervation (Wood et al., 2013). Also, the limited number of axons that traverse the degenerated distal stump form functional neuromuscular junctions (NMJ), suggesting reversibility of impairment, even after prolonged denervation (Sulaiman and Gordon, 2000).

Based on these observations, and assuming motor endplates are still capable of receiving axons, we hypothesized that coaxing growth in healthier regions of the proximal stumps via stretch would enhance functional recovery, possibly by upregulating dormant protein synthesis through tension-driven mTOR/S6 pathways (Love et al., 2017). In addition, the excision of longer patches of degenerating stump tissue prior to repair would presumably provide a more favorable regenerative environment. However, despite these possible benefits, we observed severe atrophy of distal nerves and muscle in both Graft and LETER groups, correlating with fewer axons in the distal stump and more sparsely distributed NMJs compared to repairs of acute nerve injury. These findings provide support for the notion that length and integrity of the distal stump are of primary importance in dictating functional recovery (Gordon et al., 2011).

Our implementation of both lengthening and graft-based repair in both acute and chronic models and the measurement of a comprehensive set of quantitative outcomes allows us the unique opportunity to examine relationships between key outcomes across acute and chronic injuries. Several interesting relationships were noted. Globally, a clear threshold for structural and functional outcomes was found

beyond which chronically injured nerves could not improve, irrespective of treatment strategy. These thresholds, then, may underlie an inability to recover function fully.

Several correlations of note were also identified that provide insight into key predictive outcomes. Perineurium area was strongly correlated to number of axons as well as SFI. Perineurium area is also a relatively easy parameter to measure with imaging and could be done in vivo. This comprehensive analysis of the data revealed trends that highlighted how recovery after nerve injury includes many components, and all are important for positive results following recovery. It also highlighted the stark differences between chronically injured and acutely injured nerves, which nonetheless follow the same trends.

Limitations

Nerve injuries regenerate on a very long timescale, thus even later timepoints could be considered. However, it is not always feasible to wait until functional recovery is observed, if it will ever occur at all. At the timepoints used in this study, recovery would have already been observed in an acute model of injury. Studies that have used very long time points for measuring animal recovery and studies of patients in clinical settings have both revealed that many of these chronically injured nerves show no further recovery, even years after the repair. In short, to accomplish the goals of our study, we believe our endpoint was sufficient, and we were able to see axon growth and make comparisons between treatment methods.

The SFI is not typically considered an ideal measure of sciatic nerve recovery in severe and chronic injuries (Dellon and Mackinnon, 1989; Monte-Raso et al., 2008). However, the analysis of SFI was useful to us for comparing the two groups and to examine the rats for differences during recovery. Some recovery of the muscle, though very weak, was confirmed with measurement of the dorsiflexion strength. With these results combined, we are confident in concluding that neither group had greater recovery than the other.

Conclusions and Relevance

We found that nerve lengthening was a viable method to bridge a nerve gap in a chronic injury. The nerve did not break with stretching and could be lengthened to the point an end-to-end repair was possible, even with trimming of the nerve ends. Though we found no significant improvement over the graft group, outcomes were no worse. Further experiments will be done to examine protein expression following injury, as protein expression following injury can change greatly with the passage of time, greatly slowing the rate of growth. Nerve stretching also alters protein expression but in a positive direction.

It seems likely that motor neurons require additional support in order to grow at chronic timepoints. One solution to this may be a combined treatment to stimulate the growth of motor neurons or perhaps longer periods of stretching are needed to encourage axon growth following a chronic injury. In the future, we will be exploring how the regenerative environment changes due to mechanical stimulus. By better understanding what is happening in the nerve at the molecular level, useful combined treatments might be found.

4.6 Acknowledgements

Chapter 4, in part, is currently being prepared for submission for submission for publication. My co-authors are Elisabeth Orozco, Richard Lovering, and Sameer Shah. The dissertation author is the primary author of the paper. I also acknowledge the generous support of the Department of Veterans Affairs (VA MERIT IRX001471A).

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Chapter 5: Early phase proteomic profiling of nerves lengthened following injury

5.1 Abstract

Following the transection of an axon, the still living proximal axon stump must regrow and eventually find its way back to its target endpoint. Because this process of growing back to the endpoint can take weeks, months, or even years to complete, the changing regenerative environment of an axon over time is important to understanding how best to treat nerve injuries. Also of interest is the response of axons to biomechanical stimulus, which have been shown to grow more quickly or upregulate proteins when under tension. In this study, we examined the differences of the proteomic profile five days following the start of treatment between an injured nerve that is lengthened and a nerve that is simply treated with a graft.

5.2 Introduction

Following injury, the environment of the nerve changes. Distal to the lesion, Wallerian degeneration occurs, clearing away the dead axons and forming a tube through which the proximal axons can grow (Gaudet et al., 2011). Proximally, regeneration begins. Many structural and signaling pathways are upregulated during the initial stages of regeneration (Abe and Cavalli, 2008) as the proximal axons extend and Schwann cells redifferentiate (Stoll and Müller, 1999). However, timing has an important role in these pathways and protein regulation changes over time (Jimenez et al., 2005), with some proteins being upregulated, some downregulated, and some trends reversing during the weeks following injury to eventually produce a less favorable growth environment and slower regeneration (Gordon and Tetzlaff, 2015). In a study of retinal ganglion cells following axonal injury, quantitative analysis revealed the downregulation of pathways c-*myc* and mTOR reduced the potential for regeneration over time (Belin et al., 2015). The changes in the ability of the nerve to regenerate are very important to the eventual repair of the injury.

Besides injury, the nerve also responds to biomechanical stimulus even when no injury is present. Sustained stretching of the nerve has been shown to significantly upregulate important proteins such as mTOR and S6 as well as resulting in trends of upregulation of structural protein tubulin (Love et al., 2017). Furthermore, axons have been shown to grow at much higher rates when stretched than when left untouched in vitro (Bray, 1984; Pfister et al., 2004). Though the results of tension experiments show a clear effect of it on peripheral nerves, there are still gaps in our understanding of the mechanism and how it differs from injury (Gangatharan et al., 2018).

We have shown previously that a nerve stretched to bridge a nerve gap has equal or superior outcomes to using an autograft to bridge a nerve gap (Howarth et al., 2019). Injury and stretch both upregulate proteins that are important for axonal regeneration. This has important implications for chronic nerve injury, where important protein pathways have long been downregulated after the initial stages of regeneration. The potential for stretch to enhance or reactivate regeneration pathways could increase our knowledge of how to improve both acute and chronic nerve injury regeneration. In this study, we hypothesize that proteins involved in nerve regeneration and cell proliferation will be upregulated in nerves that have been injured and stretched when compared to nerves with only an injury without stretch.

