Conditioning Electrical Stimulation to Enhance Regeneration and Reinnervation Following Peripheral Nerve Injury

by

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Abstract

Background: Peripheral nerve injury is common, identified in 3% of patients presenting to major emergency centers. Poor outcomes are attributable to the slow intrinsic rate of nerve regeneration. A strategy to accelerate this process is of paramount clinical importance. A conditioning crush lesion (CCL) – crushing a nerve one week prior to nerve cut and repair – significantly accelerates axonal extension; however, clinical translation of a CCL is impossible due to the injurious nature of a crush lesion

.

Hypothesis: Conditioning electrical stimulation (CES) is a clinically feasible method of delivering a conditioning-like effect to accelerate regeneration and improve functional nerve recovery.

Aim 1: **Determine if CES improves nerve regeneration and sensorimotor reinnervation.** In a Sprague-Dawley rat model, the regenerative capacity of CES was compared to CCL and negative controls. Our results demonstrated that CES upregulates genes necessary for regeneration, increases the length of axonal extension, and improves sensory (von Frey filaments, nerve counts) and motor (horizontal ladder, toespread width, nerve conduction studies, neuromuscular junction analysis) reinnervation outcomes. Functional recovery in animals treated with CES supersedes that of CCL-treated animals. These results support our hypothesis that pre-injury electrical stimulation delivers a pro-regenerative conditioning effect.

Aim 2: Compare the effects of CES with PES. Postoperative electrical stimulation (PES) delivered immediately after nerve repair improves patient sensorimotor outcomes and is used clinically at many institutions. To compare CES vs. PES and determine if a synergistic effect can be obtained, animals were divided into a) CES, b) PES, c) CES+PES, and d) negative control. Animals treated with CES had significantly longer lengths of nerve regeneration and improved sensorimotor recovery compared to all other cohorts; no synergistic effect was identified when combining CES + PES.

Aim 3: Investigate the effect of CES in three common clinical scenarios: primary nerve repair, nerve grafting, and distal nerve transfers. To lay a strong foundation for clinical translation, we assessed the effects of CES on promoting reinnervation following three common nerve surgeries in which clinical outcomes are inadequate. In all three studies, length of regeneration and sensorimotor reinnervation outcomes were assessed as above.

a) Nerve grafting: to mimic autologous nerve graft repair surgeries, a gap was created in the rat tibial nerve and repaired with a 0.5 cm nerve autograft. Regeneration and reinnervation outcomes of animals treated with CES were significantly greater animals treated with PES or no-stimulation.

b) Distal nerve transfer: A branch of tibial nerve was be sutured to the injured distal stump of the common peroneal nerve, in keeping with the common surgery to treat foot drop following common peroneal nerve injury. Animals treated with CES prior to nerve transfer had significantly greater motor recovery.

c) Primary nerve repair: to mimic patients presenting with a nerve injury amenable to primary repair, the tibial nerve was transected; three days later, one cohort of animals received CES and the other did not. Nerve repair was performed 2 weeks after injury. Results demonstrate an improved length of regeneration and greater sensorimotor recovery in the CES animals, despite delaying surgery. As current guidelines mandate immediate nerve repair these results suggest the need for a change in clinical practice.

Significance: Our results suggests CES significantly improves regeneration and reinnervation outcomes beyond the conditioning 'gold-standard' CCL; however, unlike a CCL, CES can be translated to the bedside. CES outcomes supersede the gold-standard adjuvant to surgical repair (PES) suggesting a need to change perioperative management of patients with peripheral nerve injury. CES consistently conferred a greater functional recovery the common surgical procedures of nerve autograft reconstruction, distal nerve transfer, and primary repair. As electrical stimulation is already established as safe in humans, randomized controlled trials to investigate CES in clinical practice are required, CES may dramatically improve clinical outcomes and quality of life for patients with peripheral nerve injury.

Preface

This thesis is an original work by Jenna-Lynn Senger. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name "Electrical Stimulation to Promote Nerve Regeneration", No. AUP00001871, November 27, 2016.

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I was responsible for literature review and co-created the manuscript. VMK Verge, KM Chan, and CA Webber all reviewed relevant literature and co-created the manuscript.

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I was responsible for study design, animal surgeries, tissue collection and processing, data analysis, and manuscript creation. VMK Verge and J Olson assisted in study design. HSJ Macandili assisted in tissue processing. KM Chan was involved in study design, data analysis and manuscript creation. CA Webber was the senior author and involved with concept formation, data analysis, and manuscript creation.

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I was responsible for study design, animal surgeries/behaviour testing, tissue collection and processing, data analysis, and manuscript creation. KM Chan was involved in study design, data analysis, nerve conduction studies, and manuscript creation. HSJ Macandili assisted in tissue processing and functional assessment. AWM Chan assisted in Western blot analysis. VMK Verge assisted in study design. CA Webber was the senior author and involved with concept formation, data analysis, and manuscript creation.

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I was responsible for study design, animal surgeries/behaviour testing, tissue collection and processing, data analysis, and manuscript creation. KM Chan was involved in study design, nerve conduction studies, and manuscript creation. CA Webber was the senior author and involved with concept formation, data analysis, and manuscript creation.

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I was responsible for study design, animal surgeries/behaviour testing, tissue collection and processing, data analysis, and manuscript creation. KM Chan was involved in study design, data analysis, nerve conduction studies, and manuscript creation. T Kwan-Wong was responsible for blinding/unblinding and data analysis. J Olson assisted in study design. CA Webber was the senior author and involved with concept formation, data analysis, and manuscript creation.

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I was responsible for study design, animal surgeries/behaviour testing, tissue collection and processing, data analysis, and manuscript creation. K Rabey performed kinematic and kinetic analysis. AW Chan performed immunohistochemistry and Western blot analysis. M Morhart assisted in animal surgeries. KM Chan was involved in study design, data analysis, and manuscript creation. CA Webber was the senior author and involved with concept formation, data analysis, and manuscript creation.

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List of Abbreviations

AFO	Ankle-foot orthotic
ANOVA	Analysis of variance
Arg1	Arginase-1
ATF-3	Activating transcription factor 3
BDNF	Brain derived neurotrophic factor
BSA	Bovine serum albumin
Cam kinase	Ca^{2+}/calmodulin-dependent protein kinase
cAMP	cyclic adenosine monophosphate
CCL	Crush conditioning lesion
CCL-2	c-c class chemokine 2
CES	Conditioning electrical stimulation
CMAP	Compound muscle action potential
CNTF	Ciliary neurotrophic factor
CP	Common peroneal
CRE	cAMP response element
CREB	cAMP response element-binding protein
db-cAMP	Dibutyryl cAMP
DNT	Distal nerve transfer
DR	Delayed repair
DRG	Dorsal root ganglion
EPAC	Exchange protein directly activated by cAMP
ERK	Extracellular signal-regulated kinase

ES	Electrical stimulation
GAP-43	Growth associated protein 43
GFAP	Glial fibrillary acidic protein
GDNF	Glial-derived neurotrophic factor
GPCR	G-protein coupled receptor
HSLAS	Health Sciences Laboratory Animal Services
HR	Heel-rise (opposite)
IC	Initial contact
IENF	Intraepidermal nerve fiber
IL-6	Interleukin-6
IR	Immediate repair
Jak	Janus-kinase
LIF	Leukemia inhibitory factor
MAG	Myelin-associated glycoprotein
MAPK	Mitogen-activated protein kinase
MUNE	Motor unit number estimation
NF200	Neurofilament-200
NCS	Nerve conduction studies
NGF	Nerve growth factor
NMJ	Neuromuscular junction
NT-3	Neurotrophin-3
NT-4/5	Neurotrophin-4/5
OBPI	Obstetrical brachial plexus injury

OCT	optimum cutting temperature
OT	Initial contact- opposite toe off
pCREB	Phsophorylated cAMP response element-binding protein
PBS	Phosphate buffered saline
PDE4	Phosphodiesterase-4
PES	Postoperative electrical stimulation
PGP9.5	Protein gene product 9.5
PKA	Protein kinase A
PLCy	Phospholipase C-gamma
PLP	Paraformaldehyde Lysine Periodate
PSA	Polysialic acid
qRT-PCR	Quantitative real time reverse transcription polymerized chain reaction
RAG	Regeneration associated genes
RCT	Randomized controlled trial
SCG	Satellite glial cells
SCb	Slow component b
SEM	Standard error of the mean
STAT	Signal Transducer which Activator of Transcription
TGF-α	Transforming growth factor alpha
TO	Toe-off
trkB	Tyrosine receptor kinase B
VEGF	Vascular endothelial growth factor
VGCC	Voltage gated calcium channel

Chapter 1

Introduction

1.1 Epidemiology of peripheral nerve injuries

Peripheral nerve injuries are heterogenous in the patient population affected, the functional deficits incurred, and the overall outcomes anticipated. Nerve injuries are formally graded using the Sunderland classification, which classifies nerve injuries based on the extent of injury to the axon and connective tissue: first degree (damaged myelin), second degree (damaged axon), third degree (damaged axon and endoneurium), fourth degree (damaged axon, endoneurium, perineurium), fifth degree (complete physiologic disruption), and sixth degree (mixed injury) (Mackinnon & Dellon, 1988). When the outer layers of connective tissue remain in continuity, as in first- and second-degree injuries, the innate regenerative mechanisms of the peripheral nervous system are sufficient to reinnervate distal targets and restore functional recovery; therefore, these patients require only close observation. More extensive injuries necessitate surgical repair to re-establish structural continuity and thereby provide a conduit to guide regenerating axons to their correct motor or sensory targets. Among patients requiring surgical management, common etiologies can be divided into four categories: severe compression neuropathies, nerve traction injuries, nerve transections injuries, and mixed injuries.

1.1.1 Compression neuropathies

Carpal tunnel syndrome, a compression neuropathy in which the median nerve is entrapped at the level of the wrist, is identified in 3.8% of the general population (Atroshi et al., 1999). Among patients diagnosed with carpal tunnel syndrome, 25% have severe symptoms that require surgical decompression (Atroshi, Gummesson, Johnsson, McCabe, & Ornstein, 2003). The number of carpal tunnel releases performed annually is rising, with a 38%

increase between 1996 and 2006 (Fajardo, Kim, & Szabo, 2012). Up to half a million patients in the United States undergo surgical carpal tunnel release each year, at a cost exceeding two billion dollars (Dale et al., 2013). Despite carpal tunnel release surgery, patients with severe disease often fail to have complete sensorimotor recovery (Gordon, Amirjani, Edwards, & Chan, 2010). Similar to carpal tunnel syndrome, other compression neuropathies involving the ulnar (cubital tunnel syndrome), radial (radial tunnel syndrome, posterior interosseous nerve syndrome, Wartenberg's syndrome), lateral femoral cutaneous (meralgia paresthetica) or the tibial (tarsal tunnel syndrome) nerves similarly cause motor and/or sensory deficits that often preclude activities of daily living and may require surgical decompression. Severe compression neuropathy results in a disruption of the blood-nerve barrier and endoneurial edema. Over time, chronic compression induces focal demyelination and Wallerian degeneration; following surgical decompression, axons must regenerate from the site of compression to their distal targets.

1.1.2 Nerve traction injuries

Obstetrical brachial plexus injuries (OBPI) are identified in 1.6-2.6/1000 live births (Coroneos et al., 2017). These injuries are typically attributable to traction placed on the brachial plexus during delivery if the infant becomes impinged in the birth canal. Risk factors include shoulder dystocia, macrosomia, gestational diabetes, or delivery with vacuum or forceps. Erb's palsy, an isolated injury to the upper plexus (C5, C6), is identified in 46% of OBPI patients and has the best prognosis, with spontaneous recovery in 80% of patients. Flail limb secondary to dysfunction of the entire brachial plexus (C5-T1) is identified in 20% of OBPIs and has poor outcomes without surgical management (Buterbaugh & Shah, 2016).

Surgical options include exploration of the brachial plexus with resection of neuromas-in-continuity and nerve autografting, or distal nerve transfers using uninjured intra- or extra-plexus donor nerves to reinnervate denervated motor targets. Though children have a greater capacity for nerve regeneration than adults, despite surgical management, functional deficits may persist (Ladak et al., 2013; Lin, Schwentker-Colizza, Curtis, & Clarke, 2009).

1.1.3 Nerve transection injuries

Traumatic peripheral nerve injuries are common. A recent review of the National Inpatient Sample database identified a mean incidence of 43.8 nerve injuries/million people annually (Karsy et al., 2019). The digital and ulnar nerves (18%) were the most frequently affected, followed by the radial (15%) and median (13%) nerves, with brachial plexus injuries identified in 15% of patients (Karsy et al., 2019). Overall, 30% of patients were unable to return to work postoperatively, 75% of patients reported regular pain, and 19% of patients developed depression or anxiety (Rasulić et al., 2017). The personal and socioeconomic costs associated with these traumatic nerve injuries are therefore significant.

Digital nerve injuries are most often the result of nerve transection, with an annual population incidence of 6.2/100,000 (Thorsen, Rosberg, Steen Carlsson, & Dahlin, 2012). Digital nerve transection requires surgical exploration and repair to restore nerve continuity. Despite successful surgical nerve repair, sequelae include cold insensitivity (2-53%), hyperesthesia and/or dysesthesia (40-67%), and numbness (>75%), with consequential impairment in activities of daily living (Dunlop, Wormald, & Jain, 2019; Thorsen et al., 2012). The majority

(80%) of patients with digital nerve injuries are unable to return to work for a median of 2 months postoperatively (Thorsen et al., 2012).

1.1.4 Mixed nerve injuries

Mixed nerve injuries are typically the result of major trauma, and include patterns of injury including nerve transection, avulsion, and traction. Concurrent soft tissue and/or bony injuries are often identified in these patients, which may delay diagnosis and management of peripheral nerve injuries, and affect recovery of strength and range of motion. In one of the largest studies of its kind, peripheral nerve injuries were identified in 2.8% of patients with upper/lower extremity trauma presenting to a tertiary care centre in Alberta (Noble, Munro, Prasad, & Midha, 1998). These results are similar to those of a large European database review that identified a 3.3% incidence of nerve injury among patients with injury to their upper limb (Huckhagel, Nüchtern, Regelsberger, & Lefering, 2018). Patients with severe peripheral nerve injuries are most commonly young males involved in high energy events such as in motor vehicle collisions or industrial accidents (Huckhagel et al., 2018). Among all trauma patients, those with peripheral nerve injuries typically have a longer stay in hospital and require prolonged inpatient rehabilitation (Huckhagel et al., 2018). Patient outcomes after brachial plexus injury are particularly poor. In a large series of patients with brachial plexus injuries, good recovery, defined as movement against resistance, was identified in only 35% of patients treated with exploration and neurolysis, 57% of patients treated with grafting, and 17-67% of patients treated with nerve transfer (Rasulić et al., 2017). Given these poor outcomes, identification of novel perioperative and surgical techniques to improve regeneration and reinnervation is of significant importance.

1.1.5 Treatment of nerve injuries

Despite differences in the patient demographics affected, the source of axonal injury, and the expected sensorimotor outcomes following compression neuropathy, nerve traction or transection injuries or massive traumas, functional recovery in all situations is dependent on the intrinsic mechanisms of nerve regeneration. As discussed in the next section, the faster regenerating axons reach their target muscle or sensory receptor, the greater the anticipated motor and sensory recovery. The longer these targets are left denervated, the lower the chance of functional reinnervation. Strategies to accelerate the rate of axon extension and to improve target reinnervation are therefore fundamental to improving outcomes following any type of peripheral nerve injury.

1.2 Limitations to Peripheral Nerve Regeneration

Despite advances in surgical techniques, outcomes for patients with peripheral nerve injuries have not significantly improved in the past 25 years (Lundborg, 2000). Even with timely surgical repair, only approximately 10% of regenerating neurons reach their target tissue, often resulting in poor patient recovery (Zochodne, 2012). To date, there remains no effective treatment that reliably confers a full recovery of premorbid motor and sensory function following peripheral nerve injury (Sabatier & English, 2015).

Successful functional recovery is directly related to the time required for regenerating motor or sensory axons to reach their target muscle or sensory receptor. The time to end-target reinnervation is affected by ineffective staggered regeneration at the site of injury, the distance between the site of injury and the distal target, the slow innate speed of axon

extension, and the declining regenerative capacity of denervated muscle and sensory receptors.

As early as the 20th century, Cajal used silver staining to visualize of axonal extension following nerve injury. He recognized that at the site of injury, regenerating axons take a tortuous route and form retrograde spirals rather than extending straight towards their target (Cajal, 1928). Cajal's findings were confirmed decades later, when it was recognized that rather than axons extending as a unified front, individual axons regenerate across the site of surgical coaptation in an irregular temporal pattern, with progressively larger numbers of axons identified immediately distal to the repair site over a four week time period in a rodent model (Brushart et al., 2002b). The process is now called 'staggered regeneration' and significant slows in the rate of axon extension, thereby delaying end target reinnervation.

The innate speed of axon extension beyond the site of surgical repair is slow, approximately 1 mm/day in humans and up to 3 mm/day in rodents. This means that when a long distance of regeneration is required, such as a proximal injury of a nerve with a distal target, the time required for regenerating axons to reach their target can be months to years. Seminal work by Fu and Gordon established that the regenerative capacity of denervated distal targets progressively deteriorates over time; when compared to acutely repaired nerve transections, a chronic nerve injury decreases the regenerative capacity of motoneuron by 66% and distal Schwann cells by 90% (S. Y. Fu & Tessa Gordon, 1995; S. Y. Fu & T. Gordon, 1995; Gordon, Sulaiman, & Boyd, 2003). To differentiate the effects of chronic axotomy from chronic denervation, a cross-reinnervation model was used to isolate the effects of

denervating the motoneurons of the proximal stump (chronic axotomy) compared to denervating the Schwann cells of the distal stump (chronic denervation). Chronic axotomy was examined by performing a tibial nerve transection up to 12 months prior to suturing the proximal stump of the chronically axotomized tibial nerve to the distal stump of an acutely transected common peroneal nerve; chronic denervation was evaluated by transecting the common peroneal nerve up to 4 months prior to coaptation of this distal stump to the proximal stump of an acutely transected tibial nerve. By three months following chronic axotomy, the total number of motor units in each muscle was 35% of controls; however, the few axons that reached muscle had increased branching patterns. As such, muscle force and weight were similar to acutely injured axons (S. Y. Fu & Tessa Gordon, 1995). Contradictory to common belief, it was therefore concluded that chronically denervated endplates could accept reinnervation. In the chronically denervated distal stump, however, axons that reached the muscle branched to the maximal capacity, however, so few motoneurons traversed through the intramuscular sheath to reach their target that motor recovery did not recover (S. Y. Fu & T. Gordon, 1995). The denervated Schwann cells of the distal stump was subsequently found to be supportive of axon regeneration for up to one month in rodent models, after which the growth-supportive phenotype of these Schwann cells progressively declines (Hoke, Gordon, Zochodne, & Sulaiman, 2002; You, Petrov, Chung, & Gordon, 1997). Denervated Schwann cells over time become atrophic, downregulate expression of neurotrophic factors, and have a decreased ability to interact with regenerating axons (Gordon et al., 2003). Together, these important observations suggest that the faster regenerating axons can reach their target, the greater the capacity for reinnervation and improved functional recovery.

Improving functional recovery following peripheral nerve repair necessitates decreasing the time between nerve injury and target reinnervation to minimize the challenges of reinnervated a chronically denervated motor target. Two promising perioperative strategies to accelerate axon extension either at the site of coaptation, or through the distal stump are postoperative electrical stimulation (PES) and the conditioning lesion (CL) respectively. The following sections describe the effects of these techniques on enhancing regeneration and reinnervation outcomes and their underlying mechanisms of action.

1.3 Electrical stimulation for enhancing peripheral nerve regeneration

Prior to the 21st century, electrical stimulation for peripheral nerve injuries was not delivered to the nerve itself, but rather to the denervated muscle bulk for maintaining contractility, or to the joint for treating of contractures (Chan, Curran, & Gordon, 2016). Only two small studies had investigated the use of electrical stimulation to the nerve itself. Nix and Hopf showed that electrical stimulation of the tibial branch to soleus following axonotmesis of the same nerve significantly improved motor recovery compared to non-stimulated controls (Nix & Hopf, 1983). Similarly, Pockett and Gavin showed improvement in reflex contractions of the ankle extensor muscles following a crush injury if the nerve was treated with PES (Pockett & Gavin, 1985). Unfortunately, the precise anatomical site of electrical stimulation is unreported in both studies.

1.3.1 What is Postoperative Electrical Stimulation?

In 2000, the Gordon lab identified postoperative electrical stimulation (PES) as an effective tool for enhancing nerve regeneration. Using a rodent model, the femoral nerve was transected and repaired with or without electrical stimulation delivered postoperatively. Electrical stimulation was delivered using two insulated wires: the cathode was secured alongside the femoral nerve and the anode was sutured to muscle. Wires were connected to a stimulator and electrical stimulation was delivered immediately following nerve coaptation using supramaximal pulses at 3 Volts of 0.1 second duration. A frequency of 20 Hz was chosen to mimic the physiologic frequency of motoneuron discharge. Outcomes of animals treated with electrical stimulation were compared to those who received immediate postoperative sham-stimulation, in which the electrodes were implanted as before but the stimulator was not activated (Al-Majed, Neumann, Brushart, & Gordon, 2000). This paradigm of delivering electrical stimulation has since become the standard for PES against which other models of electrical stimulation are compared. Stimulation for one hour immediately post-repair was as effective as continuous stimulation for one day, one week, or two weeks for improving motoneuron regeneration. Based on retrograde labelling of motoneurons, all animals that received PES had significantly accelerated axon outgrowth across the coaptation site, shortening the time required for staggered regeneration from 8-10 weeks in non-stimulated controls, to 3 weeks in animals who received PES. Further studies have demonstrated that the pro-regenerative effects of PES can be translated to improve regeneration through a chronically denervated distal stump. Chronically injured neurons treated with PES were found to have improved capacity to extend regenerating axons, while

chronically denervated Schwann cells treated with PES better supported regeneration (Elzinga et al., 2015; Huang, Zhang, Lu, Hu, & Luo, 2013).

The effects of electrical stimulation differ between motor and sensory neurons; whereas motoneuron regeneration is enhanced by any duration of electrical stimulation between 1 hour and 2 weeks (Al-Majed, Neumann, et al., 2000), sensory axon outgrowth is improved with one hour of PES, but any longer is detrimental to recovery (Geremia, Gordon, Brushart, Al-Majed, & Verge, 2007). The effects of PES are more robust in motoneurons, with a three-fold improvement in staggered regeneration, greater than the 1.7-fold increase identified in sensory neurons (Suzuki, Ochi, Shu, Uchio, & Matsuura, 1998).

1.3.2 Mechanism of Action of Postoperative Electrical Stimulation

The mechanism of action underling the pro-regenerative effects of PES has been well studied. The cell body is critical to the effects of PES; blocking sodium channels proximal to the site of electrical stimulation with tetrodotoxin obliterates the retrograde signal generated by PES, and consequently eliminates its pro-regenerative effects (Al-Majed, Neumann, et al., 2000). Important signaling pathways within the cell body include the activation of tyrosine receptor kinase B (TrkB) by its ligand brain derived neurotrophic factor (BDNF), and the cAMP pathway.

TrkB and BDNF as well as Neurotrophin-4/5 (NT-4/5) are important neurotrophic factors upregulated following nerve injury for promoting nerve regeneration. BDNF is expressed both in the distal stump by Schwann cells as well as at the cell body by the neuron itself.

Axon outgrowth is promoted by recombinant BDNF, NT-4/5 or TrkB agonists, and inhibition of neurotrophic factors by antagonistic antibodies or genetic knock out models inhibits regeneration (English, Wilhelm, & Ward, 2014). Delivery of PES following nerve transection causes a 2- to 3-fold increase in BDNF and trkB mRNA expression at 8 hours and 2 days post-nerve repair, respectively. By contrast, among non-stimulated animals, a two-fold increase in expression of these mRNA was not observed until 7 days post-repair (Al-Majed, Brushart, & Gordon, 2000). Expression of BDNF and trkB are directly linked to upregulation of GAP-43 and the cytoskeletal proteins actin and tubulin, with decreased expression of neurofilament (Al-Majed, Tam, & Gordon, 2004). Accelerated upregulation of BDNF and trkB with PES results in early expression of tubulin and GAP-43, which contribute to accelerating axonal growth at the coaptation site (Al-Majed et al., 2004).

An important downstream effect of BDNF upregulation is activation of the adenylyl cyclase pathway; it is therefore accepted that PES increases expression of cAMP. Both cAMP and its downstream target protein kinase A (PKA) are central to nerve regeneration. Numerous studies have demonstrated that upregulation of cAMP via exogenous administration of forskolin (an adenylyl cyclase stimulator), dibutyryl cAMP (a soluble cAMP analog), or rolipram (a phosphodiesterase IV inhibitor) all increase neurite extension *in vitro* (Aglah, Gordon, & Posse de Chaves, 2008). Increasing neuronal cAMP levels accelerates axonal extension and improves staggered regeneration comparable to PES (Gordon et al., 2009).

The effects of upregulating these molecular pathways are twofold: acceleration of staggered regeneration and induction of preferential motor reinnervation. As growing axons extend

through the site of repair, they do so not as a uniform front but rather individual axons extend in a sporadic sequence termed "staggered regeneration". This process is slow, requiring significantly longer for all axons to cross the site of repair than would be predicted based on the traditional belief that axon extension progresses at 3mm/day (Al-Majed, Neumann, et al., 2000; Witzel, Rohde, & Brushart, 2005). One hour of 20 Hz PES enhances the speed by which axons undergo staggered regeneration, but does not accelerate the rate of axon extension in the distal stump beyond the site of surgical repair (Brushart et al., 2002a). In addition, PES causes parent axons to emit more regenerating axon sprouts (Franz, Rutishauser, & Rafuse, 2008). It is suggested that the greater the number of sprouts, the higher the probability that at least one will innervate the appropriate basal lamina-lined epineurial tube of the denervated distal stump.

After nerve injury, the specificity of regenerating axons to their original muscle targets is poor (Brushart & Mesulam, 1980). PES induces preferential motor reinnervation, in which transected motoneurons preferentially reinnervate motor pathways when given equal access to motor and cutaneous epineurial tubes (Al-Majed, Neumann, et al., 2000; Brushart, Jari, Verge, Rohde, & Gordon, 2005). Quadriceps muscle were labelled with FluoroGold prior to femoral nerve trunk transection and repair to visualize regenerating motoneurons. Three weeks post-repair, regenerating fibers were labeled with Fluororuby. Double-labeled fibers indicated motoneurons that were regenerating down a motor pathway. Animals that did not receive PES had positive double labelling in only 40% of fibers; this was significantly less than 75% of axons in PES-treated animals. These results suggest that PES significantly improves the specificity of regeneration (Brushart et al., 2005).

1.3.3 Duration of Postoperative Electrical Stimulation

Since Al-Majed's description of brief intermittent electrical stimulation demonstrated that one hour of electrical stimulation confers regenerative outcomes comparable to longer timepoints (Al-Majed, Neumann, et al., 2000), this protocol has become the most common paradigm selected for both animal and human studies, and the standard against which other models are compared. Limited studies have further investigated the optimal duration of electrical stimulation, with few direct comparisons between the standard one hour and other timepoints. It has been suggested that as little as ten minutes of PES may be as effective in improving regeneration outcomes as the full sixty minutes (Calvey et al., 2015). Improved regeneration outcomes with a shortened period of electrical stimulation have been reported by other authors. Twenty minutes of electrical stimulation at 20 Hz delivered to the rodent sciatic nerve increased the number of regenerating axons and their myelin thickness, with improved nerve conduction studies (Huang et al., 2013) and thirty minutes of daily electrical stimulation regeneration of the facial nerve following axotomy and repair (Sharma, Moeller, Marzo, Jones, & Foecking, 2010). A more recent study, however, suggests muscle mass and force generated is improved if electrical stimulation is delivered for one hour daily for six days rather than as a one-time treatment (Koo et al., 2018). Further investigation to uncover the appropriate duration of electrical stimulation is required.

1.3.4 Clinical Translation of Postoperative Electrical Stimulation

PES has been successfully translated from the bench to the bedside in a series of randomized controlled trials (RCTs) led by the Chan laboratory. Among patients with severe carpal tunnel syndrome, one hour of PES immediately following decompression surgery

significantly improved recovery of motor unit number estimation (MUNE) by 6-8 months to a level comparable to healthy controls, whereas patients who did not receive PES had no recovery by this timepoint. Improved motor and sensory testing were further confirmed in PES patients (Gordon et al., 2010). The exciting effects of PES were confirmed in another model of compression neuropathy, cubital tunnel syndrome, in which patients treated immediately post-decompression with PES had improved MUNE, grip strength, and key pinch compared to non-stimulated controls (Power, Morhart, Olson, & Chan, 2016).

The use of PES has been expanded beyond compression neuropathy to nerve transection. In an RCT evaluating outcomes of complete digital nerve injury, patients treated with PES immediately following digital nerve repair had significant improvement in sensory recovery (Wong, Olson, Morhart, & Chan, 2015).

1.3.5 Limitations of Postoperative Electrical Stimulation

Despite the exciting outcomes observed in animal and human models of PES, motor and sensory recovery remains incomplete. The effects of this technique are limited, however, to improving regeneration only at the site of coaptation. PES does not accelerate axon regeneration once they reach the endoneurial tubes of the distal stump (Chan et al., 2016). For proximally located injuries, while accelerated staggered regeneration through the injury site is advantageous, the subsequent slow rate of axonal extension precludes timely reinnervation of end targets. A strategy to accelerate the intrinsic rate of nerve regeneration is therefore of significant clinical importance.

1.4 The Conditioning Lesion for Enhancing Peripheral Nerve Regeneration

One potential approach to accelerate nerve regeneration is a planned peripheral nerve insult (conditioning lesion) prior to a subsequent nerve injury (test lesion). While initial methodologies precluded clinical translatability, research over the past four decades has yielded valuable mechanistic insights. These studies not only provided a solid foundation to support the use of conditioning lesion (CL) in clinical settings, they have helped to identify molecular targets that could potentially be pharmacologically manipulated to enhance peripheral nerve regeneration.

This review of the conditioning has been published in *Annals of Neurology* (Senger JL, Verge MVK, Chan KM, Webber CA. 2018; 83: 691-702) (Senger, Verge, Chan, & Webber, 2018).

1.4.1 What is a Conditioning Lesion?

Injury to the peripheral nervous system triggers a well-characterized sequence of events. A "conditioning lesion" is a purposeful peripheral nerve insult prior to definitive injury and repair, with the aim of enhancing axon regeneration. Parameters that appear to affect the CL response include the: i) method of conditioning (Arntz, Kanje, & Lundborg, 1989; Dahlin & Kanje, 1992; Dahlin, Necking, Lundstrom, & Lundborg, 1992; Hollis et al., 2015; Sisken, Kanje, Lundborg, Herbst, & Kurtz, 1989; Udina et al., 2008), ii) location of the CL,(Ryoke et al., 2000) iii) magnitude of the insult, iv) duration of conditioning effect (Jenq, Jenq, Bear, & Coggeshall, 1988; Sjoberg & Kanje, 1990a), v) CL interval (Arntz et al., 1989; Forman et al., 1980; Richardson et al., 2009; Torigoe, Hashimoto, & Lundborg, 1999; Ying, Misra, & Verge, 2014) and vi) type of nerves suitable for conditioning,(Bisby & Keen, 1985; Bontioti,

Kanje, & Dahlin, 2003; Galbavy, Kaczocha, Puopolo, Liu, & Rebecchi, 2015) A summary of the findings is shown in Table 1. Given the variations in surgical techniques and methods of assessing regeneration, with many studies predating more modern techniques of analysis, direct comparisons between them is challenging. However the overall outcomes of these studies suggest that enhanced regenerative outcomes of peripheral nerves following a CL include: i) decreased latency between time of injury and initiation of regeneration (I. G. McQuarrie, 1985), ii) increased rate of axonal outgrowth by three-to-five times that of controls(Richardson & Verge, 1987; Torigoe et al., 1999), and iii) increased numbers of regenerating fibers (Hoffman, 2010).

1.4.2. Potential Clinical Importance and Feasibility

Two main barriers that have precluded clinical translation of CLs are the unpredictability of nerve injuries and the lack of an ethically acceptable and clinically feasible conditioning method that does not involve further damage to the nerve. Importantly however, although most nerve injuries are accidental and cannot be predicted ahead of time, there is an increasing number of surgical procedures in which a peripheral nerve is intentionally injured. One example is distal nerve transfer surgery following injury to a neighboring nerve, in which a healthy donor nerve is deliberately cut and rerouted to reinnervate recently denervated muscles or cutaneous sensory targets. Because this procedure carries the advantage of markedly reducing the regeneration distance, it has become a preferred option over traditional nerve graft repair. With distal nerve transfers being elective procedures, there is ample time for the donor nerve to be conditioned prior to surgery. In addition, conditioning may also be applicable in other clinical scenarios in which preplanned nerve injury occurs,

TABLE 1. Summary of the Methods of Delivery and Effects of Conditioning Lesions

Property	Conclusions
Method of conditioning	• Multiple modalities attempted; any degree of injury to the nerve promotes some degree of regeneration • Nerve cut or crush has greatest effect on regeneration and is the standard against which other modalities are tested
Location of conditioning	• Conditioning lesion must share the same cell body as test lesion • Conditioning must be performed on a peripheral nerve (test lesion may be on the associated peripheral or central axon branches) • Conditioning lesion of the axon should be performed physically close to the future site of the test lesion
Magnitude of conditioning insult	• The greater the force applied to the nerve, the greater the regenerative capacity • Complete axotomy is recommended to maximize the conditioning effect
Duration of exposure	• Single conditioning lesion is sufficient to elicit the conditioning effect, with no added benefit with multiple conditioning lesions
Conditioning interval	• 6–7 days between conditioning and test lesion recommended
Type of nerves that can be conditioned	• Upper & lower extremity • Old & young patients • Sensory & motor • Autonomic (controversial)

such as excision of nerve tumors and nerve transfers in amputees for control of myoelectric prosthesis.

Although ethically it is difficult to justify deliberately injuring a healthy nerve and subject patients to two operations, there are other potential non-invasive methods of delivering the conditioning effects. In lieu of crushing or cutting the nerve, vibration and compression have been explored as alternatives (Dahlin & Kanje, 1992; Dahlin et al., 1992). These observations suggest that it may be feasible to deploy CL in clinical settings to accelerate peripheral nerve regeneration. Finding a non-invasive delivery method that is optimal and clinically acceptable is the challenge.

1.4.3. Mechanism of Action of the Conditioning Lesion

1.4.3.1 Neuronal changes

CL influences the rate of retrograde signaling, transcription within the dorsal root ganglion (DRG), and anterograde transport (Ben-Yaakov & Fainzilber, 2009; Hanz & Fainzilber, 2006). Axonal injury instigates a biphasic retrograde response: an initial calcium influx initiates cytoskeletal rearrangement and protein synthesis, followed by a delayed phase mediated by macromolecules trafficked toward the nucleus via the dynein motor system (Rishal & Fainzilber, 2014). Further, CLs cause localized changes in protein expression that serve as retrograde signals to prime neurons to mount an enhanced cell body response to the test lesion (Ben-Yaakov & Fainzilber, 2009; Kanje, Skottner, Lundborg, & Sjoberg, 1991; Richardson et al., 2009; Ying et al., 2014). Blocking retrograde signaling suppresses the conditioning effect (Kanje et al., 1991), suggesting that retrograde signals are generated by

the CL, such that by the time the test lesion is performed, these signals have already reached the cell body and initiated the synthesis of regeneration-associated mRNAs and proteins that are transported with cytoskeletal elements down to the regenerating axon front (Sjoberg & Kanje, 1990b). Although the peripheral axon was initially thought to be solely affected(Oblinger & Lasek, 1984), subsequently it became clear that regeneration of the central axon branch of the conditioned sensory neuron is also enhanced (I. G. McQuarrie & Grafstein, 1973; Richardson & Issa, 1984; Richardson & Verge, 1987).

Conditioning accelerates the anterograde transport of newly synthesized proteins and mRNA into the axon by 2-3 times (Mar et al., 2014). These proteins are cargo of the slow component b (SCb) pathway, implicated in the conditioning effect (Maier & McQuarrie, 1990; I. G. McQuarrie & Grafstein, 1982). In contrast, the fast transport pathway is not affected by conditioning (Perry, Krayanek, & Wilson, 1987; Redshaw & Bisby, 1987). CLs augment the amount of tubulin and actin available to the developing growth cone and accelerate the SCb pathway by 20-25% (Jacob & McQuarrie, 1996). Axotomy accelerates the polymerization of actin and tubulin by 50% and 43% respectively. This shift in the monomer-to-polymer ratio decreases the total number of molecules being transported, accelerating the SCb pathway and promoting intra-axonal microtubule assembly at the axonal tip (Jacob & McQuarrie, 1996). Tyrosination of tubulin is similarly induced by conditioning, suggesting an overall dynamic shift of microtubule formation (Mar et al., 2014). Conversely, axotomy decreases local neurofilament protein synthesis which is believed to increase tubulin bioavailability by decreasing interference in tubulin transport (Tetzlaff, Leonard, Krekoski, Parhad, & Bisby, 1996). In addition to changes in tubulin, actin synthesis and transport are

likely enhanced in response to CL, as suggested by Lund et al. (2002) who described increased expression and actin polymerization in response to the injury further accelerates SCb transport (Lund, Machado, & McQuarrie, 2002). Recent insights demonstrate that local axonal synthesis of beta-actin supports a role in the regeneration response in conditioned axons, as it is implicated in both initiation and maintenance of axonal growth (Willis & Twiss, 2006). The CL, therefore, increases the availability of tubulin and actin through the SCb pathway and locally for the early formation of microtubules and microfilaments necessary to the developing growth cone.

As SCb transportation is reliant on associations with membrane-bound vesicles, an increased rate of their transport may be partially responsible for the accelerated rate of the SCb pathways. As demonstrated by Mar et al. (2014), conditioning accelerates anterograde transport of lysosomal and synaptophysin-containing vesicles by 1.6x and 1.5x respectively, but has no effect on the overall number of organelles being transported (Mar et al., 2014). Mitochondria are also affected by conditioning, with increased numbers being transported in both anterograde and retrograde directions (Mar et al., 2014). While the speed of mitochondrial movement itself remains unchanged, CLs increase the number of mitochondria arriving at the cell body and the site of injury, presumably providing ATP for the increased transcription/translation evoked by the CL. Extrasomatic/axonal *de novo* protein synthesis has been previously described (Van Minnen et al., 1997). While not specifically investigated in CLs, *de novo* protein synthesis likely occurs at the site of nerve injury in the conditioned axon to allow for rapid transition to the more robust CL-associated

regeneration state. Further research investigating the role of central vs. local protein synthesis in CL is required to fully elucidate the underlying mechanisms.

1.4.3.2. Glial Response

Nonneuronal cells distal to the test lesion are also conditioned as they become proliferative. The conditioning effect was suppressed when Schwann cells and other nonneuronal cells were eliminated from the site of injury (Sjoberg & Kanje, 1990b). The importance of the local nerve environment in the conditioning response was further demonstrated by studies showing that conditioning initially induces a 'reactive Schwann cell phenotype' (days 0-2) that does not promote axonal outgrowth, followed by the second phase (>3 days) which is characterized by migratory Schwann cells (>3 days) that, through secretion of neurotrophins, promote rapid and effective axonal regeneration (Torigoe et al., 1999).

