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Neuronal activity as a therapy for peripheral nerve injury.

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B.S., Haverford College, 2009

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An Abstract of a Thesis

Submitted to the Faculty of the James T. Laney

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Abstract

Neuronal activity as a therapy for peripheral nerve injury.

Author: Laura Jones

Moderate daily exercise is an effective way to enhance axon regeneration and improve functional recovery from peripheral nerve injury. Increased neuronal activity associated with locomotion has been hypothesized to be the basis for this facilitation, but other factors also have been suggested. Neuronal activity can be produced separately from these other potential sources of enhancement of axon regeneration using optogenetics. We hypothesized that increased neural activity would be sufficient to promote axon regeneration after peripheral nerve transection. In mice that express a light-activated cation channel, channelrhodopsin2 (ChR2), and yellow fluorescent proteins (YFP) in neurons, we activated axons in the sciatic nerve using blue light immediately prior to transection and measured axon regeneration at three time points: two, three and four weeks post transection. By two weeks after injury, optical activation enhanced axon regeneration to the same extent as electrical stimulation. Treatments with brief electrical stimulation or optical activation just prior to nerve transection and repair both resulted in larger M responses three weeks later, but the optical treatment led to nearly exclusive enhancement of regeneration of axons which were ChR2+. By four weeks after sciatic nerve transection and repair, significant reinnervation of muscle fibers had occurred in all animals studied. In mice treated optically, all of the re-occupied motor end plates were ChR2+, consistent with selective enhancement of optically activatable motor axons. These anatomical observations are consistent with our hypothesis that increased neuronal activity is adequate to promote axon regeneration after peripheral nerve injury.

Neuronal activity as a therapy for peripheral nerve injury.

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Introduction

Peripheral nerve injuries are common—damage to at least one peripheral nerve is found in almost 3% of trauma patients (Noble et al., 1998), resulting in approximately 200,000 new patients a year (Taylor et al., 2008). The number of non-traumatic peripheral nerve injuries may be much larger. The gold standard of care for patients with nerve transections is surgical: the two ends of the damaged nerve are sutured together such that there is no tension in the nerve. Despite advances in surgical techniques over the past 30 years, only 10% of patients recover movement in the injured limb (Scholz et al., 2009). This means that *at least* 180,000 Americans *each year* are left permanently debilitated due to damage to peripheral axons. This poor functional outcome is most often attributed to the slow regeneration of damaged axons, which has led researchers to seek alternative therapies to enhance axon regeneration after transection and repair.

In 1979, Hoffer et al. showed that, in recordings from a neuroma, the ineffective and unregulated regeneration of a cut nerve, motor axons continued to be active months after the initial injury and did not continue to atrophy. Sensory axons, which stopped being active shortly after transection, presumably due to a lack of input from the periphery, continued to atrophy until the neuron eventually died if regeneration was inhibited. Hoffer et al. (1979) posited that the motor axons were spared this atrophy because of the consistent spike trains originating from circuitry within the spinal cord. They theorized that there was a signal keeping the motor axons from atrophy, which the sensory axons were not getting. They put forth the following two scenarios: 1) the action potential formation that is driven in motor axons by central nervous system circuitry, which the sensory neurons no longer received after transection, *was* the signal; 2) sensory neurons were more susceptible to atrophy than motoneurons because of a lack of an essential neurotrophic factor.

Nix and Hopf (1983) followed up on scenario 1, above, by applying electrical stimulation to cut peripheral nerves to see if activity facilitated axon regeneration. They found that application of electrical stimulation at a constant rate of 4Hz over the course of recovery led to increased functional recovery of the soleus muscle in rabbits. They chose 4 Hz because the soleus muscle is composed of slow-twitch muscle fibers that inherently fire at a slow rate. Motoneurons innervating fast twitch muscle fibers tend to fire faster than those innervating slow twitch fibers (Hoffer et al., 1987, Loeb et al., 1987).

Since then, more activity-based therapies—therapies which induce action potentials in injured neurons —have been characterized. For motoneurons, these kinds of therapies include active exercising like forced running on a treadmill (Sabatier et al., 2008a) or swimming (Teodori et al., 2011), as well as more passive movements, such as in rodents who are anesthetized and attached to custom bicycle pedals (Udina et al., 2011), or direct electrical stimulation of the axons (Al-Majed et al., 2000b, Brushart et al., 2002, English et al., 2007b, Asensio-Pinilla et al., 2009, Foecking et al., 2012). All of these therapies enhance the regeneration of injured axons in peripheral nerves, but whether the increased activation of the injured neurons is the basis for this enhancement has not been rigorously tested. We hypothesize that the increased neuronal activity associated with these treatments is the likely stimulus for the enhancement of axon regeneration, but the extent to which activity must be increased, and the intensity of activity needed to promote axon regeneration is not known. Without knowledge of these requirements, the translation of these basic scientific findings to treatment of the heterogeneous population of nerve injuries observed clinically will be limited.

Unfortunately, the utility of the contrast to the two scenarios put forth by Hofer et al (1979) – activity vs. neurotrophic factors – may be questionable in today's context. Considerable evidence exists that the effectiveness of activity-based therapies is

dependent on neurotrophic factors, in particular, Brain Derived Neurotrophic Factor (BDNF) (Frostick et al., 1998, Al-Majed et al., 2000a, English et al., 2007b, Gordon, 2009, Gomez-Pinilla et al., 2011, Wilhelm et al., 2012). In animals in which the gene for BDNF is knocked out, axon regeneration is severely limited, even after being exercised or with the aid of electrical stimulation, unless exogenous BDNF is added back into the system.

Both Schwann cells and neurons with axons in the periphery express BDNF. Expression of BDNF by motoneurons is known to be increased in the first 2-3 days following nerve transection and then dramatically decreased (Al-Majed et al., 2000a, Geremia et al., 2007). Its expression is increased in Schwann cells of the distal segment of cut nerves, beginning 2-3 days after injury, and stays up-regulated for several weeks, while axons are regenerating (Frostick et al., 1998, Gordon, 2009).

Increased neural activity results in an increased expression of BDNF in neurons (Hong et al., 2008, Gomez-Pinilla et al., 2011, Zheng et al., 2011). The gene for BDNF contains at least 11 promoter regions which can be differentially spliced to form as many as 17 different mRNAs, each encoding the same protein, presumably allowing multiple levels of control of BDNF expression (Timmusk et al., 1993, Aid et al., 2007, Pruunsild et al., 2007). One of these 3' promoters of the BDNF gene, exon VI, has been shown to be stimulated in response to intracellular calcium influx (Hong et al., 2008) and generation of the cyclic AMP response element binding protein (CREB) (Zheng et al., 2011). Thus, the gene for BDNF, a critical neurotrophic factor to the success of activity-based therapies, can be regulated in an activity-dependent manner.

Because there are so many promoters of the BDNF gene, and several have been shown to be upregulated in response to a variety of stimuli (Pruunsild et al., 2007), it is challenging to tease apart whether increased neuronal activity is the only reason for increased BDNF expression and enhanced axon regeneration in activity-based

therapies. The potential consequences of activity-based therapies are widespread—exercise effects everything from oxygenation and hormonal levels, to changes in environment. Electrical stimulation is more specific, but still stimulates the entire nerve and has the potential to spread current throughout the body and certainly into adjacent tissues. Until recently, electrical stimulation was the most specific way to increase activity in transected neurons. Optogenetics—the use of mouse genetics to target expression of optically activatable channels in a cell-type specific manner—enables researchers to activate a specific cell-type without inducing changes in surrounding tissues. In this study, we have used a mouse that expresses an optically activatable cation channel, channelrhodopsin2 (ChR2), under the control of the *thy1* promoter, which is expressed in a subset of neurons, including both sensory and motoneurons with axons in the sciatic nerve. In this mouse the targeted neurons can be activated by blue light (473nM). By exposing transected neurons to blue light, we are able to restrict the effect of activity-based therapies to an identified subset of the neurons of an axotomized nerve. This specificity is also incredibly temporally sensitive because the cation channels expressed in the specific cell types are open only during exposure to the specific wavelength of light, which is only available during activation sessions. Optical activation has the additional benefit over electrical stimulation of eliminating the chance of current spread during activation because no current is introduced. Finally, electrical stimulation recruits axons based on length constant (i.e., the easier it is for electricity to pass along an axon, the sooner it will be recruited through electrical stimulation), so that axons are recruited from largest to smallest. In contrast, optical stimulation recruits axons into activity in a more physiological manner (Llewellyn et al., 2010), from smallest to largest, because the light is used to open cation channels that are more dense in smaller diameter axons than larger ones. At least for motoneurons, this size-ordered recruitment into activity is the same as observed *in vivo* (Henneman and Olson, 1965).

Our overall approach uses the technique of optical activation and mouse genetics to evaluate whether neuronal activity is sufficient to promote axons in the sciatic nerve to regenerate after peripheral nerve injury. We compared the effect of optical activation to electrical stimulation, treadmill training, and untreated controls to determine if this specific activation was enough to promote axon regeneration, and then evaluated functional recovery to see if activation of a subset of neurons led to a facilitation of axon regeneration in only those neurons that were activated, or if there was a more general increase in regeneration of all axons, even those not activated during the cell-type specific activity-based therapy sessions. We hypothesized that activating a subset of neurons would facilitate axon regeneration in those neurons alone, and not in neighboring axons that were not activated during the optical activation sessions.