5.3 Methods

Surgical procedure

10-12 week male Lewis inbred rats (n=8) were split into two groups and received autograft or device implantation surgeries as described previously (Howarth et al., 2019). Briefly, a 10mm section of the sciatic nerve was transected from the nerve. In autograft group rats (n=4), the transected nerve was flipped and re-sutured to the proximal and distal stumps of the nerve. In device implantation group rats (n=4), the proximal stump of the nerve was sutured to a lengthening device. Rats were then allowed to recover.

Tissue harvest

Rats returned to normal behavior within two days following surgery. Rats which had a device implanted had the external wired pulled on once a day, starting the second day post-surgery, to stretch the nerve 1mm/day. On the fifth day post-surgery, the wire was pulled, and rats were euthanized 60-90 minutes following this final stretch. Autograft group rats were also euthanized on the fifth day post-surgery.

Following euthanasia, a 10mm section of the sciatic nerve was harvested. The section of nerve was selected so that it was proximal to the site of the injury as well as any swelling of the nerve. In the device implantation group rats, this was nerve that was stretched but proximal to the section that was clamped. In addition, the sciatic nerve contralateral to the injury was also harvested. Following harvest, the nerves were frozen in liquid nitrogen cooled isopentane and stored at -80 ^{O}C until tissue processing.

Tissue processing

Tissue was split into three different groups: contralateral nerve (n=4), lengthened nerve (n=4), and graft nerve (n=4). In collaboration with the UCSD Biomolecular and Mass Spectrometry Facility, the tissue was processed using LC/MS. Tissue was digested in a 1:10 trypsin solution to prepare for liquid chromatography. Peptides were then separated by Ultra Performance Liquid Chromatography (UPLC), samples having been loaded in a random order, and analyzed with electronspray ionization mass spectrometry (ESI-MS) using an Orbitrap Fusion Lumos Mass Spectrometer (Thermo Scientific).

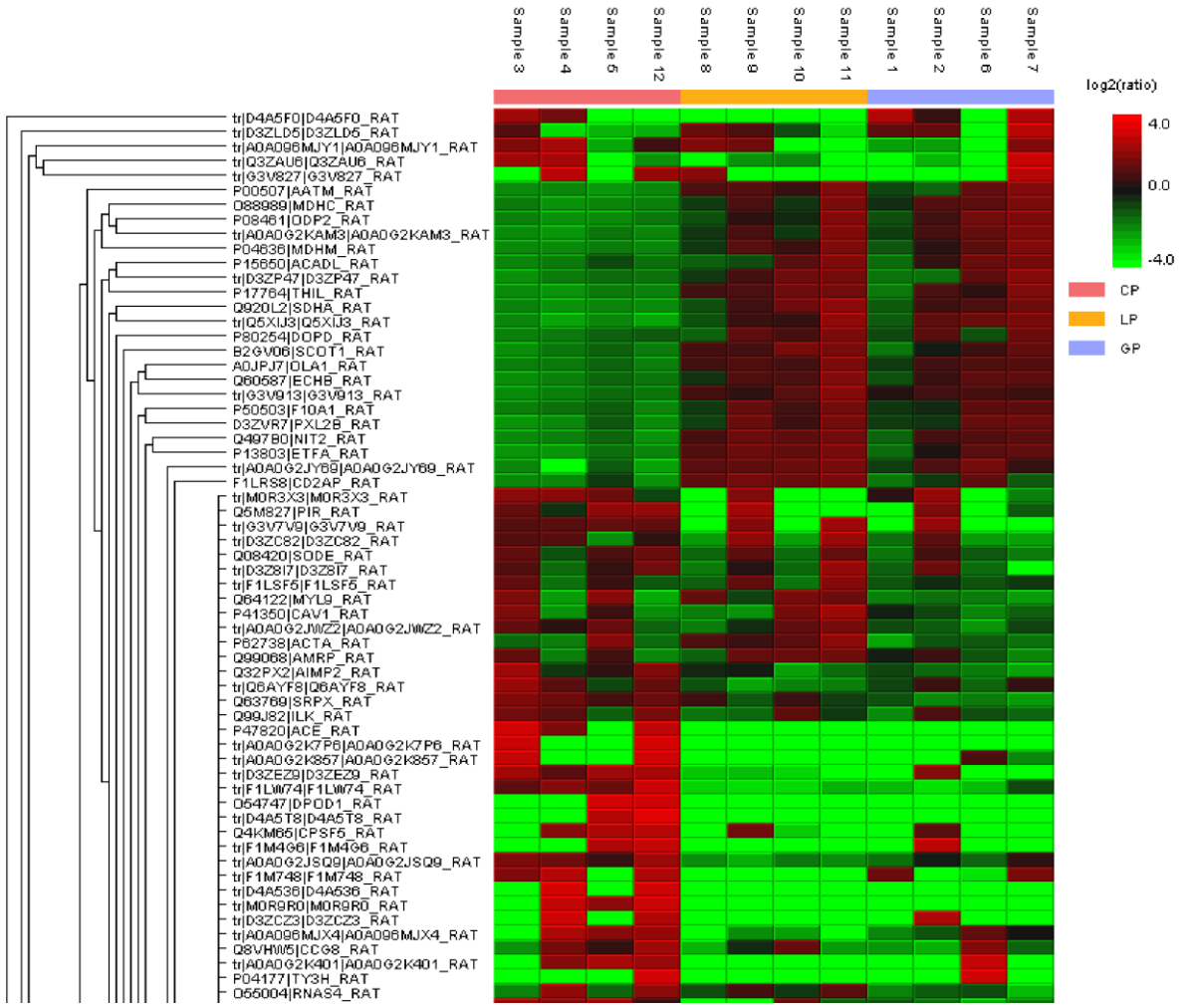

Figure 5.1: A portion of the heatmap which was produced to show expression of proteins. Proteins that were significantly upregulated (red) or downregulated (green) when comparing control nerve (left 4 columns), lengthened nerve (middle 4 columns), and graft nerve (right 4 columns) are shown. Biological replicates appeared to have similar levels of expression and injured nerve groups appeared more similar to each other than to the control nerve.

Data analysis

Data from LC/MS was analyzed using Peaks 8.5 software to determine relative protein regulation (Figure 5.1) using label-free protein quantification (Cox et al., 2014). Proteins were selected for further analysis from the total proteins detected if there was a significant difference between groups or between the lengthened and graft nerve. These proteins were then uploaded to DAVID 6.8 (NIAID/NIH). Comparisons were made by the number of proteins in different annotation categories (Functional Annotation and Gene Ontology) listed as significant, noting the groupings specified as significant.

