Conditioning electrical stimulation fails to enhance sympathetic axon regeneration

Tina Tian[1,2,3], Alandrea Harris[4,5], Jordan Owyoung[3,6], HaoMin SiMa[3], Patricia J. Ward[2,3,§]

1. Medical Scientist Training Program, Emory University School of Medicine, Atlanta, GA
2. Neuroscience Graduate Program, Laney Graduate School, Emory University, Atlanta, GA
3. Department of Cell Biology, Emory University School of Medicine, Atlanta, GA
4. Summer Opportunity for Academic Research, Emory University, Atlanta, GA
5. University of Kentucky, Lexington, KY
6. Genetics and Molecular Biology Graduate Program, Atlanta, GA

§ Corresponding author

Abstract

Peripheral nerve injuries are common, and there is a critical need for the development of novel therapeutics to complement surgical repair. Conditioning electrical stimulation (CES) is a novel variation to the well-studied perioperative electrical stimulation, both of which have displayed success in enhancing the regeneration of motor and sensory axons in an injured peripheral nerve. CES is a clinically attractive alternative not only because of its ability to be performed at the bedside prior to a scheduled nerve repair surgery, but it has also been shown to be superior to perioperative electrical stimulation in the enhancement of motor and sensory regeneration. However, the effects of CES on sympathetic regeneration are unknown. Therefore, we tested the effects of two clinically relevant CES paradigms on sympathetic axon regeneration and distal target reinnervation. Because of the long history of evidence for the enhancement of motor and sensory axons in response to electrical stimulation, we hypothesize that CES will also enhance sympathetic axon regeneration. Our results indicate that the growth of sympathetic axons is acutely inhibited by CES; however, at a longer survival time point post-injury, there is no difference between sham CES and the CES groups. There has been evidence to suggest that the growth of sympathetic axons is inhibited by a

conditioning lesion, and that sympathetic axons may respond to electrical stimulation by sprouting rather than elongation. Our data indicate that sympathetic axons may retain some regenerative ability after CES, but no enhancement is exhibited, which may be accounted for by the inability of the current clinically relevant electrical stimulation paradigm to recruit the small-caliber sympathetic axons into activity. Further studies will be needed to optimize electrical stimulation parameters in order to enhance the regeneration of all neuron types.

INTRODUCTION

Peripheral nerve injuries (PNIs) are common (Taylor et al 2008), and the current standard of care relies on the inefficient process of spontaneous axon regeneration after intraoperatively achieving a tensionless anastomosis of the cut nerve stumps with only 10% of PNI patients ever achieving full functional recovery (Scholz et al 2009). Therefore, there is a critical need for the development of novel therapeutics to complement surgical repair.

Electrical stimulation (ES) is a well-studied experimental intervention that has been shown to enhance motor and sensory axon regeneration (Asensio-Pinilla et al 2009, Gordon 2016, Gordon et al 2010, Gordon et al 2007, Gordon & English 2016, Gordon et al 2009, Huang et al 2013, Park et al 2019, Willand et al 2016). The use of ES prior to injury for nerve conditioning is a novel way to improve outcomes after PNI (Senger et al 2019, Senger et al 2020, Senger et al 2018). Previous research on the use of ES has been focused on applying the intervention perioperatively with improved outcomes in animal models and in clinical trials (Al-Majed et al 2000, Al-Majed et al 2004, Gordon et al 2010). Likewise, one hour of conditioning electrical stimulation (CES) 7 days prior to nerve injury and repair has shown significant improvement in motor and sensory axon regeneration (Senger et al 2019, Senger et al 2018). CES was also found to be more favorable than perioperative ES (PES) or CES + PES together (Senger et al 2020). Because many nerve repair surgeries are elective, CES in clinic prior to a scheduled operation may make care more accessible.

Mechanistically, ES acts similarly to a conditioning lesion (CL) through the

upregulation of neurotrophins and regeneration-associated genes (Chan 2019, Forman et al 1981, Hoffman 2010, McQuarrie 1986, McQuarrie & Grafstein 1981, McQuarrie et al 1977, Senger et al 2019, Senger et al 2018). Of note, a CL has been shown to inhibit the regeneration of sympathetic axons (McQuarrie et al 1978), and ES may induce sprouting (short, misdirected growth) of sympathetic axons as opposed to the enhanced regeneration observed in motor and sensory axons (Maehlen & Njå 1982, Saygili et al 2010, Xie et al 2007). Motor axons, understandably, have been a major focus of regeneration-related studies, yet peripheral nerves are primarily composed of sensory and sympathetic axons. For example, only 6% of the rat sciatic nerve is composed of myelinated motor axons while unmyelinated sympathetic axons make up 23% (Schmalbruch 1986).

Notably, neither a CL nor ES promotes sympathetic axon elongation (Maehlen & Njå 1982, McQuarrie et al 1978, Saygili et al 2010, Xie et al 2007), and the effect of CES on sympathetic axon regeneration is unknown. Therefore, in this study, we compared the outcomes of two clinically relevant CES paradigms on sympathetic axon regeneration and peripheral target reinnervation (Calvey et al 2015, Sayanagi et al 2021, Senger et al 2018). Because of the abundance of evidence that ES enhances motor and sensory regeneration, we hypothesize that CES will enhance sympathetic regeneration.