Activity-based therapies are novel and potentially exciting treatments for patients with peripheral nerve injuries because they are effective and easily applied. There are many aspects of these therapies, however, that have not been thoroughly researched, leaving clinicians with questions about how to most effectively implement these treatments. We believe that *the* critical feature of exercise is that it increases the activity of axotomized neurons and stimulates the regeneration of their axons. The nature of, and limits to, this effect of increased activity, however, are unknown. This study outlines our ability to optically activate motor axons in transected sciatic nerves and assay their recovery, both anatomically and functionally. This will allow clinicians to be better able to design effective, activity-based therapies for patients with a variety of peripheral nerve injuries.

Materials and Method

Animals and surgical methods. All procedures were approved by the Institutional Animal Care and Use Committee of Emory University and conformed to the Guidelines for the Use of Animals in Research of the Society for Neuroscience. All mice used were on a C57BL/6J background, were backcrossed for at least six generations, and expressed the ChR2-YFP construct under the direction of the *thy-1* promoter (Jax #007612) (Arenkiel et al., 2007). In these mice, a subset of all motoneurons with axons in the sciatic nerve express both channelrhodopsin2 (ChR2) and yellow fluorescent protein (YFP).

Experiment Name	Groups Used	Stimulation	Length of Experiment
Graft Experiment	Exercise (N = 3)	10 m/min, 5 days/wk	2 weeks
	Electrical Stimulation (N = 5)	1 hr, 20 Hz 100% intensity	
	Optical Stimulation (N = 3)	1 hr, 20 Hz 100% intensity	
	Untreated (N = 2)	None	
EMG Experiment	Electrical Stimulation (N = 4)	1 hr, 20 Hz 100% intensity	3 weeks
	Optical Stimulation (N = 3)	1 hr, 20 Hz 100% intensity	
Force Experiment	Electrical Stimulation (N = 3)	1 hr, 20 Hz 100% intensity	4 weeks
	Optical Stimulation (N = 5)	1 hr, 20 Hz 100% intensity	
	Untreated (N = 4)	None	

Experiments were conducted on adult (older than 2 months old) male mice weighing 18 – 28 g. In previous reports from this laboratory, a marked sex difference in the requirements for the effective use of exercise to promote axon regeneration was found (Wood et al, 2012). Although no similar sex difference was found for the effectiveness of brief electrical stimulation to enhance axon regeneration in cut nerves exists (Thompson et al, 2013), we chose to perform all experiments in male mice to avoid any unknown sex difference in the effects of optical stimulation. A mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg) was used for anesthesia, with additional ketamine as needed throughout the procedure.

Graft experiments. The common fibular (CF) nerve was exposed and cut 1 mm distal to its branching from the sciatic nerve. A segment of the CF nerve (8 –10 mm long) was harvested from a strain-matched wild-type (WT) donor mouse that did not contain the YFP transgene. The proximal end of the donor graft was opposed to the proximal segment of the cut nerve of the host mouse on a small rectangle of SILASTIC film (Dow Corning 501-1) and secured using 8 µl of fibrin glue (de Vries et al., 2002; MacGillivray, 2003). The distal end of the donor graft was not secured to the host's distal stump to prevent any influence by potential-target-derived molecules. All surgeries were performed bilaterally.

Treatment groups. Four treatment groups were studied: treadmill training, electrical stimulation, optical activation, and untreated controls. For both the electrical stimulation and optical activation groups, an acute stimulating cuff-type electrode (Stein et al., 1977) was placed around the common fibular nerve, bilaterally, immediately prior to transection. Optical cuffs were created using 2 ultra-miniature blue LEDs (Digikey 511-1615-1-ND) which were connected together by fine wire (California Fine Wire AS631) and solder, such that the LEDs were parallel and approximately 1 mm apart. This construct was then threaded into a silastic tube (Dow Corning 508-006) cut longitudinally to create a cuff similar to the design of the electrical cuff and held in place with a Medical Adhesive A and epoxy so that no wires or soldering points were exposed, but the light sources were uncovered. The nerves were then optically activated or electrically stimulated according to its treatment group at 20 Hz for an hour at an intensity level high enough to induce maximal responses. Similar treatments were applied to both limbs. The absolute intensity of these stimulations were different between mice, but were determined by inducing maximal participation in each mouse empirically. In untreated controls no stimulation or activation was applied. Treadmill training began the third day after nerve transection. In the treadmill training groups, mice

ran for one hour per day on a motor-driven treadmill five days a week for two weeks at 10 m/min.

After two weeks, all animals were deeply anesthetized with pentobarbital (150 mg/kg, i.p.) and perfused transcardially with 0.9% saline and 4% paraformaldehyde. Thirteen repaired nerves were harvested and mounted onto slides using VectaShield (Vector Labs) and stored at 4°C.

Axon length analysis. The confocal imaging and measurements of regenerating axon profiles were performed as previously described (English et al., 2005; Groves et al., 2005). Briefly, whole-mounted nerves were imaged at a low magnification using a Leica LSM SP8 confocal microscope. Stacks of serial optical sections (10 µm thick) were stitched together using the Leica software such that the entire length of the repaired nerve and graft was reconstructed in three dimensions. The lengths of ChR2-YFP axon profiles were measured using ImageJ software.

Statistical analysis. Data are expressed as the average ± SEM. Differences in the median lengths of axon profiles were assessed using a one-way analysis of variance (ANOVA). Median lengths were used because regenerating axon profile lengths are not normally distributed (English, 2005). The distribution of the medians, however, is parametric, so ANOVA were used to compare the medians of each group. Paired, post-hoc comparisons were made using Fisher's least significant differences (LSD), where appropriate. Significance was set at $p<0.05$.

Three week EMG experiments. In seven thy-1-ChR2 mice, the right sciatic nerve was exposed and an electrical stimulating cuff was placed around the whole sciatic nerve. Fine wire electrodes were placed into the gastrocnemius (GAST) muscle. Evoked EMG activity recorded from these electrodes in response to sciatic nerve stimulation (0.1 ms pulses at 0.5 Hz) included a short latency direct muscle (M) response produced by motor axon activation. Stimulus intensity was then slowly

increased, in increments of no more than 0.07 volts, until a maximal M response was recorded. Then the electrical stimulation cuff was replaced with an optical cuff, and the process repeated using optical activation. During the ramp of increasing optical intensity, the luminosity delivered changed in increments of less than 0.5 $\mu W/mm^2$. The order of type of stimulation presented was varied.

Once both sciatic nerves had been tested for responses to both electrical and optical stimulation, a cuff was placed around the right sciatic nerve and used to treat the nerve with *either* optical *or* electrical stimulation, for one hour at 20 Hz, at a stimulus intensity that evoked a maximal EMG response. This brief electrical stimulation protocol was developed to enhance axon regeneration by Gordon and colleagues (Al-Majed et al, 2000). It should be noted that pre-treatment testing to establish M response amplitudes, using both types of stimulation paradigms, led to an average of 600 stimulus presentations in each mouse, delivered no more frequently than two seconds apart (0.5 Hz). In contrast, the activity-based treatments applied consisted of 72,000 stimuli, delivered over an hour at a rate that was 40 times faster. After treatment, the right sciatic nerve was cut and repaired by end-to-end anastomosis, as described previously in detail (English, 2005, English et al., 2007a). Briefly, a small rectangle of SILASTIC film (Dow Corning 501-1) was placed under the sciatic nerve proximal to the separation of the sural, common fibular and tibial nerves, and then 8 µl of fibrin glue was placed on top of the SILASTIC film, surrounding the intact sciatic nerve. Once the fibrin glue had set, a small pair of sharp scissors was used to cut the sciatic nerve completely. More fibrin glue was then applied as needed to ensure stability before closing the leg.

Three weeks later, in anesthetized mice, both sciatic nerves were exposed again, but proximal to the transection site. The nerves were re-tested, using both optical and electrical activation, according to the pre-transection protocols.

Statistical analysis. Data are expressed as the average ± SEM. Differences in the averages of the M response amplitude were assessed using a one-way analysis of variance (ANOVA). Paired, post-hoc comparisons were made using Fisher's LSD, where appropriate.

Four week force experiments. The sciatic nerve was exposed as described above, except that experiments were conducted after a four week survival period. All experiments were conducted bilaterally. Prior to nerve transection, an S-shaped steel hook was placed beneath the Achilles tendon, against the calcaneus and connected directly to a custom built force transducer. The mice were stabilized mechanically by taping them to a surgical board, and the force transducer was clamped to the same board to increase mechanical continuity. Sciatic nerve stimulation resulted in contraction of the ankle extensor muscles and produced an isometric twitch measured by the force transducer. Muscle length was adjusted until sciatic nerve stimulation evoked a maximal twitch response. Fine wire EMG electrodes were placed into the belly of the GAST (GAST) muscle. The sciatic nerve was then stimulated, both electrically and optically, as described above, and twitch forces were recorded. The nerve was then treated, and cut and repaired as above. Three treatment groups were used for this experiment: Electrically stimulated (3 nerves), Optically activated (5 nerves) or untreated (4 nerves).

Four weeks later, in anesthetized mice, both sciatic nerves were exposed again, but proximal to the transection site. The nerves were re-tested for force, using both optical activation and electrical stimulation, according to the pre-transection protocols. Then GAST, soleus and intact triceps brachii muscles, as well as intact radial and both transected sciatic nerves were then harvested for histological analysis.