5.4 Results

To determine the differences between lengthening and no lengthening following injury as well as the differences between both injured groups and the uninjured nerve, we analyzed the data by examining the roles of proteins found to have a significant difference in expression between groups using DAVID. By observation, samples within groups appeared to have similar protein expression levels and the injured groups appeared to be more similar to each other than to the uninjured nerve (Figure 5.1). The injured groups, both lengthened and graft, had many similarities to each other when compared to the protein expression of the contralateral nerves. In Figure 5.2, the number of proteins from some of the top (highest significance) categories which are significantly up/downregulated when comparing all three groups and the number of proteins which are significantly up/downregulated when only comparing the injured groups shown on the same chart. We looked in more detail at the roles of proteins regulated differently between the lengthening and graft group nerves. Of note, translational and metabolic pathways, ribosomal proteins, and blood coagulation pathways appeared to be significantly upregulated in the lengthened nerve (Tables 5.1-5.3, categories of interest in bold).

5.5 Discussion

It is clear that both tension (Love et al., 2016) and injury (Belin et al., 2010; Bisby and Tetzlaff, 1992) affect the regulation of proteins in the nerve and in similar ways, such as upregulating structural proteins and transcription. While this is an interesting result in and of itself, it is difficult to know where the changes in the proteome as a result of injury end and the changes as a result of tension begin. However, we did observe the upregulation of several proteins related to transcription when the two treatments were compared. This leads us to believe that the driving forces that activate regeneration following injury are increased when tension is applied to the nerve.

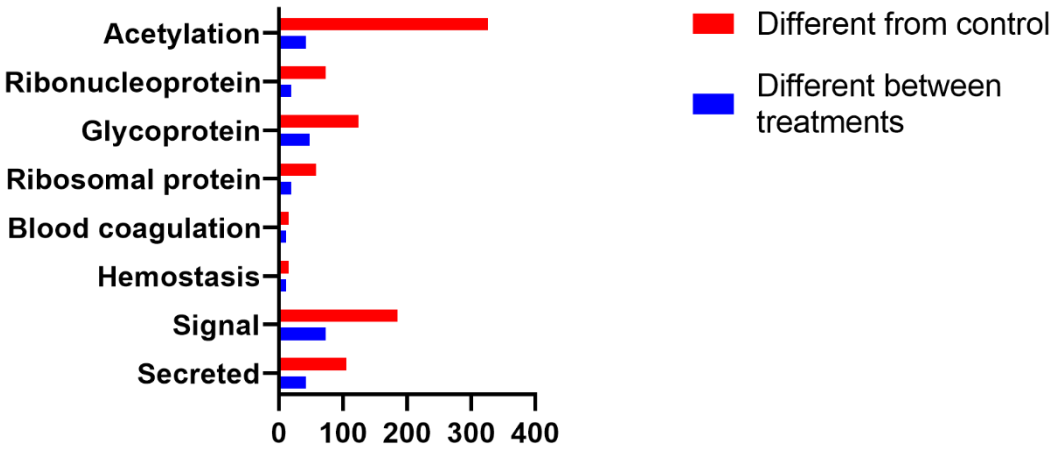

Figure 5.2: Number of proteins in top functional categories for different types of comparisons. Red bars represent number of proteins which are significantly up/downregulated when comparing all groups. Blue bars represent number of proteins which are significantly up/downregulated when only comparing injured groups to each other.

Due to the nature of the device used to lengthen the nerve, some limitations were present in the analysis. Some additional bleeding and subsequent coagulation occur due to the device's presence within the sciatic nerve bed. This is unavoidable, and as expected, an effect is seen on our subsequent analysis of the proteins. While noting the presence of these proteins/pathways, we chose to focus our efforts on proteins more likely to be involved in nerve regeneration.

Interestingly, our results from DAVID noted two proteins specifically involved in axonal regeneration to be significantly upregulated in the lengthening groups. These are calumenin, which is important for cell adhesion (Jimenez et al., 2005), and tenascin-C, which is involved in axon growth and guidance (Faissner, 1997; Schweitzer et al., 2005). These are of interest to use and will be studied further in the context of lengthening response.

Nerves are adaptable and capable of remodeling following stretch or injury (Abrams et al., 1998; Pfister et al., 2004). It is often believed that tension is harmful to nerves, and while this is certainly true in cases of extreme tension (Hentz et al., 1993), the evidence points to this not always being the case for more moderate applications of tension. Our previous research concluded that lengthening of the nerve

following an injury had the benefits of not only avoiding the use of a graft in favor of an end-to-end repair but also having as good or better outcomes when compared to an autograft, the current clinical gold standard (Howarth et al., 2019). Our findings here, combined with our previous research, lead us to believe that tension is capable of important contributions to nerve regeneration, especially increasing the amount of new protein transcription.

Future Directions

While this study allowed a new look into the role of tension following nerve injury, we have further questions to investigate. Future studies will investigate the affect of more extreme tension following injury and if/how the nerve adapts to this tension. Also of interest to us is the role of timing in nerve regeneration, so this process will also be tested at more timepoints. As mentioned, regeneration related proteins are downregulated with time, but there is a possibility lengthening could reactivate some of these pathways at chronic injure timepoints. We will next examine the differences in protein regulation of chronically injured nerves following either nerve lengthening or a graft to treat the injury to determine if lengthening could have a positive effect on chronically injured nerves.

Table 5.1: Top functional categories of proteins with significantly different expression between the graft and lengthening groups. Associated p-values are shown.

Category	P-value
Secreted	8.50E-19
Signal	4.00E-18
Hemostasis	2.50E-14
Blood coagulation	2.50E-14
Ribosomal protein	9.60E-13
Glycoprotein	5.70E-12
Ribonucleoprotein	4.00E-11
Acetylation	5.40E-11
Disulfide bonde	3.00E-09
Phosphoprotein	4.80E-09
Calcium	1.10E-07

Table 5.2: Broad gene ontology categories of proteins with significantly different expression between the graft and lengthening groups. Associated p-values are shown.

Category	P-value
Metabolic Process	1.90E-08
Immune system process	1.30E-07
Biological adhesion	2.00E-04
Developmental process	2.10E-02
Locomotion	2.60E-02
Detoxification	3.90E-02
Multi-organism process	4.20E-02
Response to stimulus	4.60E-02
Multicellular organismal process	5.30E-02
Growth	9.00E-02

Table 5.3: Top gene ontology categories (more specific than Table 5.2) of proteins with significantly different expression between the graft and lengthening groups. Associated p-values are shown.

Category	P-value
Protein activation cascade	1.10E-18
Blood coagulation	5.40E-15
Fibrinolysis	2.60E-12
Negative regulation of blood coagulation	2.80E-12
Negative regulation of hemostasis	2.80E-12
Negative regulation of coagulation	4.60E-12
Regulation of blood coagulation	5.20E-12
Negative regulation of wound healing	4.50E-11
Acute inflammatory reponse	2.20E-10
Negative regulation of response to wounding	2.80E-10
Regulation of wound healing	1.00E-09
Negative regualtion of proteolysis	1.30E-09
Proteolysis	2.70E-09
Translation	3.70E-09
Regulation of peptidas activity	5.50E-09
Peptide biosynthetic process	6.20E-09

5.6 Acknowledgements

Chapter 5, in part, is currently being prepared for submission for publication. My co-author is Sameer Shah. The dissertation author is the primary author of the paper. I would also like to acknowledge the assistance of the UCSD Biomolecular and Proteomics Mass Spectrometry Facility for the work of processing our tissue.