MATERIALS AND METHODS

Animals

Experiments were conducted on adult (2–4 months old) mice weighing 18–30 g using approximately equal males and females for each assay. Mice were randomly assigned to sham CES, 10-min CES, or 1-hour CES groups. Tg(Thy1-YFP)16Jrs transgenic (Jackson laboratory stock no. 003782) or wild type mice C57BL/6 J (Jackson laboratory stock no. 000664) were used in these experiments. Experimental groups with final numbers of mice are presented in **Table 1**. Some animals were excluded from analysis in the 2-week regeneration assay due to poor sciatic nerve section quality or

image quality. Specifically, 2 males, 1 female; 1 male; and 2 males were excluded from the sham CES, 10-min CES, and 1-hour CES groups, respectively. All experiments were approved by the Institutional Animal Care and Use Committee of Emory University (PROTO202100177). A summary of methods is presented in **Figure 1.**

Surgical procedures

Anesthesia was induced with 3% isoflurane in 1 L/min oxygen and maintained at 2% isoflurane in 1 L/min oxygen. The sciatic nerve was exposed via a small (~1 cm) skin incision along the femur followed by blunt dissection between the hamstring muscles. Two monopolar needle electrodes (Ambu, #74325-36/40) were placed on either side of the sciatic nerve and were used to deliver electrical stimulus pulses to the nerve. Using our custom Labview® software, electrical stimulation (20 Hz for one hour or 10 minutes, 0.1 ms duration pulses) was applied at a supramaximal intensity (Brushart et al 2002, English et al 2007, Park et al 2019, Thompson et al 2014). Supramaximal intensity was calculated as 1.5-times the voltage required to evoke a maximal muscle response (typically 3-4 V). Sham animals had their sciatic nerves exposed with electrodes placed for 1 hour but were not stimulated. In the 10-minute CES group, after 10 minutes of ES, the stimulation was turned off while the electrodes remained in place for another 50 minutes. All mice were anesthetized for equal amounts of time. The muscle was closed with 5-0 absorbable suture (CP Medical, 421A), and the skin was closed with 5-0 non-absorbable suture (MYCO Medical Supplies, N661-SI) **(Figure 1A)**. Seven days later, mice were re-anesthetized, and the sciatic nerve was transected with sharp micro-scissors. It was then either repaired with a nerve graft for acute axon regeneration studies or repaired by simple end-to-end anastomosis using fibrin glue, composed of fibrinogen and thrombin in a 2:1 ratio, for reinnervation studies **(Figure 1B)** as we have previously described in detail (de Vries et al 2002, Gordon & English 2016, Sabatier et al 2008, Thompson et al 2014, Wariyar et al 2022). Animals received 5 mg/kg of 1.5 mg/ml oral suspension meloxidyl (Pivetal, ANADA 200-550) upon ambulation after surgery and then once a day for 3 days postoperatively for pain control.

Mice were euthanized at 2 or 10 weeks post-PNI with an intraperitoneal (IP)

injection of 150 mg/kg 10 mg/ml Euthasol® solution (0.75ml Euthasol® [Virbac AH Inc, Fort Worth, TX, ANADA 200-071, NDC 051311-050-01] in 29.25 ml 0.9% bacteriostatic sodium chloride [Hospira, Inc., Lake Forest, IL, NDC 0409-1966-02]) with a 25-gauge 1 mL syringe (BD, 309626). Upon verification of no response to noxious stimuli, mice were exsanguinated with 0.9% NaCl (Fisher Scientific, S2711) then perfused with 4% paraformaldehyde (Sigma-Aldrich, P6148) in 0.01M phosphate buffered saline (1X PBS, Sigma-Aldrich, P4417), pH 6.9.

Notes on the choice of experimental models: Nerve grafts are frequently used in clinical scenarios in which CES would be useful. Thus, in one assay we measured axon elongation into grafts after 2 weeks of growth. A longer survival time is necessary to measure the reinnervation of peripheral targets, so we opted to perform CES followed by a transection and immediate repair. If we had used a graft, the regenerating axons would need to cross 2 coaptation sites instead of one, which could make results more difficult to interpret.

Sympathetic axon profile quantification

Sciatic nerves were collected 2 weeks post-nerve grafting while mice were under anesthesia and postfixed for 30 minutes with 4% paraformaldehyde in 1X PBS **(Figure 1C)**. Nerves were then placed in 20% sucrose with 0.02% sodium azide (Fisher Scientific, S227I-25) for cryoprotection and allowed to sink at 4°C prior to being cryosectioned at 20 µm and placed on charged slides (VWR Superfrost® Plus, 48311-703).

Sciatic nerve sections were blocked with 10% normal goat serum (VWR International, GTX73206) in 1X Tris-buffered saline (Pierce, 28358), 0.1% Tween® 20 detergent (TBST) (Fisher Scientific, BP337500) for 1 hour at room temperature (RT). Rabbit anti-tyrosine hydroxylase (TH, 1:750, Abcam Ab112) was diluted in the blocking buffer and allowed to incubate at RT in a humidity chamber overnight followed by 3x 10-minute washes with TBST. Secondary antibody goat anti-rabbit Alexa Fluor™ 647 (1:200, Invitrogen A21245) in blocking buffer was allowed to incubate at RT for 2 hours followed by 3x 10-minute washes with TBST. Dried slides were then mounted with Fluoro-Gel with Tris (Electron Microscopy Sciences, 17985-10) and imaged on a Nikon

A1R confocal microscope on the 20x objective on the resonant scan mode, 8x averaging. The entirety of the nerve section was imaged with the “ND Acquisition + Large Images” wizard.