Single Motor Unit Force calculations (SMUF). We used a version of the incremental twitch subtraction motor unit number estimation method (ITS-MUNE) that was first described by McComas et al. (1971), but used evoked forces rather than EMG

measurements. The validity of this method is described in detail by Major et al. (2007). It uses incremental stimulation of a peripheral nerve to recruit motor units and measures single motor unit twitch force from changes in twitch force elicited by small increases in stimulus intensity. Stimulation, either electrical or optical, was begun at an intensity below the threshold for force production and slowly increased in intensity until maximal twitch force was recorded. The difference in stimulation intensity between these increments was no more than 0.5 $\mu W/mm^2$ for optical stimulation and 0.07 volts for electrical stimulation. Because our stimulation steps were very small, repeated presentations of the same stimulation intensity were not needed. The resulting force trace from each stimulus trial was subtracted from that of the preceding trial, and incremental differences were then considered candidates for the force produced by a newly recruited motor unit. For such candidates to be considered single motor unit twitches, the incremental difference trace had to yield a positive peak force. Alternation of motor unit recruitment sometimes resulted in negative potentials in the incremental difference traces (Major et al., 2007). It is possible that at greater stimuli intensities, the muscle might be expected to be more stiff because the background force is higher. This higher background force could contribute to larger twitch forces being recorded from later recruited motor units, but it would be difficult to determine exactly how much. Because of this potential error in our measurements, we did not sample motor units whose twitches were extracted at stimulus intensities evoking greater than 50% of maximal whole muscle twitch tension. All incremental differences which met these criteria were then evaluated visually before final acceptance as a single motor unit twitch, as described by Major et al. (2007). The average peak force from all accepted single motor unit twitches was used as the average single motor unit force. For each mouse studied, an estimate of the single motor unit force strength activated electrically

and optically was made bilaterally, both before and after transection and repair of the sciatic nerve.

Statistical analysis. Data are expressed as the average ± SEM. Differences in the medians of the maximal force twitch and single motor unit force (SMUF) were assessed using a Mann-Whitney U test.

Analysis of muscle reinnervation. The reinnervated GAST and soleus muscles and the intact triceps brachii muscles were harvested at the end of the post-transection testing experiment in each mouse, and fixed in 4% paraformaldehyde overnight before being cryoprotected in 20% sucrose for three days. Muscles were then sectioned on a cryostat in a horizontal plane at 20 µm thickness and reacted with a monoclonal antibody to the synaptic vesicle protein, SV2 (Developmental Studies Hybridoma Bank) for 72 hours at 4º C, followed by a goat anti-mouse immunoglobulin secondary antibody conjugated to Alexafluor 647 (Life Technologies D22914), and rhodamine-conjugated α-bungarotoxin (Life Technologies B35451). In each muscle studied, fluorescent images of 50 motor endplates, marked by bungarotoxin binding, were obtained and scored for immunoreactivity to SV2, indicating the presence of a neuromuscular synapse, and YFP, indicating innervation by a light-sensitive (ChR2+) motoneuron. The percentage of reinnervated (SV2+) GAST and soleus motor endplates, as well as the percentage of those endplates containing YFP, were compared between treatment groups and to that found in the intact triceps brachii muscles. Significance of differences between groups was evaluated using ANOVA and appropriate post hoc paired testing, as described above.

RESULTS

Expression of ChR2-YFP

The ChR2-YFP construct is present in both sensory and motor axons (Figure 1A). In dorsal root ganglia (DRG), high expression patterns can be seen preferentially in larger cell bodies and their axons. Intensity of YFP expression is greater around the edge of the cell bodies (Figure 1B). In the spinal cord, however, the YFP is rarely seen in the somata of motoneurons and never to the same extent found in DRG neurons, even after signal amplification with anti-GFP antibodies. Because YFP is sometimes seen in the proximal processes of motoneuron dendrites, we assume that Chr2-YFP is very efficiently transported into the dendrites and axons of motoneurons. Expression within axons often is not uniform—small, punctate regions of the axons can be seen in whole mount nerves (Figure 1C), a pattern reminiscent of para-nodal expression. In histological sections of the sciatic nerve, YFP expression is more uniform.

Optical activation reliably elicits EMG responses in a physiological manner

In EMG recordings taken from the GAST muscle as the intensity of either electrical stimulation or optical activation, expressed as a multiple of threshold, was slowly increased (Figure 2A), optical activation recruits axons in a physiological manner (Llewellyn et al., 2010) because smaller axons tend to have a higher density of the cation channels and so are depolarized more easily than larger axons (Foutz et al., 2012). Electrical stimulation, however, recruits axons based on their length constant—meaning larger axons get recruited more easily than smaller axons because there is less resistance to electrical current passing along larger axons. Thus, a larger percentage of the total EMG response is elicited at threshold through electrical stimulation. In contrast, because only the smallest axons are recruited at threshold during optical stimulation, there is a much more gradual increase in EMG response as intensities increase above

threshold. At least for motoneurons, this size-ordered recruitment into activity is the same as observed *in vivo* (Henneman and Olson, 1965).

Optically activatable axons are responsive to 473 nm light, and in this paper were activated with cuffs containing two ultra-miniature blue LEDs. We recorded EMG activity easily following trains of activations up to 20Hz with 1ms stimulus pulses (Figure 2B). The cuffs were tested for illumination output, which was linear in the voltage range used during these experiments (Figure 2C).

Optical activation facilitates axon regeneration

The Common Fibular (CF) nerves of *thy-1-ChR2-YFP* mice were either treated with an hour of optical activation or electrical stimulation or were untreated, then cut and repaired with 10-15 mm-long grafts harvested from the CF nerve in a non-fluorescent mouse (Figure 3A). Untreated *thy-1-ChR2-YFP* animals were then either run on a treadmill for two weeks at 10m/s for an hour 5 days a week or were left untreated. Two weeks post-nerve repair, host mice were perfused, and the regeneration of *ChR2-YFP*$^{+}$ axon profiles was analyzed using confocal microscopy. We found that the lengths of axon profiles from treated animals—either electrically, optically or exercised, were significantly increased, relative to untreated mice. The omnibus test of the ANOVA of the median lengths of axon profiles after each treatment type (Figure 3B, inset) was significant ($F_{3, 9}$=14.884, p<0.01). The average median length of axon profiles from all treated nerves was significantly longer than that measured in nerves from untreated mice (LSD, $p < 0.03$). No significant difference was found in the distributions of axon profile lengths or in the median lengths of axon profiles between electrical and optically treated animals (p=0.28). The median axon profile length of treadmill trained animals was significantly longer than either stimulation treatment ($p < 0.01$). These data are consistent with previously published results, where electrical treatment and exercise

were shown to promote axon regeneration after transection (Al-Majed et al., 2000b, Brushart et al., 2002, English et al., 2007b, Sabatier et al., 2008b, English et al., 2009, English et al., 2011, Wilhelm et al., 2012).

Muscle response EMG recordings.

The right whole sciatic nerve of *thy-1-ChR2-YFP* mice was treated either with an hour of optical activation or electrical stimulation, and then cut and repaired to itself through a tensionless end-to-end anastomosis. Immediately prior to treatment, EMG recordings were made of the M response elicited by both blue light and electrical current. Three weeks post repair, mice were re-tested, and M responses were analyzed (Figure 4).

Because only a subset of all motor axons are ChR2+ in these animals, optical activation would be expected to elicit a smaller M response than electrical stimulation. We compared the ratios of the M responses evoked after electrical treatment with those elicited by optical treatment, as well as the ratios within treatments, pre- vs. post-transection. The omnibus test of the ANOVA of these ratios was significant ($F_{7, 25}=3.0665$, $p<0.02$). We found the difference in M response amplitudes elicited after electrical treatment to be significantly larger than M responses evoked in optically treated animals (LSD, $p < 0.05$).

We also hypothesized that by activating this subpopulation of motoneurons optically, enhanced regeneration and functional reformation of neuromuscular synapses will occur only in ChR2/YFP+ axons. Thus, we expected that most, if not all, of the measured regeneration to occur in optically activatable axons after optical treatment. Because electrical stimulation activates both ChR2+ and ChR2- axons indiscriminately, we expected to find a difference in amplitude between the M response elicited by electrical stimulation and optical activation. Activating the intact sciatic nerve with blue

light yielded approximately half the maximal M response amplitude of electrical stimulation in intact animals (LSD, $p < 0.02$). This same statistically significant difference between the optically evoked M response and the electrically evoked M response was seen three week after transection in electrically treated animals ($p < 0.03$). After optical treatment, the difference between optically and electrically elicited M responses was not statistically significant ($p = 0.83$). This indicates that all the axons that had regenerated after transection and formed functional neuromuscular synapses were ChR2+ after optical treatment, while only ca. 20% were optically activatable after electrical treatment.

Whole muscle force recordings.

Both whole sciatic nerves of 15 *thy-1-ChR2-YFP* mice were treated either with an hour of optical activation or electrical stimulation or were untreated, and then cut and repaired to themselves through a tensionless end-to-end anastomosis (Figure 5A). Immediately prior to treatment, force measurements were made in response to application of both blue light and electrical current to the nerve proximal to the transection. Four weeks post-repair, mice were re-tested, and force measurements were repeated. Each animal served as its own control, because each animal was tested in the same way both before and after transection and those measurements were analyzed as repeated measures.