Satellite glial cells (SGCs) represent a growing area of investigation in nerve regeneration and neuropathic pain research. Like neurons, SGCs express the nerve growth factor (NGF) high affinity receptor, tropomyosin/tyrosine related kinase A (trkA) and the low-affinity (p75) neurotrophin receptor, suggesting that SGCs and neurons interact with each other through neurotrophins (Hanani, 2005). SGCs may regulate the availability of neurotrophin to the neuron by releasing and internalizing NGF. Following peripheral nerve injury, SGCs increase production of NGF and neurotrophin-3 (NT-3) which may partially compensate for the lack of neurotrophins normally transported from the target and peripheral nerve to the ganglia before axotomy. While these levels are insufficient to reverse the cell body injury response required for regeneration (Verge, Gratto, Karchewski, & Richardson, 1996) SGC-

derived NGF converges on the injury-associated increased neuropoietic cytokine signaling to positively influence regenerative axon growth (Quarta et al., 2014). However, the cellular and molecular changes in this glial population and whether they are critically linked to the conditioning effect remain largely unknown.

Following injury, the SGC-to-neuron ratio increases and there is a six-fold increase in the number of gap junctions between surrounding SGCs. Hanani et al. (2002) suggest that these gap junctions may be responsible for the spread of neuropathic pain following injury (Hanani, Huang, Cherkas, Ledda, & Pannese, 2002). Post-injury, SGCs express heightened levels of neurotrophins, TGF-α, fibroblast growth factor2, and glial-derived neurotrophic factor (GDNF) (Hanani, 2005). In a study by Lu and Richardson (1991), SGCs were indirectly implicated in the CL response (X. Lu & Richardson, 1991). They showed that a C-parvum-induced inflammatory environment in the DRG resulted in SGC proliferation and a four-fold increase in the number of axons regenerating after dorsal root crush, comparable to that observed with a sciatic nerve CL. Interestingly, the induction of an inflammatory environment around the sciatic nerve decreased the rate of peripheral nerve regeneration, suggesting intraganglionic inflammation, at least by C-parvum alone, is not sufficient to mount the full conditioning effect.

1.4.3.3 Inflammation

Heightened inflammation induced by macrophages improves axon regeneration, and when macrophages are eliminated in the milieu, axon regeneration is significantly reduced. Hollis et al. (2015) echoed the importance of macrophages by simulating a CL using ethidium

bromide injected into the sciatic nerve (Hollis et al., 2015). The increased number of macrophages in the DRGs was associated with a robust growth of the peripheral axons. Salegio (2010) further concluded that: (i) macrophage depletion during the conditioning phase abolishes the conditioning effect, (Salegio, Pollard, Smith, & Zhou, 2010) and (ii) that while the inflammatory response triggered by a sciatic nerve CL significantly elevates the numbers of macrophages, this increased immune surveillance does not translate into enhanced systemic nerve regeneration, as it did not impact subsequent optic nerve repair (Salegio et al., 2010). Thus, the authors suggest that macrophages are critical for early activation of the conditioning effect (Salegio, Pollard, Smith, & Zhou, 2011).

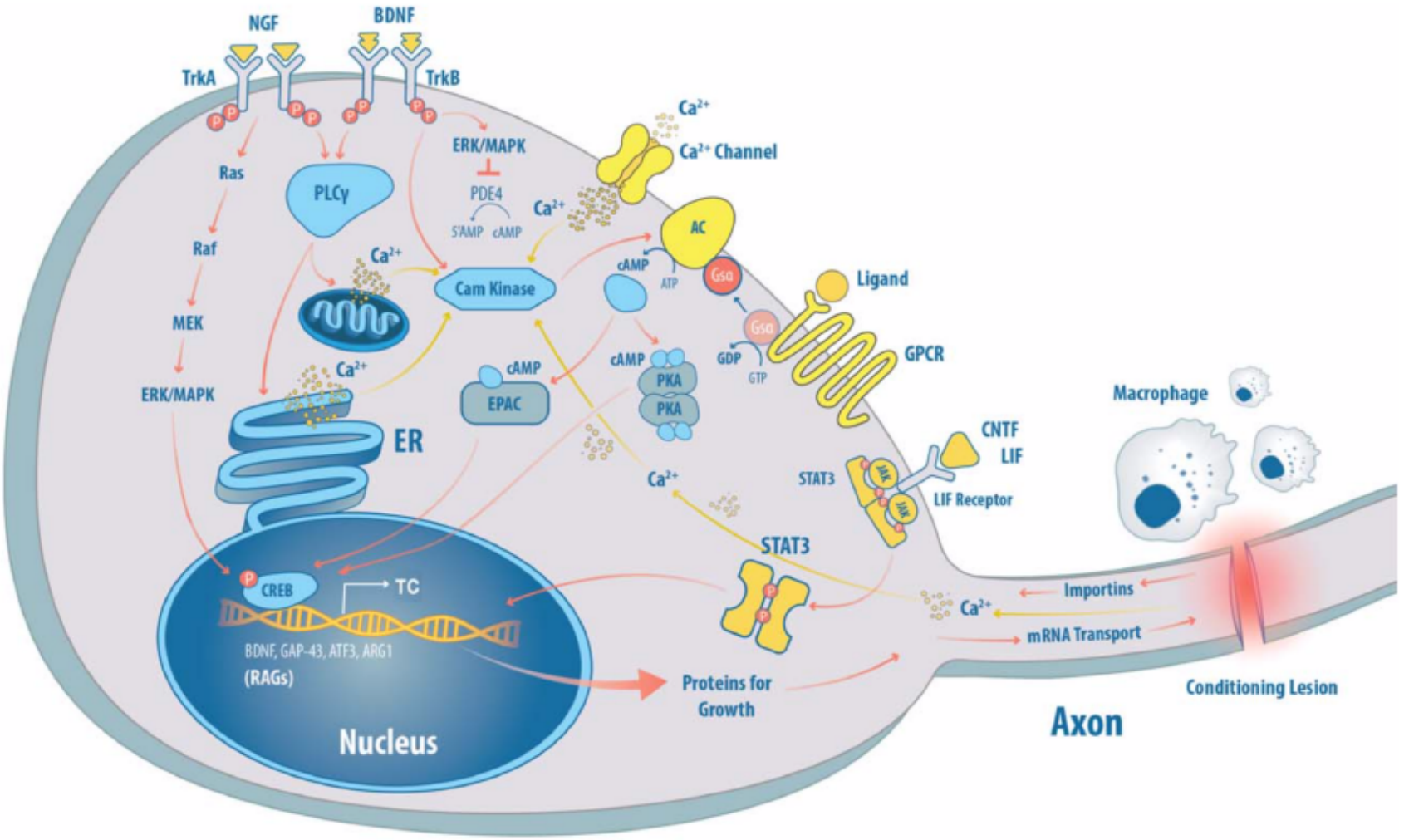
Macrophage infiltration at the DRG promotes nerve regeneration following CL. In a seminal study by Niemi et al. (2013), the number of macrophages close to the sensory neuron cell body were exogenously increased by overexpression of CCL2 (c-c class chemokine 2), a chemokine expressed by sensory neurons and Schwann cells following nerve injury (Niemi et al., 2013). The increased number of intraganglionic macrophages resulted in enhanced axon outgrowth akin to a CL, similar to the findings of Kwon et al. (2015) who also demonstrated intraganglionic injection of CCL2 to mimic the CL effect with mobilization of pro-regenerative macrophages (Kwon et al., 2015). In both studies, CCL2 knock-out animals failed to mount a conditioning effect. Together, these studies link innate immunity to enhancement of the intrinsic repair response.

Molecular Mechanisms

Cellular changes are the result of altered molecular signaling within the neuron. CLs induce positive signals that promote regeneration and eliminate negative signals that normally inhibit regeneration(Christie, Webber, Martinez, Singh, & Zochodne, 2010; Hoffman, 2010). Numerous changes in gene expression are involved in the conditioning effect. One study reported 773 probe sets were upregulated and 497 downregulated at one-day post CL, which increased to 1,867 and 1,400 respectively at 14 days, with 79-89% of probes altered by more than 20% (Blesch et al., 2012). Whereas CLs trigger changes in multiple molecular pathways, no single uniform 'conditioning pathway' has been identified (Mar et al., 2014). We summarize pathways implicated in the CL effect in Figure 1.1 and provide an overview of major signaling molecules in Table 2.

1.4.3.4 Cyclic AMP

A summary of the adenylyl cyclase pathway in response to conditioning is incorporated into Figure 1.1. Developmental neuronal cAMP levels are significantly higher than in adulthood, with the high levels underlying the ability of neurons to grow axons over non-permissive substrates such as myelin-associated glycoprotein (MAG) or myelin. Conversely, the transition to low cAMP levels are associated with an inability to overcome this inhibition (Cai et al., 2001). Perhaps CL-associated increase in cAMP levels recapitulates aspects of the developmental state, as CLs double cAMP levels in DRGs 1 day postconditioning, with high levels persisting for at least 1 week (Blesch et al., 2012), providing a rationale for the classic 7-day interval between the conditioning and the test lesion for in vivo studies.



DRG Neuronal Cell Body

Figure 1.1. Proposed Mechanisms of the Conditioning Lesion. All notations used in the figure are highlighted in the description below. At the CL injury site, fast calcium ion influx as well as delayed retrograde signals, such as importins, initiate the regenerative response within the DRG cell body. Multiple signaling pathways are known to be responsible for the CL effects. The initial calcium influx alters the membrane potential causing opening of voltage gated calcium channels (**VGCC**) to further increase intracellular calcium levels. In turn, this activates Ca^{2+}/calmodulin-dependent protein kinase (**Cam kinase**). Activated Cam kinase activates adenylyl cyclase (**AC**) that catalyzes ATP to cyclic AMP (**cAMP**). AC is also activated by G-protein-coupled receptor (**GPCR**) that in turn upregulates the G_s-α subunit (**G-stimulatory protein alpha**) via GTP. cAMP causes dimerization of protein kinase A (**PKA**) and activates (EPAC) (exchange protein directed activated by cAMP), both of which are translocated to the nucleus to phosphorylate the transcription factor cAMP response element-binding protein (**CREB**), resulting in transcription (**TC**) of regeneration-associated genes (**RAGs**) such as brain derived neurotrophic factor (**BDNF**), growth associated protein-43 (**GAP-43**), arginase-I (**ARG1**) and activating transcription factor 3 (**ATF3**).

The binding nerve growth factor (**NGF**) and BDNF to their respective tropomyosin related kinase receptor A (**TrkA**) and tyrosine receptor kinase B (**TrkB**) receptors will activate multiple intracellular signaling pathways. **TrkB** can act through the extracellular signal-regulated kinase (**ERK**)/mitogen-activated protein kinase (**MAPK**) pathway that inhibits phosphodiesterase-4 (**PDE4**) which normally breaks down cyclic-AMP (**cAMP**) to adenosine monophosphase (**5'AMP**) to maintain intraceullar cAMP levels. Further,

activated TrkA and TrkB receptors can stimulate phospholipase C-gamma (**PLCy**) to induce the release of intracellular calcium from their internal stores which will go on to further activate CamK. Finally, activated TrkA receptors can activate the Ras-Raf-MEK-ERK/MAPK pathway to phosphorylate/activate CREB.

Following conditioning, leukemia-induced factor (**LIF**) and ciliary neurotrophic factor (**CNTF**) activate Janus-kinase (**Jak**), which phosphorylates both the receptor and Signal Transducer which Activator of Transcription (**STAT**). STAT dimerizes and translocates to the nucleus where it regulates gene expression.

The end result of the CL is upregulation of RAGs that leads to the production of structural proteins needed for nerve regeneration at the extending nerve front.

TABLE 2. Summary of Molecular Mechanisms of Conditioning Lesions

Molecule	Action	Conditioning Experiments
cAMP	• Ligand binds to G protein–coupled receptors linked to adenylyl cyclase stimulatory trimeric G-protein (G_s) activates adenylyl cyclase • Adenylyl cyclase activates cAMP • Elevated cAMP allows axons to grow in the presence of myelin/MAG	• CLs cause a 2-fold increase in cAMP 1 day postconditioning, lasting at least 1 week[59,60] • Exogenous cAMP has less effect on accelerating growth rate, lower expression of RAGs[51,61]
PKA	• Binding of cAMP induces conformational change to release the catalytic domains of PKA • Activated PKA phosphorylates CREB	• Not yet studied
Exchange protein directly activated by cAMP (EPAC)	• Second pathway through which cAMP mediates its effects on CREB	• Not yet studied
BDNF	• Does not directly regulate cAMP • Binds to receptor tyrosine kinase (TrkB), which activates phospholipase C gamma, causing release of calcium from internal stores • Increased calcium enhances CREB phosphorylation by activating CREB kinase (calcium/calmodulin-dependent kinase IV)	• CLs induce a two-fold increase in BDNF 3 days post-conditioning, lasting at least 11 days.[76]
CREB	• Activated CREB dimerizes and binds to CRE to affect transcription of RAGs	• Not yet studied
ATF-3	• Downstream of CREB • Upregulation enhances number/length of neurite outgrowth	• Upregulated following CL[65] • Overexpression alone insufficient to overcome inhibitors in CNS • May contribute to dimerizing with other transcription factors (c-Jun, CREB, STAT3) [66]
Arg-1	• Downstream of CREB • Key in polyamine synthesis, necessary for overcoming myelin/MAG axon growth inhibition	• Upregulated by regulators of the conditioning response (db-cAMP, BDNF) [64] • Blockage of Arg-1 inhibits conditioning effect[67]
GAP-43	• Downstream of CREB • Hypothesized cytokine signaling pathway is involved in its effects (overexpression of GAP-43 is attenuated by JAK2 inhibitor)	• Conditioning has limited effect, likely because CLs do not affect fast transport system[54]
IL-6	• Product of adenylyl cyclase pathway & ligand for JAK/STAT3 pathway • Promotes BDNF expression • Capable of overcoming MAG/myelin inhibition in dose-dependent manner	• Exogenous cAMP upregulates IL-6, but not needed for cAMP to overcome MAG/myelin inhibition[54] • Intrathecal IL-6 suppresses inhibition and promotes regeneration of dorsal spinal axons[54] • Decreased regeneration of dorsal column axons in vitro after conditioning in IL-6 k/o animals[68]
LIF	• Induced following axotomy, ligand for JAK/STAT3 pathway	• Attenuated conditioning effect in LIF-null nice[74] • No loss of conditioning effect in LIF k/o animals[80]
JAK	• Activated by ligand-induced homo- or heterodimerization of gp130 receptor, causing phosphorylation of tyrosine residues on JAK	• Exogenous CNTF, acting through this pathway, induces conditioning effect[71]
STAT3	• JAK phosphorylates STAT3, which is transported to the cell body, translocated to the nucleus, and affects transcription	• Inhibition of JAK2 at the time of conditioning decreases pSTAT3 to inhibit the conditioning effect[69]

Arg = arginase; ATF = activating transcription factor; BDNF = brain-derived neurotrophic factor; cAMP = cyclic adenosine monophosphate; CL = conditioning lesion; CNS = central nervous system; CRE = cAMP response element; CREB = cAMP response element-binding protein; db-cAMP = dibutyryl cAMP; GAP = growth-associated protein; IL = interleukin; JAK = Janus kinase; k/o = knockout; LIF = leukemia inhibitory factor; MAG = myelin-associated glycoprotein; PKA = protein kinase A; RAG = regeneration-associated gene; STAT = signal transducer and activator of transcription.

The role of cAMP in the CL has been well described in the literature and it is suggested that the regenerative properties of CLs are at least partially dependent on protein kinase A (PKA) activity, a cAMP-activated enzyme required for both nerve maturation and regeneration (Hannila & Filbin, 2008). The binding of cAMP on the two regulatory domains of protein kinase A (PKA) induces a conformational change leading to the release the catalytic domains of the activated PKA. PKA phosphorylates and activates cAMP response element-binding protein (CREB) at serine-133, which dimerizes and binds the cAMP response element (CRE) resulting in altered transcription of numerous regeneration-associated genes (RAGs) including arginase-1, and regeneration-associated cytokines or growth factors such as interleukin-6 (IL-6)(Cao et al., 2006) and brain derived neurotrophic factor (BDNF) (Finkbeiner et al., 1997; Melemedjian et al., 2014; Tao & Aldskogius, 1998). Wei et al. (2016) has shown that despite blocking cortical PKA with Rp-cAMP, exercise training increased axonal regeneration and functional recovery in the corticospinal tract. This observation led to identification of a second pathway through which cAMP can mediate its CL effect, that may function simultaneously with PKA, namely the EPAC2 (exchange protein directly activated by cAMP) pathway (Wei et al., 2016). The role of EPAC2 in peripheral nerve regeneration and its function in the conditioning effect is an ongoing research focus. Of note is the extensive crosstalk between pathways that impact cAMP levels. cAMP may activate multiple signaling pathways with protein kinases that can also phosphorylate CREB. Alternatively, as discussed below, many of the signaling pathways activated by the increased growth factor expression can affect this pathway, either indirectly impacting cAMP levels by affecting phosphodiesterase activity or by phosphorylating CREB.

Interestingly, despite strong evidence shows that endogenous cAMP plays a role in the conditioning effect, attempts to administer exogenous cAMP to recreate this effect have proven challenging. Methods of increasing cAMP levels include exogenous administration of dibutyryl cAMP (db-cAMP), a membrane-permeable cAMP analogue, and infusion of the phosphodiesterase-IV inhibitor Mesopram. *In vi*vo infusion of db-cAMP to the DRG blocks MAG-inhibition and increases neurite length two-fold;(Cai, Shen, De Bellard, Tang, & Filbin, 1999; Neumann, Bradke, Tessier-Lavigne, & Basbaum, 2002) however, the outcomes of exogenous cAMP differ from a 'true' CL as several studies conclude that the intrinsic growth rate is not affected(Blesch et al., 2012; I.G. McQuarrie, Grafstein, & Gershon, 1977) and there is significantly lower expression of RAGs such as GAP-43, c-jun, and β-III tubulin compared to traditional conditioning (Blesch et al., 2012), Combinatorial treatments may prove beneficial. For example, significantly improved regeneration of the central sensory axon branch is seen when cAMP is combined with immediate autologous bone marrow graft, and injection of neurotrophin-3 (NT-3) (P. Lu, Yang, Jones, Filbin, & Tuszynski, 2004). Together, these data suggest other molecular pathways in addition to the adenylyl cyclase are implicated in the conditioning effect.

1.4.3. 5 Neurotrophic Factors

The injury-induced increases in BDNF are critical for induction of a regenerative response and the intrinsic ability of adult sensory neurons to regrow an axon (Geremia et al., 2010). While BDNF signaling does not directly regulate adenylyl cyclase activity and cAMP generation, it affects the PKA pathway downstream of adenylyl cyclase activation. BDNF binding to its cognate neurotrophin receptor tyrosine kinase, trkB, leads to phospholipase C

gamma activation (PLCγ),(Finkbeiner et al., 1997) resulting in increased release of calcium from internal stores. The elevated calcium concentration is linked to enhanced CREB phosphorylation through its role in activation of a CREB kinase (calcium/calmodulin-dependent kinaseIV –Ca\MKIV)-regulated pathway. Activation of the extracellular signal-regulated kinase (ERK; also known as MAPK) downstream of trkB activation can result in both phosphorylation of CREB (Finkbeiner et al., 1997), as well as the inhibition phosphodiesterase 4 (PDE4), an enzyme which hydrolyses cAMP, allowing cAMP levels to rise and initiating the pathway linked to the ability of central axons conditioned by a peripheral lesion to overcome MAG and myelin-mediated inhibition of axon growth (Gao et al., 2004).

The role of NGF in the CL response *in vivo* is less well-defined as its levels are only transiently upregulated in response to peripheral nerve lesion(Heumann et al., 1987). The transient elevation in NGF synthesis around the site of peripheral nerve injury is regulated by expression of interleukin 1β IL-1β), a cytokine involved in nerve regeneration. An initial spike in IL1β is significantly greater 1 day following with a subsequent fall in expression by day three (Ryoke et al., 2000). Immuno-neutralization of endogenous NGF increases expression of proteins associated with axonal regeneration in sympathetic neurons such as galanin, vasoactive intestinal peptide, c-jun, damage-induced neuronal endopeptidase, with coincident decreased Substance P expression. This supports NGF as a negative factor for nerve regeneration capable of suppressing axotomy-induced changes in gene expression (Shoemaker, Sachs, Vaccariello, & Zigmond, 2006). A similar role for NGF in reversing injury-associated phenotype is also observed in injured sensory neurons (Verge et al., 1996).

1.4.3.6 cAMP response element-binding protein

CREB is a transcription factor that binds to cAMP response elements (CRE) to affect transcription of over 100 downstream genes involved in cellular survival, neural plasticity, memory, and learning, including arginase-1, IL-6, neuropeptide Y, CREM (cAMP responsible element modulator), and VEGF (Cao et al., 2006). Gao et al (2004) demonstrated that blocking CREB inhibits the ability of cAMP and BDNF to overcome myelin inhibition (Gao et al., 2004). Conversely, constitutively active CREB within the DRG promotes regeneration in a manner akin to the conditioning effect. However, the results are less robust suggesting other parallel pathways are evoked during CLs. Specific genes upregulated by this pathway include ATF-3, arginase-1, and GAP-43.

Activating transcription factor-3 (ATF-3) is a member of the CREB protein family that is strongly upregulated in DRG following peripheral nerve injuries, including CLs. ATF-3 overexpression in cultures of naïve sensory neurons enhances the number and length of neurite outgrowth, mimicking trends observed in response to a CL, albeit to a lesser degree (Seijffers, Allchorne, & Woolf, 2006). Unlike CLs, elevated levels of ATF-3 alone are insufficient to overcome inhibitors in the CNS that preclude regeneration. It is therefore unlikely ATF-3 alone is responsible for the conditioning effect; rather, ATF-3 may contribute by dimerizing with other transcription factors such as c-Jun, CREB, and STAT-3 to affect nerve regeneration (Seijffers, Mills, & Woolf, 2007).

Arginase-1 (Arg-1) expression is also increased in response to nerve conditioning. Regulators of the conditioning response such as db-cAMP and BDNF, can result in increases

in Arg-1 expression by 6-7x and 2-3x respectively, in a CREB-dependent manner, with Arg-1 being a key enzyme in polyamine synthesis, a novel pathway(Gao et al., 2004) linked to overcoming myelin and MAG axon growth inhibition (Cai et al., 2002). Blockage of Arg-1 downstream polyamines inhibits the conditioning effects of cAMP and BDNF, whereas Arg-1 overexpression promotes regeneration by neutralization of myelin-induced inhibition (Cai et al., 2002).

GAP-43 expression is high during embryological development, but levels drop dramatically after maturation of the nervous system; peripheral axonal injury results in a return to elevated levels in the DRG, whereas injury to the central axon branch of sensory neurons typically does not (Cafferty et al., 2004). Infusion of the Janus kinase (JAK) 2 inhibitor, AG490, attenuates the increase in GAP-43 levels following peripheral nerve injury, implicating cytokine signaling pathways in its injury-associated expression (Qiu, Cafferty, McMahon, & Thompson, 2005). Thus, increased levels of GAP-43 affected by a peripheral CL likely prime the central branches of these axons for improved regeneration following central lesion. Interestingly, there appears to be a ceiling to the conditioning effect on GAP-43 expression, with levels not rising beyond that observed with an isolated peripheral nerve test lesion; potentially because CLs do not further enhance the fast-transport system (Tetzlaff et al., 1996).

1.4.3.7 JAK/STAT3 Pathway

Another major pathway through which the CL exerts its effect is the JAK/STAT3 pathway. Ligand induced homo- or hetero-dimerization and activation of the gp130 receptor in target

cells results in phosphorylation of key tyrosine residues on the associated JAK which serve as docking sites for STAT3 which, once activated by the JAK, dissociates, dimerizes and activates gene transcription. The relationship between the adenylyl cyclase and JAK/STAT pathways was investigated by Wu et al. (2007) using exogenous CNTF to demonstrate a conditioning effect by activation of the JAK/STAT3 pathway (Wu et al., 2007). However, they concluded that while activation of either pathway is involved in the conditioning effect, the effects are not additive. The lack of synergy suggests that perhaps these two pathways (cAMP and JAK/STAT3) work in parallel effecting similar outcomes. Downstream products of the adenylyl cyclase/CREB pathway include CNTF, IL-6, and leukemia inhibitory factor (LIF) which act as ligands to initiate the JAK/STAT3 pathway, bridging the two separate processes.

IL-6 bridges the adenylyl cyclase and the JAK/STAT3 pathways, as a downstream target of CREB in the former, and an initiating ligand in the latter (Hannila & Filbin, 2008). IL-6 is well-recognized for its role in nerve regeneration and promotes BDNF expression in injured neurons that is important for priming the neurons to respond more robustly with respect to axon regeneration (Murphy et al., 2000). IL-6 is highly responsive to changes in cAMP levels and is capable of overcoming MAG/myelin inhibition in a dose-dependent manner, with outgrowth on the inhibitory substrate equivalent to that of control non-MAG-expressing cells (Cao et al., 2006). The importance of IL-6 in mounting a conditioning response remains largely unknown, with studies reporting conflicting results. Cao et al. (2006) found that while IL-6 is upregulated by exogenous cAMP, it is not required for the ability of cAMP to overcome MAG/myelin inhibition, as evidenced by similar regenerative capacities of

neurites from IL-6 knock-out and wild-type mice when grown *in vitro* on a MAG-containing culture (Cao et al., 2006). Further, intrathecal IL-6 suppresses inhibition and promotes regeneration of dorsal spinal axons. These results, however, differ from those of Cafferty et al. (2004) who reported diminished regeneration of the dorsal column axons *in vitro* following a CL in IL-6 knock-out animals that could be restored with administration of IL-6 and neurotrophins NGF or NT-3 (Cafferty et al., 2004). There is no obvious explanation for these contrasting results, as both studies used the same strain of mice, surgical techniques and similar axonal tracing analysis. The addition of neurotrophins by Cafferty et al. (2004) is based on evidence of a cooperative effect between IL-6 and neurotrophins on axon growth (Cafferty et al., 2004). *In vitro* studies revealed two stages of adult sensory neuron outgrowth: i) a neurotrophin-dependent 'neurite outgrowth stage', characterized by axonal branching; and ii) a neurotrophin-independent 'neurite elongation phase' that requires IL-6, reminiscent of the two *in vitro* growth states described by Smith and Skene (Smith & Skene, 1997).

Leukemia Inhibitory Factor (LIF), while not detected in the intact adult nervous system, is induced following axotomy at the site of injury and in axotomized sympathetic and dorsal root ganglia (Shoemaker et al., 2006). Similar to IL-6, conclusions are mixed concerning the role of LIF in CL. While one *in vivo* study reports attenuation of the conditioning effect in LIF-null mice (Cafferty et al., 2001) another one reports no loss of the conditioning effect *in vitro* among LIF knock-out animals (Shoemaker, Sachs, Vaccariello, & Zigmond, 2005). Further research is therefore required to elucidate the full role of these cytokines in CLs.

Downstream of these cytokines activating their receptors, phosphorylation of STAT-3 in peripherally injured axons induces retrograde transport of pSTAT-3 to the cell body, followed by translocation of STAT-3 to the nucleus, a process implicated in the conditioning effect. Infusion of a JAK2 inhibitor at the time of injury decreases pSTAT3 levels and inhibits the conditioning responses(Qiu et al., 2005; Wu et al., 2007). Of note, the regenerative outcomes here are due to enhanced neurite outgrowth, with no improvement in reducing the latency period. In this context, axonal regeneration is impeded despite a prior CL when the JAK/STAT pathway was blocked (Qiu et al., 2005).

1.4.4. Thesis Objectives

Despite over four decades of basic research into elucidating how CLs accelerate and enhance peripheral nerve regeneration, translation to human applications has yet to be realized. As suggested above, there are nerve repair scenarios that would benefit from an ethically acceptable approach to nerve conditioning. Conditioning electrical nerve stimulation may hold promise for enhancing and accelerating select nerve transfer repair paradigms. Udina et al. (2008) showed that brief electrical stimulation (ES) of the intact sciatic nerve for an hour *in vivo,* prior to assaying the impact of ES on neurite outgrowth of the stimulated sensory neurons *in vitro*, resulted in a 4-fold increase in neurite outgrowth from the stimulated neurons relative to unstimulated naïve control neurons (Udina et al., 2008).

The objective of this thesis is to systematically evaluate whether pre-injury 'conditioning' electrical stimulation (CES):

a) Creates a pro-regenerative environment with improved axon extension comparable to the gold-standard crush conditioning lesion (Chapter 2).
b) Improves sensory and motor reinnervation outcomes comparable, or greater than, the gold-standard crush conditioning lesion (Chapter 3).
c) Is comparable to the current clinical practice of postoperative electrical stimulation, and whether a synergistic effect can be obtained by combining these two techniques (Chapter 4).
d) Can be used in various clinical nerve reconstruction models including nerve autograft repair (Chapter 5), distal nerve transfer (Chapter 6), and primary nerve repair (Chapter 7).

The aim of this research is to develop a strong foundational knowledge of the effects of conditioning electrical stimulation to guide future clinical trials, to gain insight into the innate pathways of peripheral nerve regeneration, and ultimately to significantly improve outcomes for patients with peripheral nerve injuries.

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Chapter 2

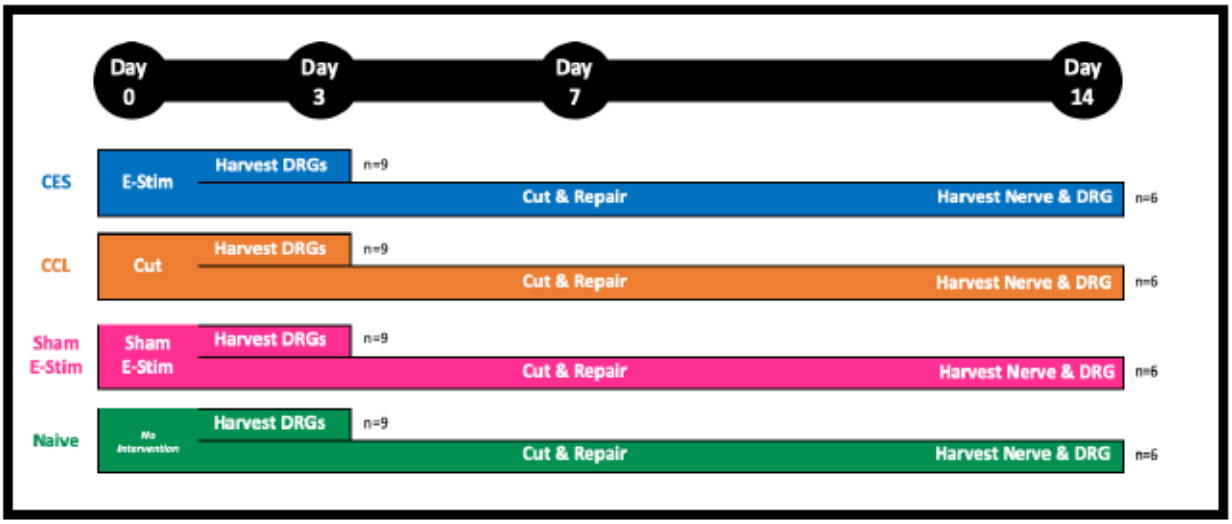
Electrical Stimulation as a Conditioning Strategy for Promoting Peripheral Nerve Regeneration in a Common Peroneal Nerve Injury Model

2.1 Preface

The crush conditioning lesion is well accepted as a highly effective technique to 'prime' the nerve for regeneration prior to injury. Crushing a nerve one week prior to nerve transection and repair significantly improves regeneration and reinnervation outcomes. Unfortunately, a crush conditioning lesion is not clinically feasible due to its injurious nature; therefore, translation to a human population has been impossible. Numerous researchers have attempted to create a conditioning-like effect using non-injurious techniques, however, regenerative outcomes have consistently remained inferior to a crush lesion. In 2008, Udina et al., suggested that electrical stimulation may be a promising conditioning technique. Authors reported that delivery of one hour of electrical stimulation to an intact nerve significantly improved neurite extension *in vitro* and axon extension through the spinal column *in vivo* in a cAMP-dependent manner (Udina et al., 2008).

Chapter 2 describes our proof of principle project, published in *Experimental Neurology* (2018; 302: 75-84), which compares the effects of conditioning electrical stimulation (CES) to the gold-standard crush conditioning lesion (CCL). A sham-ES was included to ensure that nerve dissection and manipulation in placing the stimulation wire did not inadvertently injure and thereby condition the nerve. An unconditioned nerve served as the negative control. We hypothesized that CES would cause upregulation of the regeneration associated genes (RAGs) necessary to create a 'pro-regenerative' environment in the cell body, as assessed using immunohistochemistry and quantitative real-time polymerized chain reaction. We further explored if this primed nerve microenvironment would enhance axon extension at an injury/repair site created seven days later by measuring the length of axonal outgrowth through the distal stump and quantifying the number of regenerating fibers.

The timeline below outlines the experimental paradigms: animals were conditioned with CES (blue), CCL (orange), sham-ES (pink) or no conditioning (green) followed by 1) DRG harvest at 3 days to evaluate RAG expression, or 2) a cut and repair seven days post-conditioning, with harvest of nerve tissue and DRGs at one week post-repair, to evaluate whether RAGs remain elevated and to compare length of regeneration between cohorts.



In keeping with our hypothesis of equivalence, we found that three days post-conditioning, CES caused upregulation of RAGs comparable to a CCL, and significantly greater than in negative controls. We further showed that axon extension seven days following nerve transection was similar in cohorts conditioned with electrical stimulation or a crush, and significantly longer than the sham or unconditioned cohorts. Together these results suggest that pre-injury electrical stimulation delivers a conditioning-like effect, with regenerative outcomes comparable to a crush lesion. These exciting findings form the foundation on which Chapters 3 through 7 are built.

Electrical Stimulation as a Conditioning Strategy for Promoting and Accelerating Peripheral Nerve Regeneration

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2.2 Abstract

The delivery of a nerve insult (a "conditioning lesion") prior to a subsequent test lesion increases the number of regenerating axons and accelerates the speed of regeneration from the test site. A major barrier to clinical translation is the lack of an ethically acceptable and clinically feasible method of conditioning that does not further damage the nerve. Conditioning electrical stimulation (CES), a non-injurious intervention, has previously been shown to improve neurite outgrowth *in vitro*. In this study, we examined whether CES upregulates regeneration-associated gene (RAG) expression and promotes nerve regeneration *in vivo,* similar to a traditional nerve crush conditioning lesion (CCL). Adult rats were divided into four cohorts based on conditioning treatment to the common peroneal (fibular) nerve: i) CES (1 h, 20 Hz); ii) CCL (10 s crush); iii) sham CES (1 h, 0 Hz); or iv) naïve (unconditioned). Immunofluorescence and qRT-PCR revealed significant RAG upregulation in the dorsal root ganglia of both CES and CCL animals, evident at 3-14 days post-conditioning. To mimic a clinical microsurgical nerve repair, all cohorts underwent a common peroneal nerve cut and coaptation one week following conditioning. Both CES and CCL animals increased the length of nerve regeneration (3.8-fold) as well as the total number of regenerating axons (2.2-fold), compared to the sham and naïve-conditioned animals ($p<0.001$). These data support CES as a non-injurious conditioning paradigm that is comparable to a traditional CCL and is therefore a novel means to potentially enhance peripheral nerve repair in the clinical setting.

2.3 Introduction

Peripheral nerve injury is common, identified in 3% of all clinical presentations to major emergency centers (Noble, Munro, Prasad, & Midha, 1998). Despite surgical advancements and dedicated rehabilitation, clinical outcomes have not significantly improved in the past twenty-five years (Lundborg, 2000). The slow rate of regeneration (1-3 mm/day) is insufficient to reinnervate distal targets. Extended denervation results in a local environment at the distal stump and motor/sensory targets that is not conducive to regeneration. Therefore, an intervention to accelerate peripheral nerve regeneration is of utmost clinical importance.

Forty years of animal studies have revealed that conditioning lesions, in which the peripheral nerve is damaged by a crush or cut prior to a test nerve injury, promotes nerve regeneration. The conditioning lesion instructs the neuronal cell bodies to change their gene expression pattern to an 'injured/repair' state. Regeneration-associated gene (RAG) expression is upregulated along with proteins Tα1-tubulin, β-actin, and other axonal structural proteins necessary for nerve regeneration (Lund, 2002; Richardson et al., 2009). Following nerve transection, a conditioned nerve will accelerate its regenerative capacity by up to 5-times that of unconditioned nerve (Gordon, Brushart, & Chan, 2008).

Unfortunately, the therapeutic potential of the conditioning lesion cannot be translated to the clinic because of two fundamental obstacles: a) the timing of a nerve injury is usually unpredictable, and b) one cannot justify intentionally damaging intact nerves in patients. Distal nerve transfer surgery, however is one scenario in which, at a prescheduled time, a healthy intact nerve is intentionally cut. In this elective procedure, the proximal stump of an

intact nerve (innervating a nonessential motor or sensory target) is cut and coapted to the denervated stump of an injured nerve, to reinnervate its target. These patients are, therefore, ideal candidates for conditioning, which could be delivered to the donor nerve prior to the nerve transfer procedure. How to deliver this nerve conditioning effect at the bedside without injuring the donor nerve remains a more elusive challenge.

Electrical nerve stimulation is a non-invasive technique for improving nerve regeneration that, similar to the crush conditioning lesion (CCL), upregulates RAG expression in regenerating neurons (Al-Majed, Neumann, Brushart, & Gordon, 2000; Al-Majed, Tam, & Gordon, 2004; Geremia, Gordon, Brushart, Al-Majed, & Verge, 2007). To date, electrical stimulation has only been used post-operatively. Although post-surgical electrical stimulation reduces the delay of regeneration across the injury gap (Al-Majed, Neumann, et al., 2000), it does not improve the rate of axonal extension beyond this site (Brushart et al., 2002). Accelerated axon regeneration beyond the cut site, as observed following a CCL, is required for timely distal target reinnervation. Udina et al. (2008) proposed electrical stimulation as a conditioning strategy (Udina et al., 2008). After stimulation of the intact sciatic nerve (1hour, 20 Hz), it significantly increased neurite outgrowth of the affected dorsal root ganglia (DRG) neurons when assayed *in vitro* one week later. However, the use of conditioning electrical stimulation (CES) in an *in vivo* model of peripheral nerve regeneration has not been investigated.

The goal of this study is to test the hypothesis that CES of intact nerves prior to transection and microsurgical repair upregulates RAGs and accelerates peripheral nerve regeneration in a manner similar to a CCL.

2.4 Methods

2.4.1 Animals: Adult Sprague Dawley rats (175g; Charles River laboratory), were placed under the care of the Health Sciences Laboratory Animal Services (HSLAS) at the University of Alberta. A total of 60 animals were used in these experiments: 24 animals for RAG analysis at the DRG (3 days after conditioning; n=6/group), 12 animals for DRG qRT-PCR analysis (n=3/group), and 24 animals for nerve regeneration studies and delayed DRG RAG expression (n=6/group).