In Fiji, linear regions of interest (ROIs) were created that spanned the width of the nerve sections every 500 µm distally, starting from the injury site using a custom-made macro. One linear ROI was created 250 µm proximal to the injury site and used as the control for the calculation of regenerative indices. The mean fluorescent intensities of lightly fluorescent TH+ axons were measured to identify a threshold for each nerve section. At each ROI, a “Plot Profile” was generated, after which the “Find Peaks” function under Broadly Applicable Routine (BAR) was utilized (Ferreira et al 2015). “Minimum peak amplitude” was set to “0,” and the “minimum value of maxima” was set to the previously identified threshold. The number of peaks corresponds to the number of axon profiles at that ROI. This was repeated for all ROIs in each nerve section. The number of axon profiles at each ROI was then divided by the number of axon profiles 250 µm proximal to the injury site to calculate the regenerative index. Two or three nerve sections per animal were analyzed. Technical replicates of regenerative indices were averaged for each biological replicate.

Sympathetic reinnervation of the neuromuscular junction

Neuromuscular junction reinnervation with sympathetic neurons was assessed in the gastrocnemius muscles at 10 weeks post-injury **(Figure 1E)**. Gastrocnemii were harvested from perfused mice and cryoprotected with 20% sucrose with sodium azide and allowed to sink at 4°C overnight. Afterwards, the muscle was cryosectioned at 30 µm, and sections were placed on charged glass slides. Gastrocnemii from the contralateral leg of 7 randomly selected mice, which did not receive any intervention or injury, were used as an additional control.

To evaluate NMJ sympathetic reinnervation, slides were permeabilized with 0.1% Triton®-X-100 (Fisher BioReagents, BP151-500**)** in 1X PBS then blocked for 2 hours at RT with 2% bovine serum albumin (BSA, Sigma-Aldrich, A2153), 0.1% Triton in 1X PBS. Rabbit anti-TH (1:500) was diluted in the blocking buffer, and the primary antibody was allowed to incubate for 2 nights at 4°C in a humidity chamber. After 3x 10-minute

washes with 1X PBS, secondary antibody goat anti-rabbit Alexa Fluor™ 488 (1:200, Invitrogen, A11008) was diluted in the blocking buffer and allowed to incubate at RT for 2 hours followed by 3x 10-minute washes with 1X PBS. Following the washes, α-bungarotoxin (α-BTX) Alexa Fluor™ 555 (1:500, Invitrogen, B35451) was diluted in 1X PBS and placed on the slides for 30 minutes. Slides were washed 3x 10-minutes with 1X PBS. After the slides were dried, they were mounted with Fluoro-Gel with Tris buffer and imaged on a Nikon Ti-E fluorescent microscope. A 60x oil objective was used with the following exposure times: TRITC 200 ms, FITC 80 ms. NMJs were identified by presence of α-BTX, and sympathetic reinnervation was identified by whether or not TH overlapped with α-BTX. At least 50 NMJs were evaluated per mouse with an average of 56.75 NMJs. The number of NMJs with sympathetic innervation was divided by the total number of NMJs evaluated per mouse to calculate a percentage reinnervation. The total number of NMJs analyzed was 1,816.

Retrograde labeling

Nine weeks and 4 days after nerve transection and repair, Cholera Toxin Subunit B, Alexa Fluor™ 555 retrograde fluorescent tracer (Invitrogen, C22843) was injected into the reinnervated gastrocnemius muscle **(Figure 1D)**. Each head of the gastrocnemius received 2µL of tracer delivered via a 26-gauge Hamilton syringe. These would mark the cell bodies sympathetic neurons whose axons had successfully regenerated to that muscle target. The injections were made in at least two locations in both the medial and lateral heads of the gastrocnemius.

Three days after tracer injection, mice were perfused as described above. Lumbar sympathetic ganglia L2-L5 were dissected from the level of the left renal artery to the bifurcation of the abdominal aorta and mounted onto glass slides with Fluoro-Gel mounting media with Tris buffer (Zheng et al 2017) **(Figure 1F)**. Images were taken with a 20x objective, 100 ms exposure time on the TRITC channel, with a Nikon Ti-E fluorescent microscope. Labeled sympathetic neurons were identified if the retrograde label filled the soma and extended into the proximal dendrites and if a nuclear shadow could be visualized. Labeled profiles that did not meet these criteria were not counted.

Statistical analysis

Sample sizes were predetermined based on previous work (English et al 2021, Park et al 2019, Ward et al 2021, Ward et al 2016, Wariyar et al 2022). Data are represented as mean ± standard error of the mean (SEM) or as medians with 95% confidence intervals (CI). GraphPad Prism version 9.4.0 for Windows was used to perform statistical analyses (GraphPad Software, San Diego, California USA, www.graphpad.com). Biorender.com was used to create portions of Figure 1.

For the 2-week axon regeneration experiment, 2-way repeated measures analysis of variance (ANOVA) was used to analyze the data followed by Tukey's multiple comparisons post hoc test. For NMJ reinnervation analysis, one-way completely randomized ANOVA was followed by post hoc Tukey's multiple comparisons test. Due to the nonparametric nature of cell counts and uneven variances, we used the Kruskal-Wallis test to analyze retrogradely-labeled sympathetic neurons. * or + $p < 0.05$, ++ $p < 0.01$, *** $p < 0.001$.