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Chapter 6: Quantitative assessment of triceps control following nerve transfer surgery

6.1 Abstract

Several different treatments exist for spinal cord injury which, although they do not directly address the injury, seek to improve the quality of life for individuals with these injuries. One such treatment is a nerve transfer, in which a "redundant" donor nerve can be redirected to a functionally silenced, but structurally intact recipient nerve to innervate a muscle paralyzed by the spinal cord injury. An important hypothesized benefit of selecting this treatment is the possibility of regaining more refined control of the target muscle when compared to other treatments, such as a tendon transfer. However, while control has been evaluated using task-based assessments, objective approaches to evaluating control following nerve transfers have not been deployed. Towards the development of such strategies, in this study we describe a new protocol to quantify the elbow control of patients receiving nerve transfer surgery of the teres minor branch of the axillary nerve to the triceps branch of the radial nerve. We also aim to use this method to more comprehensively evaluate neuromuscular function following this procedure.

6.2 Introduction

Within the United States, there are approximately 288,000 persons living with a spinal cord injury (SCI). Approximately 18,000 new injuries occur each year ("Spinal Cord Injury Facts and Figures at a Glance," 2018) with the majority of victims being young, healthy, and active people in their most productive years. More than half of SCIs occur within the cervical spine, resulting in compromised arm and hand function. Recovering even partial arm and hand function can have enormous impact on independence, as persons with cervical SCI are dependent upon hand and arm function for mobility and activities of daily living (Waters et al., 1996). Thus, upper extremity function is consistently rated as the most desired function in this population, above bowel and bladder function, sexual function and even walking (Anderson, 2004).

Loss of triceps muscle function is common in spinal cord injuries above the C7 level and restoration of active elbow extension is regarded as the first priority of surgical rehabilitation of upper extremity function in these patients (Moberg, 1975). Elbow extension is important for several reasons, including propulsion of wheelchair, ability to reach out in space, oppose gravity and to provide an antagonistic force to the elbow flexors, and thereby optimize hand function. For example, it has been documented that active elbow extension increases key pinch power when combined with brachioradialis transfer (Brys and Waters, 1987) and improves the control of both shoulder and elbow flexion speed (Remy-Neris et al., 2003). Restoration of elbow extension is thus regarded as a critical prerequisite to restore arm- and hand-control.

The two classic procedures used to restore triceps function in this population are posterior deltoid or biceps brachii muscle transfers into the triceps tendon. Both procedures are reliable and effective, and result in rapid restoration of elbow extension. However, disadvantages include loss of donor muscle function, mismatch of muscle architecture between donor and acceptor, the need for tendon or alloplastic interposition grafts, lengthy immobilization periods and risk of functional deterioration due to mechanical failure such as tendon elongation.

Nerve transfers avoid these concerns, and, though requiring a longer time to recovery, offer a promising alternative to traditional muscle transfers, especially in spinal cord injury patients with a structurally intact lower motor neuron. Their paralyzed muscles undergo atrophy due to inactivity, but will not degenerate and become fibrotic as they do after a primary peripheral nerve injury. Additionally, muscles paralyzed due to damage to the central nervous system usually preserve their spinal reflexes and can be functionally stimulated by external devices even decades after the original onset of paralysis, despite diminishing of the motor end plates (Ethier et al., 2012; Peckham and Knutson, 2005). Thus, transfer of motor axons that have an intact connection to the spinal cord above the level of the lesion can reinnervate paralyzed muscles after SCI.

Many methods to evaluate neuromuscular function following spinal cord injury exist, including strength, patient reported outcomes, and electrodiagnostics (Kalsi-Ryan et al., 2014). However, none of these reveal the amount of control the muscle has developed throughout recovery. In this study, we describe novel methods to quantify the control of elbow extension after nerve transfer, based on patients' ability to track a dynamic visual target. We also evaluated several standard methods of functional recovery, to place our approach in the context of a comprehensive evaluation of the outcomes of nerve transfer surgeries.

6.3 Methods

Patient recruitment

All procedures were approved and proceeded by the UCSD Institutional Review Board. Patients of either gender were enrolled, between ages 18-50 years old, who were scheduled for teres minor branch of the axillary nerve to-radial nerve transfer at the University of California, San Diego. Inclusion criteria was as follows: (1) Have cervical SCI; (2) Demonstrate stability of motor examination for at least six months; (3) Retain intact innervation to the paralyzed triceps. The stimulatable triceps strength must be at least an MRC 3/5 using methods previously described; (4) Donor nerves/muscles must be of MRC grade 4/5 (teres minor); (5) Have access to a caregiver or therapist who can assist with physical therapy.

Surgery

General endotracheal anesthesia was administered and the patient was supine with the arm abducted on an arm board. An incision was made in the axilla. Motor nerves were exposed and then stimulated to confirm both their identity and proper innervation. The donor and recipient branches were selected and cut from their respective original nerves. The cut donor nerve branch was then sutured to the cut recipient nerve fascicle to allow the brain-controlled axons to grow to the new target muscles (Bertelli et al., 2011). Skin was then closed in multiple layers with absorbable sutures and skin glue. The patient was then placed in a surgical dressing and a sling, and discharged within 24-48 hours. On the second

post-operative day, the dressing was removed. Pain control and wound care was assessed. The patients were instructed to keep the incision site clean and dry using an abduction pad under the arm and limit shoulder abduction to 30 degrees for the first 2 weeks.

COPM

The Canadian Occupational Performance Measure (COPM), which identifies issues of personal importance to the client, and detects changes in a client's perception of performance over time, was used as a personalized functional test. Detailed methods may be found in the Wangdell and Fridén report (2012). Briefly, a semi-structured interview was performed to explore the patient's activity problems or limitations due to their lack of hand function. Thereafter, the patient was asked to choose up to five key problems and use a 10-point scale (10 being highest performance and satisfaction) to rate their own level of performance and satisfaction with each of the five identified problems. Survey results pre-operatively and 18 months post operatively were compared.

Strength and control testing

Testing of elbow control and function was done pre-operatively and 6, 12, and 18 months post-operatively for Patient 2 and 18 months post-operatively for Patient 1. Six control subjects were also tested. Patients were positioned in front of table, with height adjusted for comfort. The arm above the elbow was rested in a support bracket on the table while the forearm was secured to a skid. Full range of motion for the elbow was ensured by manually extending and flexing the elbow of the subject. A goniometer was taped to the forearm and just above the elbow joint while the elbow was in neutral position. The goniometer measured the angle of the elbow. The goniometer was calibrated by measuring the angle manually at 0^{o} and 90^{o}. EMG electrodes were placed at the infraspinatus, biceps, triceps lateral, and triceps longhead muscles. Elbow angle and EMG data were input in LabVIEW on a laptop dedicated to recording the data. Setup is shown in Figure 6.1.