2.4.2 Conditioning surgery: All surgical procedures were approved by the University of Alberta Animal Research Ethics Board. Each cohort of animals was equally divided into four groups based on the type of treatment to the common peroneal (CP) nerve (also called the fibular nerve): CES, CCL, sham electrical stimulation or naïve (no conditioning). In preparation for surgery, the animals were given a dose of subcutaneous buprenorphine (0.01 mg/kg/animal) and anesthetized by oral inhalation of isoflurane (2% titrated and delivered at 1-2 L/min). A longitudinal incision was made over the right lateral lower limb from knee to mid-tibia and the CP nerve was identified at the level of the fibular neck. The nerve was carefully isolated and conditioning was performed as described below. All incisions were sutured with a two-layer closure using 4-0 Vicryl. Surgical details specific to the type of conditioning are as follows: i) CES: stainless steel wires bared of insulation at the ends were

connected to a SD-9 stimulator (Grass Instruments Co., Quincy, MA). The cathode wire was wrapped around the CP nerve at the level of the fibular neck, and the anode wire was placed into the tibialis anterior muscle. CES was performed by delivering continuous 20 Hz of 0.1 ms duration of balanced biphasic pulses, with voltage titrated to maintain a visible twitch in the lower limb extensors, for one hour (Udina et al., 2008). ii) CCL: A non-toothed fine hemostat (5 mm tip) was used to crush the CP nerve at the level of the fibular head for ten seconds. Complete axotomy was confirmed visually by examination of the nerve. iii) Sham electrical stimulation: for one hour, the electrodes were placed at the same locations as the CES group, but no current was delivered. iv) Naïve: No surgical intervention was performed. Three days following conditioning, animals were euthanized to harvest the L4 and L5 DRGs for RAG qRT-PCR and immunocytochemistry respectively. The examiner was blinded to the conditioning state of each animal.

2.4.3 Nerve transection and microsurgical repair surgery: Post-conditioning, the remaining cohort of 24 animals was left in their cages for 7 days prior to CP transection and microsurgical repair. The animals were anesthetized to surgical stage and an incision was made posterior to the lateral middle third of the palpable femur. The biceps femoris muscle was released from the vastus lateralis muscle to facilitate identification of the sciatic nerve. The sciatic nerve was traced distally and the site of trifurcation was dissected to isolate the CP nerve from the tibial and sural nerves. One centimeter distal to the trifurcation, the CP nerve was cut and immediately repaired using 9-0 silk suture under 3.5x loupe magnification (Figure 2.1). The hamstrings were re-suspended with stitches, and the skin was closed in two-layers using a running subcutaneous and horizontal mattress stitch with 4-0 Vicryl suture

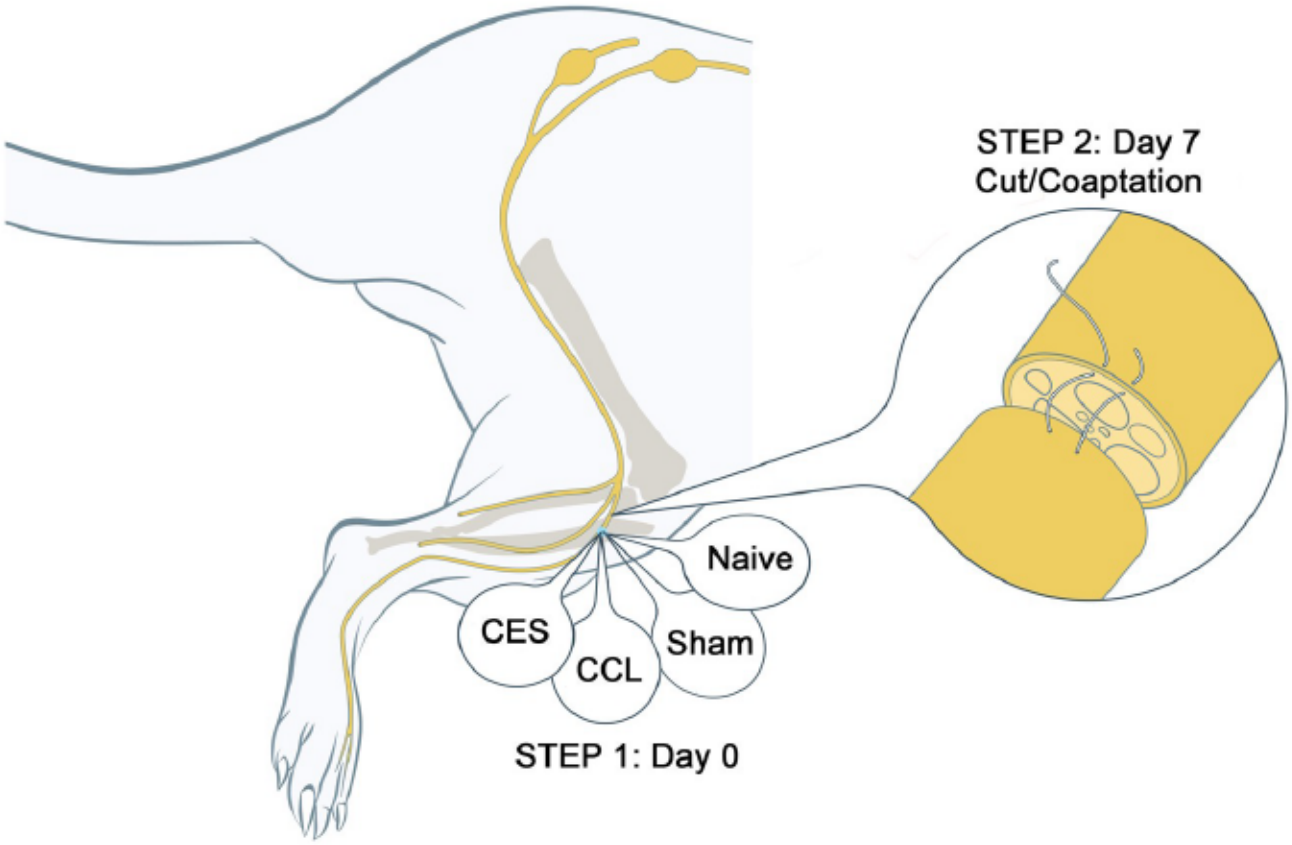


Figure 2.1: Experimental design of conditioning paradigm and cut and microsurgical repair

Step 1 (Day 0): conditioning of the common peroneal (CP) nerve with CCL, CES, or sham CES 20 mm distal to the sciatic trifurcation. The naïve group does not undergo surgery at this time. Step 2 (Day 7): the CP nerve is cut 10 mm proximal to the site of conditioning (10 mm distal to the sciatic trifurcation) and an epineurial repair is performed.

(Ethicon Inc, Somerville NJ). Animals received 0.01 mg/kg/animal buprenorphine postoperatively. One week later, the regeneration bridge, and a 1 cm segment of both the proximal and distal nerve were harvested in order to quantify the rate of regeneration. The L4 and L5 DRGs were harvested to analyze RAG expression at the neuronal cell bodies.

2.4.4 Tissue Analysis: Animals were euthanized by carbon dioxide and exsanguinated by opening the thoracic cavity and cutting the left ventricle of the heart. The sciatic nerve was re-exposed, and the site of cut/microsurgical repair was identified. A 3-cm toothpick was placed on the CP nerve to stabilize the proximal nerve, the regeneration bridge and the distal nerve regions for harvesting. The proximal sciatic nerve was then traced back to the vertebral column and the L4 and L5 DRGs were collected. Tissues were fixed in Zamboni's fixative (paraformaldehyde, picric acid and NaOH) (American MasterTech Scientific, Lodi, CA) for four hours, rinsed with 0.01 M PBS five times, post-fixed in 30% sucrose solution overnight at 4°C, then embedded and frozen in optimum cutting temperature (OCT) (Sakura Finetek, Torrance, CA) using indirect exposure to liquid nitrogen. Longitudinal sections of the nerves and DRG (12 µm) were thaw-mounted onto Superfrost Plus microscope slides (Thermo Fisher Scientific, Waltham, MA) and stored at -80°C until processing.

2.4.5 Immunofluorescence: Slides were warmed to room temperature for 30 minutes before antigen retrieval in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0; American MasterTech Scientific, Lodi CA) for 20 minutes at 80°C and then cooled to room temperature. Slides were washed three times in 0.01 M PBS for five minutes and permeabilized with 0.1% Triton-100X. Sections were then blocked in 10% normal goat

serum (MP Biomedicals, Santa Ana, CA) and 3% bovine serum albumin (BSA) (Sigma Aldrich, St. Louis, MO) in 0.01 M PBS for 90 minutes. Primary antibodies diluted in 3% bovine serum albumin in 0.01 M PBS were applied overnight at 4ºC. Nerve sections were stained with the primary antibody mouse anti-neurofilament-200 (NF200) (1:500, Sigma Aldrich). Primary antibodies for DRG analysis included rabbit anti-GAP-43 (1:500, Millipore, Billenca, MA), rabbit anti-GFAP (1:500, DAKO, Santa Clara, CA), and chicken anti-BDNF (1:500, Promega, Madison, WI). Slides were washed three times for five minutes each, and secondary antibody diluted in 3% BSA in 0.01 M PBS was applied for 90 minutes at room temperature. Secondary antibodies included Cy3-conjugated goat anti-mouse (Sigma), Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA), and donkey anti-chicken 594 (ThermoFisher, Waltham, MA). Slides were washed in PBS and mounted with a coverslip using 50% glycerol/PBS. DRG sections were counterstained with the nuclear stain NucBlue (Invitrogen) prior to mounting with Glycerol/PBS and a coverslip. The specificity of the secondary antibodies employed was confirmed in experiments where the primary antibodies were omitted, revealing an absence of nonspecific staining.

All slides processed for immunofluorescence were qualitatively examined to ensure that there were no discernible slide to slide variations for the same marker within individual experimental groups. Quantitative analysis was then performed in a blinded manner on DRG sections to detect alterations in immunofluorescence signal, as previously described (Bray et al., 2013; Wilson-Gerwing, Johnston, & Verge, 2009). To ensure reliable direct comparisons between experimental and control groups, all sections for each individual marker were processed for immunofluorescence in an identical and parallel fashion on the same day.

Immunofluorescence data was gathered from digital images taken under identical exposure conditions using the 20x objective lens (Zeiss Axio Imager fluorescence microscope). Twelve representative DRG sections were analyzed per animal (n=6 animals/condition) in a blinded manner. To quantify the intensity of immunofluorescence signal, individual neurons with visible nuclei (NucBlue-stained) were manually circled using ImageJ software (ImageJ; Rasband WS). Cells were considered positive for GAP-43 or BDNF expression as determined by measuring the level of immunofluorescence over neurons considered to be devoid of positive immunofluorescence upon scrutiny with a higher power objective. To facilitate evaluation, data was further subdivided into categories of "highly" GAP-43 or BDNF-immunopositive versus "low to moderately" immunopositive, by analyzing individual scatterplots to determine 'natural breaks' between the two populations. To assess whether the DRG neurons with detectable GFAP expression in perineuronal satellite glial cells (SGCs) was altered in response to conditioning with or without nerve transection and repair, a binary evaluation approach was employed; all DRG neurons were classified as "positive" or "negative" based on the presence or absence of GFAP immunofluorescence in their associated SGCs. A minimum of three-quarters of the SGCs surrounding the neuronal cell body was required for a positive classification.

2.4.6 Quantitative real time reverse transcription-PCR (qRT-PCR): qRT-PCR was used to quantify the expression of mRNA changes of GAP-43, BDNF and GFAP in CES (n=3) and naïve (n=3) control groups at 3 days post-conditioning. DRGs were stored in RNALater until total RNA isolation was performed using the RNeasy Kit (Qiagen, Hilden, Germany) and converted to cDNA (QuantiTect Rev Transcription Kit, Qiagen). Synthesized cDNA was

used as a template for quantifying alterations in mRNA levels with the following primers: GAP43 (F5'-ACCACCATGCTGTGCTGTATGA-3'; R5'-CTTATGAGCCTTATCCTCCGG-3'), GFAP (F5'-CGCTTCCTGGAACAGCAAAA-3'; R5'-CCCGAAGTTCTGCCTGGTAAA-3'), BDNF (Qiagen Catalog #QT00375998). PPIA served as the housekeeping gene for normalization (Qiagen Catalog #PPR06504A-200). qRT-PCR was performed in triplicate using 96-well plates; each 20 µL reaction consisted of 1 µL template, 0.6 µL sense and 0.6 µL anti-sense primers, and 10 µL SYBR Premix Ex Taq II (ThermoFisher).

2.4.7 Statistical Analysis: Results are presented as the mean ± standard error mean (s.e.m.). Groups were compared using a one-way analysis of variance (ANOVA) to identify differences in the mean between groups, followed by Bonferroni post hoc analysis. Statistical significance was accepted with a level of $p<0.05$. All statistical analyses were performed using STATA 14 (StataCorp LP, Collagen Station, Texas).

2.5 Results

2.5.1 Conditioning electrical stimulation (CES) of intact nerves upregulates regeneration associated gene expression (RAG) comparably to traditional nerve CCL

At day 0, three cohorts of six animals each had their CP nerve isolated 20 mm distal to the sciatic nerve trifurcation site. These animals were conditioned as follows; i) CES for 1 hour at 20 Hz, (ii) nerve CCL for 10 s, or (iii) sham CES for 1 hour at 0 Hz. A fourth cohort of six animals was unconditioned at day 0 (naïve animals).

Increased GAP-43 and BDNF expression have both been causally linked to the regenerative state of sensory neurons (Bomze, Bulsara, Iskandar, Caroni, & Skene, 2001; Geremia et al., 2010; Zhang, Luo, Xian, Liu, & Zhou, 2000). To determine if CES upregulates GAP-43 and BDNF expression 3 days following conditioning, as previously established in CCL, protein and mRNA levels were quantified in L4, L5 DRG neuron (Geremia et al., 2007; Karchewski, Gratto, Wetmore, & Verge, 2002). Representative DRG sections and their corresponding scatterplots demonstrated an increased GAP-43 immunofluorescence in CES and CCL compared to sham and naive controls (Fig 2.2A-D, n=3 animals analyzed per treatment group). The mean level of GAP-43 immunofluorescence per neuron was significantly elevated in the conditioned versus control groups, with CES neurons displaying a mean level of immunofluorescence of 21.6 ± 1.4 au and CCL neurons displaying 22.1 ± 2.8 au at 3 days post-conditioning versus sham ES (8.8 ± 0.5 au) or naïve (7.4 ± 0.8 au) controls ($p<0.01$) (Fig 2.2E). In a parallel manner, conditioning also significantly increased the number of DRG neurons expressing GAP-43 (Fig 2.2F). GAP-43 protein expression was observed in 88.0 ± 2.0% and 87.4 ± 1.6% of DRG neurons conditioned with CES and CCL with 70.5 ± 2.6% and 68.0 ± 6.4% of them having low to moderate immunofluorescence levels and 17.5 ± 1.2% and 19.4 ± 5.1% high levels, respectively. This was significantly higher than sham and naive cohorts, in which GAP-43 was detected in only 24.3 ± 1.1% and 18.8 ± 2.3% of neurons, respectively ($p<0.01$). Neither sham nor naive had high levels of GAP-43 immunofluorescence ($p<0.001$ compared to conditioned DRG neurons). The average percentage of GAP-43 expressing DRG neurons was statistically higher in all comparisons ('low-moderate', 'high' and total) of CES and CCL with sham or naïve (Fig 2.2F, $p<0.01$).

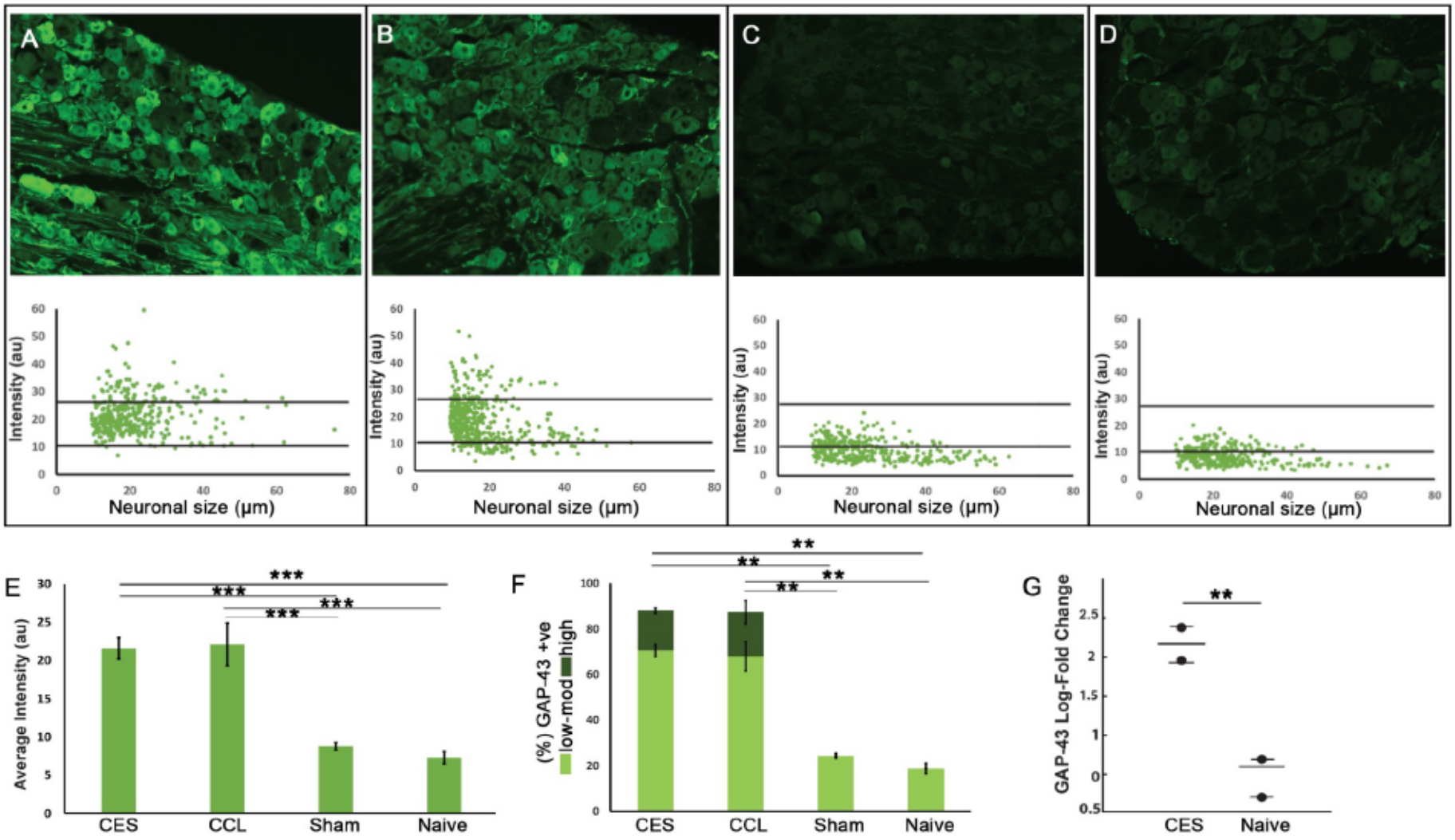
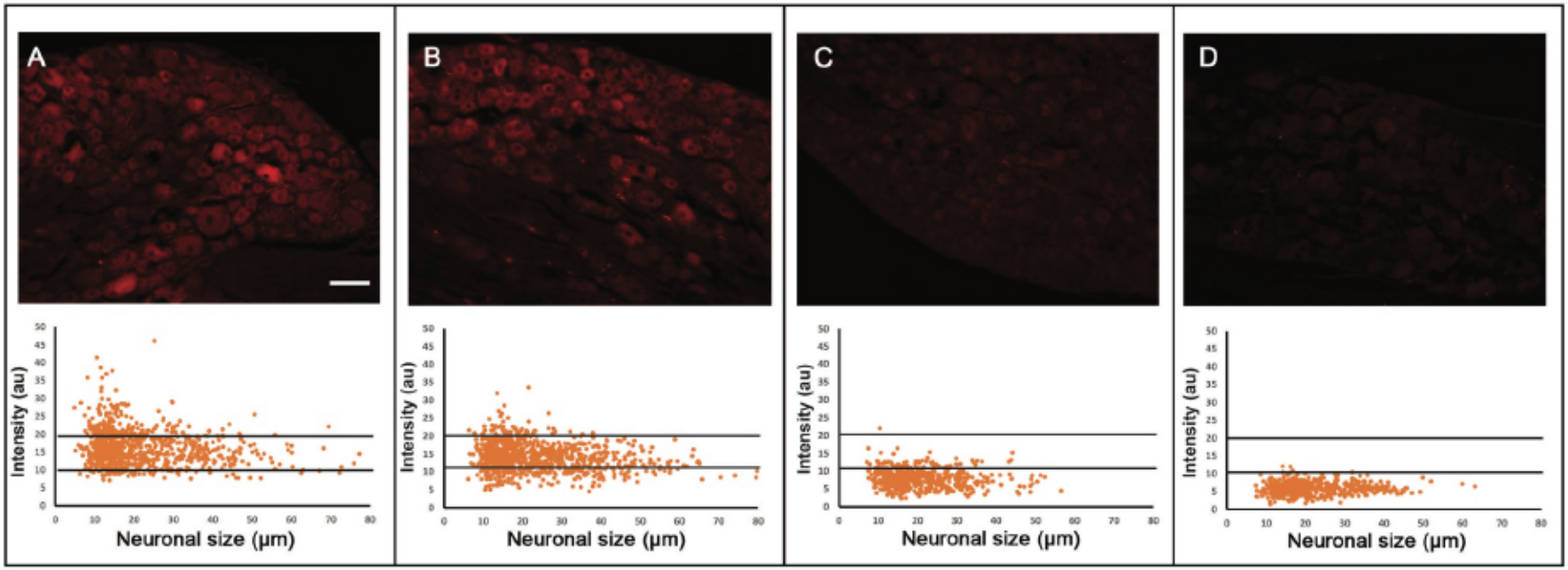

A
B
C
D
Intensity (au)
Neuronal size (μm)
E
Average Intensity (au)
***
CES
CCL
Sham
Naive
F
(%) GAP-43 +ve
low-mod
high
**
G
GAP-43 Log-Fold Change

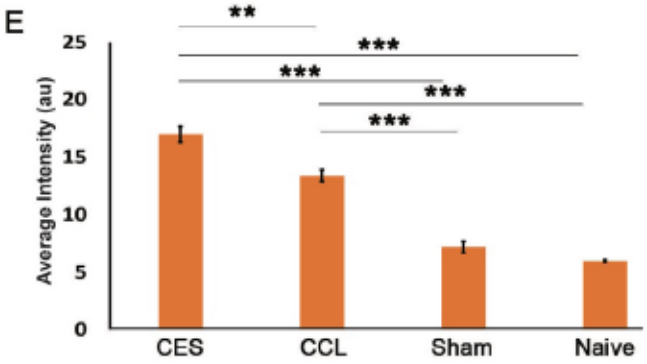
Figure 2.2: Electrical and crush conditioning three days prior upregulates GAP-43 expression at the DRG

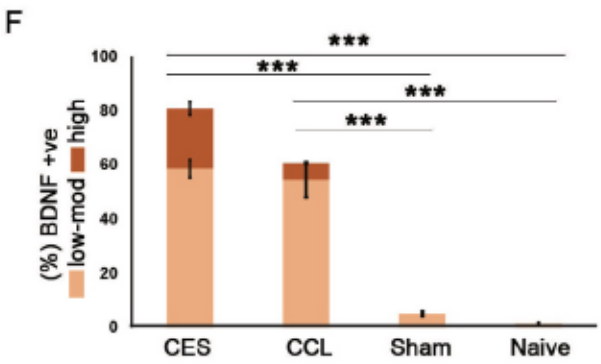
Representative photomicrographs of L5 DRG sections processed for immunofluorescence to detect GAP-43 expression, with corresponding representative scatterplots depicting relative changes in immunofluorescence signal over individual neurons as related to size, from one rat/condition (n=12 DRG sections/rat analyzed for a total of ~2,000 neurons analyzed/condition), 3 days following either CES (A), CLL (B), sham CES (C), or no conditioning/naïve (D). Elevated neuronal GAP-43 expression is apparent in DRG neurons from the two conditioned groups (A,C). Lower solid lines on scatterplots depict the threshold of labeled versus unlabeled populations while the upper solid line separates neurons with 'low to moderate' versus 'high' levels of immunofluorescence. Bar graph (E) demonstrates the average GAP-43 immunofluorescence of 3 animals per experimental group. For each n, 6 DRG tissue sections were analyzed resulting in ~2,000 neurons analyzed per group). Bar graph (F) depicts the mean percentage of neurons with low to moderate (light green) and high (dark green) levels of GAP43 immunofluorescence/condition. Statistical analysis compares changes in the intensity (E) and the incidence (F) of GAP-43 expressing neurons above threshold in each cohort (**$p<0.01$). Bar and whisker graph (J) depicts qRT-PCR results comparing relative GAP-43 mRNA in CES and naïve animal DRG neurons (**$p<0.01$, n=3). Scale bar in A represents 80 μm.

Finally, qRT-PCR also demonstrated that CES significantly increased GAP-43 mRNA expression relative to naïve controls (M_{log2}= 2.2 ± 0.3 log, n=3) (Fig 2.2G, $p<0.01$).

At three days post-conditioning, the levels of BDNF immunofluorescence and the percentage of BDNF expressing neurons were significantly greater in the conditioned compared to the unconditioned animals. Representative DRG sections and their corresponding scatterplots demonstrated an increased BDNF immunofluorescence in CES and CCL compared to sham and naïve controls (Fig 2.3A-D, n=3 animals analyzed per treatment group). The mean level of BDNF immunofluorescence per neuron was significantly elevated in the conditioned versus control groups with CES neurons displaying a mean level of immunofluorescence of 17.0 ± 0.7 au and CCL neurons displaying 13.4 ± 0.5 au versus sham ES (7.2 ± 0.5 au) or naïve (6.0 ± 0.1 au) controls ($p<0.001$) (Fig 2.3E). Further, conditioning also significantly increased the number of DRG neurons expressing detectable BDNF (Fig 2.3F). BDNF protein expression was observed in 80.9 ± 1.3% and 60.7 ± 6.7% of DRG neurons conditioned with CES and CCL, with 58.6 ± 3.4% and 54.5 ± 6.5% of them having low to moderate levels of immunofluorescence and 22.3 ± 2.4% and 6.2 ± 0.3% high levels, respectively. This was significantly higher than sham and naive cohorts, in which BDNF was detected in only 5.1 ± 0.9% and 1.5 ± 0.3% of neurons, respectively ($p<0.001$). Neither sham nor naive had high levels of BDNF immunofluorescence. The average percentage of BDNF expressing DRG neurons was statistically higher in all comparisons ('low-moderate', 'high' and total) of CES and CCL with sham or naïve (Fig 2.3F, $p<0.001$). Interestingly, the average neuronal intensity of BDNF was statistically higher in CES compared to CCL ($p<0.01$). Elevated levels of BDNF protein in response to CES were also reflected at the transcript


A
B
C
D
Intensity (au)
Neuronal size (μm)


E
Average Intensity (au)
**
***
***
***
***
***
CES
CCL
Sham
Naive


F
(%) BDNF +ve
high
low-mod
***
***
***
***
***
CES
CCL
Sham
Naive

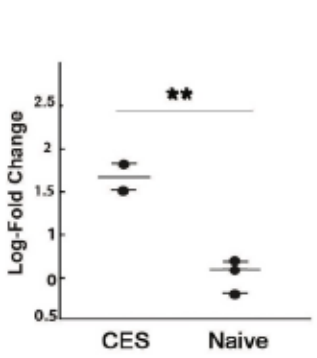

G
Log-Fold Change
**
CES
Naive

Figure 2.3: Electrical stimulation and crush conditioning three days prior upregulates BDNF expression in DRG neurons

Representative photomicrographs of L5 DRG sections processed for immunofluorescence to detect changes in BDNF expression with corresponding representative scatterplots depicting relative change in immunofluorescence signal over individual neurons as related to size, following CES (A), CCL (B), sham CES (C), and no conditioning (D). Lower solid lines of scatterplots depict the threshold of labeled versus unlabeled populations and the upper solid line separates neurons with 'low to moderate' levels of immunofluorescence from those with 'high' levels. Bar graph (E) demonstrates the average BDNF immunofluorescence of 3 animals per experimental group. For each n, 6 DRG sections were analyzed resulting in resulting in ~2,000 neurons analyzed per group). Bar graph (F) represents the mean percentage of neurons with low to moderate (light orange) and high (dark orange) levels of BDNF immunofluorescence/condition. Statistical analysis compares the intensity and the number of BDNF expressing neurons above threshold in each cohort (**$p<0.01$, ***$p<0.001$). Bar and whisker graph (J) depicts qRT-PCR result comparing relative BDNF mRNA expression in CES and naïve animal DRG (**$p<0.01$, $n=3$). Scale bar in A represents 80 μm.

level by qRT-PCR which demonstrated that CES significantly increased BDNF mRNA expression relative to naïve controls (M_{log2}=1.7 ± 0.2 log, n=3) (Fig 2.3G $p<0.01$).

2.5.2 CES activates perineuronal satellite glial cells

To determine whether the changes in the neuronal cell body response induced by CES were being communicated to perineuronal SGCs, upregulation of GFAP, known to increase following CCL, was examined (Stephenson & Byers, 1995; Woodham, Anderson, Nadim, & Turmaine, 1989; Xie, Strong, & Zhang, 2009). CES of the intact CP nerve activated the SGCs surrounding the DRG neurons, increasing levels of GFAP immunofluorescence over perineuronal SGCs in manner comparable to that observed in the CCL group (Fig 2.4 A, B, n=3 animals analyzed per treatment group). The increased GFAP displayed more prominently around large size neurons, although examples of small and medium size sensory neurons with GFAP positive SGCs were also evident. GFAP positive SGCs were found surrounding 29.9 ± 3.4% and 39.5 ± 6.6% of CES and CCL conditioned DRG neurons, respectively, which were significantly higher than sham (8.3 ± 5.4%,) and naïve animals (13.4 ± 2.1%) ($p<0.001$) (Fig 2.4A-E). Quantitative RT-PCR confirmed that CES resulted not only in increased GFAP protein, but also increased GFAP mRNA levels (M_{log2}= 3.4 ± 0.7 log, n=3) relative to naïve control isolates (n=3) ($p<0.05$) (Figure 2.4F).

2.5.3 CES and CCL-induced RAG expression remains upregulated at 14 days post-conditioning

DRGs from animals 14 days post-conditioning (7 days following nerve cut and microsurgical repair) were harvested to compare the levels of RAG expression between groups. Given that all animals sustained an injury with the cut and microsurgical repair, all DRGs demonstrated the

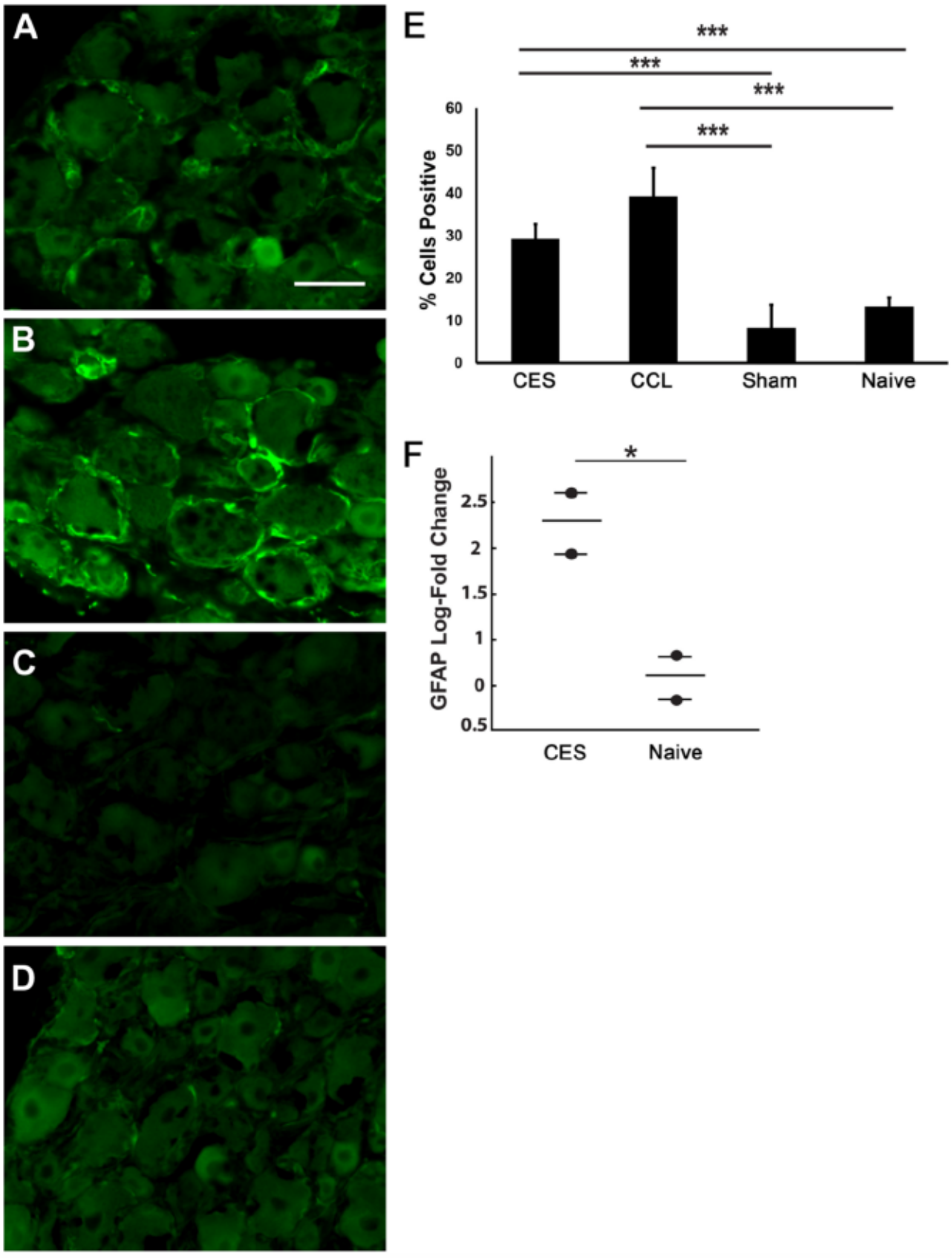
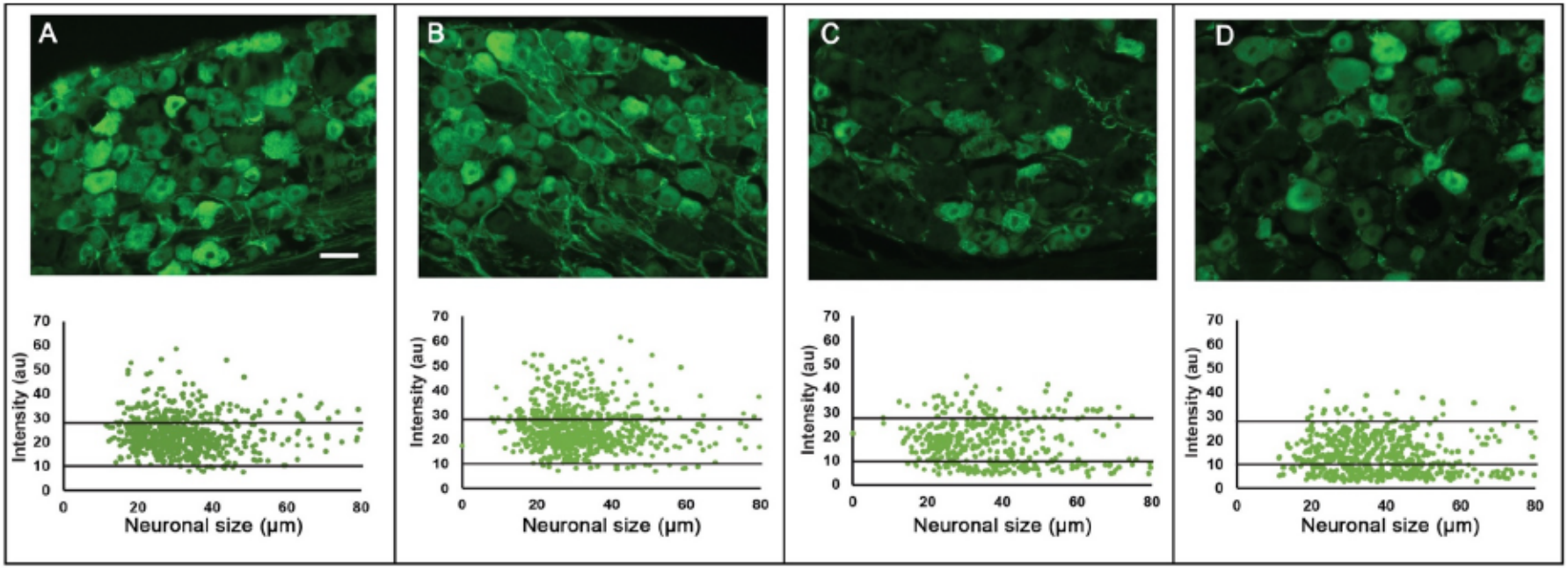
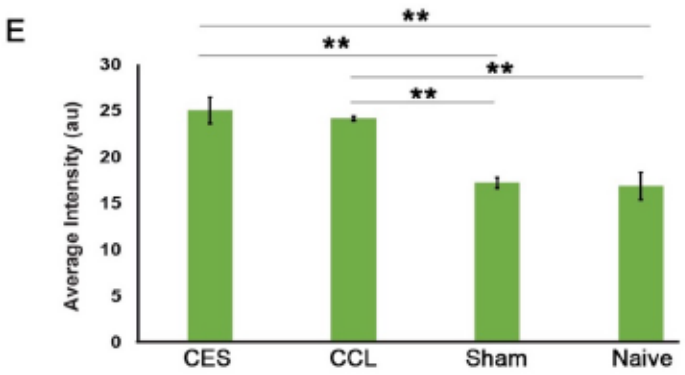

A
B
C
D
E
***
***
***
***
% Cells Positive
60
50
40
30
20
10
0
CES
CCL
Sham
Naive
F
*
GFAP Log-Fold Change
2.5
2
1.5
1
0
0.5
CES
Naive

Figure 2.4: Electrical stimulation and crush conditioning three days prior upregulates perineuronal satellite glial cell (SGC) GFAP expression

Representative photomicrographs of L5 DRG sections processed for immunofluorescence to detect changes in GFAP expression following CES (A), CCL (B), sham CES (C), and unconditioned naïve (D). Bar graph (E) reports the average percentage of DRG neurons surrounded by GFAP+ve SGC cells from 3 animals per experimental group. For each n, 6 DRG tissue sections were analyzed resulting in ~2,000 neurons counted per group). Statistical analysis compares the percentage of DRG cell bodies surrounded by GFAP+ve SGCs in each cohort (***$p<0.001$). Bar and whisker graph (F) depicts qRT-PCR result comparing relative GFAP mRNA expression in CES and naïve animal DRG (n=3; *$p<0.05$). Scale bar in A, 80 µm.