RESULTS

Sympathetic axon regeneration is acutely inhibited by CES

To test the effect of CES on acute sympathetic axon regeneration, the sciatic nerve received either sham CES, 10-minute CES, or 1-hour CES 1 week prior to nerve transection and repair with a graft **(Figure 1)**. At 2 weeks post-injury, the regenerative indices were significantly decreased with CES compared to sham CES (2-way RM ANOVA, experimental group factor $F(2,12) = 4.219$, $p = 0.040966$, post hoc Tukey's multiple comparisons test) starting at 1000 µm distal to the injury site (1000 µm sham vs 1-hr $p = 0.034888$, 1500 µm sham vs 1-hr $p = 0.000718$, 2000 µm sham vs 1-hr $p = 0.028351$) **(Figure 2A)**. The effect is greater with 1-hour CES compared to 10-minute CES, with 10-minute CES only being significantly different from sham CES at 1500 µm (sham vs 10-min $p = 0.009606$) and 2500 µm (sham vs 10-min $p = 0.036377$).

Additionally, there appears to be more axonal sprouting occurring in the 10-minute and 1-hour CES groups. This is visually apparent in the CES nerve sections **(Figure 2C-D)**, especially in the 1-hour CES nerve section **(Figure 2D)**.

Long-term sympathetic reinnervation after injury is unaffected by CES

To evaluate sympathetic axon reinnervation of peripheral targets after PNI, the sciatic nerve received either sham CES, 10-min CES, or 1-hour CES prior to transection and repair **(Figure 1A-B)**. A typical NMJ in intact muscle has robust TH expression **(Figure 3B)**, while reinnervated tissue may contain fragmented NMJs with incomplete TH expression **(Figure 3C)** or negligible TH expression **(Figure 3D)**.

The percentage of NMJs that contained sympathetic reinnervation 10 weeks post-injury was not improved by CES **(Figure 3A)**. All experimental groups showed decreased NMJ reinnervation compared to intact controls (1-way completely randomized ANOVA, $F(3,28) = 10.84$, $p = 0.000067$). The mean reinnervation percentage for all CES groups, including the sham CES group, was 71.2% compared to 90.9% for the intact control group (post hoc Tukey's multiple comparisons test, intact vs sham $p = 0.000244$, intact vs 10-min $p = 0.000291$, intact vs 1-hr $p = 0.000388$). The sympathetic innervation of the intact control muscles, 90.9% **(Figure 3A)**, is comparable to previous studies (Khan et al 2016).

The L2-L5 lumbar sympathetic ganglia traverse the distance between the aortic bifurcation into the iliac arteries to the level of the left renal artery **(Figure 4A)** (Zheng et al 2017). The large L2 lumbar sympathetic ganglia can be seen directly under the diaphragmatic crura **(Figure 4B)**. The ganglia could then be mounted directly to a slide and visualized in its entirety **(Figure 4C)** with clear identification of labeled cells **(Figure 4D)**. The numbers of retrogradely-labeled cells from the gastrocnemius in the L2-L5 lumbar sympathetic ganglia (shown as medians with 95% confidence intervals) were also not significantly different between treatment groups (Kruskal-Wallis test, $H = 5.393$, $df = 2$, p-value = 0.0675), with 10-minute CES (198, 95% CI [173, 371]) and 1-hour CES (222, 95% CI [172, 355]) groups displaying no improvement compared to the sham CES group (273, 95% CI [242, 313]) **(Figure 4E)**. Although the median number of labeled cells is higher in the sham CES group, the 10-minute and 1-hour CES groups exhibited higher variability in the number of neurons that had reached the gastrocnemius muscle.

DISCUSSION

CES is a clinically appealing therapy for the treatment of injured peripheral nerves. In animal models of PNI, CES promotes sensory and motor axon regeneration after several types of nerve repair. In an effort to define protocols that would further minimize patient discomfort and intraoperative time, a 10-minute PES protocol has also been shown to provide similar benefits to the 60-minute PES protocol after an acute injury (Calvey et al 2015, Sayanagi et al 2021). One major difference between PES and a CL is that PES enhances regeneration of axons as they cross the coaptation site, reducing a process called “staggered regeneration” (Brushart et al 2002), whereas a CL accelerates the rate of axon extension (Richardson & Verge 1987). To our knowledge, this is the first study examining the effect of CES protocols on the regeneration of sympathetic axons.

Sympathetic axon regeneration was acutely inhibited by CES as demonstrated by fewer axon profiles further away from the injury site in groups that received the intervention compared to the sham stimulated group. The effect is increased with longer electrical stimulation, indicating that sympathetic axons react in ways that are incongruous with motor and sensory axons to neuronal stimulation. Our findings also suggest increased sympathetic sprouting rather than elongation, which agrees with previous findings of sympathetic sprouting in response to ES (Maehlen & Njå 1982, Saygili et al 2010, Xie et al 2007). Interestingly, at a longer survival time post-injury, there was no difference between the experimental groups. This indicates that sympathetic axons retain some regenerative ability following CES; however, our results still indicate that CES fails to enhance the regenerative ability of sympathetic axons over time.