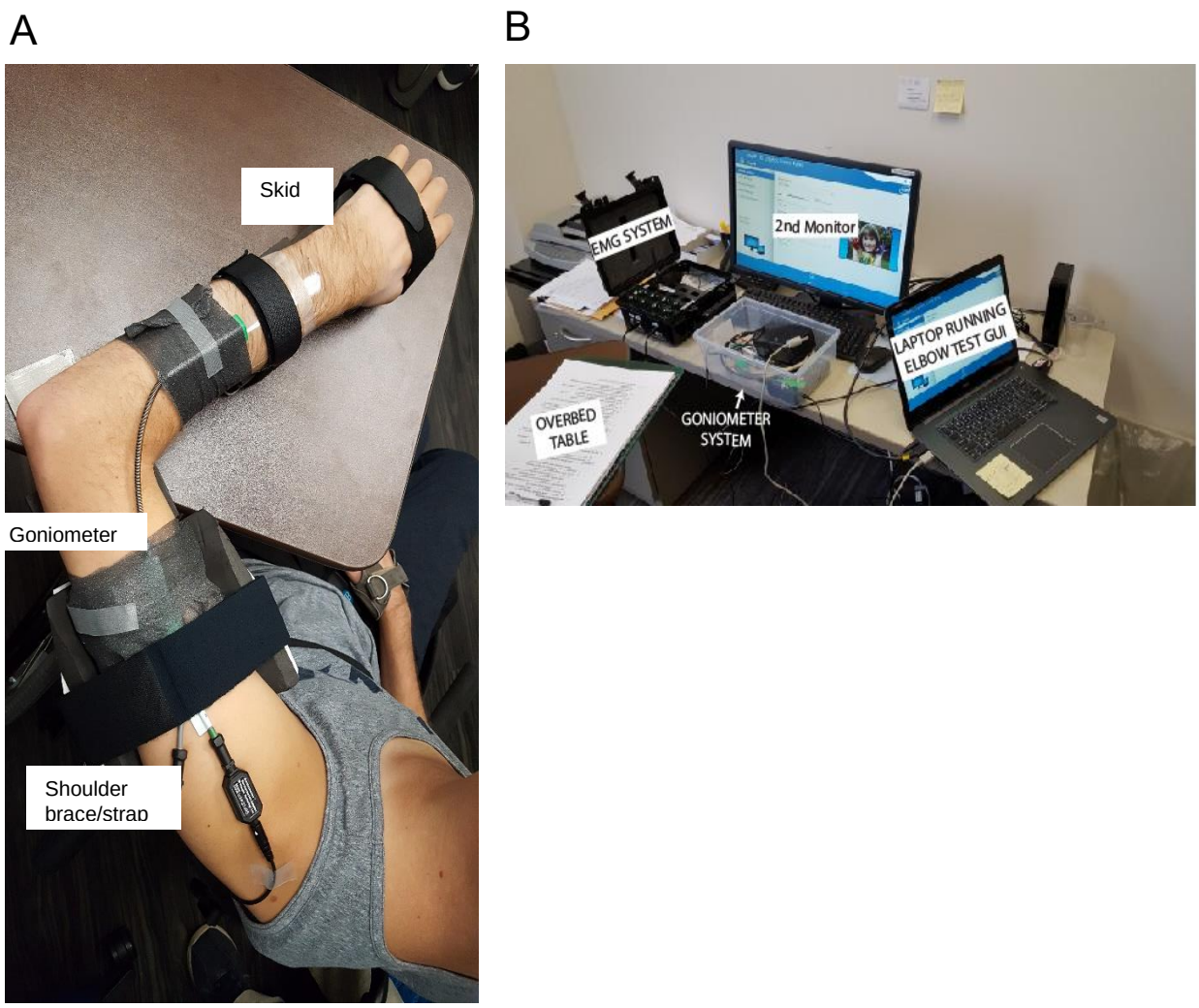


Figure 6.1: Setup of elbow control experiments. (A) Patient resting arm on table, ready for elbow angle to be measured. (B) Electronics setup, patient will follow the angle as viewed in the monitor.

When setup was complete, the patient was instructed to extend and flex the elbow. Directly within their line of sight, a screen showed a bar that moved with the change in elbow angle, and subjects were given the opportunity to learn how their movements corresponded with the bar. Subjects were then instructed to attempt to match another moving bar with their own elbow extension and flexion. Three trials of three different tests were recorded: 1) Sine wave trial, 2) Sum of sines wave trial (more challenging than the simple sine wave), 3) Step signal trial. The step trial required large changes in angle that were not always possible for patients, especially at early timepoints. Due to this, the elbow angled was changed manually by the test administrator after 5 seconds if the step could not be completed. When

these tests were complete, strength during extension (at 15º, 45º, 90º, 135º) and flexion (at 15º and 90º) was measured using a dynamometer.

Data analysis and statistics

Data was imported from LabVIEW to MATLAB R2019b. In MATLAB, the actual elbow angle compared to the target angle was plotted, as well as the EMG readings from all four muscles. Some of these plots are shown in Figure 6.2. Accuracy was measured as the root mean squared error (RMSE) between elbow angle and target angle. RMSE and extension strength were compared between patients at 18 months post-operation and control subjects, as well as different timepoints (every 6 months) for Patient 2, using one-way ANOVA in GraphPad Prism 8.

6.4 Results

Quantified control comparisons

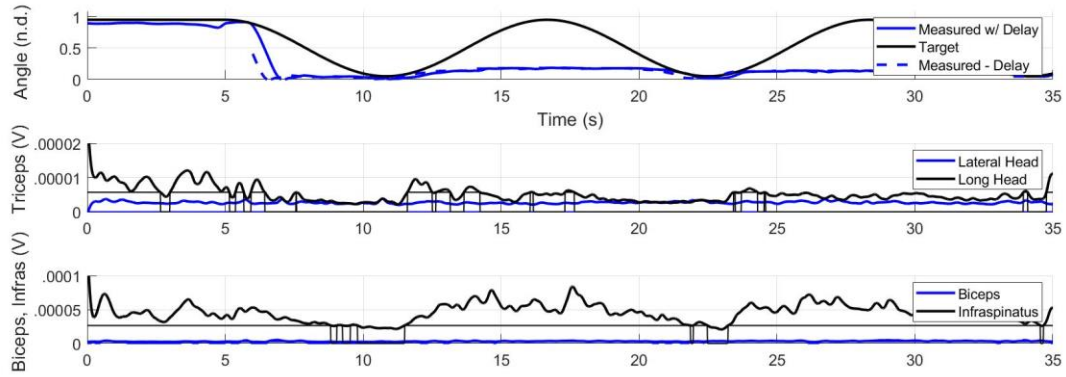
In order to determine whether elbow control improved over time following nerve transfer, we compared the RMSE of the elbow angle when compared to the target angle for all three tests at all four timepoints. We observed that Patient 2 improved in accuracy for all three tests significantly with time (Figure 6.3A).