A
B
C
D
Intensity (au)
Neuronal size (μm)
0
10
20
30
40
50
60
70
0
20
40
60
80


E
Average Intensity (au)
0
5
10
15
20
25
30
**
**
**
**
CES
CCL
Sham
Naive

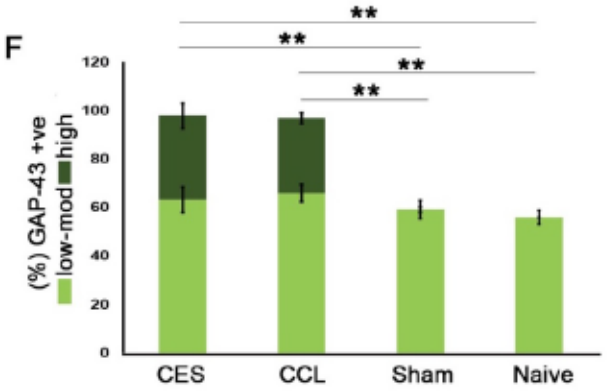
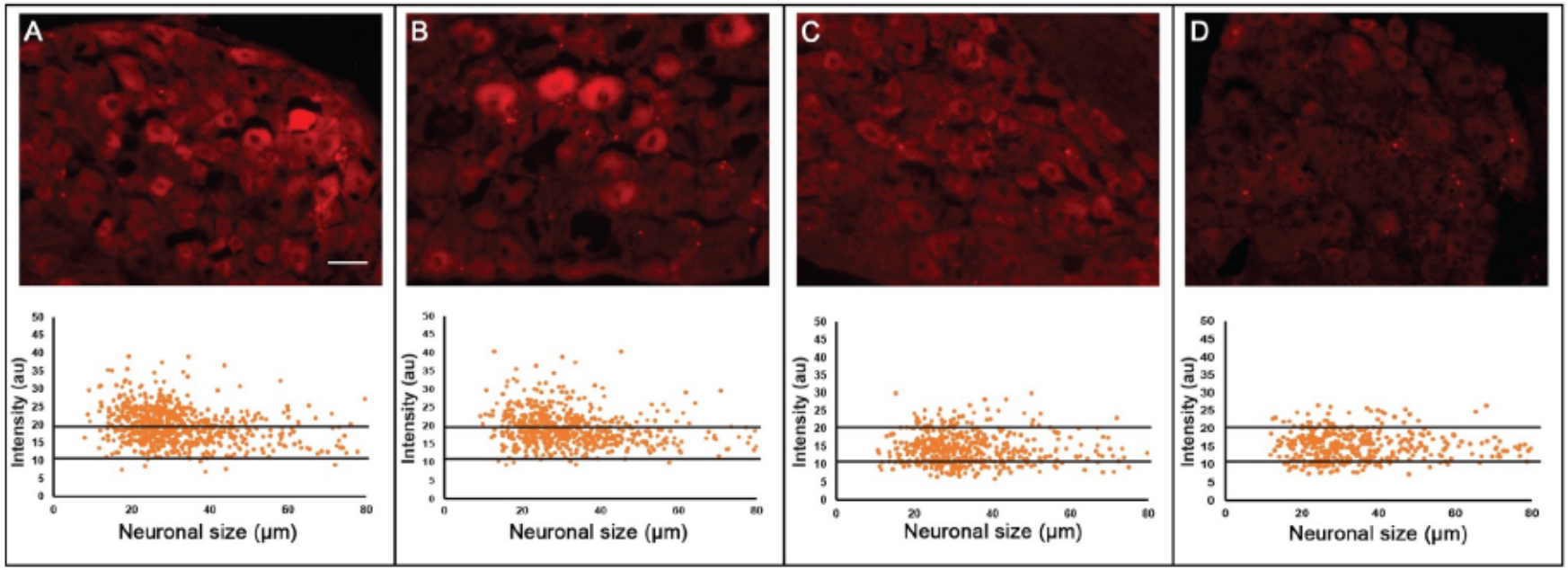

F
(%) GAP-43 +ve
low-mod
high
0
20
40
60
80
100
120
**
**
**
**
CES
CCL
Sham
Naive

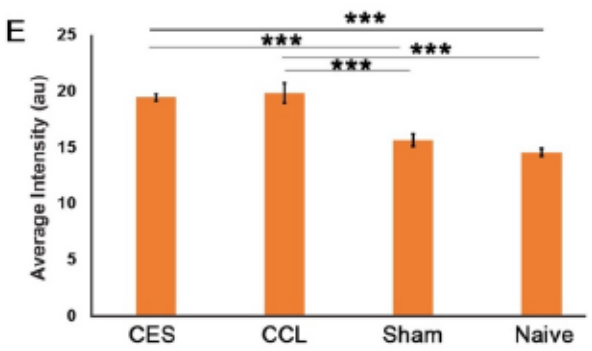
Figure 2.5: **Injured and repaired nerves subjected to prior conditioning have elevated GAP-43 expression.**

Representative photomicrographs of L5 DRG sections processed for immunofluorescence to detect changes in GAP43 expression 7-days following cut and coaptation and 14 days following conditioning. Corresponding representative scatterplots depict relative changes in GAP43 immunofluorescence over individual neurons as related to size, following CES (A), CCL (CB), sham CES (C), and no conditioning (naïve) prior to repair (D). Lower solid lines of scatterplots depict the threshold of labeled versus unlabeled populations and the upper solid line separates 'low to moderately' labeled from 'highly' labeled cells. Bar graph (E) demonstrates the average GAP-43 immunofluorescence of 3 animals per experimental group. For each n, 6 DRG tissue sections were analyzed resulting in ~2,000 neurons analyzed per group). Bar graph (F) depicts the mean percentage of neurons with low to moderate (light green) and high (dark green) levels of GAP-43 immunofluorescence/condition. Statistical analysis compares all GAP-43 expressing neurons above threshold in each cohort ($p<0.01$). Scale bar in A represents 80 μm.

predicted elevation in GAP-43, BDNF, and GFAP expression (Fig 2.5A-D, n=3 animals analyzed per treatment group); however, levels and incidence of expression were significantly higher among the conditioned cohorts. Qualitative assessment of DRG sections processed for GAP-43 immunofluorescence revealed heterogeneous patterns of expression in the sham CES and naïve control groups. There was a noticeable homogenous elevation of GAP-43 expression across all size ranges of DRG neurons in the conditioned groups (Fig 2.5A-D). The elevated expression in the two conditioned groups (CES and CCL) was also reflected in the significantly higher mean level of immunofluorescence per neuron relative to the two control groups (Fig 2.5E). The mean level of GAP-43 immunofluorescence per neuron was significantly elevated in the conditioned versus control groups with CES neurons displaying a mean level of immunofluorescence of 25.1 ± 1.4 au and CCL neurons displaying 24.2 ± 0.2 au at 14 days post-conditioning versus sham CES (17.2 ± 0.5 au) or naïve (16.9 ± 1.5 au) controls ($p<0.01$) (Fig 2.5E). In a parallel manner, conditioning also significantly increased the number of DRG neurons expressing detectable GAP-43 (Fig 2.5F). GAP-43 protein expression was observed in 97.8 ± 0.2% and 96.8 ± 1.5% of DRG neurons conditioned with CES and CCL with 63.2 ± 5.3% and 65.9 ± 3.6% of them low to moderately expressing and 34.6 ± 5.2% and 30.8 ± 2.2% high expressing, respectively. This was significantly higher than sham and naive cohorts, which expressed detectable GAP-43 in 73.3 ± 3.7% and 69.8 ± 4.3%, respectively ($p<0.01$). Despite higher expression of GAP-43 in all cohorts post-cut/coaptation (Day 14), neither sham nor naive conditioned animals had high levels of neuronal GAP-43 immunofluorescence compared to conditioned ($p<0.01$).

Similar to GAP-43, BDNF was also upregulated in the CES and CCL groups at 14 days post-conditioning as compared to sham and naïve controls. Representative DRG sections and their


A
B
C
D
Intensity (au)
Neuronal size (μm)


E
Average Intensity (au)
***
CES
CCL
Sham
Naive

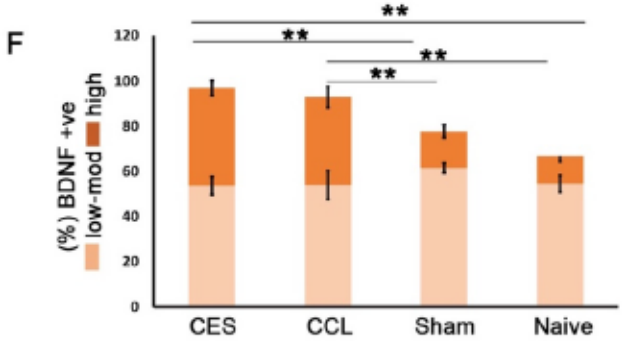

F
(%) BDNF +ve
low-mod
high
**
CES
CCL
Sham
Naive

Figure 2.6: **Injured and repaired nerves subjected to prior conditioning have elevated BDNF expression.**

Representative photomicrographs of L5 DRG sections processed for immunofluorescence to detect changes in BDNF expression 7-days post-cut/coaptation and 14 days following conditioning. Corresponding representative scatterplots depict relative changes in immunofluorescence over individual neurons as related to size, following CES (A), CCL (B), sham CES (C), and no conditioning (naïve) prior to repair (D). Lower solid lines of scatterplots depict the threshold of labeled versus unlabeled populations and the upper solid line separates 'low to moderately' labeled from 'highly' labeled cells. Bar graph (E) demonstrates the average BDNF immunofluorescence of 3 animals per experimental group. For each n, 6 DRG tissue sections were analyzed resulting in ~2,000 neurons analyzed per group). Bar graph (F) depicts the percentage of neurons with low to moderate levels of BDNF immunofluorescence (light orange) versus high levels (dark orange). Statistical analysis compares all BDNF expressing neurons above threshold in each cohort ($p<0.01$, $***p<0.001$). Scale bar in A represents 80 μm.

corresponding scatterplots demonstrated an increased BDNF immunofluorescence in CES and CCL compared to sham and naive controls (Fig 2.6A-D, n=3 animals analyzed per treatment group). The mean level of BDNF immunofluorescence per neuron was still significantly elevated in the conditioned versus control groups with CES neurons displaying a mean level of immunofluorescence of 19.4 ± 0.3 au and CCL neurons displaying 19.8 ± 0.8 au versus sham (15.6 ± 0.5 au) or naïve (14.6 ± 0.3 au) controls (Fig 2.6E, $p<0.01$ for all conditioned vs. unconditioned cohorts). Further, conditioning also significantly increased the number of DRG neurons expressing BDNF (Fig 2.6F). BDNF protein expression was observed in 94.7 ± 0.9% and 92.8 ± 1.9% of DRG neurons conditioned with CES and CCL, with 51.5 ± 4.0% and 53.9 ± 6.3% of them displaying low to moderate levels of immunofluorescence and 43.1 ± 3.3% and 38.8 ± 4.6% doing so at high levels, respectively. This was significantly higher than sham and naive cohorts, which expressed BDNF in 77.7 ± 4.6% and 66.7 ± 5.5% of DRG neurons, respectively ($p<0.01$). High levels of BDNF immunofluorescence was observed over 16.3 ± 2.8% and 12.2 ± 2.5% of sham and naive DRG neurons, respectively ($p<0.001$).

At 14 days post-conditioning, conditioned SGCs displayed higher GFAP expression compared to unconditioned cohorts. Elevated GFAP expression was observed in both the control and conditioned groups as they had undergone nerve transection and repair 7 days prior (Fig 2.7A-D). Conditioning increased the incidence of neurons being surrounded by activated SGCs with 36.4 ± 9.8% of CES neurons and 49.1 ± 10.5% of CCL neurons compared to the sham (10.0 ± 2.2%) or naive control (10.1 ± 2.5%) ($p<0.001$). The

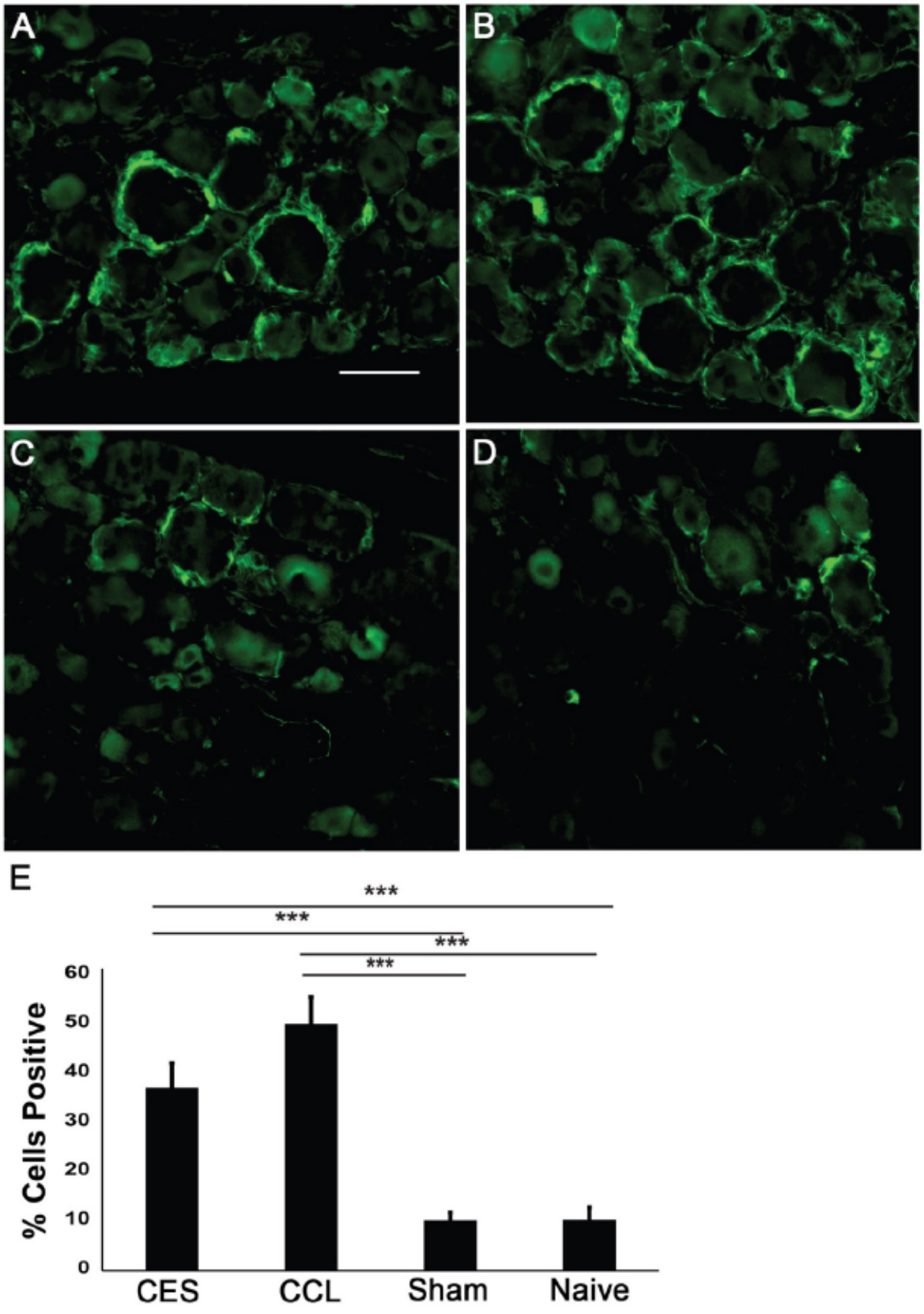

A
B
C
D
E
***
***
***
***
% Cells Positive
60
50
40
30
20
10
0
CES
CCL
Sham
Naive

Figure 2.7: Injured and repaired nerves subjected to prior conditioning have increased incidence of DRG neurons with perineuronal GFAP-positive SGCs

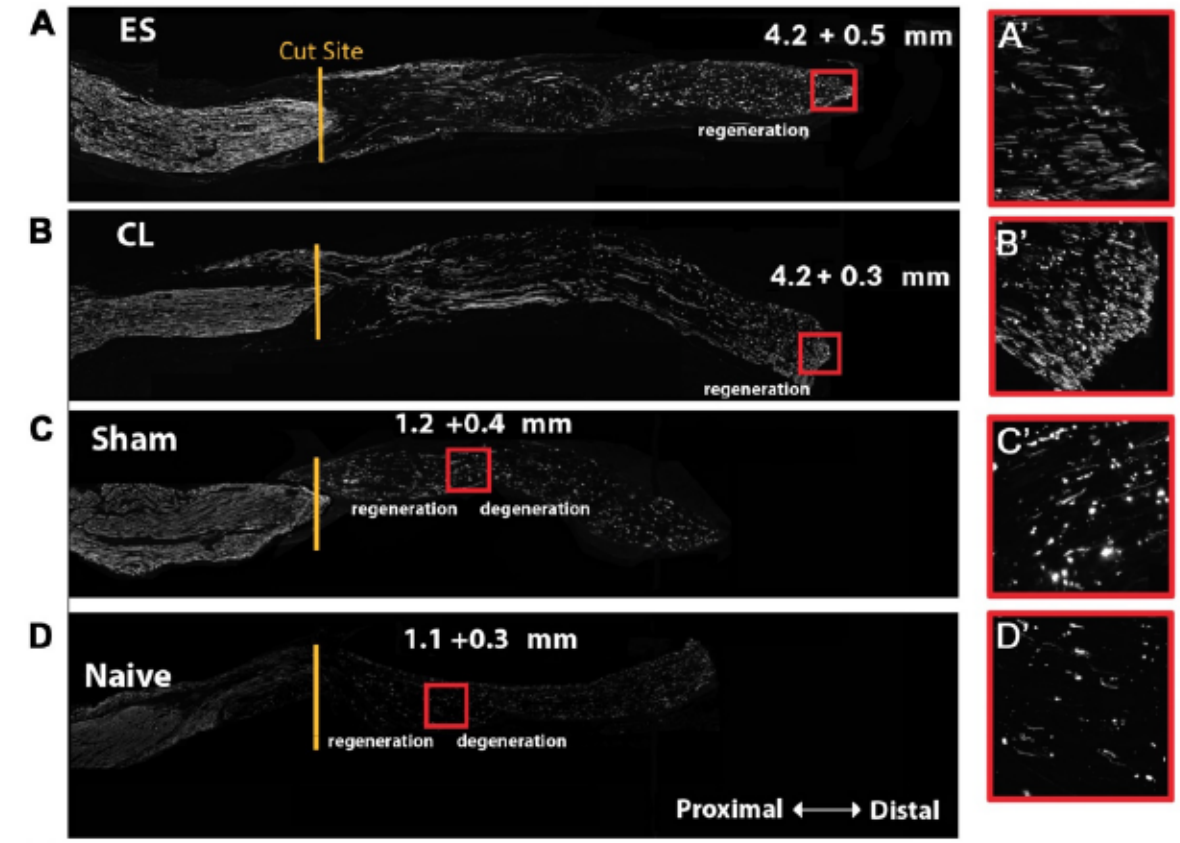
Representative photomicrographs of L5 DRG sections processed for immunofluorescence to detect changes in the levels of GFAP expressed in SGCs and incidence of neurons with GFAP-associated SGCs 7-days post-cut/coaptation and 14 days following CES (A), CCL (B), sham stimulation conditioning (C) and no conditioning (naïve) prior to repair (D). Bar graph (E) reports the average percentage of DRG neurons surrounded by GFAP+ve SGC cells from 3 animals per experimental group. For each n, 6 DRG tissue sections were analyzed resulting in ~2,000 neurons counted per group). Statistical analysis compares the percentage of DRG cell bodies surrounded by GFAP+ve SGCs in each cohort (***$p<0.001$). Scale bar in A represents 80 μm.

differences between conditioned and control groups were highly significant ($p<0.001$, Fig 2.7E).

2.5.4 CES stimulates nerve regeneration similar to CCL

One week following conditioning, the CP nerve was re-exposed and transected 10 mm proximal to the prior conditioning site. The proximal and distal nerves were immediately coapted/repaired, thereby increasing the ability of the regenerating nerve fibers to regenerate into the epineurial tubes of the distal nerve stump as it undergoes Wallerian degeneration. One week after nerve transection and microsurgical repair, the nerves were harvested and cut longitudinally to capture the proximal nerve tip, the regeneration bridge and the distal degenerating nerve stump. The sections were processed for neurofilament 200 (NF200) immunocytochemistry to label individual axons (Fig 2.8 A-D, n=6 animals analyzed per treatment group). Although the regenerating and degenerating axons both label with NF200, their morphology is unique and thus the regenerating axons are easily discerned from the degenerating fibers (Webber et al., 2011). The number of regenerating axons extending into the regeneration bridge was quantified at 250 μm intervals until less than 10 axons were observed.

CES and CCL both enhanced the extent of regeneration by 3.8-fold compared to the sham stimulated and naïve controls. The regeneration length was significantly longer in animals subjected to CES (4.2 ± 0.5 mm) and CCL (4.2 ± 0.4 mm) compared to sham conditioned (1.2 ± 0.4 mm) or naive (1.1 ± 0.3 mm) control animals (Fig 2.8 E). The differences between conditioned and control groups were highly significant for all comparisons of CES and CCL


A
ES
Cut Site
4.2 + 0.5 mm
regeneration
A'
B
CL
4.2 + 0.3 mm
regeneration
B'
C
Sham
1.2 +0.4 mm
regeneration
degeneration
C'
D
Naive
1.1 +0.3 mm
regeneration
degeneration
D'
Proximal ⟷ Distal

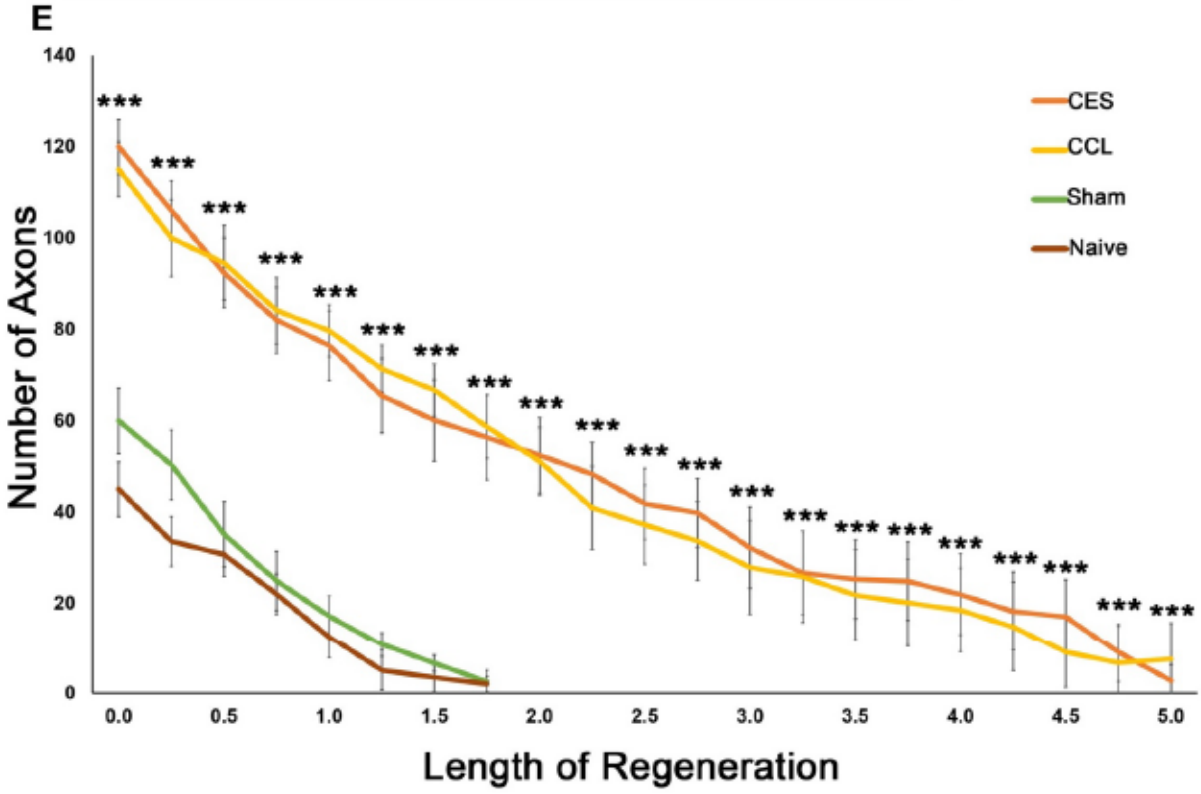

E
Number of Axons
Length of Regeneration
CES
CCL
Sham
Naive
***
0
20
40
60
80
100
120
140
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0

Figure 2.8: **Conditioning electrical stimulation promotes nerve regeneration in a manner akin to a traditional crush conditioning lesion**

to sham or naive animals ($p<0.001$); while not significantly different between conditioned groups ($p=0.67$), supporting our hypothesis of equivalence. In addition to increased length, CES and CCL conditioned animals had increased total regenerating nerve fibers compared to sham or naïve animals. At the cut site, a greater number of axons extended into the regeneration bridge following CES (120 axons) or CCL (112 axons) compared to sham (61 axons) or naïve (45 axons). This trend of significantly increased numbers of regenerating axons was present the entire length of regeneration ($p<0.001$) (Fig 2.8 E).

In conclusion, we determined that CES is sufficient to upregulate RAGs and accelerate nerve regeneration in a manner akin to a CCL.

2.6 Discussion

2.6.1 CES upregulates expression of RAGs

The classic CCL requires a prior injury to induce a neuronal cell body response characterized by elevated RAG expression and synthesis of cellular components required for effective reinnervation. This results in the neurons being primed for regeneration when a subsequent test/definitive nerve injury is performed (Al-Majed, Brushart, & Gordon, 2000; Al-Majed et al., 2004; Geremia et al., 2007; Singh et al., 2012). We demonstrate brief CES of intact nerves prior to cut and microsurgical repair upregulates DRG RAG expression without requiring axonal injury. The heightened cell body response induced in sensory neurons by CES includes increased neuronal GAP-43 and BDNF, with GFAP upregulation in perineuronal SGCs (Figs 2.2-2.7). The levels of RAG expression (increased neuronal intensity and number

of expressing neurons) remain elevated in the CES and CCL animals relative to the sham and naïve control animals after microsurgical repair (Figs 2.5-2.7).

Interestingly, at 3 days post-conditioning, CES increased both the incidence of, and the intensity at which BDNF was expressed in the DRG neurons compared to CCL (Fig 2.3 E, F); however, this distinction was not evident at 14 days. These findings concur with observations by Geremia et al., 2007 (Geremia et al., 2007) which show the effects of ES on BDNF overexpression are decreased by 3 weeks.

2.6.2 CES promotes nerve regeneration

Our results demonstrate that similar to CCL, but unlike postoperative ES (Brushart et al., 2002; Witzel, Brushart, Koulaxouzidis, & Infanger, 2016), CES accelerates nerve regeneration. Remarkably, both CES and CCL displayed an identical 3.8-fold increase in the length the axon regeneration when compared to the unconditioned animals, suggesting similar mechanisms of action (Fig 2.8). By changing the timing of electrical stimulation from postoperative to preoperative (CES), we invoked a strong cell body response akin to CCL. These findings suggest that the timing of delivery of electrical stimulation is critical to inducing specific physiological changes in the neuronal cell body of injured axons. The interactions between RAGs and the inflammatory response may be dependent on both the concentration of molecules and their temporal interactions.

A second effect of CCLs is the increased number of axons that cross the injury site. Richardson and Verge (1987) reported a 5-10 fold increase in the number of regenerated

myelinated fibers following CCL (Richardson & Verge, 1987). This study supports the hypothesis that conditioning with CCL or CES significantly increases the number of axons regenerating along the entire length of the nerve, when compared to the sham or naive. The identity of these regenerating fibers (sensory or motor) requires further investigation. We also do not know if the increased numbers of axons reflect increased number of neurons regenerating at the time point examined, and/or whether the conditioning induced branching of regenerating axons (Witzel et al., 2016). Regardless, the parallels of CES to CCL in terms of: a) enhanced RAG expression`, b) increased percentage of RAG-expressing neurons, c) increased rate of regeneration and d) an increased total number of regenerating axons, supports CES as a viable non-injurious, novel means of conditioning for peripheral nerve repair. Conditioning, either by CES or CCL, primes the regenerative response prior to the test lesion to heighten repair.

2.6.3 CES and CCL may act through related yet distinct signaling pathways

Similar regenerative success in both conditioning paradigms suggests parallel mechanisms of action; however, this may not be the case as their method of delivery is unique. CCL elicits an inflammatory response that is critical for promoting the conditioning effect (Kwon et al., 2013; Lu & Richardson, 1991; Niemi et al., 2013). It is not known if CES induces an inflammatory response; however, given the non-invasive nature of this procedure (beyond the skin lesion required to isolate the nerve) it is likely that if an immune response is evoked, it is less pronounced than traditional CCL. Importantly, the skin incision and wire placement is not sufficient to induce a conditioning effect, as there was no regeneration enhancement

following sham conditioning (Fig 2.8). Future mechanistic studies will elucidate the immune response following CES.

At a molecular level, cAMP and BDNF are critical to the CCL regeneration response. While we have yet to show a mechanistic role for these molecules in the CES-associated enhanced peripheral nerve regeneration, the molecules examined in this proof of principle study, serve an important role in gauging the state of plasticity induced by CES of the intact nerve. This may include the use of transgenic animals with conditional knockdown of key molecules such as BDNF and RNA-seq to profile and interrogate transcriptional changes.

2.6.4 CES as a potential therapy

Electrical stimulation is safe for clinical use (Barber et al., 2015; Chan, Curran, & Gordon, 2016; Gordon, Amirjani, Edwards, & Chan, 2010; Power, Morhart, Olson, & Chan, 2016; Wong, Olson, Morhart, & Chan, 2015). Post-surgical electrical stimulation reduces the delay in regenerating axons crossing the repair site ("staggered regeneration"), allowing greater numbers of axons to reach and reinnervate the target (Gordon & English, 2016). Importantly, however, a major downfall is that postoperative electrical stimulation does not increase the speed of regeneration beyond the cut site, which is critical for recovery following proximal nerve injuries (Brushart et al., 2002; Witzel et al., 2016). We show CES is a novel strategy capable of accelerating the speed of axon regeneration beyond the cut site. If these results translate to a human model, CES may allow for functional neuromuscular reinnervation following proximal nerve injuries.

2.6.5 Future Directions

We have demonstrated that CES has a remarkable impact on the processes of nerve regeneration both at the level of the cell body response, with upregulation of RAGs, as well as on the regenerative capacity and rate of regeneration of the axons themselves. Our results support that CES can be used as a preoperative intervention to induce a conditioning effect equivalent to the traditional CCL, without physically harming the nerve. Although the outcomes between CES and CCL are similar, further investigation is required to elucidate the cellular and molecular mechanisms responsible for this phenomenon. Studies to directly compare the regenerative effects of conditioning versus post-operative electrical stimulation or therapies combining both approaches are warranted to determine which paradigm translates to better behavioral and neurophysiological improvements. From a clinical viewpoint, electrical nerve stimulation is already used post-operatively and has been shown to be safe and well-tolerated. Therefore, the implementation of CES as a pre-operative conditioning paradigm is highly clinically feasible.

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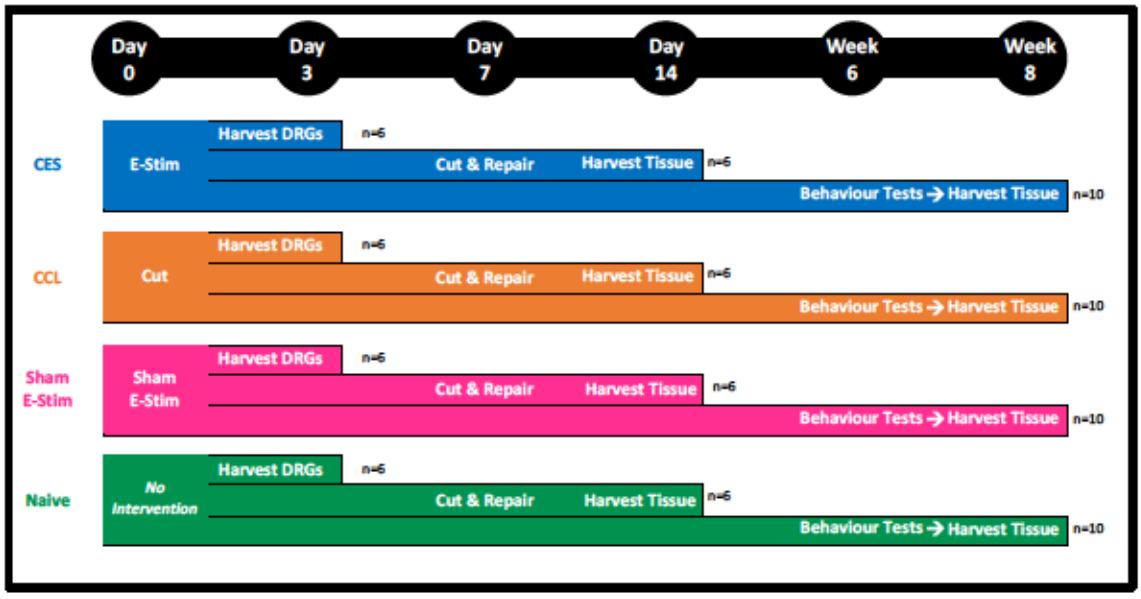
Chapter 3

Conditioning Electrical Stimulation Promotes Functional Nerve Regeneration

3.1 Preface

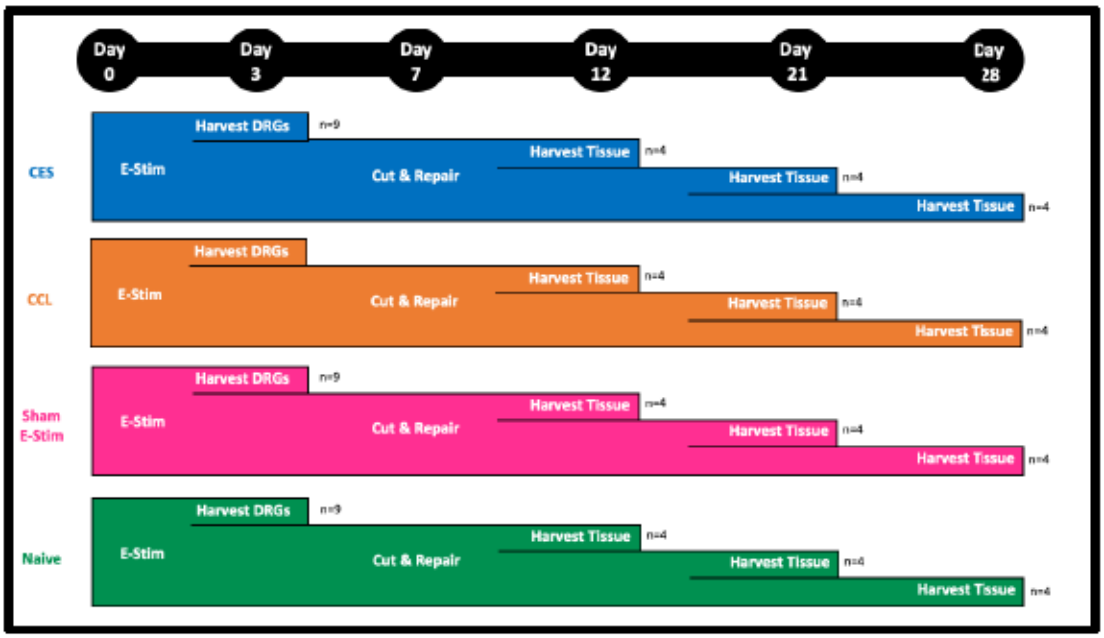
Results from our pilot project described in Chapter 2 strongly suggest that CES enhances nerve regeneration comparably to CCL, with similar upregulation of RAGs and enhanced axon extension when compared to control animals. We therefore investigated whether improved regenerative outcomes confer greater sensory and motor reinnervation.

The common peroneal nerve has a very small dermatomal distribution; therefore, evaluation of sensory outcomes is challenging in this nerve injury model. As such, we chose to evaluate sensorimotor outcomes of CES in a tibial nerve injury model. Previous studies have suggested that the pro-regenerative effects of electrical stimulation differ between individual nerves in the lower extremity. Our first aim therefore was to compare the effects of CES (blue), CCL (orange), sham-ES (pink) and no conditioning (green) on axon extension in a tibial nerve injury model, as seen in the timeline below. Similar to our results in the common peroneal nerve, CES induced a conditioning effect in the tibial nerve



In a second set of animals, sensory (von Frey filaments, intraepidermal nerve fiber density) and motor behavior tests (horizontal ladder test, gait analysis) were performed biweekly between 6 and 8 weeks of regeneration to assess functional recovery, as seen in the timeline below. Tissue analysis of muscle and footpad biopsies specimens was performed at 8 weeks of regeneration. For all outcomes evaluated, animals treated with CES had significantly improved sensorimotor recovery when compared not only to negative controls, but also to animals treated with the gold-standard crush conditioning.

To determine why animals treated with CES had improved reinnervation outcomes compared to those who received a CCL, we 1) ruled out changes in expression of regeneration associated genes at the DRG following conditioning, and 2) evaluated the length of axon extension at multiple timepoints, as seen in the timeline below. We found that while regenerating axons treated with CES and CCL have similar growth initially, axon extension is halted at the crush site in the CCL animals. By contrast, CES does not appear to damage the nerve, and therefore extension proceeds without delay.



Conditioning Electrical Stimulation Promotes Functional Nerve Regeneration

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3.2 Abstract

Peripheral nerve regeneration following injury is often incomplete, resulting in significant personal and socioeconomic costs. Although a conditioning crush lesion prior to surgical nerve transection and repair greatly promotes nerve regeneration and functional recovery, feasibility and ethical considerations have hindered its clinical applicability. In a recent proof of principle study, we demonstrated that conditioning electrical stimulation (CES) had effects on early nerve regeneration, similar to that seen in conditioning crush lesions (CCL). To convincingly determine its clinical utility, establishing the effects of CES on target reinnervation and functional outcomes is of utmost importance. In this study, we found that CES improved nerve regeneration and reinnervation well beyond that of CCL. Specifically, compared to CCL, CES resulted in greater intraepidermal skin and NMJ reinnervation, and greater physiological and functional recovery including mechanosensation, compound muscle action potential on nerve conduction studies, normalization of gait pattern, and motor performance on the horizontal ladder test. These findings have direct clinical relevance as CES could be delivered at the bedside before scheduled nerve surgery.

3.3 Introduction

Motor and sensory recovery following nerve injury is limited by the rate of peripheral nerve regrowth (1-3 mm/day), and the decreased potential of muscle reinnervation after prolonged atrophy. Consequently, recovery from peripheral nerve injury is often incomplete, resulting in long-term disability with profound personal and socioeconomic costs (Lundborg, 2000). To date, there remain no clinically feasible methods of accelerating the rate of nerve regeneration. In a rodent model however, a conditioning crush lesion (CCL) is well documented to significantly accelerate peripheral nerve regeneration. In this model, crushing a nerve 7 days prior to a nerve injury promotes nerve regeneration up to five-fold (Richardson & Verge, 1987; Torigoe, Hashimoto, & Lundborg, 1999). There is an associated upregulation of regeneration-associated genes (RAGs), such as growth associated protein-43 (GAP-43), brain-derived neurotrophic factor (BDNF), phosphorylated cAMP response element binding protein (pCREB) and glial fibrillary acidic protein (GFAP). This shifts the neuron into a regenerative state and increases the production and transport of necessary structural proteins for axon extension resulting in greater sensorimotor regeneration and functional recovery (Bisby, 1985; Hoffman, 2010; Richardson et al., 2009; Ying, Misra, & Verge, 2014). Unfortunately, CCL is not clinically feasible, and thus translation to the bedside is not possible.

An alternative technique for enhancing nerve regeneration and functional recovery that is currently being used clinically is postoperative electrical stimulation (PES) (A. A. Al Majed, Brushart, & Gordon, 2000a; A.A. Al Majed, Neumann, Brushart, & Gordon, 2000b). However, unlike CCL, PES does not accelerate the rate of nerve regeneration but rather

reduces the delay caused by staggered regeneration at the site of injury (Brushart et al., 2002). A seminal observation by Udina et al (2008) determined that the same electrical stimulation parameters 7 days prior to axotomy enhanced dorsal root ganglion (DRG) neurons neurite outgrowth to the same extent as CCL. Because PES has already been shown to be safe and well tolerated in a number of clinical trials (Barber et al., 2018; Gordon, Amirjani, Edwards, & Chan, 2010; Wong, Olson, Morhart, & Chan, 2015), electrical stimulation may be an appealing strategy of delivering a CCL-like treatment to patients with peripheral nerve injury. We therefore proposed using the well-established technique of electrical stimulation in a different context, to determine if it we could non-injuriously induce a conditioning-like effect.