The average ratios of Post vs. Pre twitch forces (±SEM) are shown as a measure of the extent of recovery in Figure 5B. The omnibus test of the ANOVA of these ratios was significant ($F_{5, 30}=3.925$, $p<0.01$). The extent of recovery in the group treated with brief electrical stimulation was significantly less than that of either animals receiving optical treatment or untreated animals (LSD, $p<0.03$). Both optically treated and untreated controls recovered to similar levels ($p>0.05$) regardless of whether testing was done optically or electrically.

We also analyzed differences in the ratio of maximal twitch forces evoked optically or electrically in the different groups as a measure of the extent of regeneration of ChR2+ axons (Figure 5C). Based on assumptions from our three week survival time data, we expected to find that this measure of the percentage of optically activatable axons would be similar to that in untreated mice after electrical treatment, but increased in mice whose nerves had been treated optically prior to transection. What we found, however, was quite different. The omnibus test of the ANOVA for these data was significant ($F_{3, 36}$=3.036, $p<0.05$). Both optical and electrical treatments change the percentage of optically activatable axons four weeks after transection (LSD, $p<0.04$), but they both do so by decreasing the percentage of ChR2+ axons. The ratio between optically activatable and electrically stimulated twitch forces in untreated controls was unchanged from intact levels ($p=0.417$).

Treatments do not affect Single Motor Unit Force (SMUF)

We studied the forces produced by single motor units extracted by step-wise analysis (Major et al., 2007) in our different treatment groups. Forces produced by all of the extracted single motor units in each group were pooled and used to generate cumulative histograms (Figure 6). Regardless of treatment, the median of forces produced by single units in reinnervated muscles are significantly smaller than the medians measured in the same mice prior to sciatic nerve transection and repair ($U = 2072$, $p<0.01$). Prior to transection, the median of motor unit forces produced by optical activation is not significantly different than the median generated in response to electrical activation ($U = 16{,}386$, $p<0.02$). Motor unit forces are, on average, significantly smaller in all of the reinnervated muscles than in the same muscles prior to sciatic nerve transection and repair.

Motor endplate reinnervation

The re-occupation of motor endplates by terminals of regenerating axons was studied in both GAST muscles of optically treated, electrically treated, and untreated mice. The intact right triceps brachii muscle was studied as a control. Cryostat sections of muscles were reacted with an antibody to the synapse-specific protein, SV2, to mark synaptic terminals and α-bungarotoxin to mark the locations of aggregations of acetylcholine receptors at motor endplates. Fifty motor endplates were analyzed in each muscle and scored for the presence of immunoreactivity to SV2 and the presence of ChR2-YFP. Two such endplates, one containing YFP and one without YFP are shown in Figure 7A-D.

We analyzed the percentage of ChR2+ axons vs. all reinnervated motor endplates using ANOVA ($F_{7, 32}$=7.37, $p<0.01$). After optical treatment, no significant difference was found between the percentage of endplates that were SV2+ and the percent that were ChR2+ (LSD, $p=0.178$). Essentially all reinnervated motor endplates were ChR2+. In all other comparisons of total endplate reinnervation to ChR2+ reinnervation, significant differences were found (LSD, $p<0.05$), meaning that significant motor endplate reinnervation by ChR2- axon terminals had occurred. This finding is consistent with our hypothesis that optical treatment increases the regeneration of ChR2+ axons and muscle fiber reinnervation selectively.

Discussion

The main finding of this study is that brief optical activation of the sciatic nerve enhanced regeneration of axons. Two weeks after transection, the median lengths of axon profiles in all treatment groups were significantly longer than untreated negative controls. Exercised animals had median axon profile lengths which were significantly longer than both electrically and optically treated mice. There was no difference in median axon profile lengths between electrically and optically treated groups. The longer median axon profile lengths in exercised animals lengths was not surprising, given that exercised animals were treadmill trained so much more frequently than all of the other animals studied. We interpret these findings to mean that optical activation can facilitate growth in optically activatable axons

Three weeks after transection, we continued to see evidence of an enhancement after electrical and optical treatment. Based on analyses of M responses, we were able to further explore the sufficiency of activity in axon regeneration. We found that three weeks after optical treatment, regeneration of optically activatable axons was preferentially enhanced over ChR2-negative axons. This selectivity of enhancement was not found in electrically treated nerves. Total M response amplitudes in animals electrically treated immediately before transection were significantly greater than those in optically treated animals. This finding has led us to conclude that electrical stimulation is more efficient at enhancing axon regeneration and functional neuromuscular reinnervation, likely because it acts on all available motoneurons and not a subset. The lack of a significant difference between optically and electrically evoked M responses after optical treatment, however, has led us to conclude that optical activation selectively enhances regeneration in only optically activatable axons.

At four weeks after sciatic nerve transection and repair, we evaluated the effect of optical activation of regenerating axons on axon regeneration and muscle

reinnervation using elicited twitch forces and motor endplate reformation. At this survival time a different outcome was obtained. We found no significant difference in the extent of functional muscle reinnervation between optically or untreated animals. We also found a decrease in the extent of functional muscle reinnervation after electrical treatment. The percentage of ChR2+ reinnervation is also different than we would have expected. We looked at the ratio of optically vs. electrically evoked force twitches as a measure of the percentage of optically activatable motor units. Untreated controls had a much higher percentage of ChR2+ axons as measured through force recordings. There was no difference in this ratio after either electrical or optical treatment, and the percentage of ChR2+ axons in these treatment groups was measured as lower than intact or untreated controls.

There are several possible reasons why these data are inconsistent with our hypotheses:

1) We assumed, based on preliminary studies, that the optically activatable motor axons in *thy-1-ChR2* mice represented a relatively small subset of the total number of motor axons, so that substantial differences in the twitch forces produced by optical activation vs. electrical stimulation of axons in the sciatic nerve would exist. Changes in this large difference in twitch tensions due to optical treatment of regenerating axons would be readily detected. Based on the ratio of twitch tensions elicited by optical vs. electrical stimulation (see fig. 5C), or the percentage of motor endplates covered by YFP+ synaptic terminals (see Fig. 7E), we concluded that a much larger proportion of the motor axons in the sciatic nerve of these mice contained ChR2 and YFP. With such a high ratio of innate ChR2+ motor axons, it is unlikely that we would have been able to discern an increase in the percentage of these axons among those regenerating in the sciatic nerve using either twitch forces as outcome measures, regardless of treatment.

We previously estimated that the percentage of motoneurons that contained ChR2-YFP in these animals was closer to 40% based on analysis of motor end plate innervation. These preliminary data are consistent with the pre-transection M-response data in our three week experiment in which a similar ratio of optically vs. electrically elicited EMG responses was reported. It is unclear whether these data were collected on an insufficient number of mice, yielding inaccurate results, or if the transgene is unstable and is expressed more thoroughly over several generations of back-crossing, leading to the larger proportion of motor axons that express ChR2-YFP encountered in later experiments, but mice across the age range used, which were used in later experiments, expressed more ChR2 than was measured in the same line of mice previously. 2) We assumed that a longer time difference would be necessary to see differences in functional recovery, which was wrong. A shorter time frame would likely have been a better test. Untreated axons are more likely to have caught up to those stimulated to produce enhanced axon regeneration by this point, which is what we are likely seeing (Al-Majed et al., 2000b, English, 2005). Future experiments should be done with a two week survival time to compare to the anatomical data presented above.

3) It is possible that the surgery itself might act as a stimulus to optically activatable neurons because of the high light intensity that is shone on them for the duration of the surgery. All reasonable caution was used in keeping the axons from being exposed to unnecessary light, but during the surgery itself it was unavoidable. Because our untrained controls in earlier experiments did not seem to respond to the light exposure during surgery, it is unlikely that this is confounding our data, but the physiological recordings took much longer than the graft repair surgery, so it is still possible that over the longer time course the stimulation paradigm was less significant to regeneration than the inadvertent activation by the surgical lights. To ensure this is not

occurring, filters to cancel out the blue light could be placed over the surgical lights to inhibit incidental activation during this time.

4) It is possible that ChR2+ neurons are more active even without the presence of light (i.e. during recovery). The cation channel is expressed in these axons in addition to normal voltage-gated sodium channels and could cause the cell to be generally more depolarized than ChR2-negative cells, leading to increased activity in the neurons both before and after transection, regardless of our input. It is unlikely that a higher level of spontaneous activity effected axon regeneration more than our treatment because no regeneration was enhanced in ChR2+ axons in untreated controls after two weeks of recovery (Figure 3). This means that spontaneous neuronal firing due to transgene expression was not effective at enhancing regeneration after injury.

5) Because we recorded force data from the GAST muscle without cutting the calcaneus tendon; it is possible that we were unable to accurately measure the maximal force twitch responses from the muscles, either electrically or optically.

Single motor unit forces were not affected by treatment. Four weeks after transection, the median SMUF for all treatment groups was the same, which was significantly smaller than intact animals. We have concluded that treatment does not affect motor axon sprouting or efficiency at reinnervating motor end plates.