To determine how the results at 18 months post-operation compared to the accuracy of control subjects, we compared Patient 1 and 2's results to that of the average result of the control subjects. No significant difference was found when comparing the sines and sum of sines trials. However, there was a significant difference found when comparing the results of the step trials (Figure 6.3B).

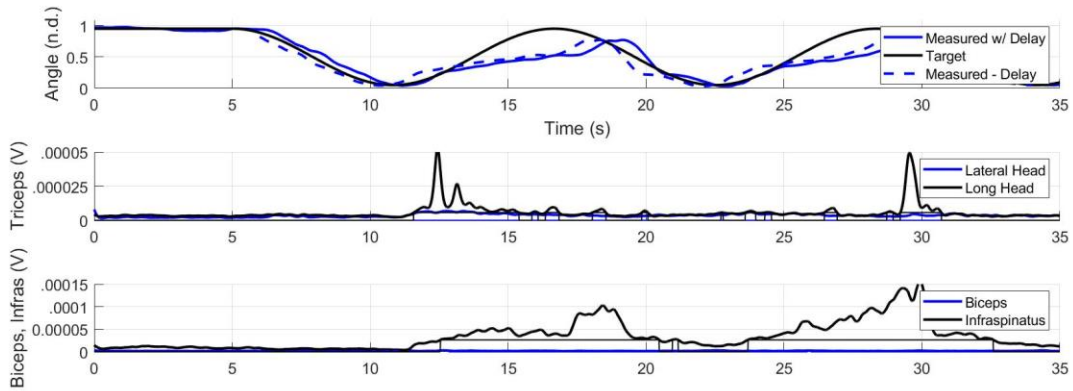
Other patient outcomes

To achieve a comprehensive evaluation of the patients, post-operatively, we examined other outcomes. Elbow flexion and extension strength were measured at different angles using a dynamometer to determine change in strength over time. COPM was measured by patient self-reporting and was used

6 month



12 month



18 month

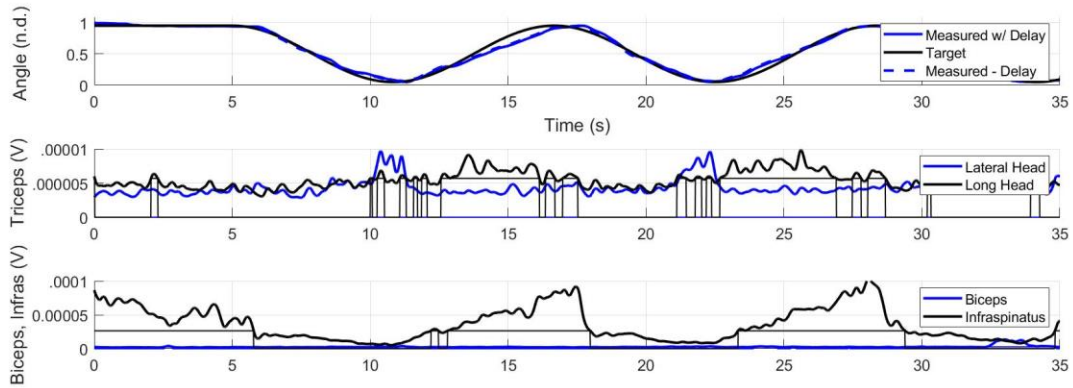


Figure 6.2: The change in elbow control of Patient 2, 6, 12, and 18 months following surgery. The graphs show a sine curve (black) that the patient attempted to follow (blue).

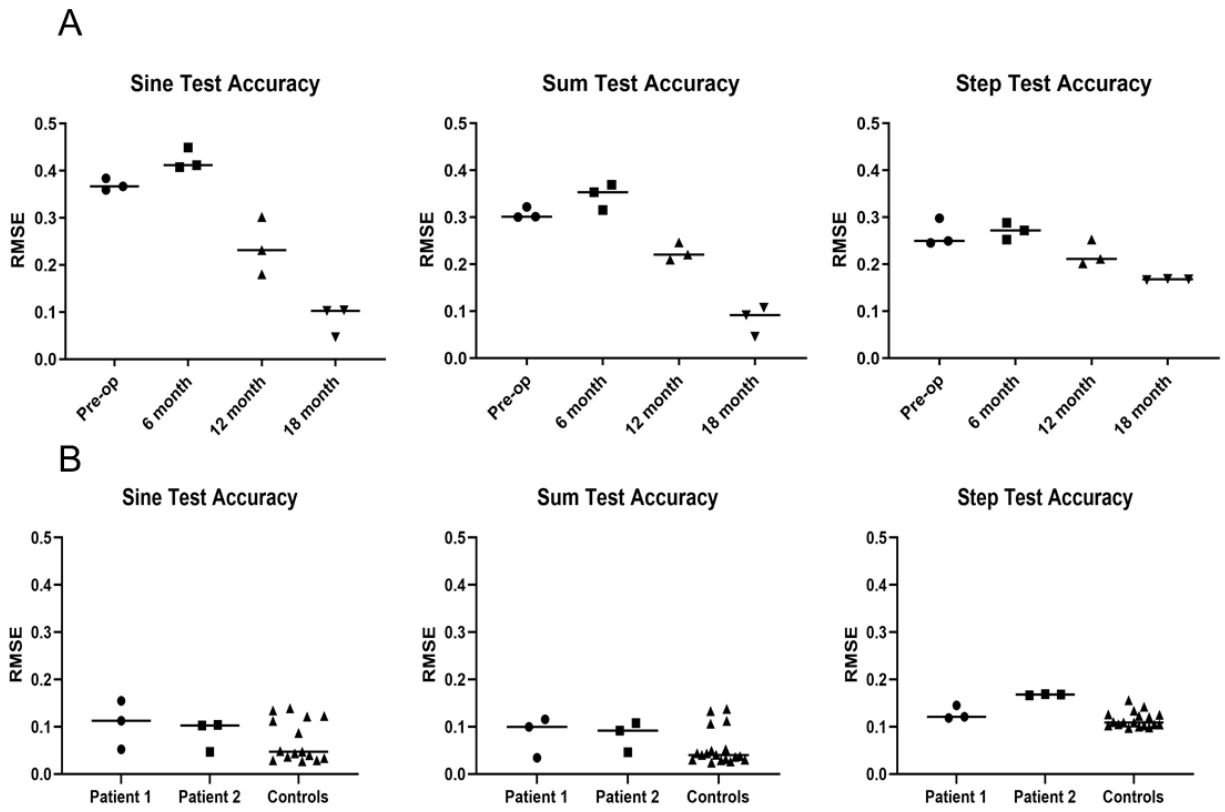


Figure 6.3: (A) Quantification of test accuracy of Patient 2 over time. Significant improvement was made over time in all testing categories. (B) Quantification of Patient 2's results as compared to Patient 1 and control subjects. Difference between patients and control subjects was only significant in the step test results.

to examine patient satisfaction after recovery. In a comparison of elbow extension strength, control subjects exhibited much higher strength than the patients (Figure 6.4A). Patient 2 self-reported a higher performance and satisfaction 18 months post-operatively when compared to pre-operatively (Figure 6.4B).