Our study uses the current clinical paradigm of electrical stimulation that has reached patient populations: 1 hour of stimulation at 20 Hz with 0.1 ms pulses. This increases the clinical relevance of our experiments. ES recruits into activity the largest diameter axons first due to their lower stimulus threshold and typically activates smaller-caliber fibers, such as sympathetic axons (Glatte et al 2019), at much higher stimulus intensities and longer durations (Llewellyn et al 2010). Thus, the current clinical paradigm may not be recruiting sympathetic axons into activity. Although less likely, it is

also possible that an initial increase in higher-caliber axonal regeneration outcompetes the smaller-caliber sympathetic axons for available endoneurial tubes, but they eventually find regenerative pathways at later time points. McQuarrie et al. (1978), also observed a “lag” in the regenerative front of sympathetic axons following a conditioning lesion. More recent studies on superior cervical ganglia have shown enhancement of sympathetic neurite outgrowth in response to a conditioning lesion (Sachs et al 2010, Shoemaker et al 2005). Our results appear to be incongruous with those found in the superior cervical ganglia, likely due to differential molecular profiles amongst sympathetic neurons (Furlan et al 2016). Therefore, the response of lumbar sympathetic ganglia, where the sympathetic neurons that contribute to the sciatic nerve reside, may not be comparable to that of the superior cervical ganglia.

An additional strength to this study was the use of a nerve graft for the acute regeneration experiments, which more closely parallels clinical scenarios in which CES would be used: elective nerve repair surgery (Senger et al 2018). These elective surgeries would often involve the use of autologous nerve grafts or nerve transfers (Seddon 1963, Siemionow & Brzezicki 2009), which is more analogous to the use of a nerve graft in mice.

One limitation of our acute regeneration study is that tyrosine hydroxylase is present in other neuronal cell types, notably C-low threshold mechanoreceptors (C-LTMRs), which is a small population of dorsal root ganglia neurons (Brumovsky 2016). C-LTMRs normally process innocuous mechanical and cooling sensations as well as affective touch (Le Pichon & Chesler 2014, Li et al 2011, Löken et al 2009, Olausson et al 2002, Zimmerman et al 2014). This concern could possibly be circumvented by a primary antibody against dopamine β-hydroxylase (DBH), which converts dopamine to norepinephrine, the main neurotransmitter for postganglionic sympathetic neurons (Kaufman & Friedman 1965); however, our current immunohistochemistry protocols have not allowed for clear visualization of DBH-positive axons in the sciatic nerve. This can be due to the relatively lower amount of *DBH* mRNA present in the distal axons compared to *TH* mRNA (Aschrafi et al 2019, Gervasi et al 2016). This limitation primarily affects the short-term regeneration assay; therefore, the long-term reinnervation and retrograde tracing assay was also performed. Utilizing counts of

retrogradely-labeled neurons in the lumbar sympathetic ganglia allowed for a more specific measure of sympathetic regeneration without the confound of neurons from the dorsal root ganglia. The combination of these assays supports our main conclusion that CES fails to promote sympathetic axon regeneration.

Most clinical situations that could entertain the possibility of CES as an adjunctive intervention would involve the use of a nerve transfer from a healthy donor nerve to an injured nerve, such as a spinal accessory nerve to suprascapular nerve transfer to reanimate the shoulder in brachial plexus palsy (Bertelli & Ghizoni 2016, Seruya et al 2015). Additionally, the ability of CES to increase the speed of motor and sensory regeneration and axonal elongation could be beneficial in situations involving a planned oncological resection (Javeed et al 2021, Senger et al 2019). Whether or not CES induces pro-regenerative effects in a proximal nerve that has been chronically injured is unknown. Future research on methods of improving outcomes in chronic PNI is needed.

For novel therapeutics to enhance the regeneration of all neuron types more efficiently, including sympathetic neurons, future study directions could focus on altering the ES parameters to better enhance sympathetic growth, a combinatorial ES paradigm to separately enhance motor and sensory growth versus sympathetic growth, or a combinatorial pharmacologic approach with ES to target genes or proteins that may enhance the growth of sympathetic neurons. Enhancing sympathetic regeneration following PNI is critical for the return of homeostasis mechanisms in vasculature, skin, muscle, bone, and all other organs of the body (Carnagarin et al 2018, Elefteriou et al 2014, Khan et al 2016, Lacroix 1989, Lee et al 2010, Lundberg et al 1985, Roatta & Farina 2010). Additionally, aberrant sympathetic outgrowth in the form of sprouting has been implicated in many neuropathic pain states (Chung et al 1996, Xie et al 2007, Zhang et al 2004, Zhang & Strong 2008). Therefore, exploring approaches that can be combined with ES in a complementary fashion to optimally enhance the regrowth of all neurons will be a critical step in improving patient outcomes after peripheral nerve injury.

CONCLUSIONS

Despite the success of CES in enhancing the regeneration and functional recovery of motor and sensory axons after PNI (Senger et al 2019, Senger et al 2020, Senger et al 2018), CES fails to enhance sympathetic axon elongation and fails to improve sympathetic reinnervation of distal tissues. CES is still a valuable therapeutic option that reduces anesthesia and operating room time as it is an intervention that can be performed at the bedside. However, there remains an urgent need to develop novel therapeutic options that enhance the regeneration of all axon types, which will be an essential step to further improve patient outcomes.

ACKNOWLEDGEMENTS

This project was supported by the NIH National Institute of Neurological Disorders and Stroke under award number K01NS124912 and in part by a developmental grant from the NIH-funded Emory Specialized Center of Research Excellence in Sex Differences U54AG062334, the National Institute of General Medical Sciences predoctoral training grant in Genetics T32GM008490, and the Medical Scientist Training Program of Emory University, School of Medicine. Icons from BioRender licensed under agreement number EN24YVUSME.