Although the force data four weeks after transection are not clear, we drew conclusions from our histological analysis of motor end plate reinnervation similar to our previous work. We found no significant difference in the total percentage of endplates reinnervated after the different treatments, indicating that the untreated control did have similar amounts of regeneration four weeks after transection, however, in all *but* the optical treatments, significantly fewer of the synaptic terminals studied did not contain YFP. After optical treatment, there was no significant difference in the proportion of

SV2+ and YFP+ terminals noted. This outcome can be interpreted to mean that after optical treatment all of the reinnervating motoneurons were ChR2+, while after electrical or no treatment a statistically significant subset of motor end plates was reinnervated with ChR2- axons. We assume, based on SV2+ proportions, that a similar number of motoneurons reinnervate the GAST muscle in all of our treatment groups, and thus concluded that in the optically treated group, there was a selective enhancement of regeneration and muscle fiber reinnervation by the activated motoneurons.

We think optical activation of transected axons is a viable therapy in mice based on our graft experiment, our data taken three weeks after transection and the histological analysis done four weeks after transection. Unfortunately, the *thy1-ChR2-YFP* mice we used for our four week time point were likely not the best genetic model to continue our exploration into functional recovery. By repeating this experiment in a mouse that expresses the ChR2-YFP transgene more sparsely, I think we would significantly increase the differences we were looking for, making them easier to see amongst the noise inherent in the measurements used.

We hypothesized that neuronal activity is sufficient to facilitate axon regeneration and functional recovery after nerve transection. We have concluded, based on our experiments, that our hypothesis was correct. We also concluded that this enhancement of axon regeneration and functional recovery is specific to those axons being activated because in optically treated nerves, enhancement of axon regeneration was found only for optically activatable axons. Based on this supporting evidence, physical therapists and physicians might see an enhancement of peripheral nerve regeneration after injury if they activated the injured neurons. The type of activation should be tailored to the type of injury or injured axon. Only those axons that are activated are likely to show enhanced regeneration, so only activity should be as specifically or as broadly applied as befits the injury.

Future studies should focus on either fully characterizing this mouse model, or using a more stable one. Determining the source of the large inter-animal variability in the force data would allow for motor unit number estimation. All experimental assays should be done on all three time points. More conceptually, other types of specific activation should be explored including that of sensory neurons, or through the use of other activation paradigms (such as DREDDs). Inhibiting activity (either through optogenetics or DREDDs, or either globally or specifically) would allow us to determine the necessity of neuronal activity in the enhancement of regeneration after peripheral nerve injury.

Figure Legends

FIGURE 1: A. Image of a whole mount of the L4 dorsal root ganglion, dorsal root, and ventral root from a Thy1-ChR2 mouse. Panels B and C are higher magnification images of the regions in the white boxes in panel A. B. ChR2-YFP is found in the cell bodies of ganglion cells, especially at the cell membrane (arrow). YFP is in axons in both the ventral root (VR) and dorsal root (DR). C. Higher concentration of ChR2-YFP is found in punctate regions (arrow heads).

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FIGURE 5: A. Schematic of experimental design. Nerves were cut and repaired by end-to-end anastomosis. Twitch forces elicited by optical and electrical nerve stimulation prior to and 4 weeks after transection were measured. B. Mean (+SEM) percent recovery, the ratio of post-transection to pre-transection maximal force, elicited both optically and electrically, is shown for the three treatment groups: Untreated, Optically treated, and electrically treated. *=$p<0.05$ vs. electrical treatment C. The average ratio of forces generated optically vs. electrically is used as a measure of the percentage of motor axons which were ChR2+ in intact mice and four weeks after sciatic nerve transection and repair in different treatment groups. * =$p<0.05$ vs. Intact and Untreated.

FIGURE 6: An array of six single motor unit twitch forces (SMUF) which were extracted from incremental stimulation of the sciatic nerve of a single mouse (inset). All peak forces were pooled for each stimulus protocol within each treatment group and used to generate the cumulative histograms shown in panels A and B, for electrically and optically evoked SMUFs, respectively.

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References

Aid T, Kazantseva A, Piirsoo M, Palm K, Timmusk T (2007) Mouse and rat BDNF gene structure and expression revisited. J Neurosci Res 85:525-535.

Al-Majed AA, Brushart TM, Gordon T (2000a) Electrical stimulation accelerates and increases expression of BDNF and trkB mRNA in regenerating rat femoral motoneurons. Eur J Neurosci 12:4381-4390.

Al-Majed AA, Neumann CM, Brushart TM, Gordon T (2000b) Brief electrical stimulation promotes the speed and accuracy of motor axonal regeneration. J Neurosci 20:2602-2608.

Arenkiel BR, Peca J, Davison IG, Feliciano C, Deisseroth K, Augustine GJ, Ehlers MD, Feng G (2007) In vivo light-induced activation of neural circuitry in transgenic mice expressing channelrhodopsin-2. Neuron 54:205-218.

Asensio-Pinilla E, Udina E, Jaramillo J, Navarro X (2009) Electrical stimulation combined with exercise increase axonal regeneration after peripheral nerve injury. Exp Neurol 219:258-265.

Brushart TM, Hoffman PN, Royall RM, Murinson BB, Witzel C, Gordon T (2002) Electrical stimulation promotes motoneuron regeneration without increasing its speed or conditioning the neuron. J Neurosci 22:6631-6638.

English AW (2005) Enhancing axon regeneration in peripheral nerves also increases functionally inappropriate reinnervation of targets. The Journal of Comparative Neurology 490:427-441.

English AW, Chen Y, Carp JS, Wolpaw JR, Chen XY (2007a) Recovery of electromyographic activity after transection and surgical repair of the rat sciatic nerve. J Neurophysiol 97:1127-1134.

English AW, Cucoranu D, Mulligan A, Sabatier M (2009) Treadmill training enhances axon regeneration in injured mouse peripheral nerves without increased loss of topographic specificity. The Journal of Comparative Neurology 517:245-255.

English AW, Schwartz G, Meador W, Sabatier MJ, Mulligan A (2007b) Electrical stimulation promotes peripheral axon regeneration by enhanced neuronal neurotrophin signaling. Dev Neurobiol 67:158-172.

English AW, Wilhelm JC, Sabatier MJ (2011) Enhancing recovery from peripheral nerve injury using treadmill training. Ann Anat 193:354-361.

Foecking EM, Fargo KN, Coughlin LM, Kim JT, Marzo SJ, Jones KJ (2012) Single session of brief electrical stimulation immediately following crush injury enhances functional recovery of rat facial nerve. Journal of rehabilitation research and development 49:451-458.

Foutz TJ, Arlow RL, McIntyre CC (2012) Theoretical principles underlying optical stimulation of a channelrhodopsin-2 positive pyramidal neuron. J Neurophysiol 107:3235-3245.

Frostick SP, Yin Q, Kemp GJ (1998) Schwann cells, neurotrophic factors, and peripheral nerve regeneration. Microsurgery 18:397-405.

Geremia NM, Gordon T, Brushart TM, Al-Majed AA, Verge VM (2007) Electrical stimulation promotes sensory neuron regeneration and growth-associated gene expression. Exp Neurol 205:347-359.

Gomez-Pinilla F, Zhuang Y, Feng J, Ying Z, Fan G (2011) Exercise impacts brain-derived neurotrophic factor plasticity by engaging mechanisms of epigenetic regulation. Eur J Neurosci 33:383-390.

Gordon T (2009) The role of neurotrophic factors in nerve regeneration. Neurosurg Focus 26:E3.

Henneman E, Olson CB (1965) Relations between Structure and Function in the Design of Skeletal Muscles. J Neurophysiol 28:581-598.

Hoffer JA, Stein RB, Gordon T (1979) Differential atrophy of sensory and motor fibers following section of cat peripheral nerves. Brain Res 178:347-361.

Hoffer JA, Sugano N, Loeb GE, Marks WB, O'Donovan MJ, Pratt CA (1987) Cat hindlimb motoneurons during locomotion. II. Normal activity patterns. J Neurophysiol 57:530-553.

Hong EJ, McCord AE, Greenberg ME (2008) A biological function for the neuronal activity-dependent component of Bdnf transcription in the development of cortical inhibition. Neuron 60:610-624.

Llewellyn ME, Thompson KR, Deisseroth K, Delp SL (2010) Orderly recruitment of motor units under optical control in vivo. Nat Med 16:1161-1165.

Loeb GE, Marks WB, Hoffer JA (1987) Cat hindlimb motoneurons during locomotion. IV. Participation in cutaneous reflexes. J Neurophysiol 57:563-573.

Major LA, Hegedus J, Weber DJ, Gordon T, Jones KE (2007) Method for counting motor units in mice and validation using a mathematical model. J Neurophysiol 97:1846-1856.

McComas AJ, Sica RE, Campbell MJ, Upton AR (1971) Functional compensation in partially denervated muscles. Journal of neurology, neurosurgery, and psychiatry 34:453-460.

Nix WA, Hopf HC (1983) Electrical stimulation of regenerating nerve and its effect on motor recovery. Brain Res 272:21-25.

Noble J, Munro CA, Prasad VS, Midha R (1998) Analysis of upper and lower extremity peripheral nerve injuries in a population of patients with multiple injuries. J Trauma 45:116-122.

Pruunsild P, Kazantseva A, Aid T, Palm K, Timmusk T (2007) Dissecting the human BDNF locus: bidirectional transcription, complex splicing, and multiple promoters. Genomics 90:397-406.