6.5 Discussion

When evaluating the results of a surgical procedure to restore neuromuscular function, many factors should be considered. These include patient satisfaction as well as a comparison of the function to what would be considered normal. Because so many outcomes are subjective, it is important to quantify when possible and include a comprehensive examination of many factors to better understand the recovery of the patient.

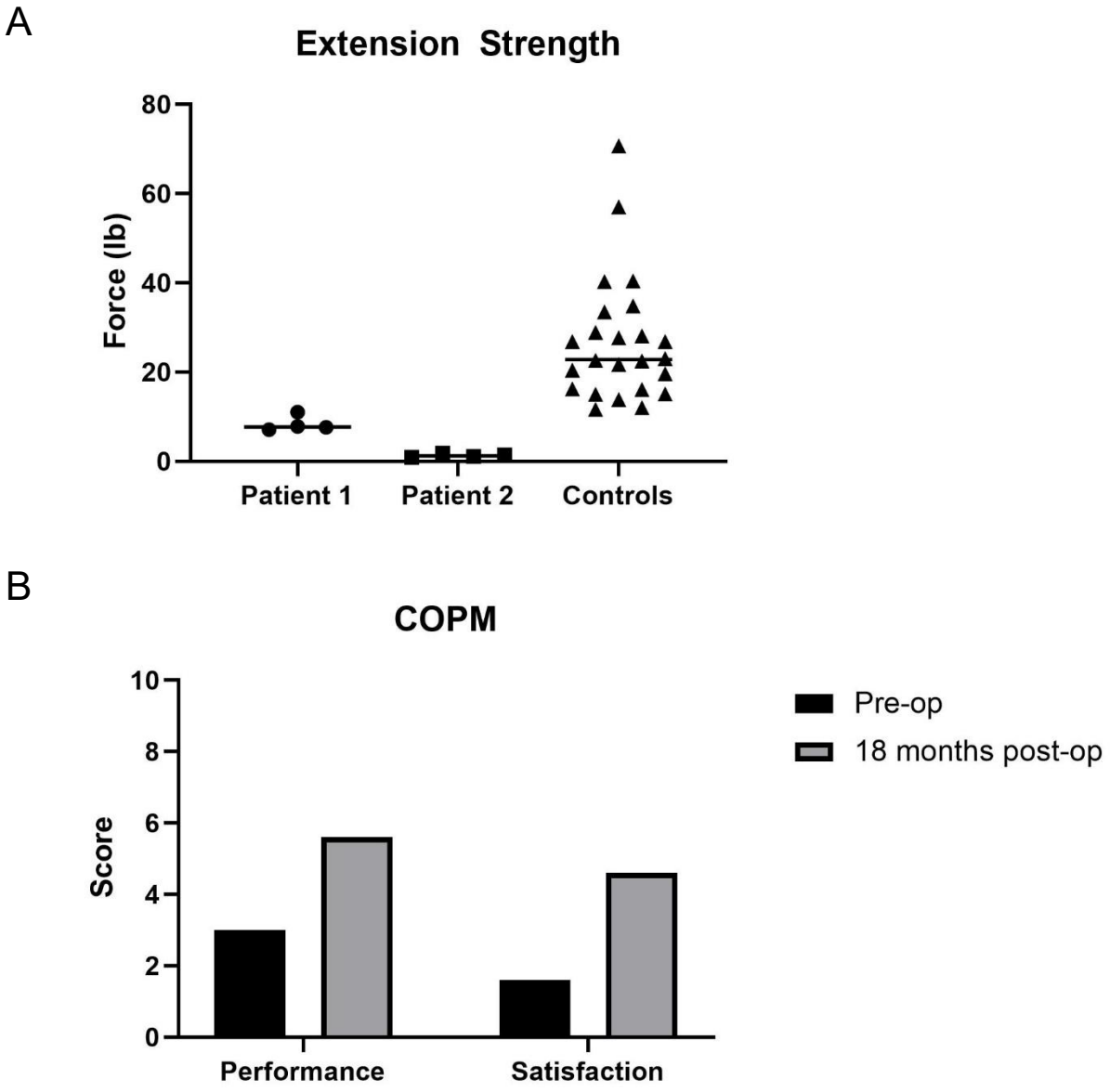


Figure 6.4: (A) Elbow extension strength comparison post-surgery for Patient 1 and 2 in comparison to control subjects' strength. (B) Change in COPM measures of performance and satisfaction of Patient 2, 18 months post-surgery.

Main findings from quantifying control

We found that our tests were sensitive enough to reveal interesting information about the recovery of the patients over time. Patient 2 had an interesting recovery, where range of motion and accuracy both recovered as the time following surgery increased. Patient 2 was also more satisfied with

his daily activity performance 18 months following the surgery, as evidenced by the higher COPM score. If only strength had been tested, the likely reasons for this satisfaction would not have been clear, which is one reason why a comprehensive evaluation is useful and necessary.

When comparing the results of patients' control 18 months post-operation to the results of the control subjects, the results are similar. Two tests showed no significant differences between patients and control subjects; however, the step trials did show a significant difference. This is likely because this test requires more strength and control to make the large changes in elbow angle very quickly, which the other tests do not require, being smooth curves. This test allowed us to identify a key difference between patients and control subjects, which was then corroborated by the results of the elbow extension strength testing.

Future Directions

Though we examined the results of only two patients in this study, we plan to test much larger patient sample sets in the future, as well as compare our results to patients who receive other treatments to regain elbow extension following spinal cord injury. Given that our data shows sensitivity in changes over time and in differences between patients and control subjects, we believe that our comprehensive method of evaluation would not only be informative on the best method for treatment (using the same methods to compare to the results of other procedures, such as tendon transfers) but also in understanding the important factors involved in neuromuscular recovery following SCI.

6.6 Acknowledgements

Chapter 6, in part, is currently being prepared for submission for publication. My co-authors are Antoinette Domingo, Shawn O'Connor, Justin Brown, and Sameer Shah. The dissertation author is the primary author of the paper. I would also like to acknowledge the generous grant from the Craig H. Neilsen Foundation

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Chapter 7: Conclusions

7.1 Summary of findings

Peripheral nerve injuries are an important area of research. Many suffer from the unfavorable outcomes of recovery following nerve injury. The aim of this dissertation was to examine new methods for treating nerve injuries in the hope that the findings from this research could be used to improve the lives of those affected by these injuries.