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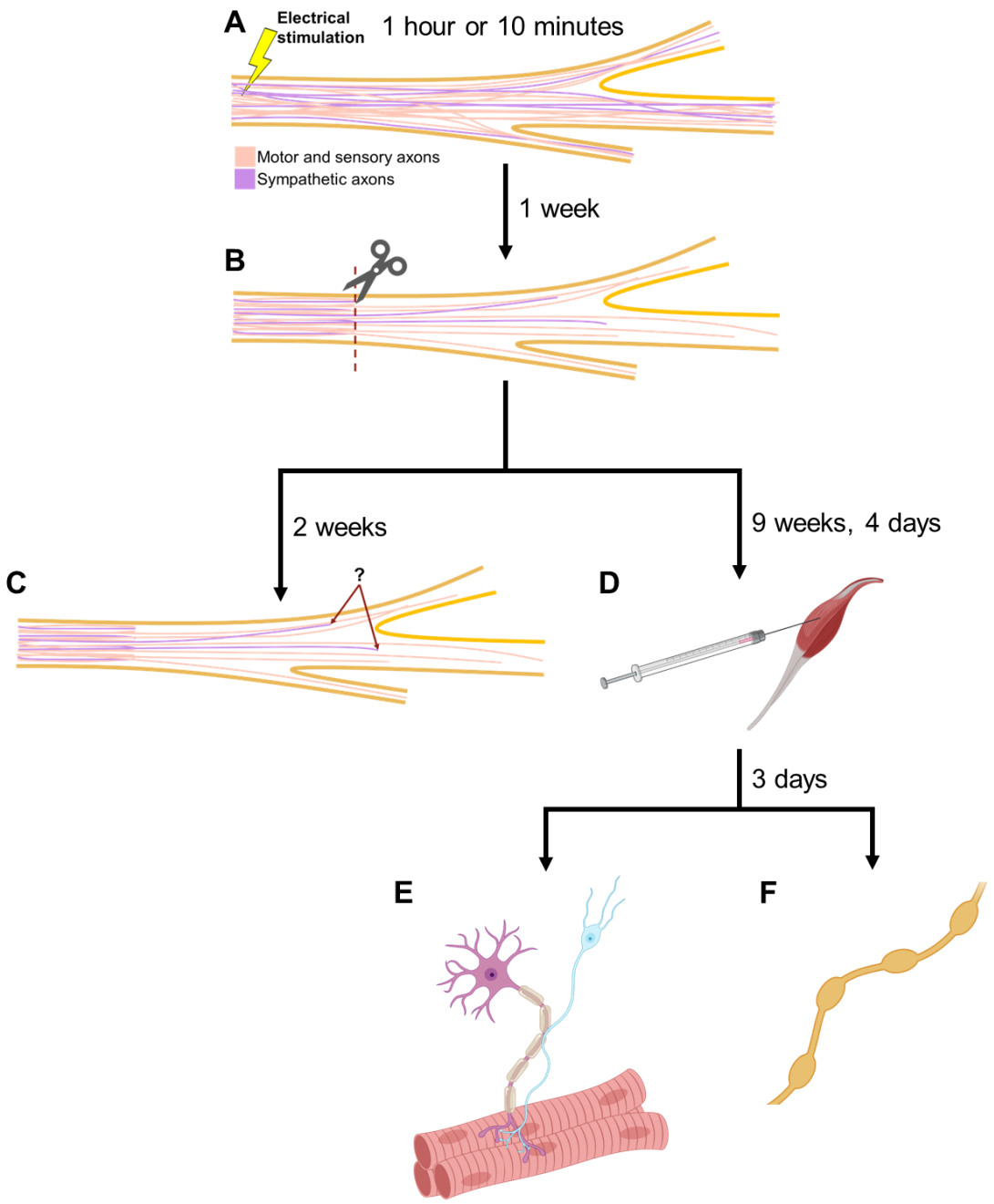

Figure 1: Summary of methods. (A) Sciatic nerves were electrically stimulated for either 1 hour or 10 minutes. (B) One week later, the nerves were transected and repaired with a sciatic nerve graft from a littermate or simply transected and repaired with fibrin glue. (C) The mice who received a nerve graft had their sciatic nerves harvested 2 weeks later for immunohistochemical staining for tyrosine hydroxylase. Axon profiles were counted. (D) The mice who had a simple transection repair surgery survived for another 9 weeks and 4 days, when their gastrocnemii muscles were injected with cholera toxin subunit B Alexa Fluor 555. (E) Three days post-injection, the mice were perfused. Their gastrocnemii muscles were harvested for evaluation of neuromuscular junction reinnervation, (F) and their L2-L5 lumbar sympathetic ganglia were harvested for retrogradely-labeled neuron counts. *D-F created with BioRender.com.*

Experiment	Treatment	n =	Strain
Axon regeneration (2 weeks)	Sham CES	2 males 3 females*	Tg(Thy1-YFP)16Jrs
	10-minute CES	2 males 3 females*	Tg(Thy1-YFP)16Jrs
	1-hour CES	1 male 4 females*	Tg(Thy1-YFP)16Jrs
Target reinnervation and retrograde labeling (10 weeks)	Sham CES	4 males 4 females	C57BL/6J
	10-minute CES	5 males 4 females	C57BL/6J
	1-hour CES	4 males 4 females	C57BL/6J

Table 1: Detailed breakdown of experimental groups. The n represents the final number of animals included in each analysis. *1-2animals were excluded due to poor section or image quality.