Sabatier MJ, Redmon N, Schwartz G, English AW (2008a) Treadmill training promotes axon regeneration in injured peripheral nerves. Exp Neurol 211:489-493.

Sabatier MJ, Redmon N, Schwartz G, English AW (2008b) Treadmill training promotes axon regeneration in injured peripheral nerves. 211:489-493.

Scholz T, Krichevsky A, Sumarto A, Jaffurs D, Wirth GA, Paydar K, Evans GR (2009) Peripheral nerve injuries: an international survey of current treatments and future perspectives. J Reconstr Microsurg 25:339-344.

Stein RB, Nichols TR, Jhamandas J, Davis L, Charles D (1977) Stable long-term recordings from cat peripheral nerves. Brain Res 128:21-38.

Taylor CA, Braza D, Rice JB, Dillingham T (2008) The incidence of peripheral nerve injury in extremity trauma. American journal of physical medicine & rehabilitation / Association of Academic Physiatrists 87:381-385.

Teodori RM, Betini J, de Oliveira LS, Sobral LL, Takeda SY, de Lima Montebelo MI (2011) Swimming exercise in the acute or late phase after sciatic nerve crush accelerates nerve regeneration. Neural plasticity 2011:783901.

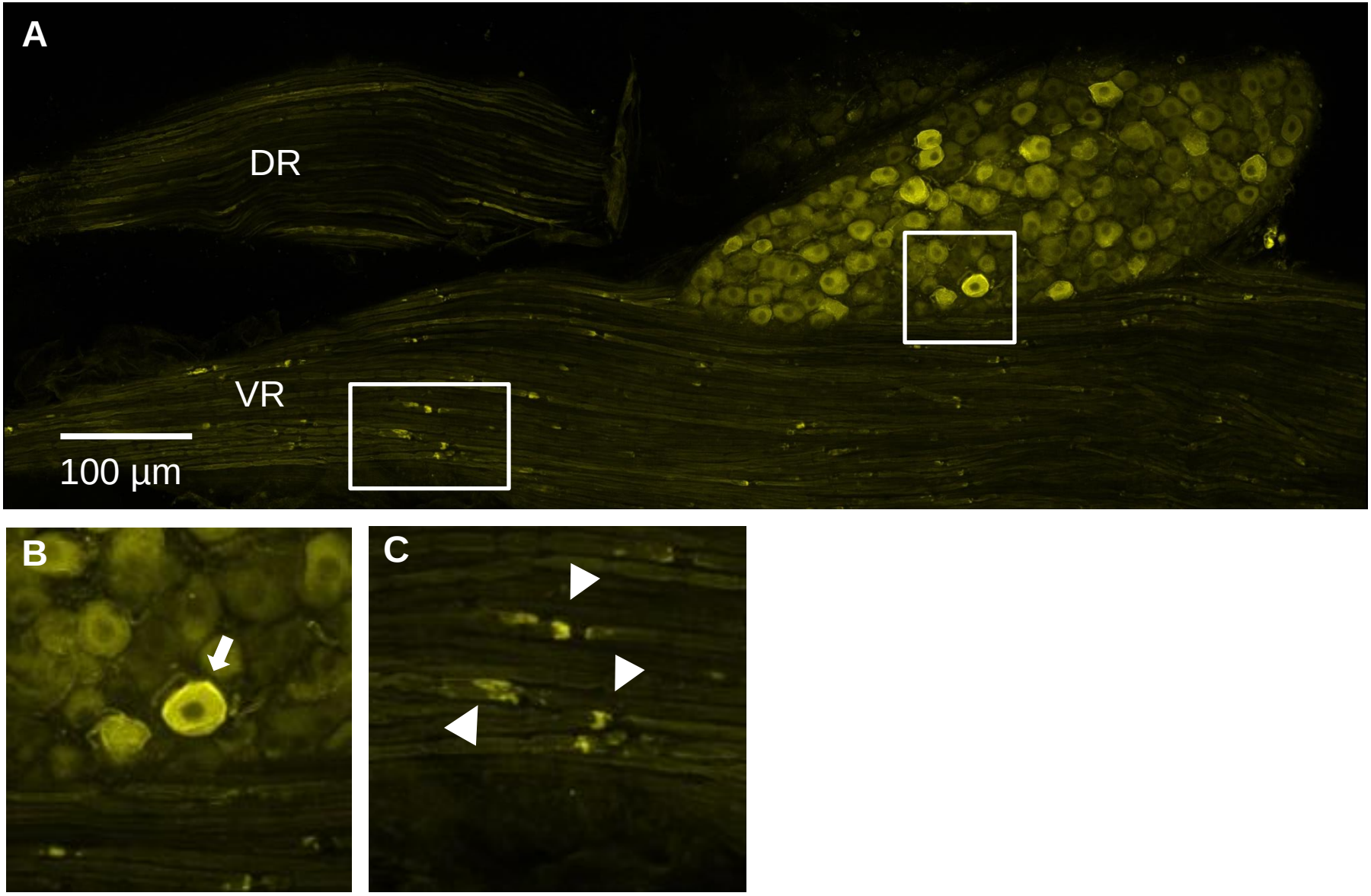
Timmusk T, Palm K, Metsis M, Reintam T, Paalme V, Saarma M, Persson H (1993) Multiple promoters direct tissue-specific expression of the rat BDNF gene. Neuron 10:475-489.

Udina E, Puigdemasa A, Navarro X (2011) Passive and active exercise improve regeneration and muscle reinnervation after peripheral nerve injury in the rat. Muscle & nerve 43:500-509.

Wilhelm JC, Xu M, Cucoranu D, Chmielewski S, Holmes T, Lau KS, Bassell GJ, English AW (2012) Cooperative roles of BDNF expression in neurons and Schwann cells

are modulated by exercise to facilitate nerve regeneration. J Neurosci 32:5002-5009.

Zheng F, Zhou X, Luo Y, Xiao H, Wayman G, Wang H (2011) Regulation of brain-derived neurotrophic factor exon IV transcription through calcium responsive elements in cortical neurons. PLoS One 6:e28441.



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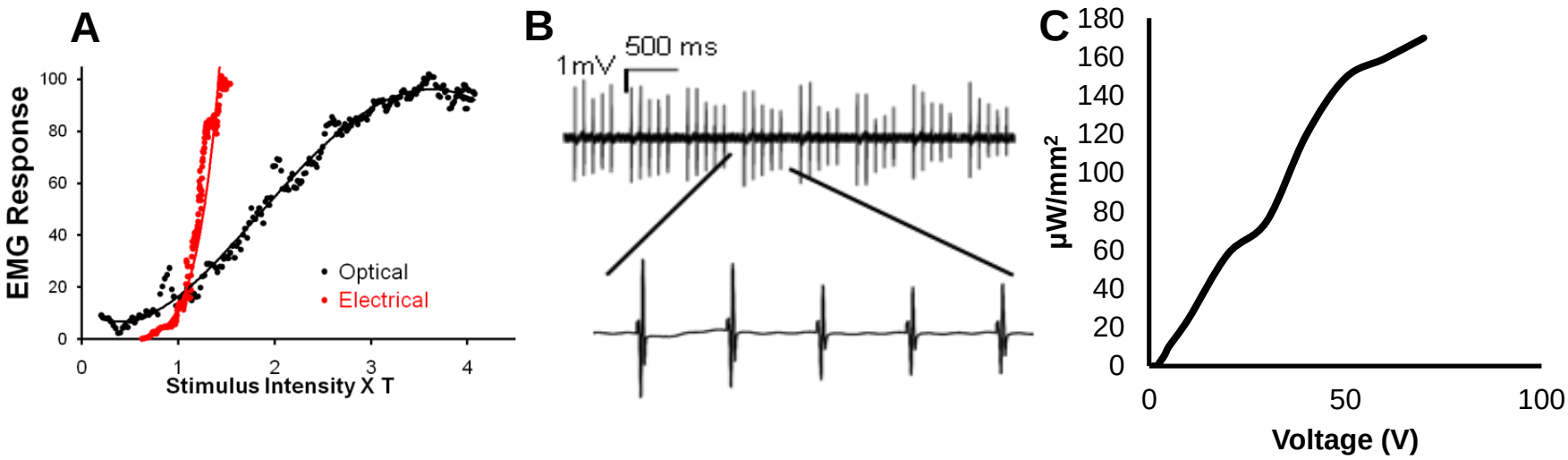