In a recent proof of principle study, we demonstrated that conditioning electrical stimulation (CES), delivered prior to nerve transection and microsurgical repair of the fibular (common peroneal) nerve, upregulates RAGs and accelerates peripheral nerve regeneration in a manner similar to a CCL (Senger et al., 2017). While these early findings seem promising, it remains to be determined whether these improvements in regeneration translate to enhancement in reinnervation and, of great practical relevance and clinical importance, function. Therefore, the goal of this study is to test the hypothesis that, in addition to increasing the rate of nerve regeneration, CES also enhances motor and sensory functional recovery. We found that while CES had a similar effect on accelerating nerve regeneration as CCL, it produced even greater function recovery.

3.4 Methods

3.4.1 Animals: Healthy adult male Sprague Dawley rats (200g; Charles River laboratory) were placed under the care of Health Sciences Laboratory Animal Services at the University of Alberta. They were housed 2 animals per flat-bottomed betachip-lined cages, under 12-hour on/off light conditions, with *ad libitum* standard rat chow and water. All experimental procedures were approved by the University of Alberta Animal Research Ethics Board, and surgeries performed in a dedicated animal surgical facility.

3.4.2 Experimental Design: Animals were randomly divided into four equal cohorts determined by the type of conditioning applied to the tibial nerve: a) conditioning electrical stimulation (CES), b) CCL, c) sham electrical stimulation controls (Sham-ES), and d) unconditioned control animals. Experimental groups were as follows: 24 animals were used for tibial nerve regeneration studies (culled 14 days post-conditioning, n=6/cohort), 40 animals were used for behavioral testing (culled 8 weeks post-conditioning, n=10/cohort), 36 animals were used for regeneration-associated gene (RAG) analysis at the DRG (culled 3 days post-conditioning, n=5/cohort for immunohistochemistry, n=4/cohort for Western blot analysis); and 36 animals were used for tibial nerve regeneration studies comparing 5, 14 and 21 days regeneration (n=4 animals/cohort),

3.4.3 Conditioning surgery: Prior to skin incision, all animals were anesthetized with inhalational isoflurane (2%, titrated at 1-2 L/min to maintain a surgical anesthetic plane), and received a single 0.01 mg/kg dose of subcutaneous buprenorphine. Opioids were chosen rather than nonsteroidal anti-inflammatories, as inflammation has been implicated in

mounting a conditioning effect (Niemi, DeFrancesco-Lisowitz, Cregg, Howarth, & Zigmond, 2016; Niemi et al., 2013). A longitudinal incision was made over the lateral aspect of the right hind paw to gain access to the superficial posterior compartment of the leg. The gastrocnemius muscle was elevated through blunt dissection and the tibial nerve was isolated as it emerged between the gastrocnemius head bifurcation. Nerve conditioning was performed as previously described (8): a) CES: stainless steel wires with ends bared of insulation were connected to an SD-9 stimulator (Grass Instruments Co., Quincy, MA). The cathode wire was wrapped around the tibial nerve at the level of the gastrocnemius head bifurcation and the anode wire was placed into the belly of the tibialis anterior muscle. A continuous train of electrical stimulation (20 Hz of 0.1 ms duration balanced biphasic pulses) as previously described in PES was delivered for 1 hour with voltage titrated to maintain a visible twitch in the lower limb flexors (A.A. Al Majed et al., 2000b; Geremia, Gordon, Brushart, Al Majed, & Verge, 2007); b) CCL: the tibial nerve was crushed at the level of the gastrocnemius muscle head bifurcation using a non-toothed fine hemostat (5 mm tip) for 10 seconds; c) sham-ES: two wires were positioned using the same landmarks as CES and left in place for 1 hour, but no current was delivered; d) unconditioned: no surgical intervention was performed prior to the test lesion and microsurgical repair. Animals were reassessed the day following all surgical intervention.

3.4.4 Nerve transection and microsurgical repair surgery: A nerve repair surgery was performed on all animals except those in which the DRGs were isolated for RAG expression 3 days post-conditioning. Animals recovered from conditioning for seven days prior to tibial nerve transection and microsurgical repair. The animals were anesthetized with inhaled

isoflurane and a single dose of buprenorphine (0.01 mg/kg) provided analgesia. An incision was made posterior to the lateral middle third of the palpable femur, and the sciatic nerve was identified and isolated. The sciatic nerve was traced distally to its site of trifurcation and, 1 cm distal to this landmark, the tibial nerve was isolated and transected. Immediately following the axotomizing transection, an epineurial repair was performed using 9-0 silk suture under 3.5x loupe magnification. The hamstring muscles were re-suspended and the skin closed using 3-0 Vicryl suture (Ethicon Inc, Somervile, NJ). Animals received 0.01 mg/kg subcutaneous buprenorphine the following day.

3.4.5 Tissue Processing: Animals were euthanized at the appropriate time points for the outcome studied: 3 days post-conditioning (RAG analysis), 5, 14 and 21 days post-coaptation (length of regeneration studies), and 8 weeks post-nerve repair (behavioural studies). Euthanasia was accomplished by carbon dioxide inhalation followed by exsanguination by left cardiac ventricle puncture.

The tibial nerve was re-exposed and the site of the microsurgical repair identified. The nerve was carefully dissected from the fibular and sural nerve branches as well as from surrounding scar and soft tissues. Once harvested, the nerve was placed on a 3 cm segment of toothpick to stabilize the proximal stump, regenerating tip, and distal stump. The proximal sciatic nerve was then traced back to its vertebral column origins for accurate identification and extraction of the L4 and L5 DRGs. Tissues were fixed in Zamboni's fixative (paraformaldehyde, picric acid, NaOH, American MasterTech Scientific, Lodi, CA) for four hours, rinsed with 0.01M phosphate buffered saline (PBS) (Thermo Fisher Scientific, Waltham, MA) five times, post-

fixed in 30% sucrose solution (Thermo Fisher Scientific, Waltham, MA) overnight at 4°C, then frozen in Optimum Cutting Temperature (OCT) (Sakura Finetek, Torrance, CA) using indirect exposure to liquid nitrogen. Both the nerve and DRGs were cut into 12 µm sections (nerve was cut longitudinally) and thaw-mounted on Superfrost Plus microscope slides (Thermo Fisher Scientific, Waltham, MA). Slides were stored at -80°C until processing.

The gastrocnemius muscles were harvested from the bilateral lower limbs, from the insertion point of the Achilles tendon onto the calcaneus to the attachment points of the two muscle heads on the proximal tibia. The ipsilateral muscle was represented as the percentage weight of the uninjured contralateral control limb. To prepare for NMJ immunocytochemistry, the muscle from the injured limb was fixed in Zamboni's solution overnight, rinsed five times with PBS, sunk in 30% sucrose overnight (4°C), then flash-frozen using liquid nitrogen in OCT. Sections of 20 µm were cut in the cryostat and stored at -80°C.

Footpads of the injured limb were collected at 8 weeks post-nerve repair. A 3 mm biopsy punch (Acuderm Inc, Fort Lauderdale, FL) was used to collect tissue from plantar footpad which was fixed in 2% paraformaldehyde, lysine, periodate (PLP) fixative for 16-20 hours at 4°C, rinsed 5x in Sorensen's phosphate buffer, then cryoprotected overnight at 4°C in 20% glycerol (Thermo Fisher Scientific, Waltham, MA)/0.1 M Sorensen's phosphate buffer. Tissue was frozen in OCT and cut into 20 µm sections on Superfrost-Plus microscope slides. Slides were stored at -80°C until processing.

3.4.6 Behavioural and physiologic outcomes: Animal behaviour testing was performed at 6.5, 7, 7.5, and 8 weeks following nerve repair. All tests were performed by the same

examiner who was blinded to the conditioning of the animals. Sensory reinnervation was analyzed using von Frey filaments. Motor testing included gait analysis for toe spread, horizontal ladder task for motor dexterity, and nerve conduction studies for compound muscle action potentials (CMAPs).

Sensory testing was performed using von Frey filaments at all four time-points post-nerve repair. Animals received thirty minutes of acclimatization after being placed in a plexiglass cage with wire mesh flooring. Increasing force was placed on the plantar aspect of the animal's injured foot within the distribution of the tibial dermatome, using calibrated von Frey monofilaments (1.4-15 g). Each paw was probed five times with each monofilament for a 3 second duration with enough force to cause bending of the monofilament. A positive result was recorded when the animal withdrew his paw for three consecutive probes of an individual monofilament.

Gait analysis was performed at 7 and 8 weeks to analyze the function of the intrinsic foot muscles innervated by the tibial nerve. The animals were placed in a specially-designed transparent narrow walkway measuring 48 cm in length, designed with an adjustable mirror underneath that allows for simultaneous visualization of the lateral view of the animal and its plantar paw. A ruler projected in the mirror from the walking path served as reference. Video was taken of the animals walking the length of the track three times per testing session. The video was then analyzed and individual screenshots were taken of each footstep when the animal was weight-bearing on that limb. Only images where the position of all toes could be adequately visualized were analyzed. A minimum of 10 images per foot were analyzed

per testing session. The distance between the first and last toe was measured on the injured right, and control left sides. Toe spread of the injured side was reported as a percentage of the uninjured contralateral control.

The horizontal ladder task was performed a total of four times, at 6.5, 7, 7.5, and 8 weeks post-nerve repair. The horizontal ladder itself consisted of an elevated (48 cm) horizontal ladder composed of clear plexiglass. At each test session, the animal was evaluated on three separate attempts at crossing the ladder. Placement of ladder rungs was changed between each testing session to prevent the rats from learning the course. Videos taken of the animals crossing the horizontal ladder were analyzed in a blinded fashion on a frame-by-frame basis. Each placement of the foot on a ladder rung was graded on a scale of 0-6 based on the scoring system developed by Metz and Whishaw (Metz & Whishaw, 2009): a total miss (score 0), deep slip (score 1), slight slip (score 2), replacement (score 3), correction (score 4), partial placement (score 5) or correct placement (score 6). The average score of the injured limb was calculated for each attempt on the ladder.

Nerve conduction studies (NCS) were performed on both lower limbs at 8 weeks post-nerve repair. Animals were anesthetized using inhaled isoflurane. Supramaximal electrical stimulation was performed at the knee and the recording leads were placed in the plantar footpad to record from the intrinsic foot muscles. The location of the lead was adjusted to detect the CMAP with the maximum amplitude and sharpest rise time. This was used to quantify the extent of successful muscle reinnervation in the foot.

3.4.7 Immunofluorescence: Slides were warmed to room temperature prior to antigen retrieval in a 60ºC citrate buffer (10 mM sodium citrate, 0.05% Tween-20, pH 6.0; Thermo Fisher Scientific, Waltham, MA) for 20 minutes and then they were cooled to room temperature. After washing the slides three times in 0.01 M PBS for five minutes, slides were permeabilized with 0.1% Triton-100X (Thermo Fisher Scientific, Waltham, MA) for 10 min. Individual sections were blocked in 10% normal goat serum (MP Biomedicals, Santa Ana, CA) and 3% bovine serum albumin (BSA) (Sigma-Aldrich, St Louis, MO) in 0.01 M PBS for 90 minutes. Primary antibodies were diluted in a solution of 0.01 M PBS containing 3% BSA, and applied to tissue sections overnight at 4ºC. DRG sections were stained with the primary antibodies: rabbit anti-GAP-43 (1:400, Millipore), rabbit-anti-GFAP (1:500, DAKO, Santa Clara, CA), chicken anti-BDNF (1:500, Promega, Madison WI), and rabbit anti-phosphorylated CREB (1:500, Cell Signaling Technology, Danvers, MA). Nerve sections were stained with 1:500 mouse anti-neurofilament-200 (NF200) (Sigma-Aldrich, St Louis, MO). Muscle sections were first labeled with 1:500 mouse anti-NF200 to stain the innervating nerve and then stained with (1:1000 dilution) conjugated anti-α-bungarotoxin for 20 min. Footpad intraepidermal nerve fibers were labeled with 1:1000 rabbit anti-protein gene product 9.5 (PGP9.5) (Encor Biotechnology Inc, Gainesville, FL). The next day, slides were washed three times for five minutes each with 0.01 M PBS, and the secondary antibodies, diluted in a solution of 0.01 M PBS containing 3% BSA, was applied for 90 minutes at room temperature. Secondary antibodies included Cy3-conjugated goat anti-mouse (Sigma-Aldrich), Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA), and donkey-anti-chicken 594 (Thermo Fisher Scientific). DRG slides were counterstained with nuclear stain NucBlue (Thermo Fisher Scientific). All slides were then

mounted with a coverslip using 50% glycerol in 0.01 M PBS. The specificity of secondary antibodies was employed by experiments in which the primary antibody was omitted, revealing the absence of nonspecific staining.

All slides were qualitatively assessed to ensure there was no discernable slide-to-slide variation for the same marker within individual treatment groups. Quantitative analysis was performed in a blinded manner on DRG sections to identify alterations in immunofluorescence signal. To ensure reliability in the comparison between experimental groups, all sections for each antibody were processed for immunofluorescence in an identical, parallel manner. Digital images were taken under identical fluorescence exposures using the 20x objective lens of a Zeiss Axio Imager fluorescence microscope. Twelve representative DRG sections were analyzed for each animal (n=5 animals/cohort). The intensity of the immunofluorescence was quantified by identifying neurons with NucBlue-stained nuclei and manually circling the perimeter of each individual DRG neuron using ImageJ software (ImageJ; Rasband WS). Cells were deemed positive for GAP-43 or BDNF expression as determined by the level of immunofluorescence of neurons considered to be devoid of positive signal upon scrutiny at higher magnification. Data was further subdivided into 'low to moderately' GAP-43 or BDNF immunopositive and 'highly' immunopositive based on natural breaks identified on analysis of scatterplots (data not shown) representing the different animals and treatment groups. Expression of pCREB and GFAP was analyzed using binary evaluation; DRG neurons were classified as "positive" or "negative" based on the presence or absence of positive staining in the nuclei or satellite glial cells (SGCs), respectively.

Nerve tissues were cut longitudinally (12 μm) to preserve the proximal stump coapted to the distal stump. Sections were processed for immunohistochemistry against neurofilament-200 (NF-200) to label individual axons. Under fluorescent microscopy, the unique morphological patterns were identified to differentiate the regenerating axons from the degenerating ones. The length of axonal extension and the number of regenerating axons were measured (250 μm intervals) from the site of repair to the most distal point of regeneration containing a minimum of 10 axons.

Muscle sections were evaluated with confocal microscopy to identify innervated neuromuscular junctions (NMJs) determined by NF200, to identify the innervating axon, and α-bungarotoxin, to label the acetylcholine receptors. The mean innervated number of NMJs of each cohort was quantified by averaging the total number of NMJs in 6 separate tissue sections per animal.

Footpad sections were imaged at 40x magnification capturing 5-6 adjacent fields for a total of 10-15 fields per animal. A z-stack of 1 μm steps was imaged for each field, and using ImageJ software, the intraepidermal nerve fiber (IENF) density was determined by counting the number of positively stained axons (PGP9.5) crossing the dermal-epidermal junction (number of IENF/mm).

3.4.8 Western blot analysis: Cellular protein was isolated from DRGs collected three days following conditioning by homogenizing samples with RIPA buffer and protease inhibitor. Protein concentrations were determined with a BCA Protein Assay Kit (Pierce) and 25 μg of

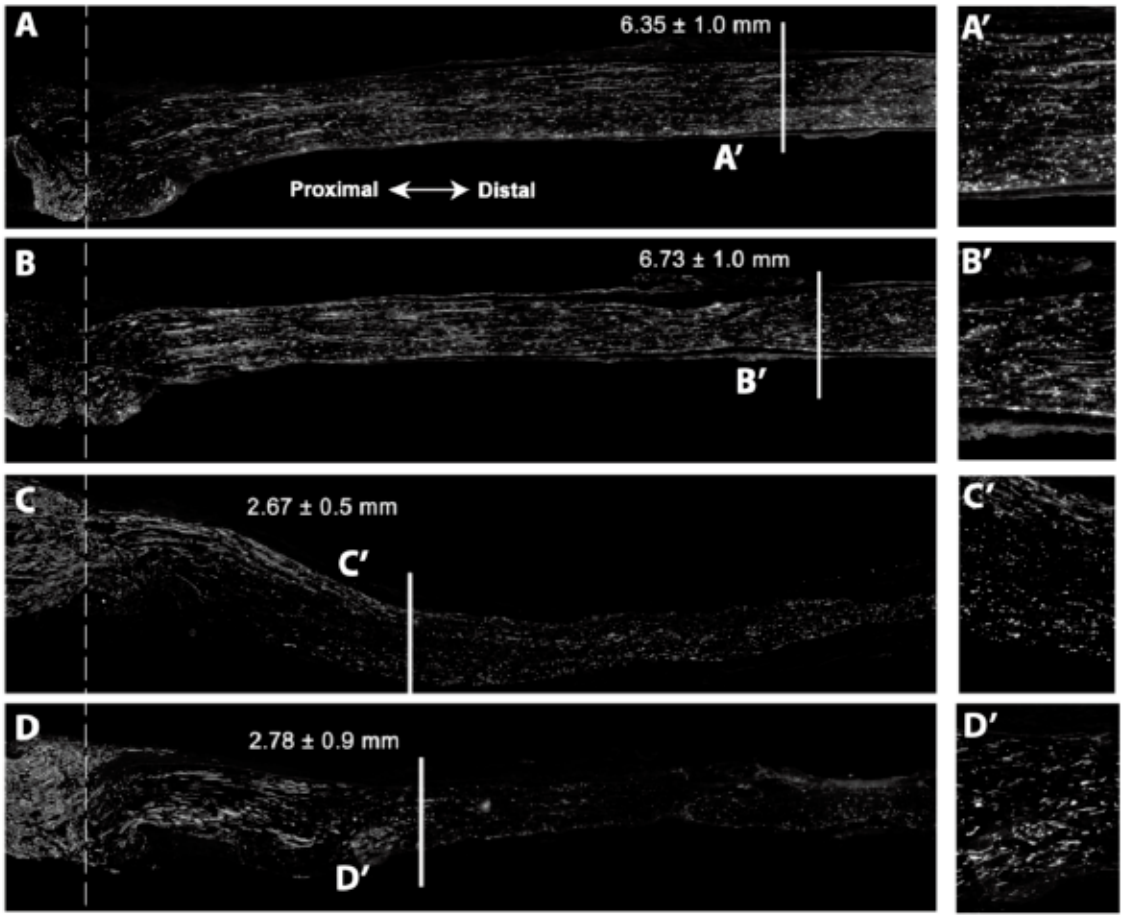
solution (protein, β-mercaptoethanol, RIPA buffer and protease inhibitor) was loaded into each well of precast gel cassettes (Mini-Protean TGX Stain-Free Protein Gel, 4-20%). Proteins were transferred overnight onto PVDF membrane (Millipore) and bands were imaged for total protein. The membrane was placed in blocking solution (5% BSA in Tris-buffered saline) for one hour, then overnight in the primary antibodies rabbit anti-GAP43 (1:1000) or rabbit anti-BDNF (1:1000), in a solution of 2.5% BSA in Tris-buffered Saline. Following secondary antibody (Goat anti-rabbit IgG HRP, 1:20,000), the blot was exposed to enhanced chemiluminescent (ECL; Lumi-Light Plus, Roche Diagnostics) for signal detection and band images were captured. Antibody expression was normalized to total protein.

3.4.9 Statistical analyses: Results are presented as the mean ± standard error mean (s.e.m). Differences in individual animals of each cohort were analyzed using a one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test to compare means between groups. A two-way ANOVA was performed to compare sensory and motor behavioral performance between cohorts. In cases where a significant interaction between the factors: time and treatment allocations was found, post hoc analysis using Tukey test was done. Statistical significance was accepted with a level of $p<0.05$. All statistical analyses were performed using STATA 14 (StataCorp LP, Collagen Station, Texas).

3.5 Results

3.5.1 CES and CCL of the tibial nerve enhances the length and magnitude of axonal regeneration

The regenerative potential of CES, CCL, sham-ES, and unconditioned animals were compared (n=6 animals/cohort). One week following conditioning, the tibial nerve was re-exposed and a complete nerve axotomy was performed 10 mm proximal to the previous conditioning site. The proximal and distal stumps were immediately repaired with two epineurial stitches; this coaptation was performed to guide regenerating nerve fibers and to mimic the clinical treatment for complete peripheral nerve injuries. One week following nerve transection and repair (14 days after conditioning), animals were euthanized and the tibial nerves were harvested and processed for neurofilament (NF200) immunofluorescence to measure the extent of nerve regeneration (Figure 3.1). Impaired to the sham-ES and unconditioned animals. The length of axonal extension for CES animals (6.35 ± 1.0 mm) was similar to CCL (6.73 ± 1.0 mm), and significantly longer than sham-ES (2.67 ± 0.5 mm) or unconditioned (2.78 ± 0.9 mm) control nerves. This difference was highly significant for all length comparisons of CES and CCL to sham-ES or unconditioned control animals ($p<0.001$) (Figure 3.1E, x-axis). There was no difference between the CES and CCL groups ($p=0.287$). Similarly, both CES and CCL had an increased number of total regenerating nerve fibers compared to the unconditioned control cohorts (Figure 3.1E, y-axis, $p<0.001$ for all). At the cut site (Figure 3.1A-D, dashed lines), the number of regenerating fibers entering the distal nerve stump was 132 ± 9 axons in CES and 113 ± 10 axons in CCL whereas a significantly lower number of axons entered the regeneration site in the sham-ES (62 ± 9 axons) or unconditioned animals (68 ± 5 axons) ($p<0.001$ for all). There were significantly


A
6.35 ± 1.0 mm
A′
Proximal ⟷ Distal
A′
B
6.73 ± 1.0 mm
B′
B′
C
2.67 ± 0.5 mm
C′
C′
D
2.78 ± 0.9 mm
D′
D′

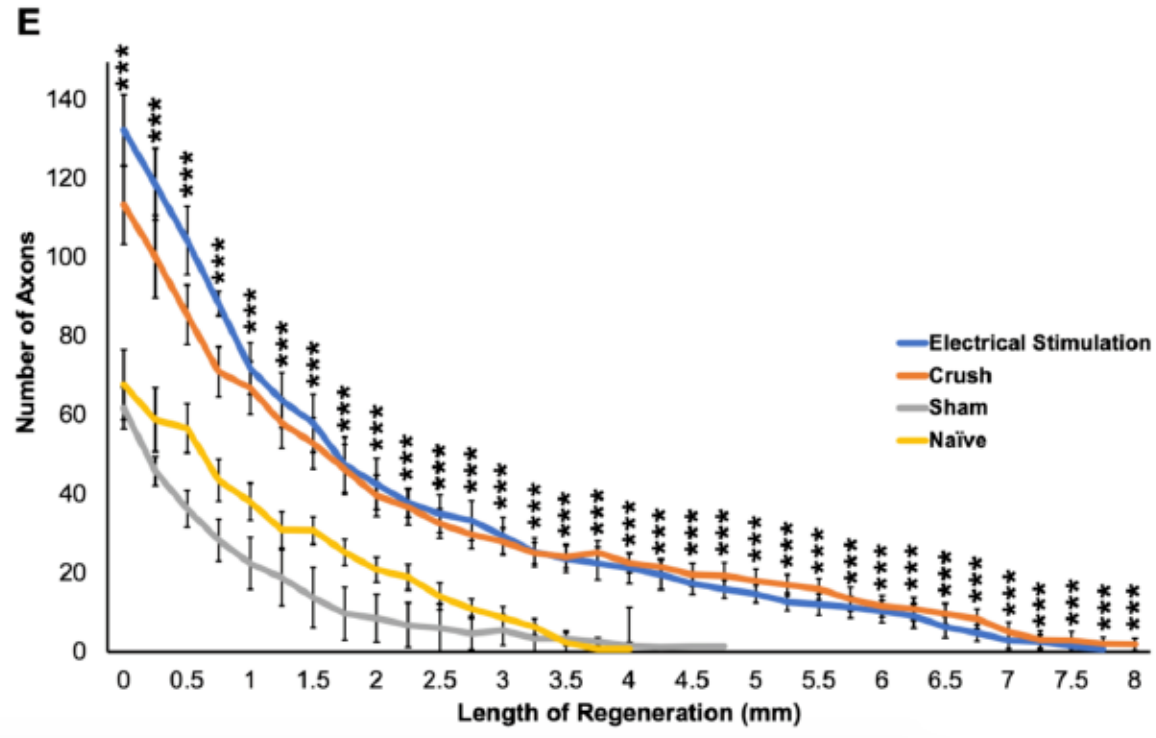

E
Number of Axons
0
20
40
60
80
100
120
140
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
5.5
6
6.5
7
7.5
8
Length of Regeneration (mm)
Electrical Stimulation
Crush
Sham
Naïve

Figure 3.1: **CES promotes nerve regeneration as efficiently as CCL**

Representative photomicrographs of NF200 labeled longitudinal tibial nerve sections at the regeneration site on day 14. These nerves were conditioned by CES (A), CCL (B), sham-ES (C) or no conditioning (D) on day 0 and then transected and repaired on day 7. Regenerating axons were identified based on their linear morphology which is distinct from the punctate appearance of NF200-positive degenerating axons. Dashed white lines depict the site of cut and coaptation and the solid vertical white lines indicate the distal-most point of regeneration (defined as the distal-most site where a minimum of 10 axons were counted). Green inset (A') depicts a magnified view of the axons at the distal tip of regeneration (arrows) with staining distal to this point being NF200-positive axons undergoing Wallerian degeneration (arrowheads). Line graph (E) depicts the average length of axonal regeneration (x-axis) and the number of regenerating axons (y-axis). Axons conditioned with CES (blue line) or CCL (orange line) had a similar number of axons present at each 250 μm interval distal to the injury site which was considerably more than sham-ES (grey line) or the unconditioned cohorts (yellow line). At all sites in which axon length and number were measured, the CES and CCL animals were significantly higher than the sham or unconditioned controls (***$p<0.001$, n=6 animals/cohort).

greater numbers of CES and CCL regenerating axons identified over the entire length of regeneration compared to the control nerves ($p<0.001$ for all).

3.5.2 CES of the tibial nerve improves sensory innervation greater than CCL

Forty animals (n=10/cohort) were examined between 6.5 and 8 weeks after cut and microsurgical repair to compare functional outcomes with or without conditioning (Figure 3.2A). At 6.5 weeks of regeneration, von Frey filament tests revealed that CES animals responded to 8.5 ± 1.0 g whereas both sham-ES and unconditioned animals were unresponsive to all filament weights ($p<0.001$); CCL animals required 16.5 ± 3.4 g of mechanical stimulation to elucidate a positive response ($p=0.06$ compared to CES). At 7 weeks, the average weight required to evoke three consecutive positive responses in the CES animals was 8.5 ± 1.0 g, which showed a trend of increased responsiveness compared to CCL (9.8 ± 1.8 g, $p=0.077$), sham-ES (10.3 ± 1.9 g, $p=0.054$) or unconditioned animals (13.8 ± 1.3 g, $p<0.01$). By 7.5 weeks, animals in the CES cohort had significant sensory improvements, with responsiveness to 4.5 ± 0.5 g, which was significantly less than in CCL (6.5 ± 0.5g, $p<0.05$), sham-ES (9.3 ± 0.7 g, $p<0.01$) or unconditioned animals (15.3 ± 3.8 g, $p<0.001$) (Figure 3.2B). Similarly, at 8 weeks, CES animals continued to improve, requiring an average of only 3.0 ± 0.6 g, significantly less than that required by animals in the CCL (6.5 ± 1.0 g, $p<0.05$), sham-ES (9.3 ± 0.7 g, $p<0.001$) or unconditioned cohorts (10.8 ± 1.5 g, $p<0.001$). Two-way ANOVA of weeks 7 and 8 revealed a significant interaction between time and treatment groups ($p<0.05$). Post hoc analysis revealed that the CES group performed significantly better compared to the other groups ($p<0.05$). These data strongly suggest increased sensitivity of the CES animals compared to all other cohorts. These behavioral

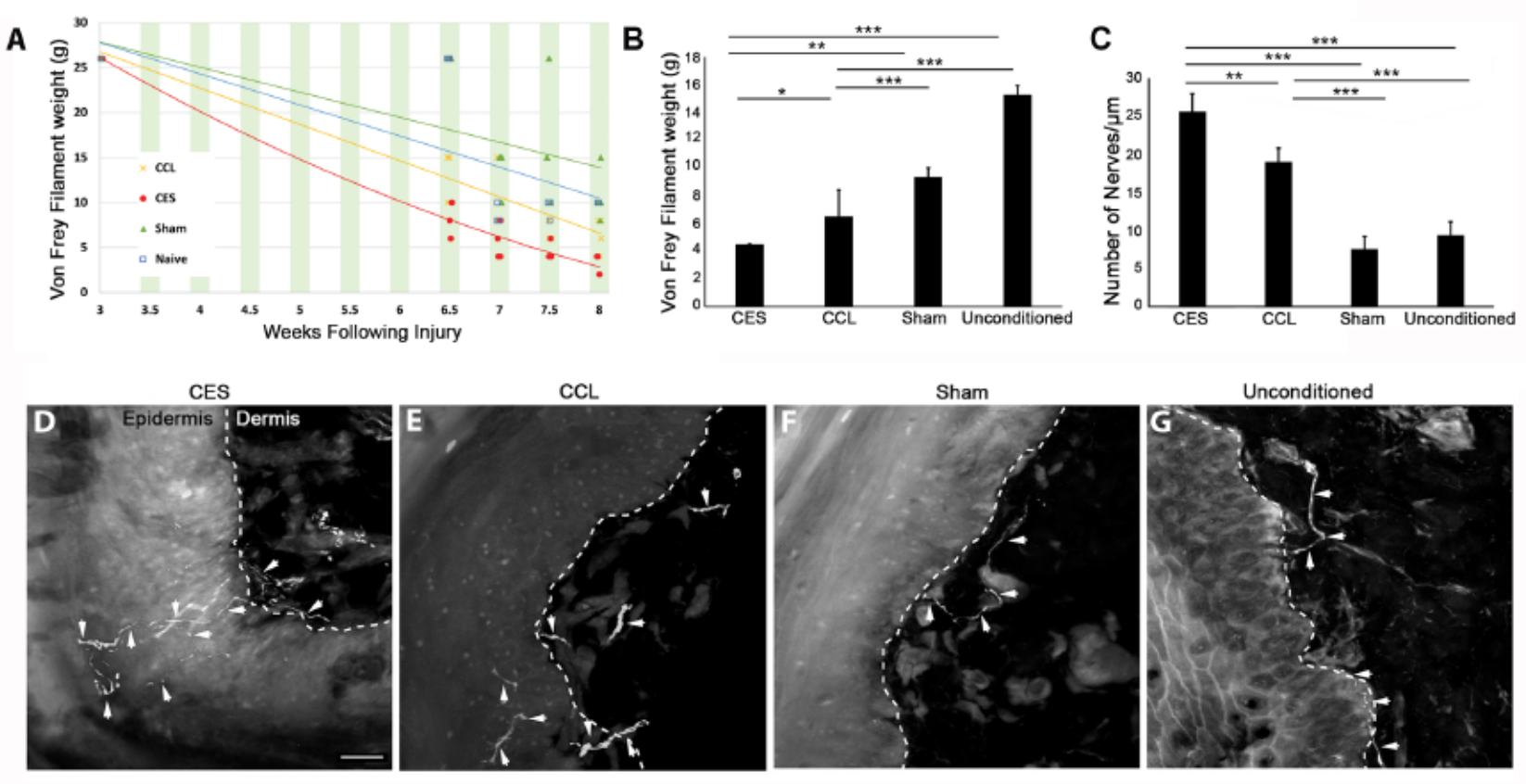

A
Von Frey Filament weight (g)
Weeks Following Injury
CCL
CES
Sham
Naive
B
Von Frey Filament weight (g)
CES
CCL
Sham
Unconditioned
C
Number of Nerves/µm
CES
CCL
Sham
Unconditioned
CES
CCL
Sham
Unconditioned
D
Epidermis
Dermis
E
F
G

Figure 3.2: CES promotes significantly greater sensory reinnervation than CCL

(A) Regression analysis of von Frey tests at 6.5-8 weeks demonstrates greater sensory recovery in CES animals compared to CCL, sham-ES, and unconditioned controls. Two-way ANOVA comparing filament weight and time at weeks 7 and 8 confirm significantly improved reinnervation in the CES cohort compared to CCL ($p<0.05$). (B) Representative histogram of a single time point demonstrates that 7.5 weeks after cut and coaptation, CES conditioned tibial nerves had significantly greater sensory recovery compared to CCL (*$p<0.05$), sham-ES (**$p<0.01$) and unconditioned (***$p<0.001$) animals (One-way ANOVA). (C-G) At 8 weeks post-nerve transection and repair, skin biopsies were harvested from the plantar footpads of each animal and tissues (12 µm sections) were processed for intraepidermal nerve fiber innervation. (D-G) Z-stacks (1 µm) were imaged with confocal microscopy and the total number of PGP9.5 label axons (arrowheads) were counted as they crossed the dermal-epidermal junctions (dashed lines) of CES (D), CCL (E), sham-ES (F) and unconditioned (G) samples. (C) Quantification revealed there was significantly more nerve reinnervation per µm of epidermis in CES compared to CCL (**$p<0.01$), sham-ES (***$p<0.001$) and unconditioned (***$p<0.001$) footpads (One-way ANOVA). Scale bar is 25 µm; n=10 animals/cohort for all sensory tests.

differences are corroborated by evidence from microscopic evaluation of injured footpads confirming greater epidermal reinnervation in CES animals. At 8 weeks, CES animals had a greater number of sensory intraepidermal nerve fibers (IENFs) crossing the dermal-epidermal junction (25.5 ± 1.6 axons/ μm) compared to CCL (19.3 ± 1.3 axons/ μm, $p<0.01$), sham-ES (7.5 ± 1.1 axons/ μm, $p<0.001$) or unconditioned controls (8.9 ± 1.9 axons/ μm, $p<0.001$) (Figure 3.2C-G).

3.5.3 CES of the tibial nerve improves motor outcomes greater than CCL

Increased sensory reinnervation was accompanied by improved functional outcomes. Gait analysis at 7- and 8-weeks post-nerve repair revealed that CES significantly improved reinnervation of the foot interosseous muscles which are responsible for toe spread (Figure 3.3A). At 7 weeks post-nerve repair, the average toe spread of the injured paw among CES animals was 55.2 ± 1.6% of their uninjured paw, which was greater than the recovered toe spread in the CCL group at 45.7 ± 4.2%; this trend however failed to show significance ($p=0.08$). CES had significantly greater toe spread recovery compared with sham-ES (29.5 ± 0.4%; $p<0.001$) or unconditioned animals (40.1 ± 1.8%; $p<0.001$). However, by 8 weeks post-nerve repair, toe spread recovery was significantly greater in the CES (58.3 ± 1.6%) compared with the CCL group (48.1 ± 7.9%; $p<0.01$) (Figure 3.3B, C). Toe spread recovery of the sham-ES and unconditioned controls remained largely unchanged from the week prior, at 28.8 ± 1.1% ($p<0.001$) and 39.3 ± 7.4% respectively ($p<0.01$). Regression analysis and two-way ANOVA of weeks 7 and 8 revealed a significant interaction between time and treatment groups ($p<0.05$, Figure 3.3A). Post-hoc analysis revealed that in the CES group, the restoration of toe spread distance was significantly better than the other groups.

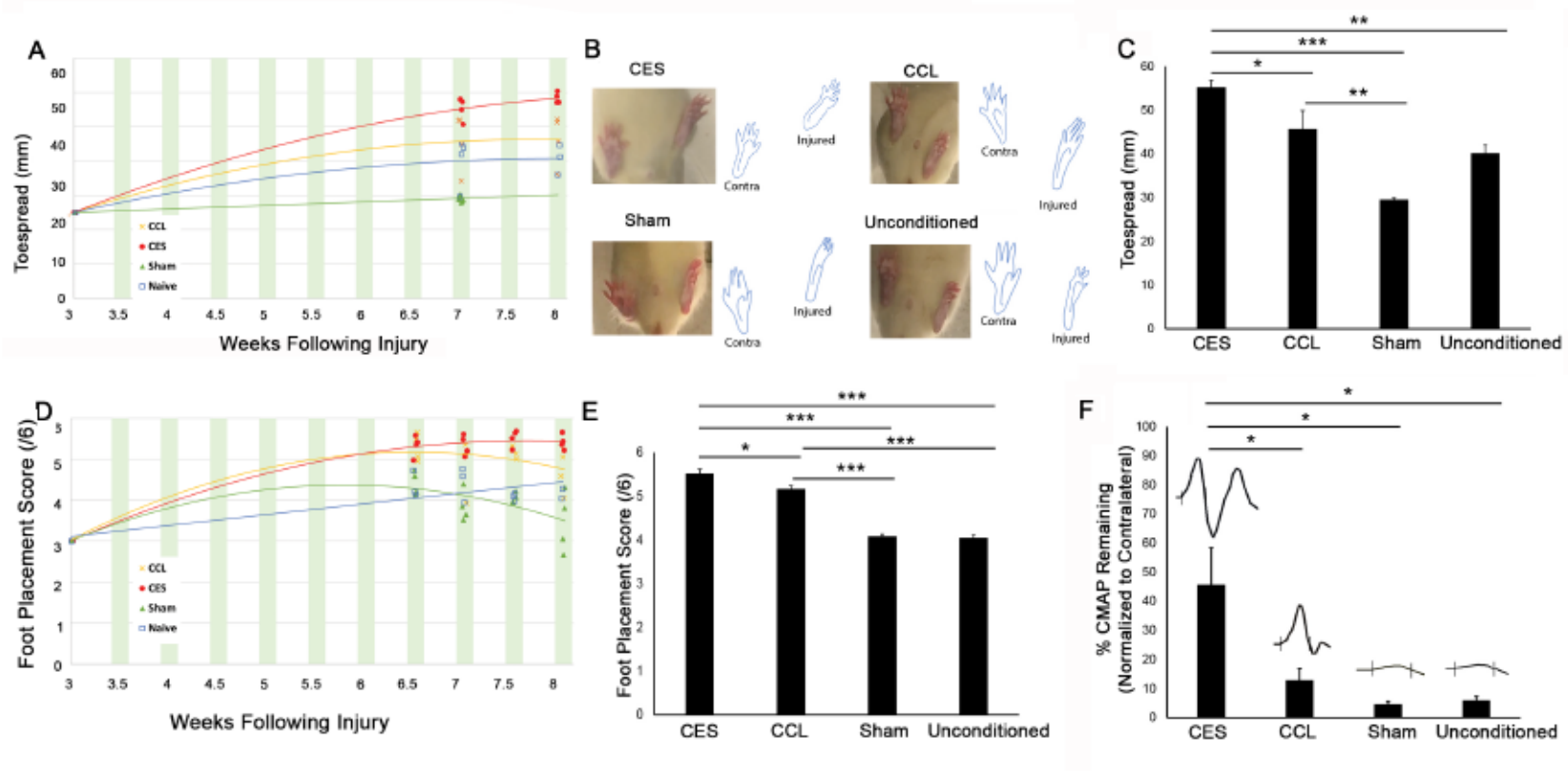

A
Toespread (mm)
Weeks Following Injury
CCL
CES
Sham
Naive
B
CES
CCL
Sham
Unconditioned
Contra
Injured
C
Toespread (mm)
CES
CCL
Sham
Unconditioned
D
Foot Placement Score (/6)
Weeks Following Injury
E
Foot Placement Score (/6)
F
% CMAP Remaining
(Normalized to Contralateral)

Figure 3.3: CES improves functional recovery to a greater extent than CCL

Functional recovery was assessed 6.5, 7, 7.5 and 8 weeks following tibial nerve cut and coaptation in animals conditioned by CES, CCL, sham-ES, or no conditioning (n=10/cohort). Toe spread measurement between the first and fifth digit of the left (injured) foot was normalized to the contralateral control foot. (A) Regression analysis at 7 and 8 weeks revealed CES had significantly wider toe spread compared to all other cohorts ($p<0.05$; Two-way ANOVA). (B) Representative photographs and camera lucida tracing of CES, CCL, sham-ES, and unconditioned feet are shown. (C) Representative histogram of a single time point revealed that at 7 weeks CES animals had greater toe spread compared to CCL (*$p<0.05$), sham-ES (***$p<0.001$) and unconditioned (**$p<0.01$) animals (One-way ANOVA). (D-E) The horizontal ladder task was performed to determine the number of correct foot placements and foot slips from the injured limb. (D) Regression analysis from horizontal ladder testing at 6.5, 7, 7.5 and 8 weeks is shown. Statistical analysis confirmed that at 7 and 8 weeks, CES animals had improved foot placement compared to all other cohorts ($p<0.05$; Two-way ANOVA) revealed greater. (E) Representative histogram of a single time point revealed that at 7 weeks, CES had greater success at this motor task compared to CCL (*$p<0.05$), sham-ES (***$p<0.001$) and unconditioned (***$p<0.001$) control animals (One-way ANOVA). (F) Compound muscle action potentials (CMAPs) were recorded at 8 weeks and ipsilateral muscles were normalized to the contralateral control. CES had increased CMAP amplitude compared to CCL, sham-ES, and unconditioned animals (*$p<0.05$; One-way ANOVA).