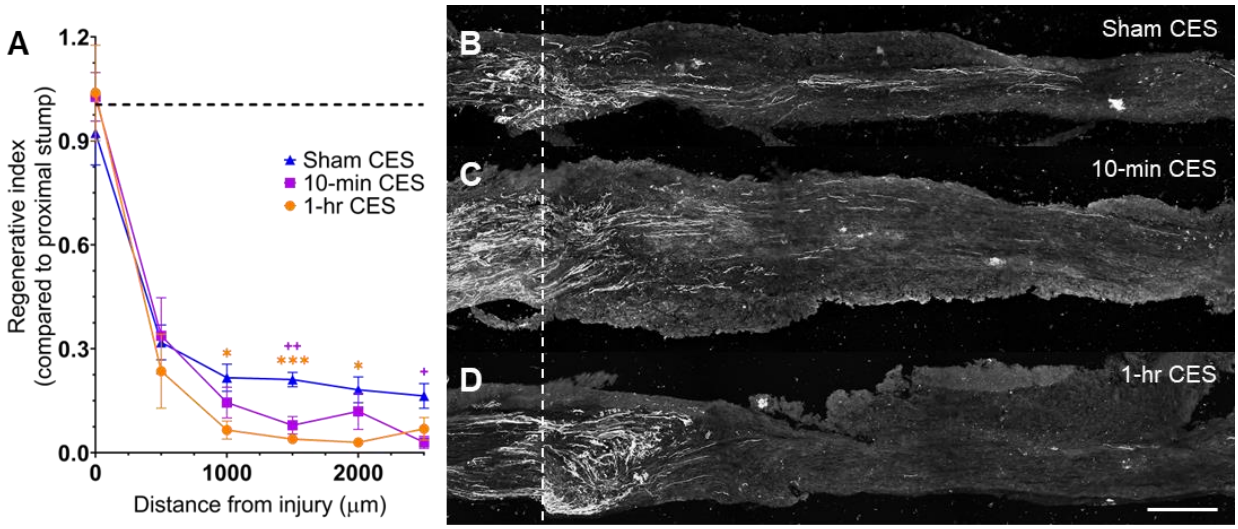

Figure 2: Conditioning electrical stimulation reduces sympathetic axon growth acutely after injury. (A) Plot of regenerative indices calculated based on number of axon profiles present every 500 µm distal to the injury site relative to the number of axon profiles 250 µm proximal to the injury site. Two-way repeated measures ANOVA with post hoc Tukey's multiple comparisons test. * = Sham CES vs 1-hr CES, + = Sham CES vs 10-min CES. * or + $p < 0.05$. ++ $p < 0.01$. *** $p < 0.001$. Black dotted line at regenerative index = 1.0. Data shown as mean ± SEM. Sham n = 5, 10-min n = 5, 1-hr n = 5. (B) Representative sciatic nerve section after sham CES. (B) Representative sciatic nerve section after 10-min CES. (C) Representative sciatic nerve section after 1-hr CES. All sections were stained for tyrosine hydroxylase. White dotted line indicates injury site. Scale bar = 250 µm.