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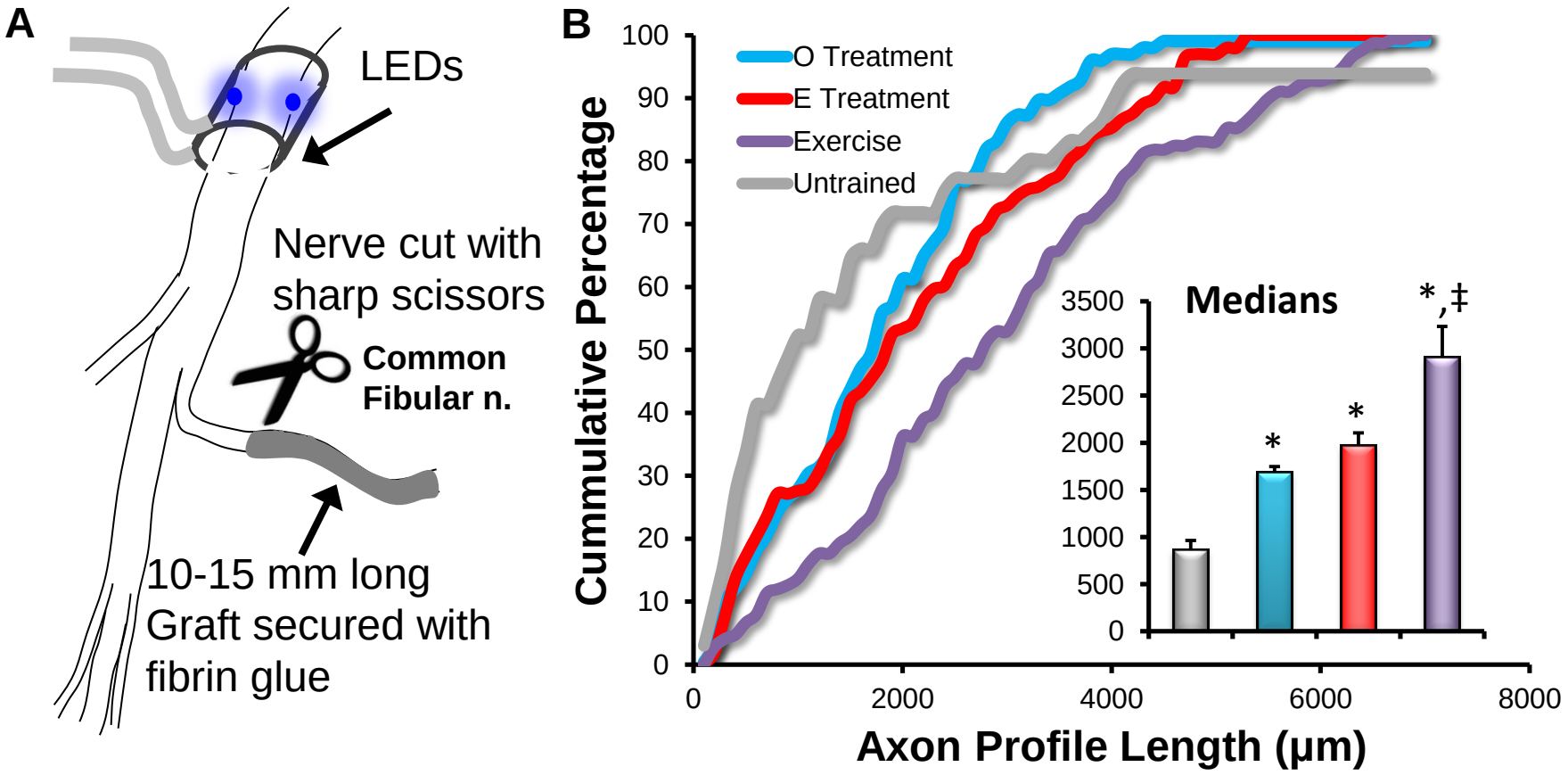

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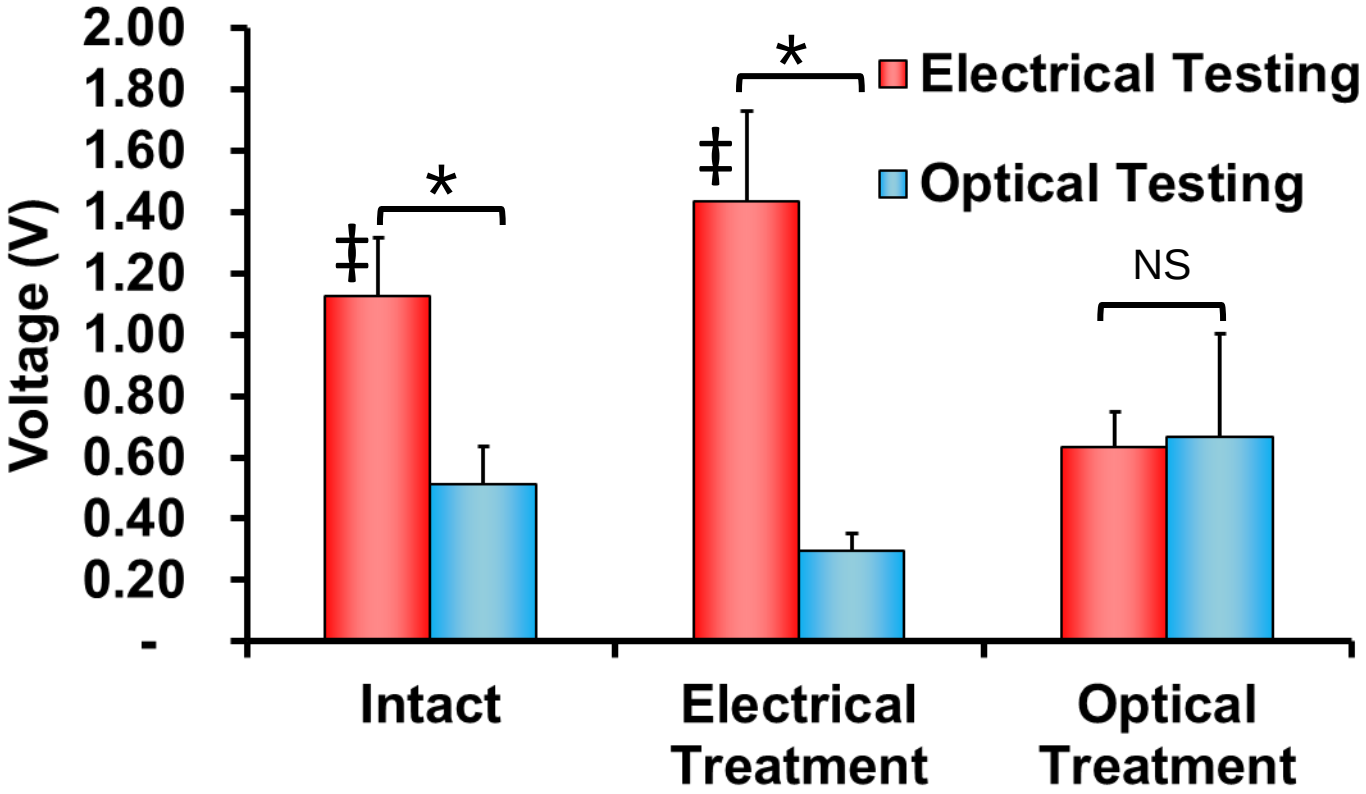

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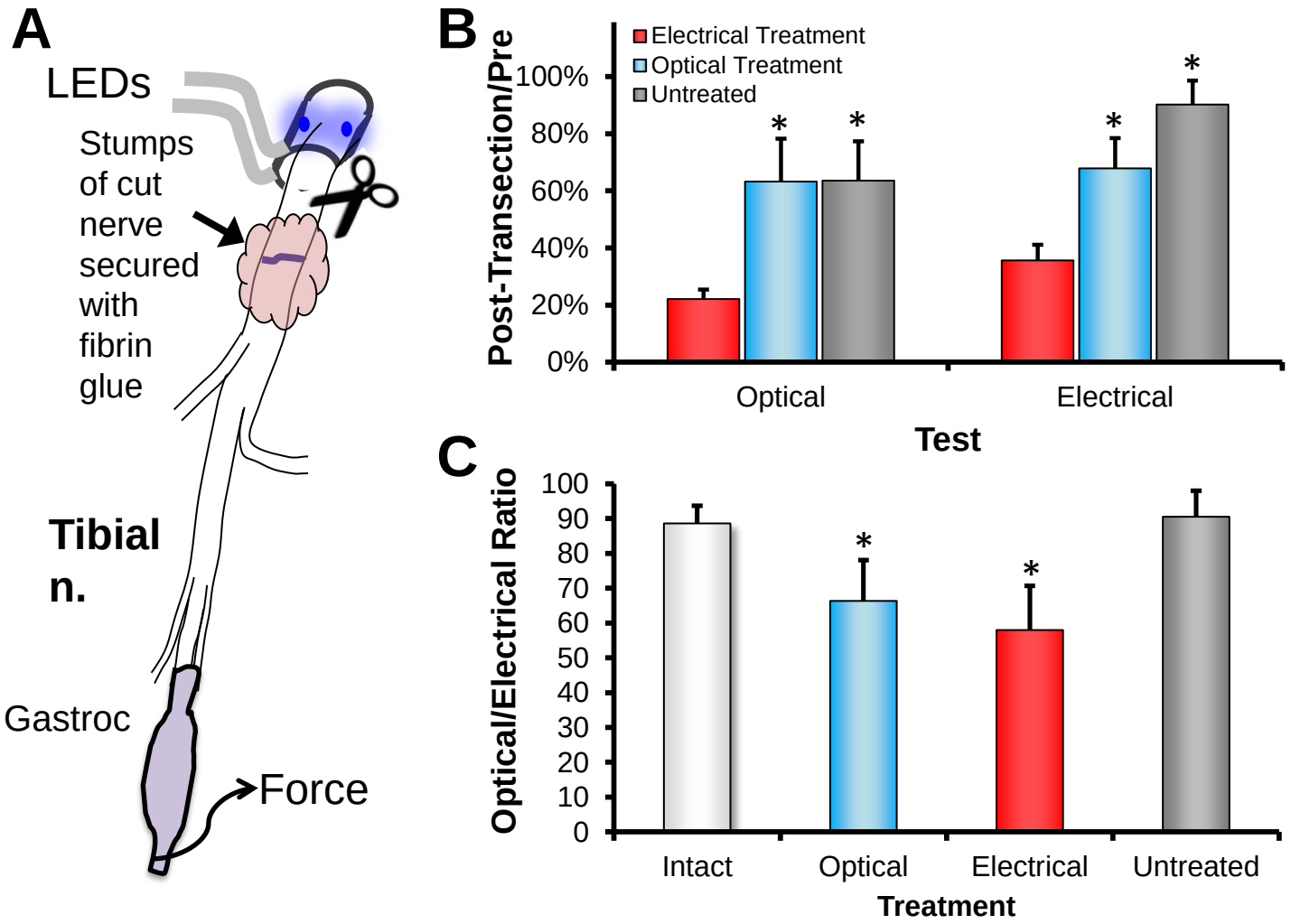