These data strongly suggest greater reinnervation to the intrinsic foot muscles in the CES animals and therefore wider toe spread compared to all other cohorts.

Horizontal ladder performance was improved in CES animals compared to all other cohorts (Figure 3.3D). At 6.5 and 7 weeks, the average paw placement score for the injured limb was 5.3 ± 0.1 out of a maximum possible score of 6 in the CES animals while the average score for CCL animals was 4.9 ± 0.3 ($p=0.218$). CES animals had a higher foot placement score compared to sham-ES (4.5 ± 0.3; $p<0.05$) and unconditioned animals (3.8 ± 0.2; $p<0.001$). At 7.5 weeks post-nerve repair, a significantly greater foot placement score was observed in CES animals (5.5 ± 0.1) as compared to CCL (5.2 ± 0.1; $p<0.05$), sham-ES (4.08 ± 0.1; $p<0.001$) or unconditioned controls (4.0 ± 0.1; $p<0.001$) (Figure 3.3E). At 8 weeks post-nerve repair, CES animals again performed superiorly (5.4 ± 0.1) to the CCL (4.7 ± 0.1; $p<0.05$), sham-ES (4.2 ± 0.1; $p<0.001$) or unconditioned (3.5 ± 0.4; $p<0.01$) cohorts. Two-way ANOVA of weeks 7 and 8 regression analysis revealed a significant interaction between time and treatment allocations ($p<0.05$, Figure 3.3D). Post-hoc analysis revealed that animals in the CES group performed significantly better than those in the other groups. These data indicate that at 8 weeks, the CES animals have improved foot placement scores compared to CCL and the two control cohorts.

As a final measure of functional improvement, nerve conduction studies were performed at 8 weeks post-nerve repair. Animals that had previously been conditioned with electrical stimulation had greater CMAP amplitudes compared to those that did not receive conditioning or CCL (Figure 3.3F). The average CMAP amplitude for the injured limb of

the CES animals was 45.7 ± 12.7% that of the uninjured leg. This was significantly higher than that recorded from the CCL group (12.9 ± 4.0 %; $p<0.05$), sham-ES (4.7 ± 0.9 %; $p<0.05$) or unconditioned control (6.0 ± 1.2 %; $p<0.05$) cohorts.

Functional studies were confirmed by tissue analysis. Gastrocnemius muscles were harvested from both the injured and contralateral limbs (Figure 3.4A-D) in all experimental groups and examined for evidence of reinnervation indirectly by muscle mass (4E) and directly by assessment of reinnervated NMJs (4F-J). Animals conditioned with CES had restored significantly more muscle mass in the gastrocnemius muscle compared to all other cohorts (Figure 3.4E). The average percentage of muscle mass (normalized to contralateral limb) in the CES cohort was 64.4 ± 4.3%, which was significantly greater than the CCL (54.2 ± 1.9%, $p<0.05$), sham-ES (49.4 ± 2.0%, $p<0.01$), or unconditioned control groups (41.2 ± 3.6%, $p<0.01$). Increased muscle mass of CES following nerve transection and repair suggests reinnervation, which rescues muscle atrophy. Reinnervation of the gastrocnemius muscle was confirmed by quantifying reinnervated NMJs (Figure 3.4F-J). Gastrocnemius muscle (50 mm^2 per tissue section) from each animal was stained with α-bungarotoxin to label the acetylcholine receptors, and NF200 to identify the innervating motor axons (together representing innervated NMJs as shown in Figure 3.4G'). Significantly more innervated NMJs were identified in CES (19.1± 0.2) compared to CCL (13.4 ± 1.3, $p<0.05$), sham-ES (4.5 ± 0.4, $p<0.001$) or unconditioned animals (4.0 ± 0.1, $p<0.001$) (Figure 3.4J).

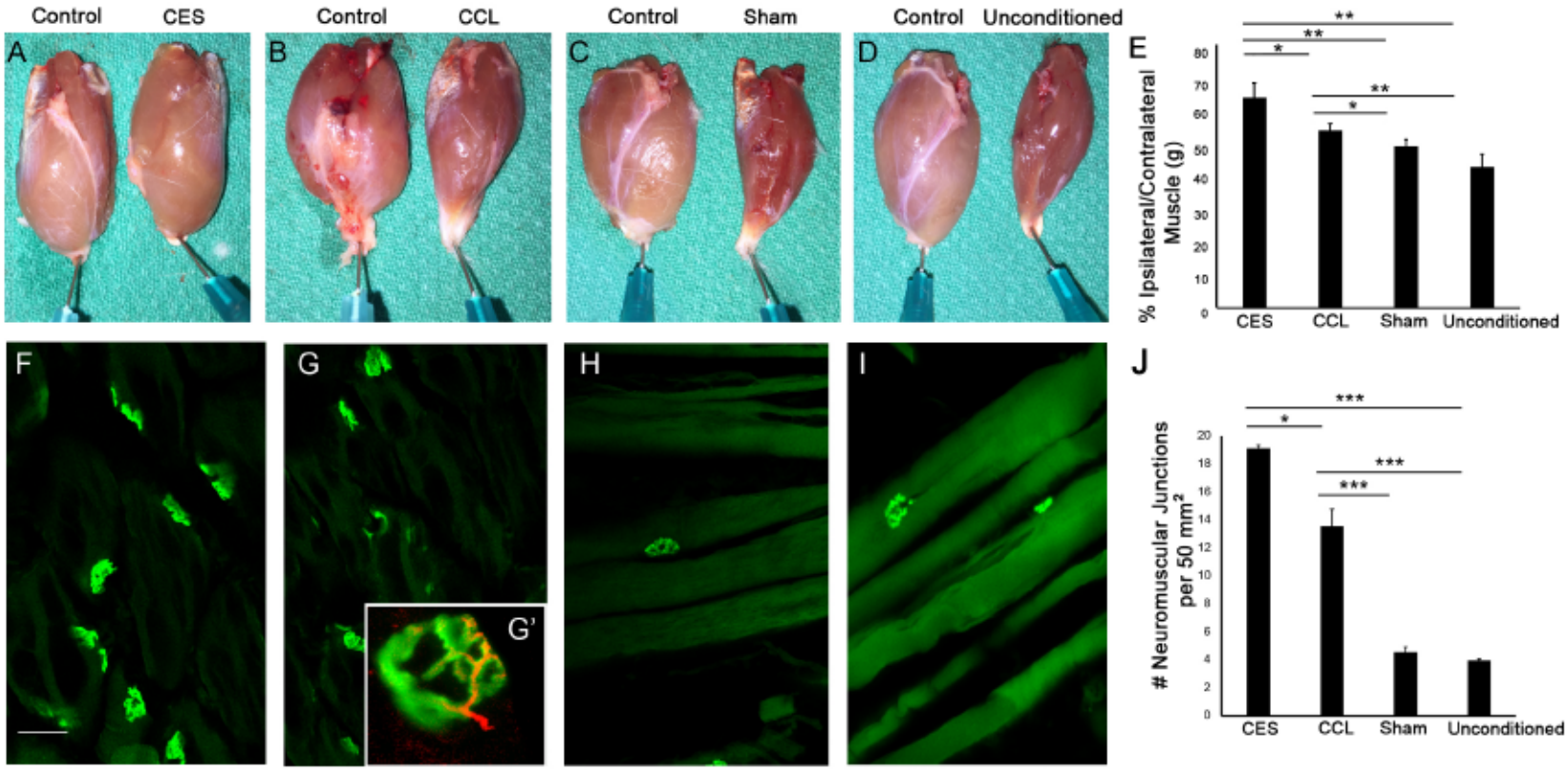

Control
CES
Control
CCL
Control
Sham
Control
Unconditioned
A
B
C
D
E
% Ipsilateral/Contralateral Muscle (g)
CES
CCL
Sham
Unconditioned
F
G
G'
H
I
J
Neuromuscular Junctions per 50 mm²
CES
CCL
Sham
Unconditioned

Figure 3.4: CES promotes muscle reinnervation to a greater extent than CCL

(A-D) Eight weeks post-nerve transection and repair, the gastrocnemius muscles from both the injured and uninjured limbs were weighed. (E) When normalized to their contralateral controls, it was determined that the weight of the ipsilateral muscle from the CES nerves (A) had significant muscle mass recovery compared to CCL (B, *$p<0.05$), sham-ES (C, **$p<0.01$) and unconditioned (D, **$p<0.01$) animals (n=10 animals/cohort). (F-I) Alpha-bungarotoxin immunocytochemistry of the ipsilateral gastrocnemius muscles were processed to identify the acetylcholine receptors at the NMJs. (J) Quantification revealed CES muscle fibers (F) had significantly more acetylcholine receptors than CCL (G, *$p<0.05$), sham-ES (H, **$p<0.001$) and unconditioned (I, ***$p<0.001$) muscles. All NMJs were confirmed to be innervated by NF200 (red, represented in G'). Scale bar in F is 20 µm.

3.5.4 Tibial nerve CES and CCL similarly upregulate expression of regeneration-associated genes (RAGs)

As CES consistently demonstrated superior behavioral and functional outcomes at 2 months of regeneration compared to CCL, we hypothesized the two conditioning paradigms may have different effects on the regenerative potential of sensory neurons. One explanation for the improved regeneration of CES beyond that of CCL is an increased expression of RAGs in the CES DRGs, measured by immunohistochemistry and Western blot analysis. At three days post-conditioning, DRGs were harvested to assess the neuronal cell body response, specifically the upregulation of GAP-43, BDNF and pCREB as well as activation of GFAP in their surrounding satellite glial cells.

Immunofluorescent labeling revealed CES and CCL similarly increased GAP-43 expression compared to controls as illustrated in the representative DRG sections (Figure 3.5A). CES and CCL had a significantly increased percentage of DRG neurons expressing moderate and high levels of GAP-43 compared to controls (Figure 3.5B). GAP-43 protein expression was observed in 86.8 ± 0.4% of DRG neurons conditioned with CES, and 86.0 ± 1.5% of DRG neurons conditioned with CCL; among these positive neurons, 75.9 ± 1.5% and 79.8 ± 1.7% of DRG neurons had low to moderate immunofluorescence and 10.9 ± 1.8% and 6.2 ± 2.4% of DRG neurons had high levels, respectively. This was significantly higher than the sham-ES or unconditioned control cohorts, in which GAP-43 was detected in only 13.1 ± 2.7% and 13.7 ± 1.7% of DRG neurons, respectively, with only 0.08 ± 0.08% and 0.04 ± 0.03% of these neurons showing high levels of GAP-43 immunofluorescence ($p<0.001$ for all).

Western blot analysis confirmed similarly increased expression of GAP-43 protein in CES and CCL compared to negative controls ($p<0.05$) (Figure 3.5C).

BDNF protein expression was similarly increased in the corresponding DRGs whose tibial nerves were subjected to CES and CCL. Representative DRG sections with corresponding analysis of neuronal BDNF immunofluorescence signal demonstrated that the percentage of DRG neurons expressing BDNF compared to sham-ES and unconditioned control cohorts (Figure 3.5D-E). CES and CCL upregulated BDNF expression such that 78.9 ± 3.1% and 61.8 ± 5.3% of DRG neurons had detectable levels of expression as compared to the relatively low percentages of BDNF positive neurons in sham-ES (9.2 ± 2.9%) or unconditioned (19.1 ± 5.8%) animals. Among positive neurons, CES and CCL had 'low to moderate' BDNF immunofluorescence in 60.5 ± 1.4% and 52.4 ± 3.5% of neurons respectively, greater than sham-ES (9.0 ± 2.8%) and unconditioned animals (19.1 ± 5.8%) ($p<0.001$ for both). Interestingly, a significantly greater proportion of CES DRG neuronal cell bodies were 'highly' positive for BDNF (18.4 ± 2.3%) compared to those DRGs conditioned with CCL (9.4 ± 2.1%; $p<0.05$). Both CES and CCL had a greater percentage of highly positive BDNF DRG neurons compared to the sham-ES and unconditioned animals (0.20 ± 0.1% and 0.04 ± 0.1% respectively). Western blot analysis confirmed increased expression of BDNF in CES and CCL ($p<0.05$) compared to controls (Figure 3.5F).

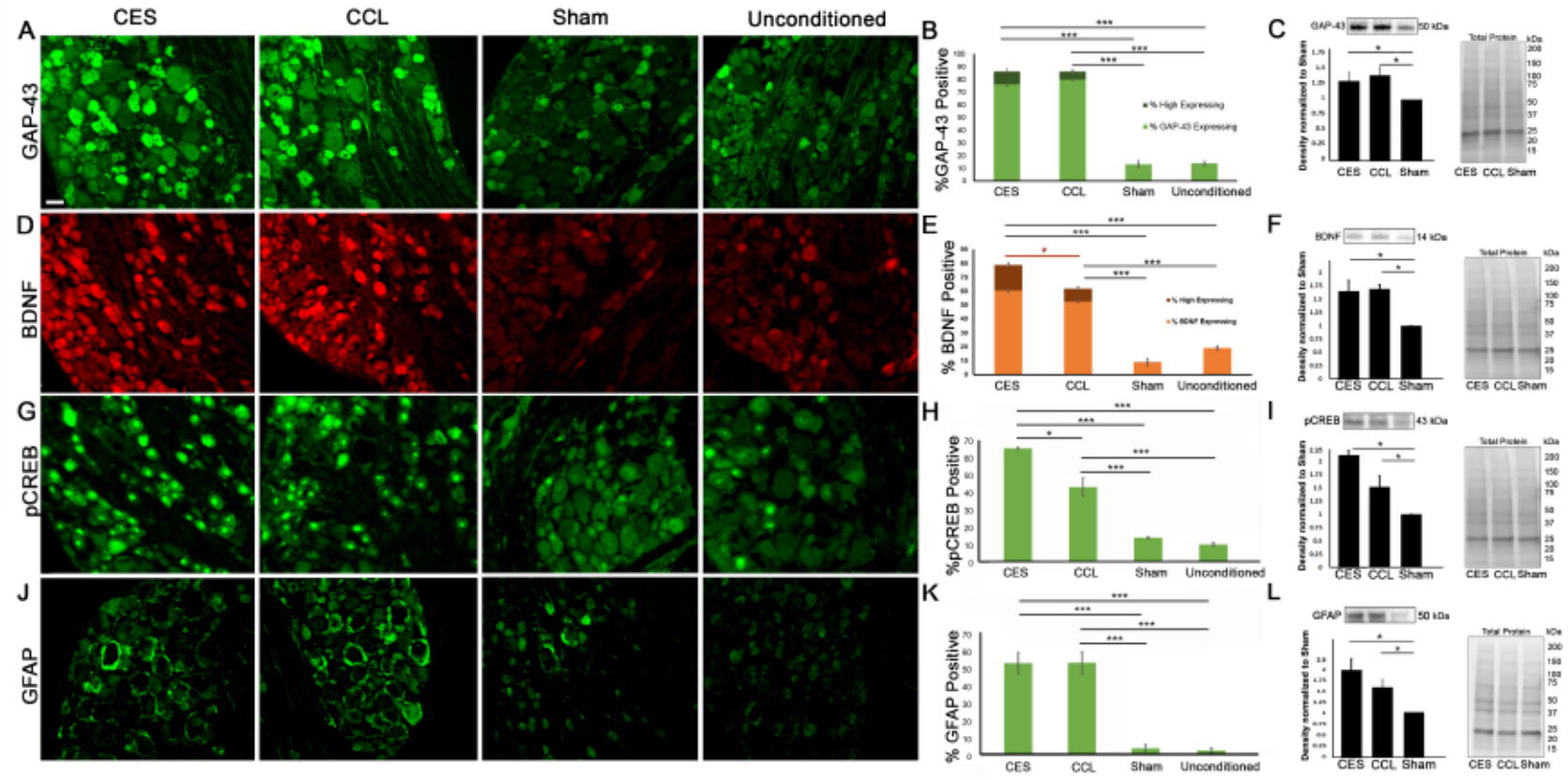

CES
CCL
Sham
Unconditioned
A
GAP-43
B
%GAP-43 Positive
% High Expressing
% GAP-43 Expressing
C
GAP-43
50 kDa
Total Protein
Density normalized to Sham
CES CCL Sham
D
BDNF
E
% BDNF Positive
F
BDNF
14 kDa
G
pCREB
H
%pCREB Positive
I
pCREB
43 kDa
J
GFAP
K
% GFAP Positive
L
GFAP
50 kDa

Figure 3.5: CES and CCL upregulate similar levels of RAG expression

Representative photomicrographs of L5 DRG sections processed for immunofluorescence to detect GAP-43 (A) and BDNF (D), pCREB (G) and GFAP (J) expression three days following CES, CCL, sham-ES, or no conditioning. Elevated neuronal GAP-43 (A) and BDNF (D) expression is apparent in all sizes of DRG neurons from the two conditioned groups. Bar graph (B) depicts the mean percentage of neurons with low to moderate (light green) and high (dark green) levels of GAP-43 immunofluorescence per cohort; bar graph (E) depicts the mean percentage of neurons with low to moderate (light orange) and high (dark orange) levels of BDNF immunofluorescence per cohort. Statistical analysis compares the incidence of GAP-43 and BDNF expressing neurons in each cohort (***$p<0.001$). (G) CES significantly increased the level of pCREB in DRG neurons compared to CCL, sham-ES, and no conditioning. (H) Bar graphs demonstrates the average pCREB immunofluorescence of 5 animals per experimental group. Statistical analysis compares the percentage of pCREB DRG neurons in each cohort (*$p<0.05$, ***$p<0.001$). (J) Elevated GFAP expression is apparent in SGCs from the two conditioned groups. (K) Bar graph reports the average percentage of DRG neurons surrounded by GFAP-expressing SGC cells from 5 animals per experimental group. Statistical analysis compares the percentage of DRG cell bodies surrounded by GFAP-expressing SGCs in each cohort (***$p<0.001$). Western blot analysis confirmed significant upregulation of (C) GAP-43, (F) BDNF, (I) pCREB, and (L) GFAP protein in CES and CCL cohorts when normalized to the amount of total protein in each lane (*$p<0.05$ for all). Scale bar represents 50 μm.

Increased expression of phosphorylated CREB (pCREB) was also observed among the conditioned cohorts (Figure 3.5G-H). The percentage of CES DRG neurons with pCREB positively stained nuclei was 64.8 ± 0.7%, which was significantly higher than in the CCL group which had positive pCREB nuclear staining in only 42.8 ± 5.3% ($p<0.05$). By contrast, sham-ES and unconditioned control animals had positive nuclear staining in 13.8 ± 0.7% and 9.7 ± 1.1% of cells, respectively, significantly less than CES ($p<0.001$) or CCL ($p<0.001$) (Figure 3.5H). Western blot analysis confirmed both CES and CCL had significantly higher pCREB levels compared to sham-ES animals ($p<0.05$ for both, Figure 3.5I).

To investigate the response of the perineuronal SGCs to conditioning, GFAP upregulation was evaluated (Figure 3.5J-K). CES yielded nearly identical upregulation of perineuronal SGC GFAP expression (52.8 ± 6.1%) compared to the traditional CCL (52.9 ± 6.4%) and were significantly higher than in the sham-ES (3.4 ± 2.1%) or unconditioned (2.1 ± 1.4%) control animals ($p<0.001$ for both) (Figure 3.5K). Western blot analysis confirmed both CES and CCL had significantly higher GFAP levels compared to sham-ES animals ($p<0.05$ for both, Figure 3.5L).

Together, these results suggest there is a comparable rise in the expression of RAGs in CES and CCL animals, suggesting similar conditioning paradigms were instigated. These results, however, do not explain the differences in sensory and motor outcomes between CES and CCL animals.

3.5.5 Regeneration in CCL is arrested by the conditioning site

In a further effort to determine the mechanism underlying improved functional recovery in the CES animals despite similar length of regeneration at one week and comparable expression of RAGs, we hypothesized that the difference was attributable to the injurious nature of a crush lesion. In our model, the crush lesion was delivered distal to the future site of cut and coaptation, therefore the regenerating axons had to traverse two injury sites: the site of transection, and the site of the CCL. By contrast, CES does not overtly damage the nerve. We therefore theorized that improved functional and behavioral outcomes of the CES animals may be because regenerating axons need only cross a single injury site. To determine the discrepancy in reinnervation outcomes between CES, CCL and unconditioned controls, the length of axonal extension was measured after 5, 14, and 21 days of regeneration (Figure 3.6). In keeping with our seven-day data (Figure 3.1), five days CES and CCL had similar rates of regeneration (4.1 ± 0.4 mm and 3.9 ± 1.5 mm, respectively) which were significantly more than the sham-stimulation negative control (1.6 + 0.1 mm; $p<0.05$). However, at 14 days post-coaptation, the relationship between CES and CCL changed; CES axons extended significantly longer (19.1 ± 1.7 mm) than CCL (8.8 ± 1.0 mm; $p<0.01$) or sham-ES (10.5 ± 1.0 mm; $p<0.01$). At this timepoint, CCL-conditioned axons were arrested at the site of crush conditioning (10 mm distal to the cut/coaptation site). By 21 days post-coaptation, the length of regeneration of CCL again extended significantly longer (17.8 ± 1.0 mm) than sham-ES (12.8 ± 0.1 mm; $p<0.001$); however, they remained significantly shorter than CES axons (22.2 ± 1.0 mm; $p<0.05$). Notably, the CES axons extended beyond the distal tip of the 2.2 cm of harvested tibial nerve (Figure 3.6).

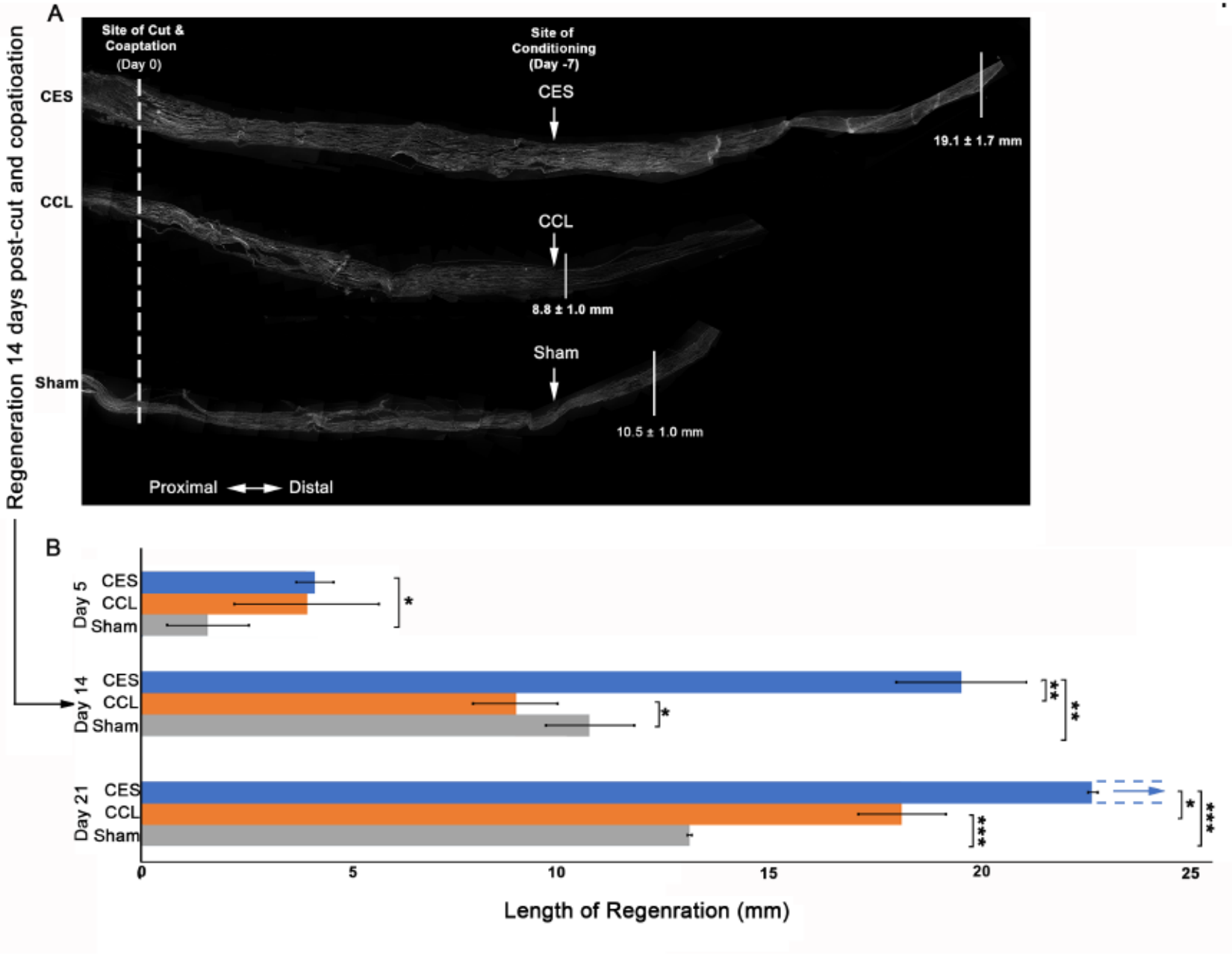

A
Site of Cut & Coaptation (Day 0)
Site of Conditioning (Day -7)
CES
CCL
Sham
19.1 ± 1.7 mm
8.8 ± 1.0 mm
10.5 ± 1.0 mm
Proximal
Distal
Regeneration 14 days post-cut and copatioation
B
Day 5
Day 14
Day 21
0
5
10
15
20
25
Length of Regenration (mm)

Figure 3.6: Traumatic conditioning (CCL) delays regeneration

Representative photomicrographs of NF200-labelled longitudinal tibial nerve sections demonstrate the length of nerve regeneration at 7, 14, and 21 days post-coaptation in CES, CCL, and sham-ES animals. The white dotted line demarcates the site of cut & surgical coaptation, and the solid white lines delineate the distal-most point of axon regeneration per conditioning. Arrows indicate the site of conditioning (day -7) located 10 mm distal to site of surgical coaptation (day 0). At day 5, CES and CCL have similar lengths regeneration, both significantly longer than sham-stimulation (*$p<0.05$). At day 14, axonal extension in CCL is arrested at the site of crush conditioning, whereas CES axons that do not have a second injury site to surpass continue to extend significantly longer than CCL (**$p<0.01$) or sham (*$p<0.05$). By day 21, CCL axons have passed the conditioning site and are regenerating faster than sham-ES (***$p<0.001$); however, these axons fail to catch up to CES axons which have regenerated the entire length of the 2.2 cm tibial length harvested. Thus CES regenerated farther than CCL (*$p<0.05$) and sham-ES (***$p<0.001$) at 21 days following microsurgical nerve repair.

3.6 Discussion

Despite numerous advances in surgical techniques for peripheral nerve surgery, patient outcomes have not improved in the past two decades; this is largely attributable to the slow innate rate of nerve regeneration. Improving patient outcomes requires identification of a method to accelerate this process. A large body of convincing evidence supports CCL as an effective means of enhancing nerve regeneration in animal models. However, this form of conditioning poses technical challenges that render it clinically infeasible. Over the past forty years, efforts have been made to identify a non-invasive method of delivering a conditioning-like effect. Unfortunately, all strategies attempted to date including vibration, freezing, ethidium bromide, and nerve compression, have failed to achieve the same magnitude of regeneration obtained by traditional CCL (Bondoux-Jahan & Sebille, 1986; L. B. Dahlin & Kanje, 1992; L.B. Dahlin & Thambert, 1993; Hollis et al., 2015).

Herein we identify CES as a novel form of nerve conditioning that, without mechanically injuring the nerve, exceeds regenerative outcomes observed with CCL. This is the first conditioning strategy that has achieved regeneration and reinnervation outcomes that supersede 'gold-standard' crush conditioning. More important, however, is the clinical feasibility of this technique. While electrical stimulation (ES) is previously well-described as a postoperative technique for promoting peripheral nerve regeneration (A. A. Al Majed et al., 2000a; A.A. Al Majed et al., 2000b; Geremia et al., 2007; Gordon et al., 2010; Gordon, Brushart, & Chan, 2008); its use as a preoperative conditioning modality *in vivo* is novel. As a postoperative intervention, the capacity of ES to enhance motor and sensory outcomes is limited by its underlying mechanism of action. PES only enhances regeneration across the

site of repair (staggered regeneration) after which the rate of regeneration returns to that of an unconditioned animal (Gordon et al., 2009). In contrast, traditional crush conditioning accelerates the rate of regeneration along the entire length of the distal stump by upregulating regeneration associated genes and expediting transportation of cytoskeletal elements actin and tubulin from the cell body to the growth cone. The first study investigating the use of ES as a conditioning lesion reported improved neurite extension comparable to CCL when outgrowth was measured *in vitro* (Udina et al., 2008). In a proof of principle study, we recently demonstrated that CES of the fibular nerve upregulates RAGs and, in response to a nerve transection and coaptation (test lesion), increases axonal elongation *in vivo* (Senger et al., 2017). Translation to a human clinical trial, however, requires a thorough investigation of the motor and sensory reinnervation outcomes to determine if the enhanced regeneration observed in the preliminary fibular nerve study results in improvements in functional outcomes.

To this end, in our current study, we demonstrated that not only does CES improve regeneration following tibial nerve transection and microsurgical repair, it also improves functional outcomes beyond that achievable with gold-standard CCL.

3.6.1 CES promotes nerve regeneration

Studies by Franz et al (2008) demonstrated that the effects of electrical stimulation are variable in different nerves (Franz, Rutishauser, & Rafuse, 2008); therefore, we sought to compare the effects of CES on the tibial nerve with our previous observations in the fibular nerve (8). Although the total length of regeneration was greater following CES of the tibial

(> 6.0 mm) compared to the fibular nerve (4.2 mm), so too was the innate regenerative capacity of their unconditioned controls (~2.7 mm, tibial; ~1.1 mm, fibular nerve) (Figure 3.1). Although the regenerative capacity of the tibial nerve seems to be greater than the fibular nerve, the net benefits of conditioning, when normalized to their controls was 3.8-fold in the fibular nerve compared to ~2.5 fold in the tibial nerve. Taken together, these data suggest that the benefits of conditioning may be greater for nerves with a lower innate regenerative capacity. Despite differences in the length of regeneration, the number of axons crossing the coaptation site in CES (~120 axons) for both tibial and fibular nerves was greater than that of controls (~50 axons). This observation suggests that the inherent differences between the nerves are more likely to be attributable to the speed of regeneration rather than the magnitude of axonal sprouting. This observation is of significant clinical importance, as it suggests that CES may be of particular importance for patients with injuries to nerves with a poor innate regenerative capacity, such as the ulnar nerve (He et al., 2014).

3.6.2 CES promotes sensory and motor reinnervation

This is the first report of a minimally invasive conditioning intervention that meaningfully enhances sensory and motor functional outcomes compared to traditional crush conditioning. Our results suggest CES significantly improves sensory reinnervation compared to both negative controls and CCL animals at 7-8 weeks (Figures 3.2). Animals conditioned with ES had increased sensitivity to mechanical force which was supported by significantly higher IENF density counts in footpad biopsy specimens compared to CCL animals. Similarly, CES improved motor reinnervation outcomes beyond CCL animals, as indicated by toe spread and foot placement analyses (Figure 3.3), muscle mass recovery of the gastrocnemius

muscle, and NMJ reinnervation (Figure 3.4). These behaviour tests were confirmed electrophysiologically, with nerve conduction studies revealing significantly greater CMAP amplitude in the CES cohort.

These considerable improvements in sensorimotor functional outcomes are seemingly discordant with regenerative outcomes at one week, which demonstrates similar lengths of nerve regeneration in the CES and CCL cohorts. Unlike the noninjurious CES, however, CCL regenerating axons must cross two injury sites, the test lesion and the distal conditioning lesion site, which delays reinnervation. Despite similarities in length of axonal extension at 5 and 7 days, when CCL axons reached the site of conditioning 10 mm distal to the site of nerve coaptation, at 14 days post-repair, the speed of axonal extension in the CCL cohort was delayed whereas CES axons continued to extend. This delay to accommodate staggered regeneration in the CCL animals was so significant, that sham-ES axons caught up (Figure 3.6). By 21 days of regeneration, axons in the CCL cohort surpassed the second (conditioning) site of injury, and extended beyond unconditioned nerves, but did not extend as far as the CES nerves. As such, axons in the CES cohort reached the distal end-targets first, allowing for earlier sensory and motor reinnervation. The similar pattern of RAG upregulation in both CES and CCL animals (in both tibial and fibular nerves) supports our findings of comparable growth rates (Figure 3.5) (Senger et al., 2017).

3.6.3 Potential mechanisms responsible for the conditioning effects

As CCL evokes a significant immune response, inflammation has been credited, at least in part, to activate the pathways responsible for RAG upregulation (Kwon et al., 2013; Kwon

et al., 2015; Sjoberg & Kanje, 1990). However, inflammation following CES is likely substantially less and thus its role in nerve regeneration in this paradigm is unclear. Typically, GAP-43 has been characterized as a 'nerve injury marker', GFAP as a 'glial cell injury marker', whereas BDNF and pCREB have been categorized as 'pro-regenerative markers' induced by nerve injury. Our findings suggest that nerve injury is not the only paradigm that regulates their expression. A potential mechanism for this that has gained increasing prominence is chromatin accessibility. In the CNS, this has been shown to play an important role in neuronal activity induced gene expression in dentate gyrus neurons (Su et al., 2017). Increased chromatin accessibility was also thought to result in enhanced regeneration of retinal neurons in young mice (Jorstad et al., 2017). Similarly, in the peripheral nervous system, chromatin regulators were found to be capable of altering the expression of a large set of RAGs following conditioning lesion (Loh et al., 2017). Together, these findings suggest that chromatin accessibility as a potent epigenetic mechanism is worthy of further exploration. Further studies investigating the common and divergent intracellular signaling cascades activated by CES and CCL, and potentially the resultant gene expression changes by unique chromatin regulation may elucidate their underlying mechanisms to promote nerve regeneration.

3.6.4 CES as a potential therapy

The importance of these findings lies in the direct translatability of CES to the bedside. Since postoperative ES has already been shown to be safe and well-tolerated by patients (Gordon et al., 2010; Wong et al., 2015), preoperative delivery of electrical stimulation (ie: CES) would be acceptable to clinicians and patients alike. There are at least three clinical

paradigms in which CES could be applied to improved patient outcome: 1) distal nerve transfers, 2) chronic nerve repair surgery 3) targeted muscle reinnervation for myoelectric prosthesis control.

In distal nerve transfers surgery, a "donor" nerve branch to a redundant muscle is transected and coapted to the distal end of a non-functional "recipient" nerve. The classic example is an Oberlin's transfer, in which the ulnar nerve branch to the flexor carpi ulnaris (FCU) muscle is transected and coapted to the distal stump of the musculocutaneous nerve branch to biceps brachii, in order to restore elbow flexion. Because flexor carpi radialis alone can flex the wrist, FCU is a 'redundant' muscle and loss of innervation has no functional effects. Similar transfers have been described to treat numerous nerve injuries in the upper and lower extremities. Nerve transfer surgery is elective, therefore the time of the transection of the donor nerve is scheduled; as such, patients could undergo percutaneous CES to the donor nerve in clinic one week prior to surgery, priming it for regeneration to enhance reinnervation of the target muscle.

Peripheral nerve injury is particularly common following major polytrauma necessitating emergent management of life or limb threatening injuries. In these situations, nerve injuries are often overlooked on initial exam, resulting in delays in repair beyond the suggested time-frame of three to six months post-injury (Jonsson et al., 2013). CES may be used as a tool to restore the regenerative capacity in these chronically denervated nerves. Furthermore, since postoperative ES is known to have different effects from CES, in that it reduces the delay caused by staggered regeneration without changing the speed of axonal outgrowth (Brushart

et al., 2002), an interesting future option is to examine whether there is a synergistic effect when combining the two treatment modalities.

Targeted muscle reinnervation is a novel technique for improving the motor function of amputees. This surgery includes the transfer of viable nerves of an amputated limb into specifically selected muscles allowing EMG-induced signals to provide voluntary 'spontaneous' movements of a prosthesis. CES prior to nerve transfer may enhance NMJ reinnervation, and therefore the overall function of the prosthetic device.

CES is a noninjurious, clinically feasible method of enhancing nerve regeneration and sensorimotor functional recovery. This study presents comprehensive evidence that CES upregulates RAGs and enhances axonal growth similar to a CCL. We further reveal CES induces sensory and motor reinnervation and behavioral outcomes that supersede those obtainable by gold-standard conditioning methods. The importance of these findings, however, lies in their direct clinical applicability to improve outcomes in numerous peripheral nerve surgical challenges, as electrical stimulation is already established as safe and well tolerated. The use of CES in these clinical situations merits thorough investigation with randomized control trials, as it will likely significantly improve patient outcomes.