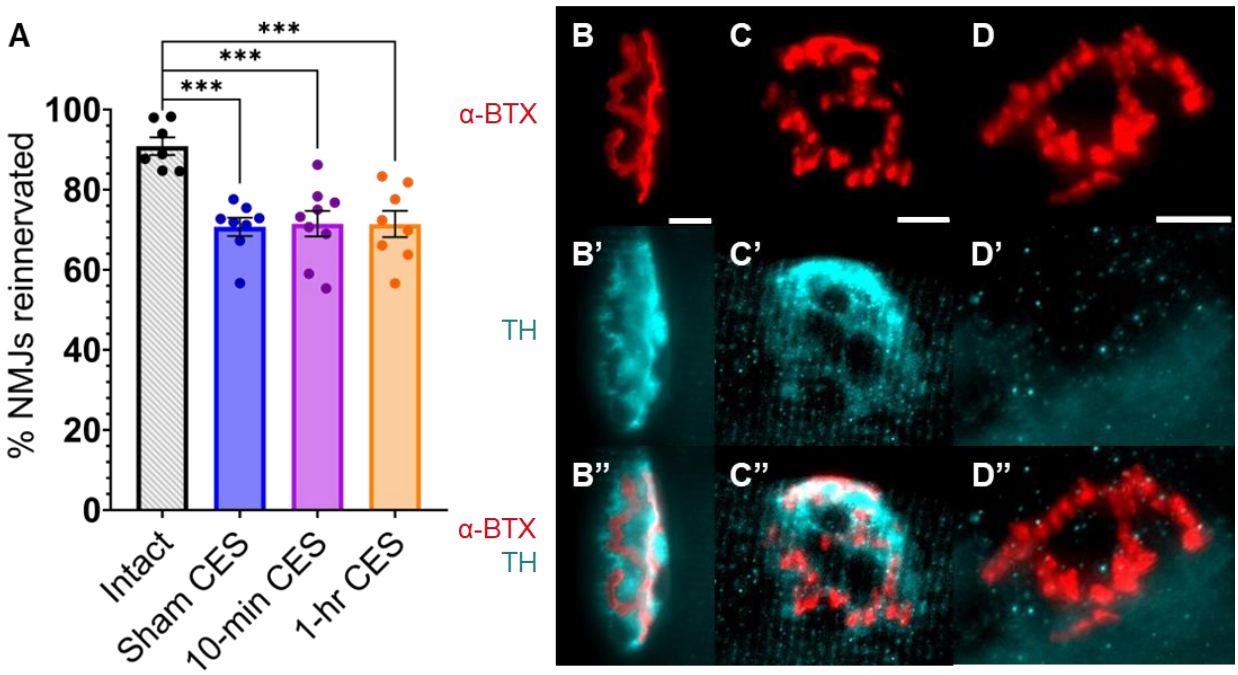

Figure 3: CES does not improve sympathetic reinnervation at the neuromuscular junction (NMJ). (A) Percentage of NMJs in the gastrocnemius with sympathetic contribution compared to uninjured (Intact) muscles. One-way completely randomized ANOVA with post hoc Tukey's multiple comparisons test. ***$p < 0.001$. Data shown as mean ± SEM. L Control n = 7, Sham n = 8, 10-min n = 9, 1-hr n = 8. (B-B") NMJ from intact muscle with sympathetic innervation. (C-C") Fragmented NMJ post-PNI in the gastrocnemius with sympathetic innervation. (D-D") Fragmented NMJ post-PNI in the gastrocnemius without sympathetic innervation. α-BTX = α-bungarotoxin, TH = tyrosine hydroxylase. Scale bars = 10 µm.

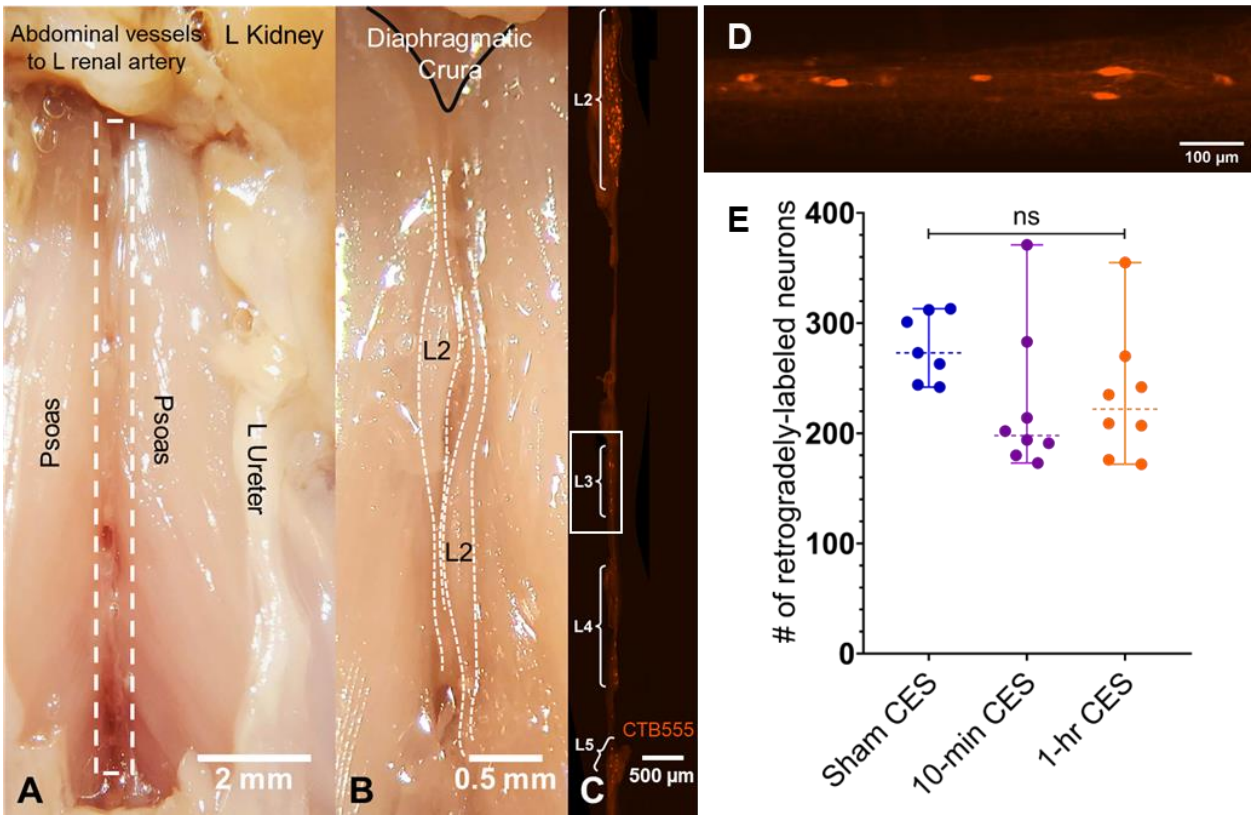

Figure 4: The number of retrogradely-traced sympathetic neurons is not affected by conditioning electrical stimulation. (A) Gross dissection of the lumbar sympathetic ganglia in a perfused mouse. (B) Magnified view of the bilateral L2 lumbar sympathetic ganglia. (C) Unilateral L2-L5 lumbar sympathetic ganglia with neurons retrogradely-labeled with cholera toxin subunit B Alexa Fluor 555 (CTB555). (D) Magnified image of the L3 ganglion with retrogradely-traced neurons. (E) Scatterplot of retrogradely-labeled neurons. Kruskal-Wallis test was not significant. Data shown as medians with 95% confidence intervals. Sham n = 8, 10-min n = 9, 1-hr n = 8.