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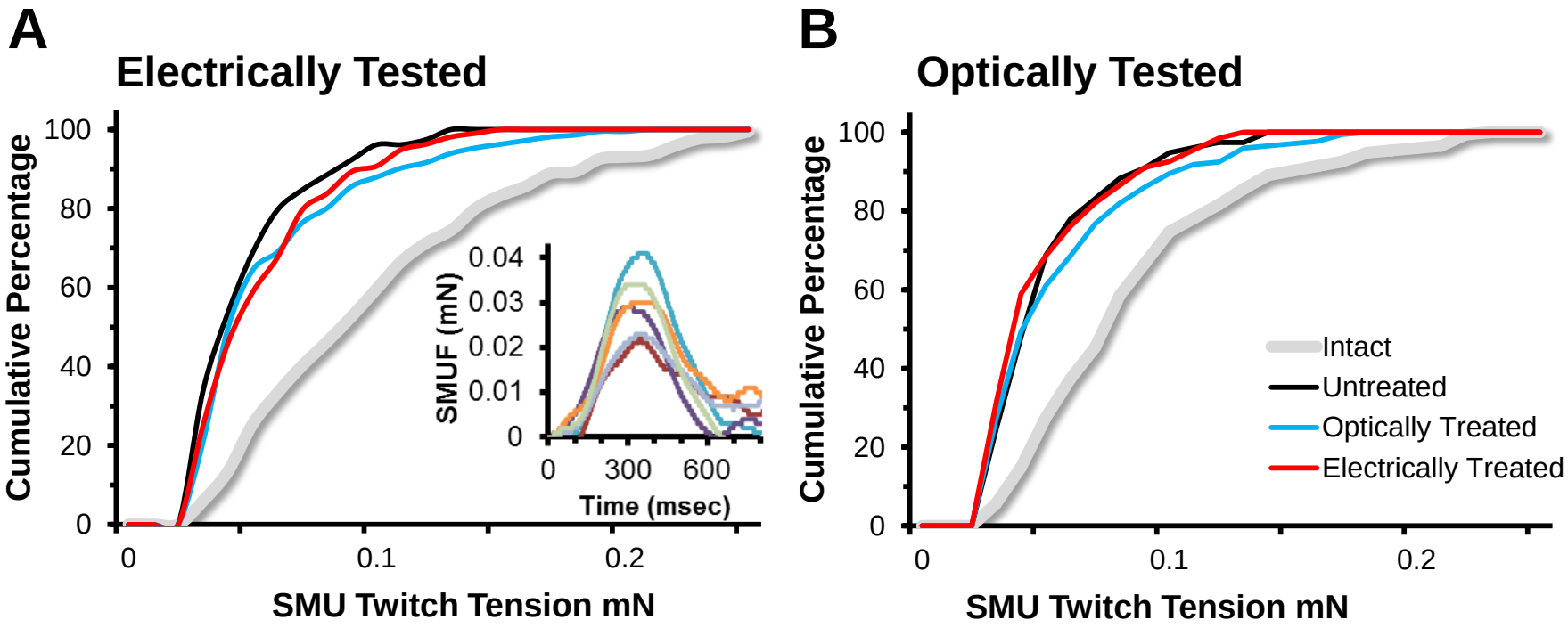

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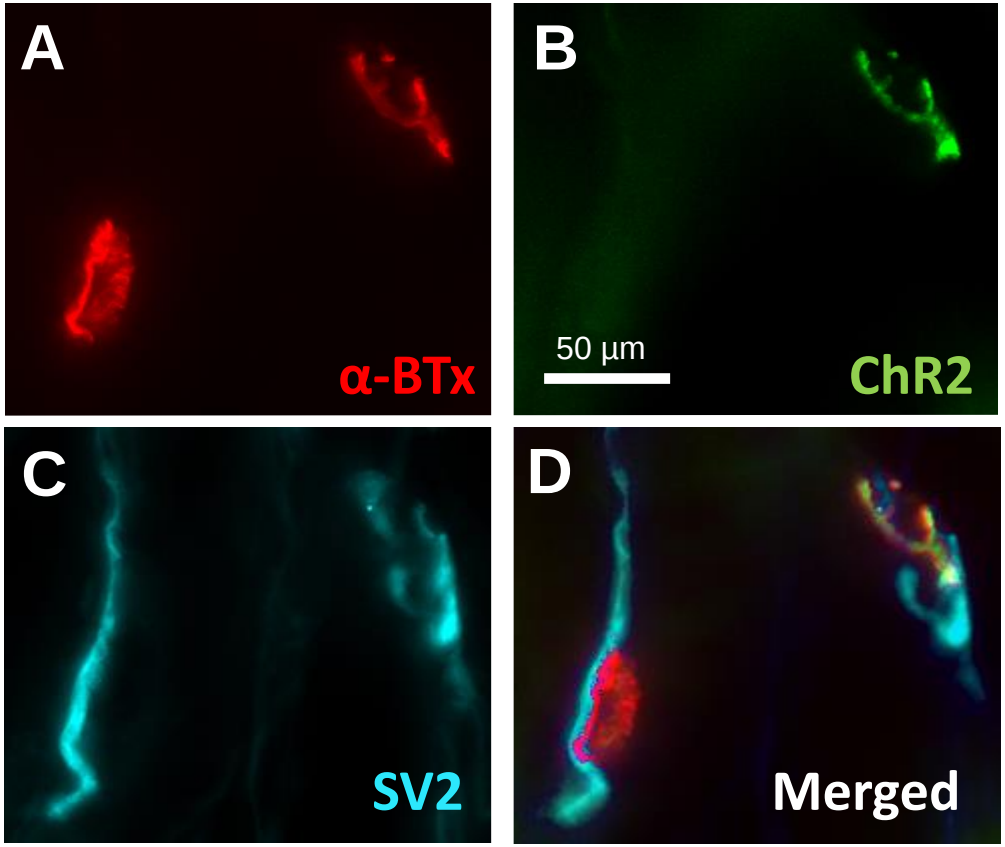

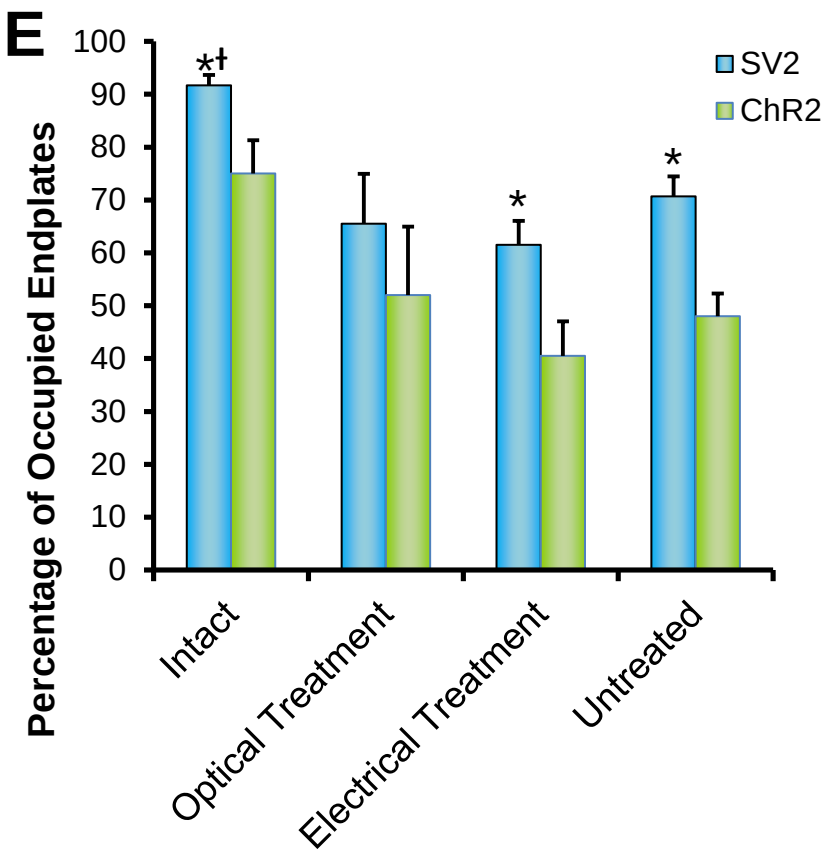

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