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Chapter 4

Conditioning Electrical Stimulation is Superior to Postoperative Electrical Stimulation, Resulting in Enhanced Nerve Regeneration and Functional Recovery

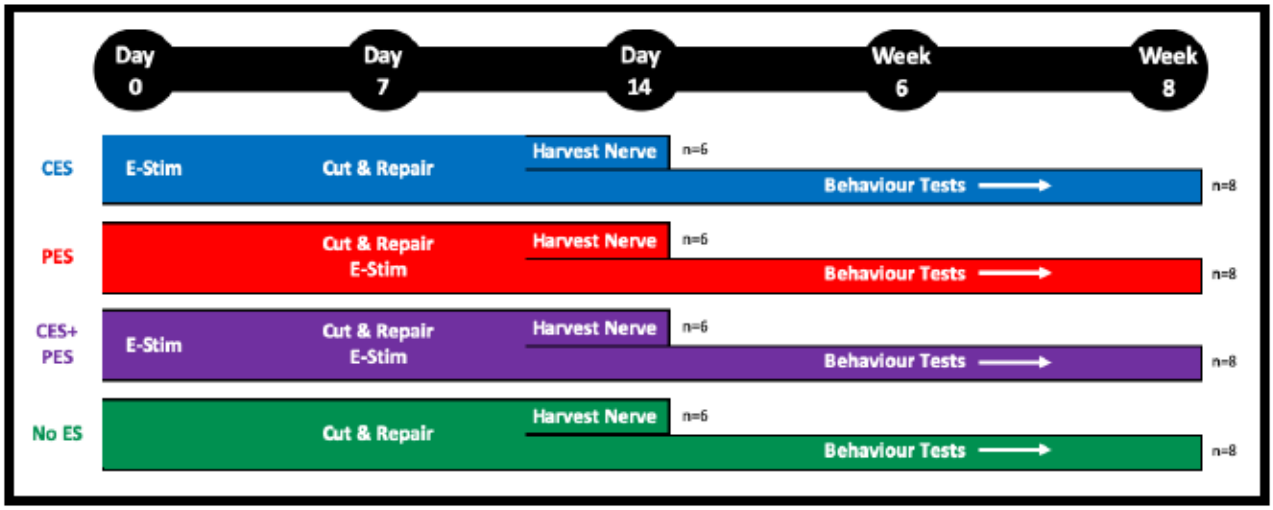
4.1 Preface

In Chapters 2 and 3, conditioning electrical stimulation (CES) is introduced as an effective technique for improving regeneration and reinnervation outcomes following peripheral nerve injury. Electrical stimulation is, however, already used in a clinical setting. Postoperative electrical stimulation (PES) is a common adjuvant therapy to surgical nerve repair in many centers. Both animal data and human clinical trials suggest PES is safe and well-tolerated by patients; therefore, it is likely CES will be well accepted by patients as a preoperative clinical tool. A direct comparison of outcomes is therefore of significant clinical importance to guide patient management.

Though both CES and PES use electrical stimulation at the same frequency, and duration, the effects of preinjury/conditioning electrical stimulation differ significantly from postoperative. Unlike a conditioning lesion, PES does not accelerate the intrinsic rate of axon extension, but rather enhances staggered regeneration at the site of surgical coaptation. A direct comparison of regeneration and reinnervation outcomes following CES and PES is of significant clinical importance to guide patient management. Furthermore, given their complementary mechanism of action, we sought to determine whether a synergistic effect could be obtained by combining these two techniques.

The following timeline depicts the experimental methods used to compare outcomes of these two clinically feasible techniques and to determine whether a synergistic effect can be obtained by combining CES and PES. Animals received electrical stimulation either a) 7 days prior to tibial nerve transection and repair (CES, blue), b) immediately following

transection and repair (PES, red), or c) both 7 days prior to, and immediately following transection and repair (CES + PES, purple); a negative control group received no electrical stimulation (green). Nerves were harvested after seven days of regeneration in one cohort of animals, and behaviour testing for sensorimotor recovery was performed between 6-8 weeks of regeneration in a second set of animals.



Conditioning Electrical Stimulation is Superior to Postoperative Electrical Stimulation, Resulting in Enhanced Nerve Regeneration and Functional Recovery

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4.2 Abstract

Background: Postoperative electrical stimulation (PES) improves nerve regeneration by decreasing staggered regeneration at the coaptation site. By contrast, conditioning (preoperative) electrical stimulation (CES) accelerates axon extension. Given that both techniques can be delivered at the bedside, a direct comparison of outcomes is of significant clinical importance. In this study, we compared regeneration and reinnervation outcomes of CES, PES, a combination of CES and PES, and a no-stimulation control.

Methods: Sprague Dawley rats were randomly divided into: i) CES, ii) PES, iii) CES + PES, and iv) no stimulation. CES was delivered one week prior to nerve cut/coaptation, and PES was delivered immediately following nerve repair. Length of nerve regeneration was assessed at 7 days post-coaptation (n=6/cohort), and behavioral testing was performed biweekly between 6-8 weeks post-coaptation (n=8/cohort).

Results: Animals treated with CES had significantly longer axon extension and improved sensorimotor recovery compared to all other cohorts. CES treated axons extended 8.5 ± 0.6 mm, significantly longer than PES (5.5 ± 0.5 mm), CES + PES (3.6 ± 0.7 mm), or no stimulation (2.7 ± 0.5 mm) ($p<0.001$). Sensory recovery (von Frey filament testing, intraepidermal nerve fiber reinnervation) ($p<0.001$) and motor reinnervation (horizontal ladder, gait analysis, nerve conduction studies, neuromuscular junction analysis) ($p<0.05$ - $p<0.001$) were significantly improved in CES animals.

Conclusion: CES significantly improves regeneration and reinnervation beyond the current clinical paradigm of PES. The combination of CES and PES does not have a synergistic effect. CES alone therefore may be a more promising treatment to improve outcomes in patients undergoing nerve repair surgeries.

4.3 Introduction

Outcomes following peripheral nerve injury are directly related to the time required for regenerating motor and sensory axons to reach their target endplates. Prolonged denervation impedes full functional recovery (Fu & Gordon, 1995a, 1995b); therefore, sensorimotor reinnervation outcomes could be improved by accelerating the intrinsic rate of nerve regeneration. A highly promising technique to this end is the conditioning lesion. Traditionally, this refers to a crush injury delivered to a nerve one week prior to a transection and repair 'test' lesion. It has been shown that a crush lesion primes the nerve such that at the time of the second 'test' lesion, the latency period is shortened and axon growth is accelerated by up to five times faster than their intrinsic rate of growth (Richardson & Verge, 1987; Senger, Verge, Chan, & Webber, 2018). Unfortunately, the injurious nature of a crush conditioning lesion precludes translation to a clinical setting. We have recently showed that one hour of conditioning electrical stimulation (CES) delivered to a nerve seven days prior to injury and repair induces a conditioning effect comparable to a crush, but in a non-injurious, thus clinically feasible manner (Senger et al., 2019; Senger, Chan, Verge, & Webber, 2017).

While the use of electrical stimulation prior to injury for conditioning is clinically novel, postoperative electrical stimulation (PES) has been well described to improve outcomes in animal models and through human clinical trials. PES is the only adjunct to surgical nerve repair that has been successfully translated to the clinic (Barber et al., 2018; Gordon, Amirjani, Edwards, & Chan, 2010; Wong, Olson, Morhart, & Chan, 2015). Unlike a conditioning lesion, however, PES does not accelerate the rate of axon extension, but rather

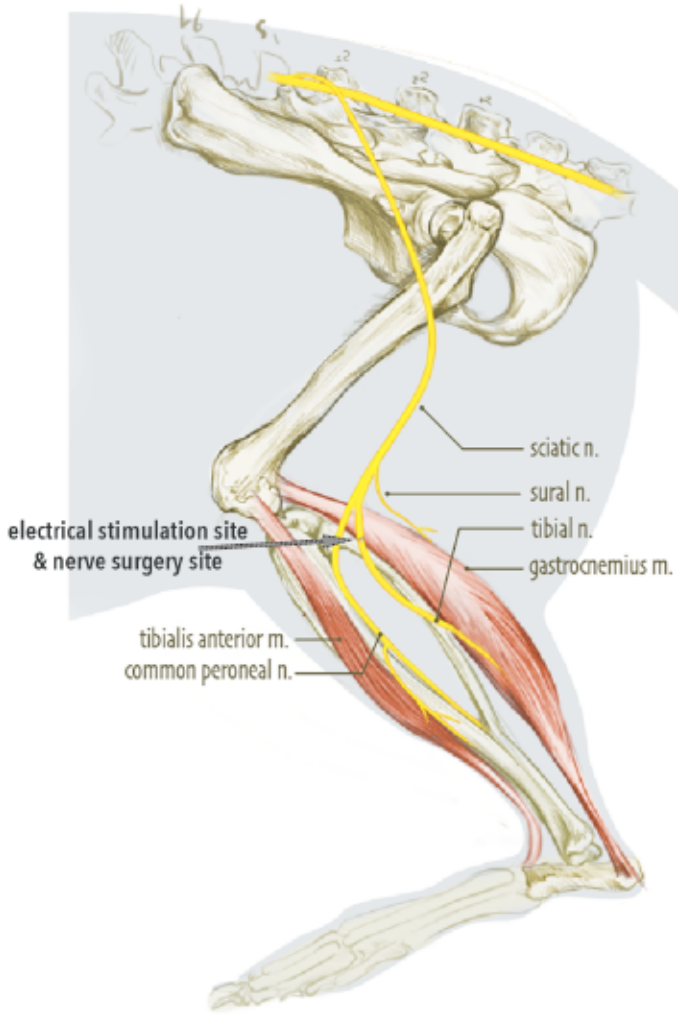
enhances regeneration of axons as they cross the site of surgical coaptation, a process called "staggered regeneration" (Brushart et al., 2002). Given the differences in their mode of action, and since both PES and CES can be delivered at the bedside, a direct comparison of outcomes is of significant clinical importance. We therefore compared length of axon extension and sensorimotor behavioral outcomes in transected nerves treated with CES or PES. Furthermore, given their complementary mechanisms of action, we sought to determine whether the combination of CES and PES would have a synergistic effect.

4.4 Methods

4.4.1 Animals: The reporting of our animal work meets the ARRIVE guidelines (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010). Adult male Sprague Dawley rats (n=122, Charles River laboratory) weighing 200 grams were placed under the care of Health Sciences Laboratory Animal Services (HSLAS) at the University of Alberta. The animals were randomly placed into the following cohorts: 24 animals for nerve regeneration analysis (n=6/cohort) and 32 animals for behavioral testing (n=8/cohort). All surgical procedures were approved by the University of Alberta Animal Research Ethics Board.

Within each cohort, animals were randomly divided into four groups based on the intervention delivered to the tibial nerve: i) CES seven days prior to nerve transection and repair, ii) nerve transection and repair followed immediately by PES, iii) CES seven days prior to nerve transection and repair followed immediately by PES, and iv) nerve transection and repair alone (Figure 4.1). Prior to all surgical interventions, animals were anesthetized

A


sciatic n.
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B

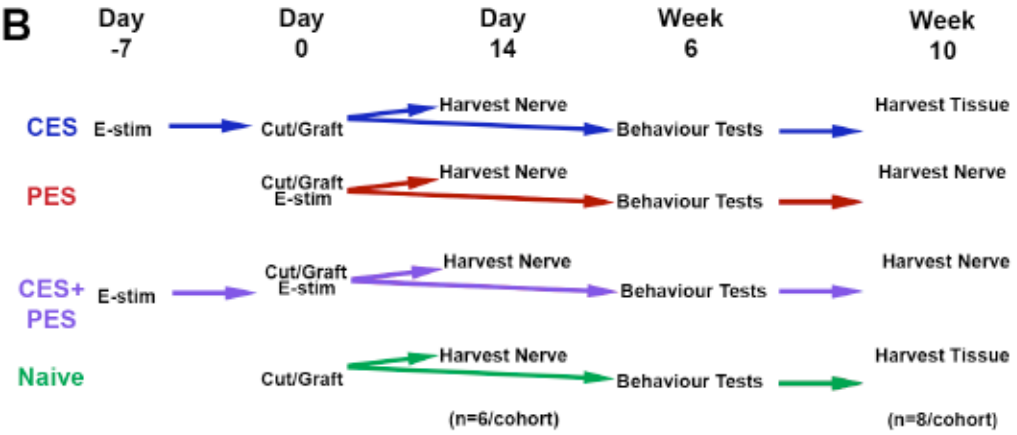
Day -7
Day 0
Day 14
Week 6
Week 10
CES
E-stim
Cut/Graft
Harvest Nerve
Behaviour Tests
Harvest Tissue
PES
Cut/Graft
E-stim
Harvest Nerve
Behaviour Tests
Harvest Nerve
CES+ PES
E-stim
Cut/Graft
E-stim
Harvest Nerve
Behaviour Tests
Harvest Nerve
Naive
Cut/Graft
Harvest Nerve
Behaviour Tests
Harvest Tissue
(n=6/cohort)
(n=8/cohort)

Figure 4.1 Experimental design of surgical and treatment paradigms.

(A) Artistic illustration of the investigations to determine which combination of CES +/- PES promotes the greatest extent of nerve regeneration. Animals were divided into four cohorts: CES, PES, CES + PES, and no ES. CES was delivered to the tibial nerve 7 days prior to nerve transection and repair. PES was delivered to the tibial nerve proximal to the site of nerve injury and coaptation, immediately following repair. CES + PES received electrical stimulation at both time points and negative control animals received neither stimulation paradigm. (B) The following timeline depicts the experimental methods used to compare outcomes of these clinically feasible techniques. Animals received electrical stimulation either a) 7 days prior to tibial nerve transection and repair (CES, Day -7), b) immediately following transection and repair (PES, Day 0), or c) both 7 days prior to, and immediately following transection and repair (CES + PES). A negative control group received no electrical stimulation. Nerves were harvested after seven days of regeneration in one cohort of animals (n=6/cohort), and behaviour testing for sensorimotor recovery was performed between 6-8 weeks of regeneration in a second set of animals (n=8/cohort).

with inhaled isoflurane (2% titrated at 1-2 L/min for maintenance of a surgical anesthetic plane) and received 0.01 mg/Kg of subcutaneous buprenorphine. All surgical procedures were performed under 3.5x loupe magnification. At the completion of all procedures, the skin was closed with 4-0 Vicryl (Ethicon Inc, Somerville, NJ).

4.4.2 Conditioning electrical stimulation (CES): CES was performed as previously described (Senger et al., 2019; Senger et al., 2017). Briefly, a longitudinal incision was made over the lateral aspect of the right lower limb. The tibial nerve was identified in the posterior compartment of the leg as it emerged between the two heads of the gastrocnemius muscle. A stainless-steel wire with bared ends was wrapped around the nerve, and the return electrode was placed into the tibialis anterior muscle; these were connected to the cathode and anode ports of an SD-9 stimulator (Grass Instruments Co., Quincy, MA) respectively. Continuous electrical stimulation was delivered at 20 Hz and a 0.1 ms duration for one hour, with voltage titrated to maintain a visible foot twitch.

4.4.3 Nerve transection and microsurgical repair: An incision was made along the lateral femur, and the hamstring muscles were dissected to identify the sciatic nerve and isolate its tibial branch. One centimeter distal to the sciatic nerve trifurcation, the tibial nerve was sharply transected, and an epineurial repair was performed using 9-0 silk suture. The hamstring muscles were resuspended with 4-0 Vicryl suture and the skin was repaired with a two-layer closure (Figure 4.1A).

4.4.4 Postoperative electrical stimulation (PES): Immediately following nerve coaptation, a stainless-steel wire bared of insulation was placed immediately proximal to the site of repair (cathode) and a second into the quadriceps muscle (anode). Electrical stimulation was performed using the same parameters as the conditioning surgery (one hour of continuous stimulation at 20 Hz) (Al-Majed, Neumann, Brushart, & Gordon, 2000; Al-Majed, Tam, & Gordon, 2004; Chan, Curran, & Gordon, 2016; Geremia, Gordon, Brushart, Al-Majed, & Verge, 2007; Gordon, 2016)

4.4.5 Tissue Analysis: Animals were euthanized either seven days following nerve repair (nerve regeneration cohort) or upon completion of behavior testing at eight weeks post-nerve repair (functional outcome cohort). Euthanization was accomplished by carbon dioxide asphyxiation followed by exsanguination through a puncture of the left cardiac ventricle. The tibial nerves were harvested as previously described (Senger et al., 2019) and fixed in Zamboni's fixative (paraformaldehyde, picric acid, NaOH; American MasterTech Scientific, Lodi, CA) for four hours, rinsed with 0.01 M phosphate buffered saline (PBS) (ThermoFisher Scientific, Waltham, MA) five times, post-fixed in 30% sucrose solution overnight at 4°C, and frozen in Optimum Cutting Temperature (OCT) (Sakura Finetek, Torrance, CA) by indirect exposure to liquid nitrogen. Tissue chucks were cut into 12 μm sections, thaw-mounted on Superfrost Plus microscope slides, and stored at -80 °C until processing. Bilateral gastrocnemius muscles were harvested and weighed for comparative purposes; the weight of the injured muscle was reported as a percentage of the uninjured contralateral muscle to control for discrepancies in animal size. The muscle from the injured limb was fixed in Zamboni's solution overnight, rinsed five times in PBS, sunk in 30%

sucrose overnight, then flash-frozen in OCT using liquid nitrogen. Plantar footpads were collected from the injured limb eight week following nerve repair using a 3 mm biopsy punch (Acuderm Ic, Fort Lauderdale, FL) and fixed in 2% paraformaldehyde, lysine, periodate (PLP) fixative for 16-20 hours at 4°C. Tissue sections were rinsed five times in Sorenson's phosphate butter, cryoprotected overnight in 20% glycerol/0.1M Sorensen's phosphate buffer, frozen in OCT, and cut into 20 μm sections on Superfrost-Plus microscope slides.

4.4.6 Immunofluorescence: After slides were warmed to room temperature, antigen retrieval was performed for 20 minutes in 60°C citrate buffer (10 mM sodium citrate, 0.05% Tween-20, pH 6.0; Thermo Fisher Scientific, Waltham, MA). After slides cooled, they were washed three times in 0.01 M PBS for five minutes/wash, and permeabilized for 10 minutes with 0.1% Triton-100X (Thermo Fisher Scientific, Waltham, MA). Tissue sections were blocked for 90 minutes in 10% normal goat serum (MP Biomedicals, Santa Ana, CA) and 3% bovine serum albumin (BSA) (Sigma-Aldrich, St Louis, MO) in 0.01 M PBS. Primary antibodies diluted in 0.01 M PBS with 3% BSA were left on tissues overnight at 4°C. Nerve sections were stained with 1:500 mouse anti-neurofilament-200 (NF200) (Sigma-Aldrich, St Louis, MO). Muscle sections were first labelled with mouse anti-NF200 (1:500) to stain the innervating nerve, then with conjugated anti-α-bungarotoxin (1:1000, 20 mins) to stain the acetylcholine receptors. Footpads were stained with anti-protein gene product 9.5 (PGP9.5, 1:1000) for identification of intraepidermal nerve fibers.

On the second day, the slides were washed three times for 5 minutes each in 0.01 M PBS. Secondary antibodies were diluted in 0.01 M PBS with 3% BSA and applied for 90 minutes

4.4.7 Behavioral and Physiologic Outcomes: Sensory and motor behavior testing was performed at 6.5, 7, 7.5, and 8 weeks following nerve cut/coaptation. Analysis of all behavior testing was performed by the same examiner blinded to the group allocation of the animals. Sensory recovery was evaluated using von Frey filament testing, and motor recovery was assessed with gait analysis for toe spread, the horizontal ladder test for motor dexterity, and nerve conduction studies for compound muscle action potentials (CMAPs).

Von Frey filament testing evaluated reinnervation of the footpad. Animals were placed in a plexiglass cage with wire mesh flooring and allowed to acclimatize for thirty minutes. Von Frey monofilaments of progressively greater weight (1.4 – 15 g) were used to probe the animal's paw. Each filament was used to probe the paw for three seconds, five times per filament. Three consecutive paw withdrawals to an individual monofilament constituted a positive response. No response was recorded as 26 g.

Gait analysis to evaluate intrinsic foot muscle reinnervation was performed by placing the animal in a specially-designed walkway constructed from transparent plexiglass with an adjustable mirror underneath to visualize the plantar paw. Animals were filmed walking the length of the track three-times per session. The video was analyzed, and individual screenshots were taken of each of weight-bearing footstep when all toes were visible. Ten images of both the affected and contralateral control footpads were analyzed per testing session; the distance between the first and last toes was measured. The toe spread of the injured foot was reported as a percentage of the uninjured contralateral limb to control for differences in animal size.

The horizontal ladder test consisted of an elevated horizontal ladder composed of clear plexiglass with metal ladder rungs arranged in a different pattern each testing session to prevent learning. The animals were filmed over three attempts to cross the ladder at each testing session, and videos were analyzed on a frame-by-frame basis. Using a 0-6 scoring system (Metz & Whishaw, 2009), a score was given for each individual foot placement on the ladder rung, with a score of 0 being a complete slip, score 1 a deep slip, score 2 a slight slip, score 3 foot replacement, score 4 foot correction, score 5 partial placement, and score 6 correct placement. The average score of the injured limb was calculated for each attempt.

Nerve conduction studies were performed at 8 weeks post-nerve repair. Animals were anesthetized with inhaled isoflurane and the lower limbs were shaved for lead placement. The recording electrode was placed in the footpad, the reference electrode into the toe, supramaximal electrical stimulation was delivered at the popliteal fossa. Maximal CMAP with the sharpest rise time was identified by adjusting the recording lead. Results were reported as the CMAP percentage of the injured side compared to the uninjured contralateral control.

4.4.8 Statistical analysis: Results are presented as the mean ± standard error mean. Groups were compared using a one-way analysis of variance (ANOVA) to identify differences in the mean between groups, followed by Bonferroni post hoc analysis. Statistical significance was accepted with a level of $p<0.05$. All statistical analyses were performed using STATA 14 (StataCorp LP, Collagen Station, Texas).

4.5 Results

4.5.1 CES enhances the length and magnitude of axonal regeneration greater than PES ± CES

Length of regeneration one week following nerve cut and coaptation was evaluated in animals treated with CES, PES, the combination of CES + PES, and a no ES negative control (Figure 4.1B). To assess the extent of regeneration, repaired nerves were harvested, sectioned, and stained for NF200 immunofluorescence. Animals treated with CES had a significantly longer length of regeneration (8.5 ± 0.6 mm) compared to PES (5.5 ± 0.5 mm, $p<0.001$), CES + PES (3.6 ± 0.7 mm, $p<0.001$), or no ES negative controls (2.7 ± 0.5 mm, $p<0.001$) (Figure 4.2). Nerves treated with CES + PES ($p<0.01$) or PES alone ($p<0.05$) had a significantly longer length of regeneration as well as number of axons when compared to naïve controls. In addition to the increased extent of nerve growth, the CES animals had significantly more axons extending from the site of surgical repair (142.9 ± 9.6 axons) compared to all other cohorts (PES 117.5 ± 6.7 axons, CES + PES, 96.2 ± 7.5 axons, no ES 67.8 ± 5.2 axons). Furthermore, manual axon counts at each 250 μm interval distal to the repair site demonstrated that CES animals had significantly more axons than animals treated with PES, CES + PES, or a no ES controls ($p<0.01$ to $p<0.001$ for all intervals).

4.5.2 CES of the tibial nerve improves sensory innervation greater than PES ± CES

Thirty-two (n=8/cohort) animals were evaluated at 6.5, 7, 7.5, and 8 weeks following tibial nerve transection and repair to compare functional outcomes (Figure 4.1B). Sensory outcomes included von Frey filament testing and intraepidermal nerve fiber density (IENFD) counts. At 6.5 weeks post-nerve cut/coaptation, the average filament weight required to

elucidate three consecutive positive sensory responses was 7.4 ± 0.4 g in CES animals; this was significantly less than in animals treated with PES (14.4 ± 2.1 g; $p<0.01$), CES + PES (14.1 ± 2.3 g; $p<0.05$), or no ES (nonresponsive to all filaments tested; $p<0.001$) (Figure 4.3A). At 7 weeks CES animals required 6.6 ± 0.6 g of force, significantly less than PES (12.9 ± 1.0 g; $p<0.001$), CES + PES (10.9 ± 1.1 g; $p<0.01$), or the no ES cohorts (17.2 ± 2.0 g; $p<0.001$). Sensory recovery in the CES cohort remained improved at 7.5 and 8 weeks post-coaptation: CES animals (4.6 ± 0.4 g; 3.1 ± 0.4 g) responded to smaller monofilaments compared to PES (9.1 ± 0.6 g; 8.9 ± 0.4 g; $p<0.001$), CES + PES (8.3 ± 1.1 g; 8.6 ± 0.4 g; $p<0.001$) or no ES cohorts (17.2 ± 2.2 g; 13.0 ± 1.2 g; $p<0.001$). No difference between the PES and combination CES + PES cohorts was observed at any time point however PES was significantly higher than naïve at 6.5, 7.5 and 8 weeks ($p<0.01$), whereas CES+PES was significantly higher than naïve at all time points ($p<0.001$-$p<0.0001$) (Figure 4.3A).

Von Frey behavioral outcomes were corroborated by quantification of footpad reinnervation 8 weeks post-nerve transection and repair. IENFD was determined by counting the number of nerves crossing the dermal-epidermal junction. Significantly more nerves from the CES cohort (22.6 ± 1.0 nerves/μm) crossed the dermal-epidermal junction compared to those treated with PES (4.9 ± 0.3 nerves/μm; $p<0.001$), CES + PES (6.7 ± 0.9 nerves/μm; $p<0.001$) or the no ES control cohorts (3.5 ± 0.2 nerves/μm; $p<0.001$) (Figure 4.3B,C). The PES cohort was significantly higher than the naïve animals ($p<0.05$) but they were not significantly different from the CES+PES group of animals.

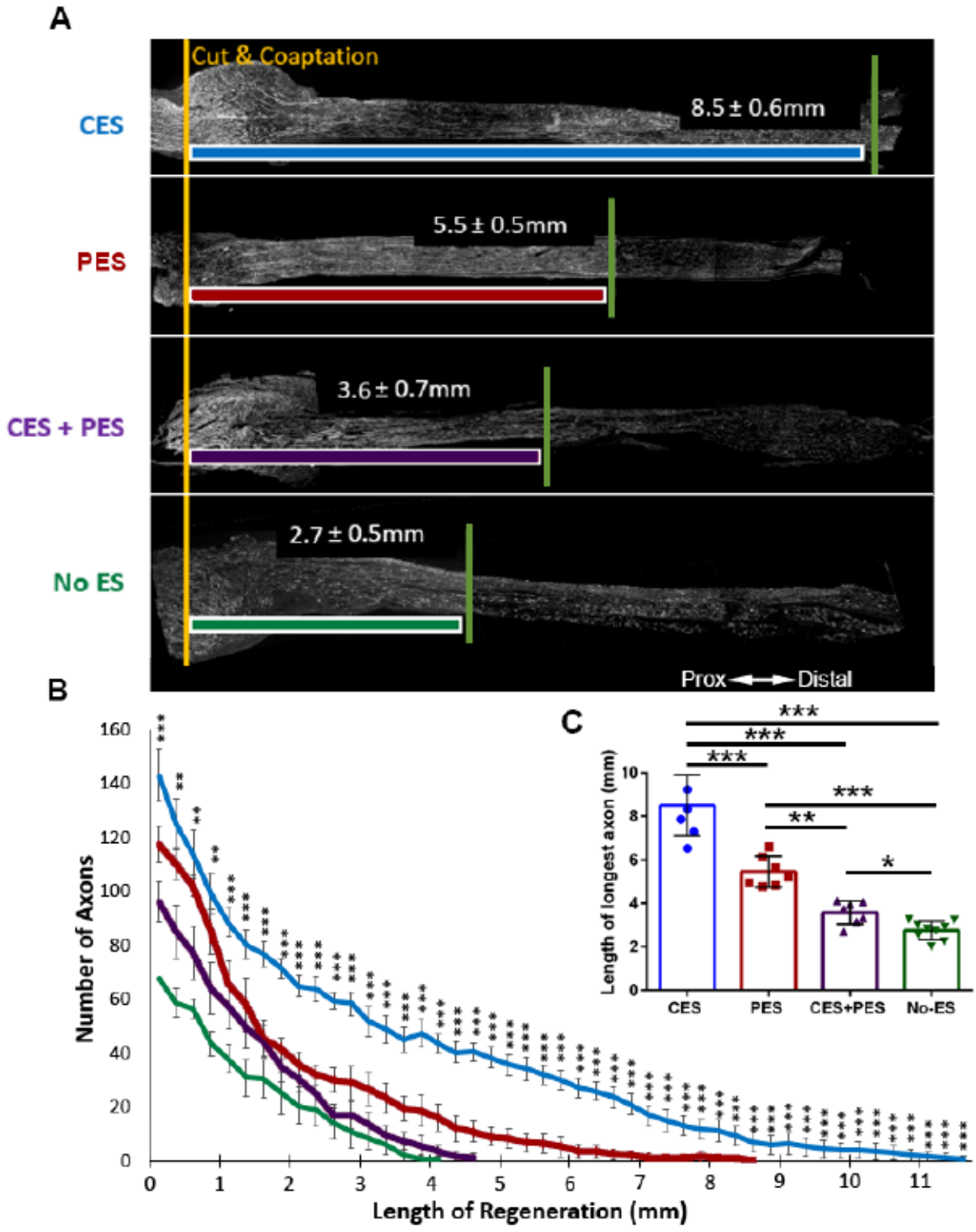
A
Cut & Coaptation
CES
8.5 ± 0.6mm
PES
5.5 ± 0.5mm
CES + PES
3.6 ± 0.7mm
No ES
2.7 ± 0.5mm
Prox
Distal
B
Number of Axons
Length of Regeneration (mm)
C
Length of longest axon (mm)
CES
PES
CES+PES
No-ES

Figure 4.2 CES promotes nerve regeneration greater than PES or CES + PES

A) Representative photomicrographs of NF-200 labeled longitudinal sections (12 μm thick) of the tibial nerve at 7 days of regeneration. Yellow line depicts the site of cut and coaptation, and green lines indicate the distal-most point of regeneration to which a minimum of 10 axons extended. Positive immunostaining beyond the green line depicts neurofilament undergoing Wallerian degeneration which is easily identified under the microscope. (B) Line graph depicts the average length of axonal regeneration on the x-axis and number of regenerating axons on the y-axis. Nerves treated with CES (blue) had a significantly longer length of axon extension and a greater number of axons present at every 250 μm intervals, when compared to nerves treated with PES (red), CES + PES (purple), and negative controls (green). (C) The average length of regeneration demonstrated that at one week, the CES cohort was superior to PES, CES+PES, and no-ES ($p<0.001$). ANOVA statistical analysis was performed followed by Bonferroni post-hoc analysis (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

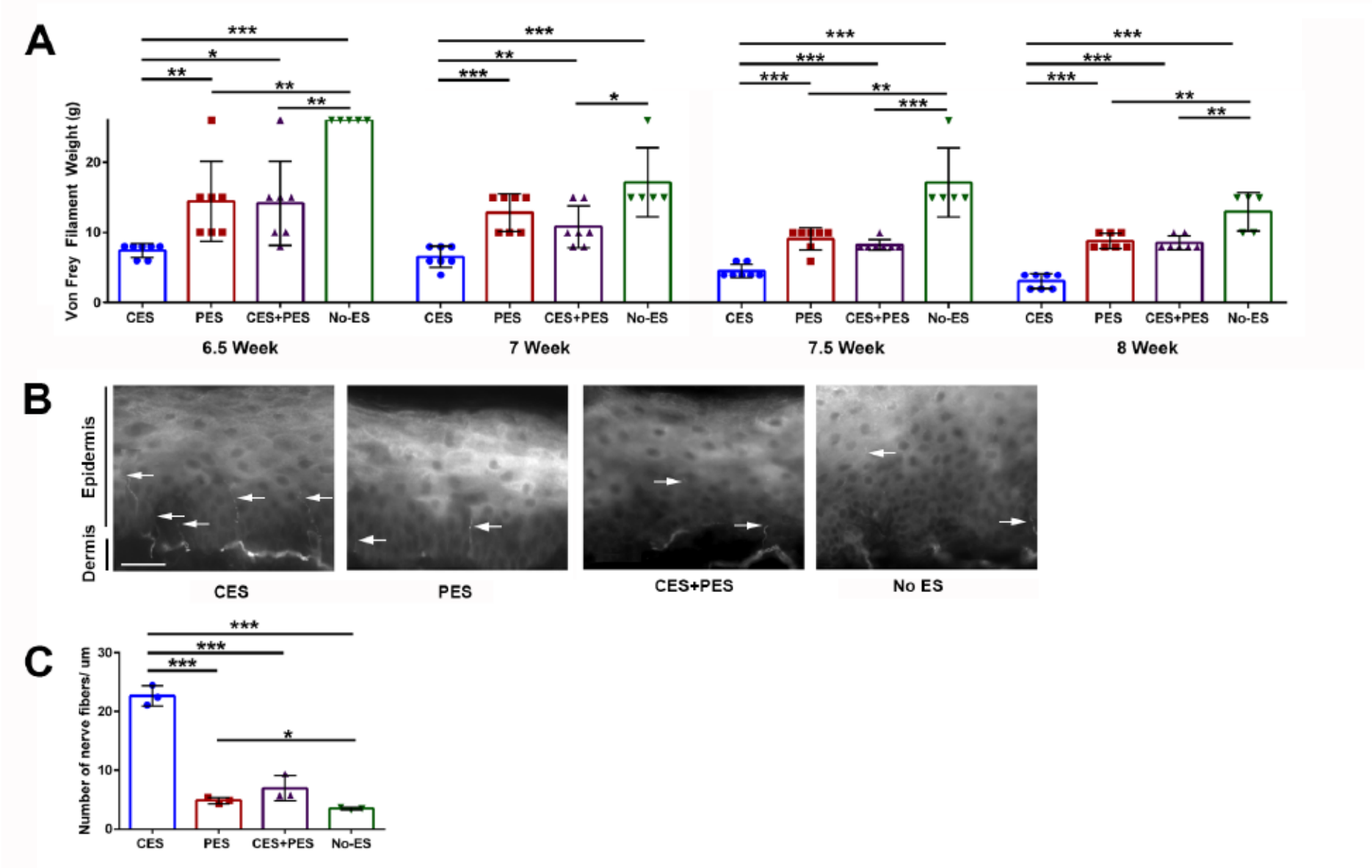
A
Von Frey Filament Weight (g)
0
10
20
CES
PES
CES+PES
No-ES
6.5 Week
7 Week
7.5 Week
8 Week
B
Epidermis
Dermis
CES
PES
CES+PES
No ES
C
Number of nerve fibers/ um
0
10
20
30
CES
PES
CES+PES
No-ES

Figure 4.3: CES improves functional recovery greater than PES or CES + PES

For all graphs, CES is depicted in blue, PES in red, CES + PES in purple, and no ES negative controls in green. (A) Scatterplot histograms depict the results of von Frey filament testing at 6.5, 7, 7.5, and 8 weeks of regeneration. At all timepoints, animals treated with CES had significantly improved sensory recovery when compared to animals treated with all other cohorts. (B, C) Footpad biopsies were collected at 8 weeks of regeneration and processed for intraepidermal nerve fiber innervation. (B) Representative skin sections (12 μm) show the PGP9.5 labelled axons crossing the dermal-epidermal junction. (C) Significantly more PGP9.5 labelled axons crossed the dermal-epidermal junction in specimens obtained from CES-treated animals when compared to PES (***$p<0.001$), CES + PES (***$p<0.001$) or negative controls (***$p<0.001$). ANOVA statistical analysis was performed followed by Bonferroni post-hoc analysis (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

4.5.3 CES of the tibial nerve improves motor innervation greater than PES ± CES

Thirty-two animals (n=8/cohort) were evaluated at 6.5, 7, 7.5, and 8 weeks following tibial nerve transection and repair to compare functional outcomes. Motor outcomes included gait analysis, performance on the horizontal ladder test, and gastrocnemius muscle weight. Gastrocnemius muscle sections were further evaluated immunohistochemically for neuromuscular junction reinnervation.

Gait analysis to evaluate toe spread, a measure of reinnervation to the intrinsic muscles of the foot, was performed at 6.5, 7, 7.5, and 8 weeks following nerve injury repair (Figure 4.4A). Toe spread of the injured limb was expressed as a percentage of the contralateral uninjured control limb. At 6.5 weeks of regeneration, animals in the CES cohort had significantly wider toe spread (62.8 ± 1.0 %) compared to PES (34.7 ± 2.4%; $p<0.001$), CES + PES (36.1 ± 1.6%, $p<0.001$), or animals that received no ES (36.5 ± 1.5% $p<0.001$). At 7, 7.5, and 8 weeks of regeneration, toe spread in the CES cohort (64.5 ± 1.9%; 66.3 ± 2.0%; 66.0 ± 1.3%) was significantly wider than the PES (42.8 ± 5.1%; 44.6 ± 3.4%; 48.7 ± 2.8%, $p<0.001$), CES + PES (39.5 ± 3.2 %; 43.9 ± 2.8%; 45.9 ± 3.3%, $p<0.001$), and no ES controls (33.1 ± 2.7%; 33.3 ± 2.7%; 32.0 ± 1.8%, $p<0.001$). Representative photomicrographs of ipsilateral and contralateral hindpaws at 8 weeks are represented in Figure 4.4B. No difference between the PES and combination CES + PES cohorts was observed at any timepoint, however PES as well as CES+PES were improved from naïve animals at both 7.5 and 8 weeks of regeneration (PES $p<0.05$ at 7.5 weeks, $p<0.001$ at 8 weeks; CES+PES $p<0.05$ at 7.5 week, $p<0.01$ at 8 weeks) (Figure 4.4A, B).

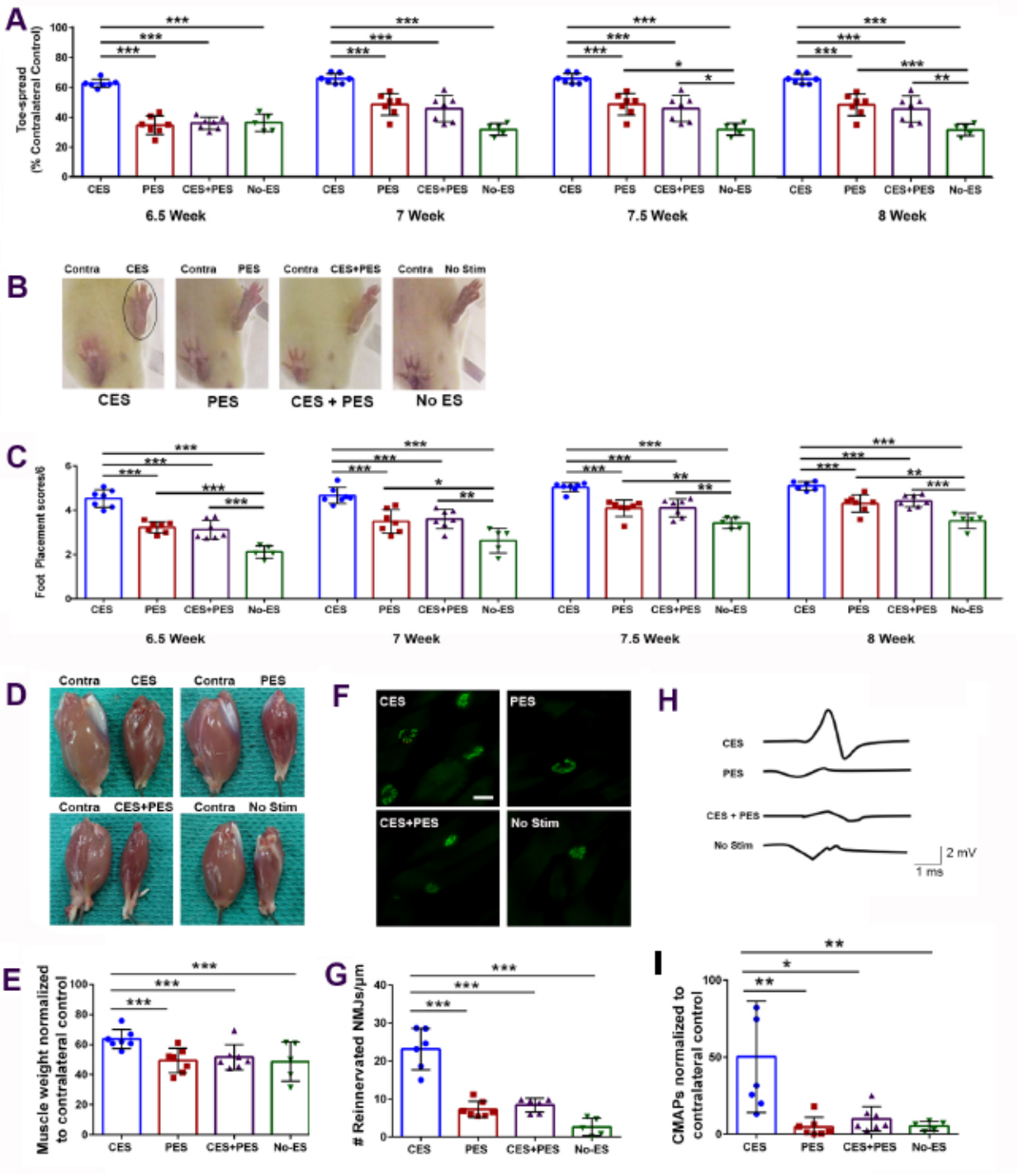
A
Toe-spread (% Contralateral Control)
CES
PES
CES+PES
No-ES
6.5 Week
7 Week
7.5 Week
8 Week
B
Contra
CES
PES
CES + PES
No ES
C
Foot Placement scores/6
D
Contra
CES
PES
CES+PES
No Stim
F
CES
PES
CES+PES
No Stim
H
CES
PES
CES + PES
No Stim
2 mV
1 ms
E
Muscle weight normalized to contralateral control
G
Reinnervated NMJs/µm
I
CMAPs normalized to contralateral control

Figure 4.4: CES improves muscle reinnervation greater than PES or CES + PES (A) Scatterplot histograms depict the width of toe spread at 6.5, 7, 7.5, and 8 weeks of regeneration. Toe spread width normalized to contralateral control in animals treated with CES was significantly wider than animals treated with PES (***p<0.001), CES + PES (***p<0.001), or no-stimulation (***p<0.001), indicating improved reinnervation of the intrinsic muscles of the foot. (B) Representative pictures of toe spread of the ipsilateral (black circle) and contralateral foot at 8 weeks of regeneration. (C) Scatterplot histograms depict the average foot placement scores on the horizontal ladder at 6.5, 7, 7.5, and 8 weeks of regeneration. Animals treated with CES had significantly higher foot placement scores at all timepoints compared to PES (***p<0.001), CES + PES (***p<0.001), or negative controls (***p<0.001). (D) Representative gastrocnemius muscles from the injury and contralateral uninjured limbs. (E) Muscle weight of the injured limb was normalized to the contralateral control. Animals treated with CES had significantly greater muscle weight compared to all other cohorts (*p<0.05; **p<0.01). (F, G) Representative photomicrographs of neuromuscular junctions labelled with alpha-bungarotoxin obtained from the gastrocnemius muscle of animals. All NMJs were confirmed to be innervated by NF200 (data not shown). Animals treated with CES had significantly more innervated acetylcholine receptors compared to all other cohorts (***p<0.001). (H-I) Compound muscle action potentials (CMAPs) were recorded at 8 weeks of regeneration and normalized to the contralateral control limb. Animals treated with CES had a greater CMAP amplitude compared to PES (**p<0.01), CES + PES (*p<0.05), and negative controls (**p<0.01). Scale bar in F is 20 μm. ANOVA statistical analysis was performed followed by Bonferroni post-hoc analysis (*p<0.05, **p<0.01, ***p<0.001).