In this dissertation, I examined the role of tension in regeneration of the peripheral nerves. I found that tension is not detrimental in end-to-end repairs. In acute injuries, end-to-end repairs performed after lengthening to bridge large nerve gaps had equal or better results when compared to gaps repaired using an autograft. In chronic injuries, I found the results of a lengthening and an isograft to be equivalent, though lengthening the nerve did avoid the need to harvest a graft. By examining protein regulation using proteomics, I found that lengthening positively affected transcription following injury. Finally, I examined a new, more comprehensive, method of assessment for recovery following nerve transfer surgery.

7.2 Future directions

Nerve lengthening

While our results showed promise for peripheral nerve lengthening as a regenerative strategy, there is still work to be done. While preliminary results for much longer nerve gaps (currently being tested in rabbits) are positive, further experiments must be done to have sufficient data to confirm our hypothesis that rabbits with lengthened nerves will perform equal or better to those with an autograft. The ability to lengthen a nerve over a longer distance has important clinical implications. If a nerve could be lengthened any distance, as long as it is done carefully enough (by taking maximum strain of the nerve into account), this strategy could be used for surgeries that otherwise have would not have been thought to be feasible. These include situations such as a nerve transfer that cannot occur because the donor and

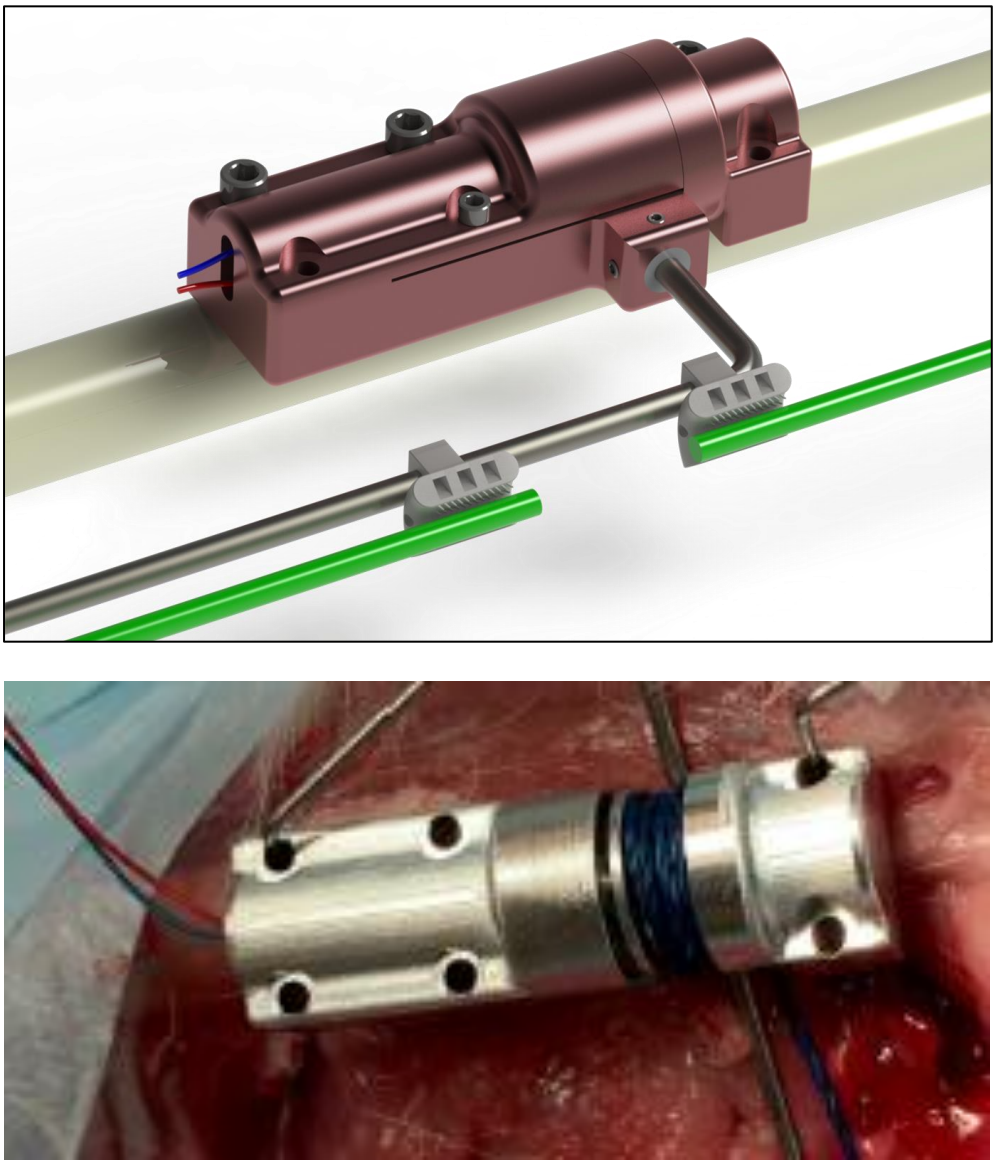

Figure 7.1: New motor for lengthening device which will perform actuation automatically by pulling the guidewire around a spool in set increments. The top image shows a CAD rendering of the motor attached to a nerve clamping/guidance system, very similar to what was used to perform the experiments detailed in Chapters 3 and 4. The bottom image is the motor attached to the femur in a cadaver.

recipient nerves are too far apart, a very large nerve gap that would likely have very poor outcomes if a long graft was used, or a nerve that is stretched to its target muscled and coapted directly into the muscle when the distal stump was found to be unsuitable for nerve regeneration or repair surgery.

Before the device can be used clinically, improvements must be made. One such improvement which is already in development is the addition of a motor (Figure 7.1). A motor would solve the two

main limitations of manual actuation: accuracy and external components. A prototype motor for this system has been designed which pulls the wire on a reel and is capable of pulling the wire in precise increments. The motor is also within protective casing and can be implanted entirely internally. This would make the device much more attractive in a clinical setting, because of both the ease of accurate actuation and the much lower risk of infection if the device is completely internal.

Chronic injuries

There are still gaps in our understanding of chronic nerve injury. While we know that nerve injuries that receive a delayed repair are less likely to achieve good functional results than nerve injuries that receive immediate repair, the precise reasons for these outcomes are still debated. We found that lengthening in a chronic model was equivalent to an isograft but can still only hypothesize on the reasons for this result. The very poor quality of the distal stump months after Wallerian degeneration begins is certainly a factor in the results, and chronic injury is one example of where a direct muscle coaptation of the nerve following lengthening could be beneficial. Alternatively, strategies for preventing muscle atrophy and endplate degeneration may also be useful when combined with nerve regeneration strategies. Further research must be done to determine if combined treatments, including combining current treatments with lengthening, will be effective in improving functional outcomes of chronically injured nerves. Additional analysis of the regenerative environment of chronic injury could aid in selecting the appropriate paths to continue research.