Performance on the horizontal ladder test is a measure of the motor reinnervation and dexterity of the injured limb. A score of 0-6 was awarded for each foot placement on the ladder rungs, with a higher score indicating a more accurate placement (Figure 4.4C). At 6.5 weeks following nerve cut and repair, foot placement scores were significantly higher in the CES cohort (4.5 ± 0.2) compared to the PES (3.2 ± 0.1, $p<0.001$), CES + PES (3.1 ± 0.2, $p<0.001$), or no ES (2.1 ± 0.1, $p<0.001$) cohorts. Similar trends were observed at 7, 7.5, and 8 weeks of regeneration: animals treated with CES (4.7 ± 0.1; 5.0 ± 0.1; 5.1 ± 0.1) had significantly higher foot placement scores than PES (3.5 ± 0.2; 3.5 ± 0.1; 4.3 ± 0.1, $p<0.001$), CES + PES (3.6 ± 0.2; 3.6 ± 0.2; 4.4 ± 0.1, $p<0.001$), or the no ES control (2.6 ± 0.3; 2.6 ± 0.1; 3.5 ± 0.2, $p<0.001$). No difference between the PES and the combination CES + PES cohorts was observed at any timepoint; however, PES as well as CES + PES were improved compared to naïve animals at both 7.5 and 8 weeks of regeneration (PES $p<0.05$ at 7.5 weeks, $p<0.001$ at 8 weeks; CES+PES $p<0.05$ at 7.5 week, $p<0.01$ at 8 weeks) (Figure 4.4C).

The gastrocnemius muscles of the injured and contralateral uninjured lower limbs were weighed to assess muscle atrophy (Figure 4.4D). The injured limb's muscle weight was reported as a percentage of the uninjured limb's muscle weight; this is to account for differences in animal size. Animals in the CES cohort had significantly greater preservation of muscle bulk (64.4 ± 5.9%) compared to PES (49.4 ± 4.2%, $p<0.001$), CES + PES (51.7 ± 2.0%, $p<0.001$), and no ES (47.3 ± 4.3, $p<0.001$) (Figure 4.4D, E). Gastrocnemius neuromuscular junction reinnervation similarly suggested greater motor recovery in animals treated with CES (23.1 ± 2.9 innervated NMJ/50 mm^2) compared to PES (7.3 ± 1.2 NMJ/50

mm^2, $p<0.001$), CES + PES (8.4 ± 0.1 NMJ/50 mm^2, $p<0.001$), or no ES controls (2.6 ± 1.2 NMJ/50 mm^2, $p<0.001$) (Figure 4.4 F, G). No difference was identified in gastrocnemius muscle weight or NMJ reinnervation between PES, CES + PES, and naïve animals.

Nerve conduction testing was performed at eight weeks to assess innervation of the muscles of the foot. The average CMAP amplitude of the injured limb was analyzed as a percentage of the contralateral uninjured leg to control for variations in animal size. In keeping with motor behavioral tests, animals in the CES cohort had significantly greater CMAP amplitude (50.3 ± 13.7%) compared to PES (4.8 ± 2.4%, $p<0.01$), CES + PES (10.0 ± 3.0 %, $p<0.01$), and no ES animals (4.6 ± 1.4%, $p<0.05$) (Figure 4.4 H, I). There was no difference between PES, CES + PES or unstimulated animals.

4.6 Discussion

4.6.1 CES has a greater pro-regenerative effect than PES

CES is an effective technique for upregulating regeneration associated genes and accelerating axon extension following surgical nerve repair (Senger et al., 2019; Senger et al., 2017). The improved regeneration outcomes conferred by CES result in enhanced sensory and motor recovery (Senger et al., 2019). To guide best patient management, the effects of CES must be directly compared to regeneration and reinnervation outcomes of current adjuvant therapies. To date, PES remains the only perioperative technique to improve nerve regeneration outcomes that has been successfully integrated into clinical practice.

The effects of PES are targeted specifically to the site of surgical coaptation. After nerve injury, axons regenerate across the injury site in a 'staggered' distribution rather than as a unified front, which delays axon extension. Delivery of one hour of PES decreases staggered regeneration, and thereby accelerates target reinnervation (Brushart et al., 2002). The regenerative effects of PES are however limited, as this technique does not affect the rate of axon extension along the distal stump (Brushart et al., 2002). A conditioning lesion, by contrast accelerates the intrinsic rate of nerve regeneration. These differences in mechanism may underlie our results that suggest nerves treated with CES had significantly greater impact on regeneration and functional recovery compared to nerves treated with PES. The fact that CES accelerates the rate of nerve regeneration distal to the site of injury is of even greater importance in humans as the length of regeneration required is significantly longer than in a rodent.

4.6.2 The combination of PES + CES does not have a synergistic effect

Given the complementary mechanisms of action of CES and PES, we hypothesized that the combination of both techniques would have a synergistic effect in enhancing nerve regeneration. Our regeneration and sensorimotor reinnervation outcomes, however, suggest the combination of CES and PES is inferior to CES alone (Figures 4.2 - 4.4). Further investigations are required to determine why the conditioning effect is downregulated by an hour of electrical stimulation following nerve repair. It is possible that stimulating the nerve twice damages the regenerating axons. By comparing outcomes of one hour, three hours, one day, there days, and seven days of continuous PES, Geremia et al. (2007) demonstrated that too much electrical stimulation is harmful to sensory nerve regeneration. They found that a

duration of stimulation longer than an hour decreased sensory recovery (Geremia et al., 2007). Unlike sensory neurons, however, the ideal parameters for delivering PES to motoneurons is less well understood. Previous studies suggest that at one hour of PES confers similar outcomes to several hours, or even days of PES (Al-Majed, Neumann, et al., 2000). There does, however, appear to be a threshold after which electrical stimulation is detrimental to regenerating motoneurons, with previous studies showing that daily PES for four weeks did not confer any benefit (Asensio-Pinilla, Udina, Jaramillo, & Navarro, 2009). To determine why one hour of PES decreases the conditioning effect requires further investigation, particularly in identifying the molecular pathways and the cellular transportation systems that are upregulated by CES as compared to the physiologic effects in a nerve treated with both CES and PES.

4.7 Conclusions

In summary, CES may be a clinically feasible preoperative modality to significantly improve regeneration and reinnervation outcomes in nerve repair. We anticipate that clinical trials will confirm the safety and efficacy of CES in a human patient population and may therefore alter current clinical practices.

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Chapter 5

Conditioning Electrical Stimulation Enhances Nerve Autograft Regeneration and Functional Recovery Beyond That of Postoperative Electrical Stimulation

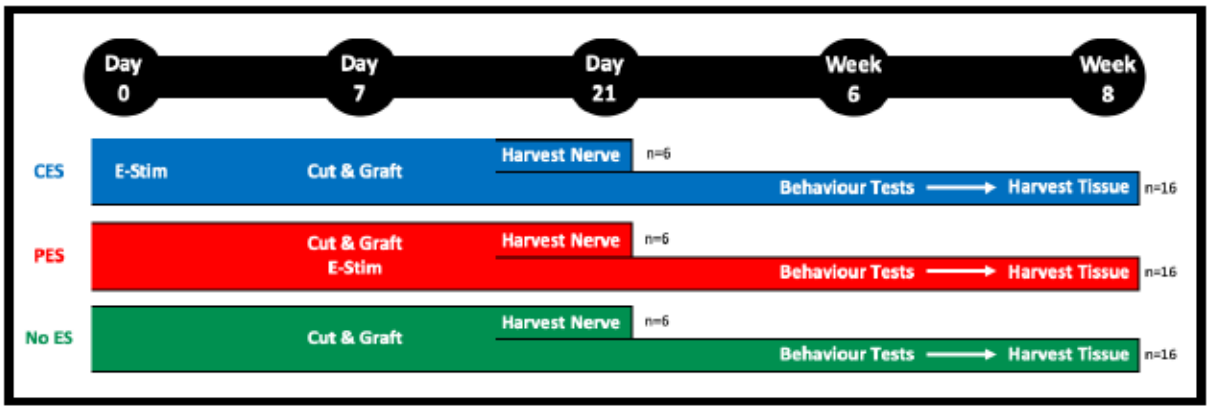
5.1 Preface

Though a tension-free repair is crucial for nerve regeneration, primary closure is often not possible when a section of nerve is damaged. Autograft repair is the most common surgical option to overcome the nerve gap, with significantly better outcomes than allograft repair or use of a nerve conduit. The effects of PES on enhancing regeneration outcomes in a nerve graft model are controversial and poorly understood in the literature; the effects of CES have never been evaluated in a nerve graft reconstruction model. Given the worldwide prevalence of nerve autografting, and the feasibility of delivering either pre-operative or postoperative electrical stimulation in this patient population, we aimed to determine the best treatment paradigm for this patient population.

Though our results in Chapter 4 suggest that the pro-regenerative effects of CES supersede those of PES, the nerve autograft confers the unique challenge of two sites of surgical coaptation (both at the proximal and distal aspects of the graft). We questioned whether the effects of PES would be augmented, with enhanced staggered regeneration at both sites and, by contrast, if the second site of repair would affect the accelerated rate of axon extension conferred by conditioning. To lay a strong foundation for translation to a clinical setting, we investigated the effects of CES and PES in a rodent model of nerve autograft reconstruction.

As shown in the following timeline, animals received electrical stimulation 7 days prior to grafting (CES, blue), immediately following grafting (PES, red), or no electrical stimulation (green). Nerves were harvested to evaluate length of axon extension at two weeks of

regeneration, and behavioural testing for sensorimotor recovery was performed weekly between 6 and 14 weeks of regeneration.



Animals treated with CES had significantly longer axon extension within the nerve graft when compared to animals treated with PES or negative controls. Furthermore, animals treated with CES had significantly greater sensory and motor reinnervation as evidenced by behavioural, electrophysiologic, and immunohistochemical outcomes. These results suggest that CES may be an effective preoperative tool for improving regeneration through nerve autograft reconstructions.

Conditioning Electrical Stimulation Enhances Nerve Autograft Regeneration and Functional Recovery Beyond That of Postoperative Electrical Stimulation

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5.2 Abstract

Background: Postoperative electrical stimulation (PES) improves nerve growth by reducing staggered regeneration at the coaptation site. By contrast, conditioning electrical stimulation (CES) accelerates axon extension. Given that both techniques can be delivered at the bedside, a direct comparison of outcomes is of clinical importance. In this study, we evaluated outcomes of these electrical stimulation paradigms in a model of nerve autograft repair to guide patient management.

Methods: Regeneration (n=6/cohort) and reinnervation (n=16/cohort) outcomes of CES and PES in a 5 mm nerve autograft model were compared. Sprague Dawley rats were divided into: i) CES, ii) PES, and iii) no stimulation cohorts. CES was delivered one week prior to nerve cut/grafting, and PES was delivered immediately following nerve grafting. Length of nerve regeneration (n=6/cohort), and behavioural testing (n=16/cohort) were performed at 14 days and 6-14 weeks post-repair, respectively.

Results & Conclusions: Animals treated with CES had significantly improved nerve regeneration and sensorimotor recovery compared to PES or negative controls. CES treated axons extended 5.9 ± 0.7 mm, significantly longer than PES (3.8 ± 0.2 mm), or no stimulation (2.5 ± 0.2 mm) ($p<0.01$). Unlike PES-treated animals, the CES animals had significantly improved sensory recovery (von Frey filament testing, intraepidermal nerve fiber reinnervation) ($p<0.001$) and motor reinnervation (horizontal ladder, gait analysis, nerve

conduction studies, neuromuscular junction analysis) ($p<0.01$). Thus, CES is a promising clinically-feasible treatment to improve nerve autograft repair outcomes.

5.3 Introduction

Outcomes following peripheral nerve injury are strongly influenced by the time required for regenerating motor and sensory axons to reach their target tissue. Functional outcomes can therefore be improved by accelerating the intrinsic rate of nerve regeneration. Although a conditioning crush lesion (CCL) delivered to a nerve one week prior to transection and nerve repair has been shown to be capable of markedly accelerating nerve regrowth, it cannot be translated to the clinic due to its injurious nature (Senger, Verge, Chan, & Webber, 2018). Recently, however, we demonstrated that one hour of conditioning electrical stimulation (CES) delivered to a nerve seven days prior to injury and repair induces a conditioning effect comparable to a CCL, but in a non-injurious, thus clinically feasible manner (Senger et al., 2019; Senger, Chan, Verge, & Webber, 2017; Zigmond & Echevarria, 2019).

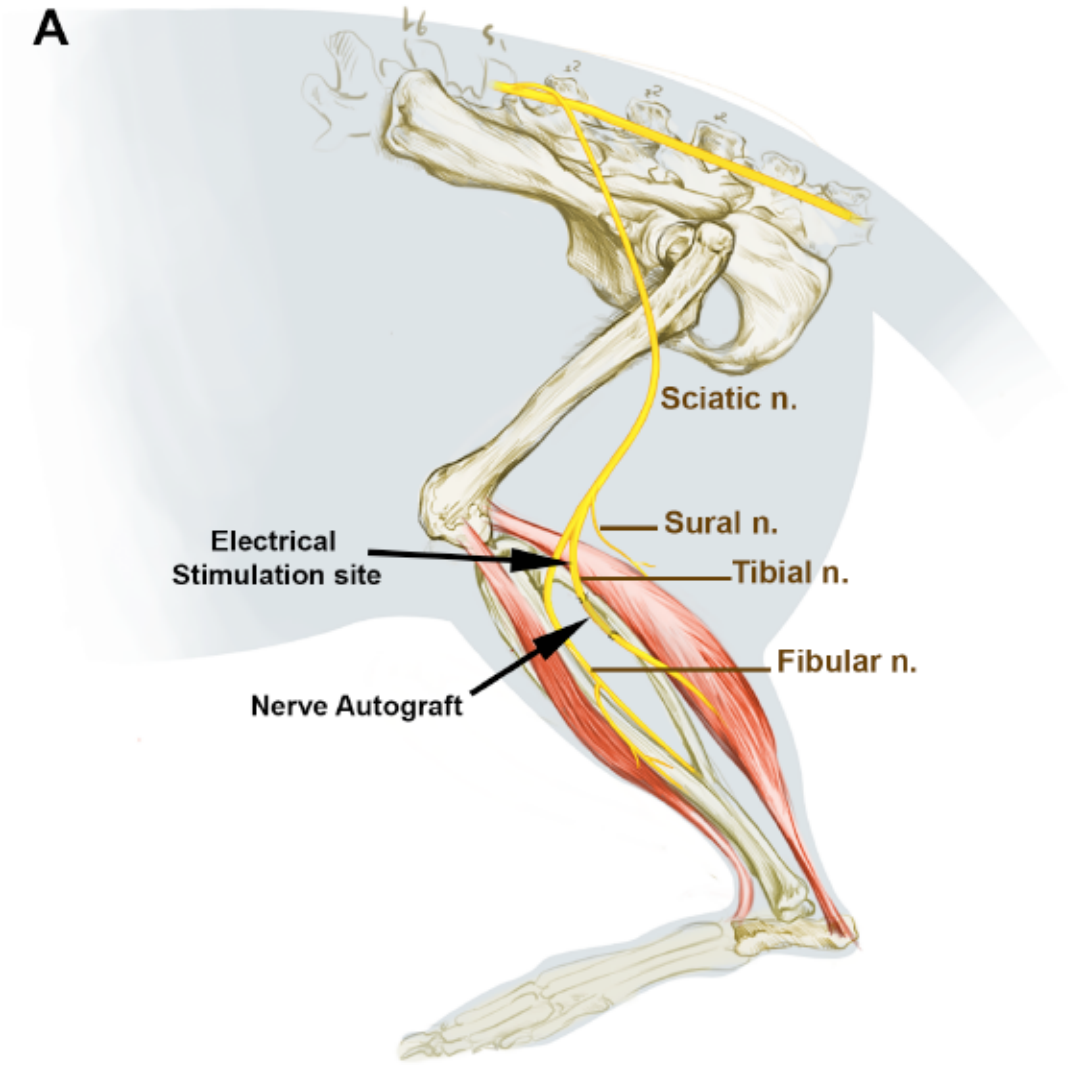
While the use of CES is novel, postoperative electrical stimulation (PES) is well described to improve outcomes in animal and human models of nerve injury, and is the only adjunct to nerve repair that has been successfully translated to the clinic (T. Gordon, Amirjani, Edwards, & Chan, 2010; Wong, Olson, Morhart, & Chan, 2015). PES, unlike a conditioning lesion (CCL or CES). However, unlike a conditioning lesion (CCL or CES), PES does not accelerate the rate of axon extension, but rather enhances regeneration of axons as they cross the site of surgical coaptation, a process called "staggered regeneration" (Brushart et al., 2002). As both PES and CES are clinically feasible modalities, a direct comparison of outcomes is of clinical importance. To inform future clinical trials, the goal of this study is to compare length of axon extension and sensorimotor behavioral outcomes of CES and PES

in an animal model of nerve autograft repair, a protocol that is widely used worldwide. Based on the knowledge of their effects on nerve regrowth, we hypothesize that CES is more efficacious than PES in enhancing the rate of nerve regeneration and return of motor and sensory function after autograft nerve repair.

5.4 Methods

5.4.1 Animals: Adult male Sprague Dawley rats weighing 200 g (Charles River laboratory) were placed under the care of Health Sciences Laboratory Animal Services (HSLAS) at the University of Alberta. We divided 60 animals into 3 separate cohorts of CES prior to an autograft repair, PES immediately following an autograft repair, and the negative control in which there was no electrical stimulation (no-ES) intervention before or after the autograft repair (Figure 5.1A). Length of nerve regeneration through a graft was measured 14 days following coaptation in 18 animals (n=6/cohort). Behavioral testing of 48 animals was performed between 6-14 weeks and animals were culled 10 or 14 weeks after the autograft repair surgery (n=8/cohort for each timepoint). All surgical procedures were approved by the University of Alberta Animal Research Ethics Board.

Prior to surgery, animals were anesthetized with inhaled isoflurane (2% titrated at 1-2 L/min for maintenance of a surgical anesthetic plane) and received 0.01 mg/kg of buprenorphine subcutaneously. All surgical procedures were performed under 3.5x loupe magnification. At the completion of all procedures, the hamstring muscles were resuspended and the skin was closed with 4-0 Vicryl (Ethicon Inc, Somerville, NJ).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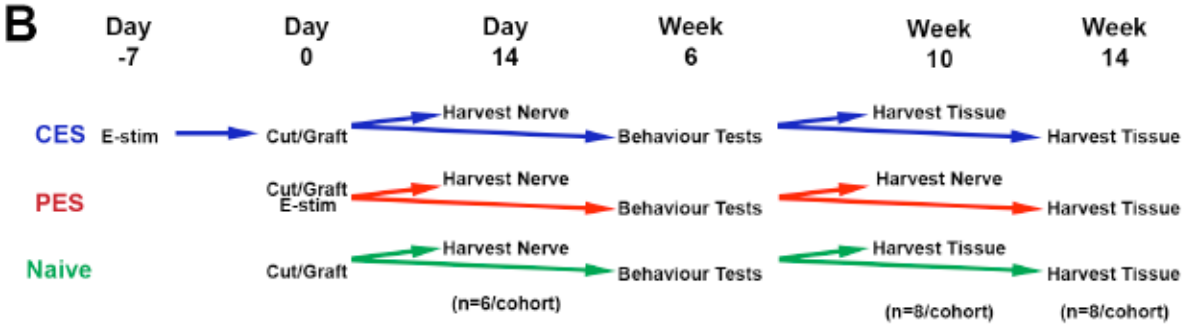
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Figure 5.1 Experimental design of surgical and treatment paradigms.

A) Artistic illustration of the investigation to describe the nerve autograft repair surgeries. Illustration of the sciatic nerve and its trifurcation into the fibular (common peroneal), sural and tibial (used in autograft repair) nerves. The site of electrical stimulation (CES or PES) is shown. (B) Animals were divided into three cohorts, CES, PES and no-ES. Two weeks following nerve repair of animals +/1 CES, PES treatment, 24 animals were harvested to determine the extent of nerve regeneration (n=6/cohort). The remaining animals underwent behavioral tests and were euthanized at 10 and 14 weeks (n=8/cohort).

5.4.2 Conditioning electrical stimulation (CES): CES was performed as previously described (Senger et al., 2019). Briefly, a longitudinal incision was made over the lateral aspect of the right lower limb. Within the posterior compartment of the leg, the tibial nerve was identified as it emerged between the two heads of the gastrocnemius muscle. The bared end of a stainless-steel wire (cathode) was wrapped around the nerve, and another wire (anode) was placed into the tibialis anterior muscle; these were connected to a SD-9 stimulator (Grass Instruments Co., Quincy, MA). Continuous electrical stimulation was delivered at 20 Hz with a 0.1 ms duration over a one-hour timespan, with voltage titrated to maintain a visible foot twitch.

5.4.3 Nerve autograft repair: An incision was made along the lateral femur and the hamstring muscles were dissected to identify the sciatic nerve, which was traced distally to isolate the tibial nerve. One cm distal to the site of trifurcation, a 5 mm segment of the tibial nerve was excised. The nerve segment was reversed 180 degrees, and the proximal and distal cut sites were coapted with 9-0 silk suture. The hamstring muscles were resuspended with 4-0 Vicryl and the skin was repaired with a two-layer closure.

5.4.4 Postoperative electrical stimulation (PES): Immediately following nerve grafting, a stainless-steel wire (cathode) bared of insulation was placed proximal to the site of repair, and the anodal wire was buried in the quadriceps muscle. PES was performed using the same parameters as the conditioning surgery (one hour of continuous stimulation at 20 Hz).

5.4.5 Tissue Analysis: Animals treated with an autograft repair were euthanized either 14 days following nerve repair (nerve regeneration cohort) or upon completion of behavioral testing at 10 or 14 weeks post-grafting (functional outcome cohorts). Euthanization was accomplished by carbon dioxide asphyxiation followed by exsanguination through a puncture to the left cardiac ventricle.

Tibial nerves were harvested as previously described (Senger, Verge et al. 2018), with fixation in Zamboni's fixative (paraformaldehyde, picric acid, NaOH; American MasterTech Scientific, Lodi, CA) for four hours, rinsed with 0.01M phosphate buffered saline (PBS) (ThermoFisher Scientific, Waltham, MA) five times, post-fixed in 30% sucrose solution overnight at 4°C, and frozen in Optimum Cutting Temperature (OCT) (Sakura Finetek, Torrance, CA) with indirect exposure to liquid nitrogen. Tissue chucks were cut into 12 μm sections, thaw-mounted on Superfrost Plus microscope slides, and stored at -80°C until processing.

The gastrocnemius muscles were harvested from both legs in their entirety and weighed for comparative purposes. The weight of the injured muscle, a function of muscle atrophy, was reported as a percentage of the uninjured contralateral muscle to control for discrepancies in animal size. Ipsilateral gastrocnemius muscle was fixed in Zamboni's solution overnight, rinsed five times in PBS, sunk in 30% sucrose and flash-frozen in OCT.

Plantar footpads were collected 8 weeks post nerve repair using a 3 mm biopsy punch (Acuderm Ic, Fort Lauderdale, FL) and fixed in 2% paraformaldehyde, lysine, periodate (PLP) fixative overnight. Tissue sections were rinsed five times in Sorenson's phosphate butter, cryoprotected overnight in 20% glycerol/0.1 M Sorensen's phosphate buffer, frozen in OCT, and cut into 20 μm sections.

5.4.6 Immunofluorescence: After slides were warmed to room temperature, antigen retrieval was performed for 20 minutes in 60ºC citrate buffer (10 mM sodium citrate, 0.05% Tween-20, pH 6.0; Thermo Fisher Scientific, Waltham, MA). After the slides cooled, they were washed three times in 0.01 M PBS for five minutes/wash, and permeabilized for 10 minutes with 0.1% Triton-100X (Thermo Fisher Scientific, Waltham, MA). Tissue sections were blocked for 90 minutes in 10% normal goat serum (MP Biomedicals, Santa Ana, CA) and 3% bovine serum albumin (BSA) (Sigma-Aldrich, St Louis, MO) in 0.01 M PBS. Primary antibodies diluted in 0.01 M PBS with 3% BSA were left on tissue overnight at 4ºC. Nerve sections were stained with mouse anti-neurofilament-200 (NF200; 1:500) (Sigma-Aldrich, St Louis, MO). Muscle sections fixed in Zamboni's were labelled with mouse anti-NF200 (1:500) to stain the innervating nerve, then with conjugated anti-α-bungarotoxin (1:1000, 20 mins). Footpads were stained with anti-protein gene product 9.5 (PGP9.5, 1:1000) for identification of intraepidermal nerve fibers overnight at 4ºC. Secondary antibodies included Cy3-conjugated goat anti-mouse (Sigma-Aldrich), Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA), and donkey-anti-chicken 594

(Thermo Fisher Scientific). DRG tissues were stained with nuclear stain NucBlue (Thermo Fisher Scientific). All slides were mounted with a coverslip using 50% glycerol in 0.01 M PBS.

Morphological identification of regenerating NF-200 axons were examined to measure a) the length of axonal extension, and b) the number of regenerating axons at 250 μm intervals from the site of repair to the most distal point of regeneration (minimum of 10 axons must be observed at that distance in order to continue measuring the next distance interval). The operator was blinded to the treatment condition for all tissue analyses and quantification.

Muscle sections and footpads were evaluated using confocal microscopy. The number of innervated neuromuscular junctions in sections of the gastrocnemius muscle was determined, with NF-200 labelling the axons and α-bungarotoxin the acetylcholine receptors. The number of neuromuscular junctions present in each standardized size of tissue was quantified; six sections were analyzed per animal. The tibial-nerve innervated footpads were evaluated to identify PGP9.5-labelled axons crossing the dermal-epidermal junction (three sections were evaluated per animal). A z-stack of 1 μm steps was obtained for each field and intraepidermal nerve fiber (IENF) density was determined by counting the number of axons crossing per 1 mm of dermal-epidermal junction.

5.4.7 Behavioural Outcomes: Sensory and motor behavior testing was performed weekly between 8-14 weeks following nerve grafting, by the same examiner blinded to the test group

of the animals. Calibrated von Frey monofilaments (1.4–15 g) were used to evaluate sensory reinnervation to the plantar footpad. Three consecutive paw withdrawals to an individual monofilament constituted a positive response.

Motor outcomes included gait analysis for toe spread, horizontal ladder test for motor dexterity, and nerve conduction studies for compound muscle action potentials (CMAPs). Gait analysis to evaluate intrinsic foot muscle reinnervation was performed by placing the animal into a specially-designed walkway constructed from transparent plexiglass with an adjustable mirror underneath to visualize the plantar paw. Animals walked the length of the track three-times/session while being filmed. The video was analyzed and individual screenshots were taken of each weight-bearing hindpaw where all toes were visible. Ten images of both the affected and contralateral control footpads were analyzed per testing session; the distance between the first and last toes were measured. Results were reported as the percentage of toe spread of the injured side compared to the uninjured contralateral control.

The horizontal ladder test consisted of a 48 cm elevated horizontal ladder composed of clear plexiglass with metal ladder rungs arranged in a different pattern each testing session to prevent learning. The animals were filmed over three attempts to cross the ladder at each testing session, and videos were analyzed on a frame-by-frame basis. Using the 0-6 scoring system developed by Metz and Whishaw (Metz and Whishaw 2009), a score was given for each individual foot placement on the ladder rung, with 0 being a complete slip, 1 included

a deep slip, 2 included a slight slip, 3 included foot replacement after a slip, 4 included a foot correction, 5 included a partially successful foot placement, and 6 included correct placement. The average score of the injured limb was calculated for each attempt.

Nerve conduction studies were performed at 10, 12 and 14 weeks following nerve autograft repair surgery. Animals were anesthetized with inhaled isoflurane and the lower limbs were shaved for lead placement. Recording leads were placed in the plantar footpad, and supramaximal electrical stimulation was delivered at the knee. Maximal CMAP with the sharpest rise time was recorded. Results were reported as the CMAP percentage of the injured side compared to the uninjured contralateral control.

5.4.8 Statistical analysis: Results are presented as the mean ± standard error mean (s.e.m). Groups were compared using a one-way analysis of variance (ANOVA) to identify differences in the mean between groups, followed by Bonferroni post hoc analysis when a significant difference was found. Statistical significance was accepted with a level of $p<0.05$. All statistical analyses were performed using STATA 14 (StataCorp LP, Collagen Station, Texas).

5.5 Results

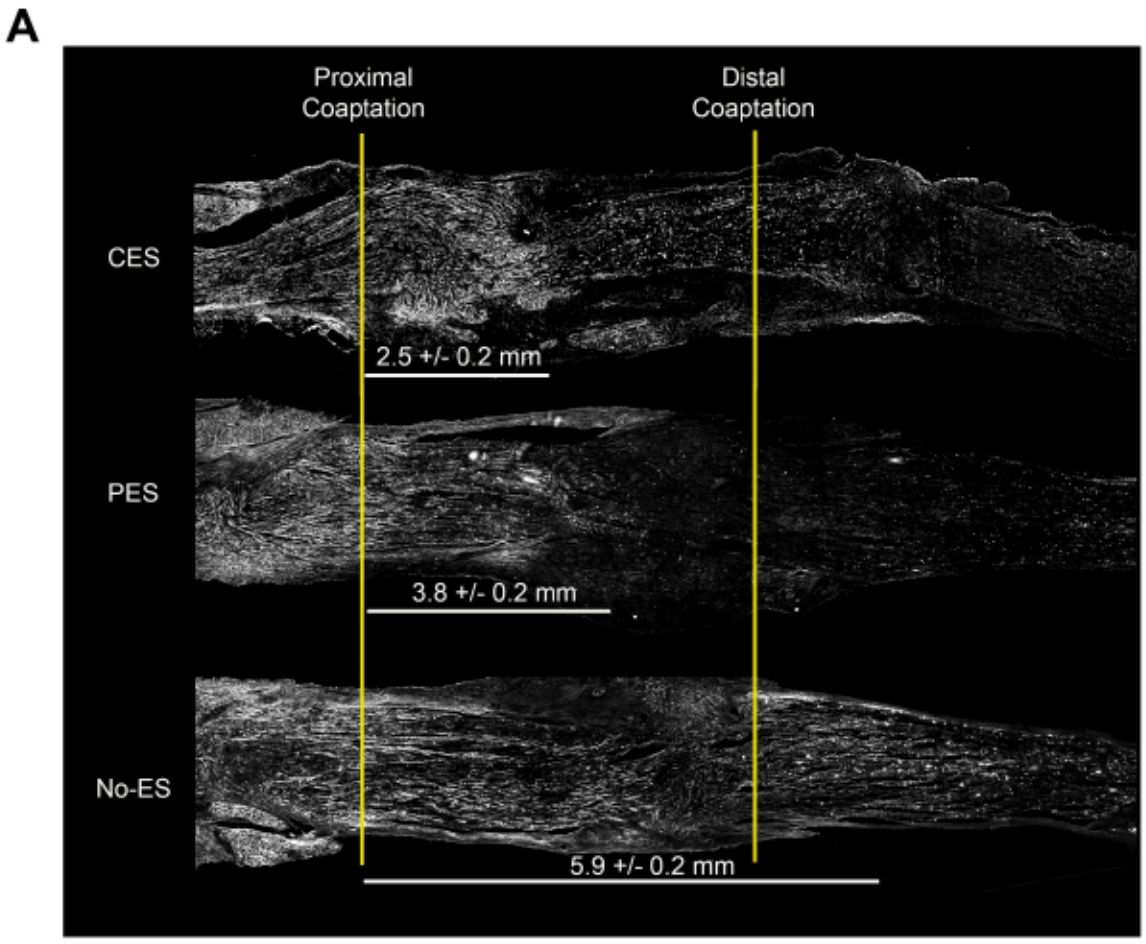
5.5.1 CES improves regeneration outcomes in a nerve autograft model

Length of regeneration two weeks following tibial nerve autograft repair surgery was compared between animals treated with CES, PES, and a no-ES negative control. Each tibial

nerve and its graft were harvested, and following cryostat sectioning, the tissues were stained with NF200 immunofluorescence to assess the extent of axon regeneration. Animals treated with CES prior to grafting had a significantly longer length of axon extension at two weeks of regeneration (5.9 ± 0.2 mm) when compared to animals who received PES after grafting (3.8 ± 0.2 mm, $p<0.01$) or animals that received no-ES (2.5 ± 0.2 mm, $p<0.001$). Length of axon extension in animals treated with PES was significantly longer than negative control ($p<0.001$) (Figure 5.2). Our previous studies showed sham-ES and no-ES were equivalent in all analyses and therefore a sham-ES treatment group was not included in our study (Senger et al., 2019; Senger et al., 2017).

5.5.2 CES improves sensory reinnervation outcomes in a nerve autograft model

Sensory outcomes included von Frey filament testing and IENF density counts. Weekly von Frey monofilament testing between 6-14 weeks of regeneration revealed significantly greater sensory recovery at all time points in animals treated with CES when compared to the PES and the no-ES cohorts. At six weeks of regeneration, animals treated with CES responded to 7.8 ± 0.5 g of mechanical stimulation, which was significantly less than PES (19.3 ± 2.6 g, $p<0.001$) or no-ES (22.1 ± 2.5 g, $p<0.001$). This trend persisted, and animals treated with CES had significantly improved sensory recovery compared to PES and no-ES at seven (5.5 ± 0.6, 10.6 ± 1.1, 12.4 ± 2.4, $p<0.001$), eight (5.8 ± 0.4, 11.3 ± 0.7, 13.1 ± 0.4, $p<0.001$), nine (5.25 ± 0.3, 9.3 ± 0.4, 10.1 ± 0.9, $p<0.001$), ten (4.25 ± 1.0, 9.3 ± 0.6, 9.6 ± 1.1, $p<0.001$), eleven (5.25 ± 0.5, 11.9 ± 0.9, 12.1 ± 1.0, $p<0.001$), twelve (4.9 ± 0.5, 8.0 ± 0.2, 12.5 ± 1.1, $p<0.001$), and thirteen (4.75 ± 0.7, 8.2 ± 0.4, 9.7 ± 0.3, $p<0.001$) weeks of regeneration


A
Proximal Coaptation
Distal Coaptation
CES
2.5 +/- 0.2 mm
PES
3.8 +/- 0.2 mm
No-ES
5.9 +/- 0.2 mm

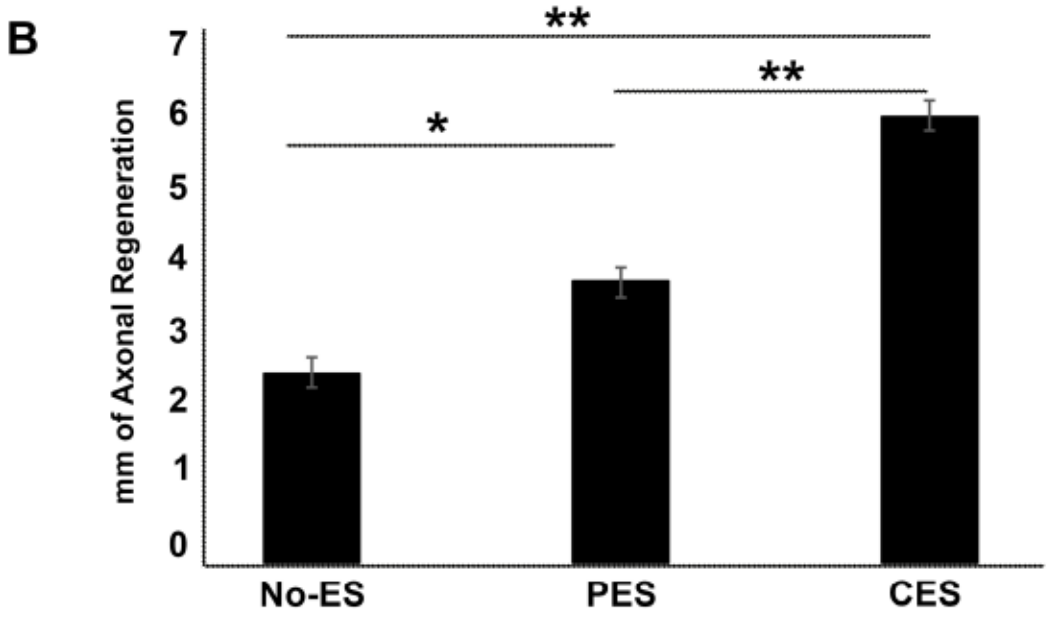

B
**
**
*
mm of Axonal Regeneration
0
1
2
3
4
5
6
7
No-ES
PES
CES

Figure 5.2: CES improves regeneration in a 0.5 cm nerve autograft reconstruction. Representative photomicrographs of NF200 labelled longitudinal sections of grafted tibial nerve at 14 days of regeneration. Yellow lines depict the proximal and distal coaptation sites abutting the interposed 0.5 cm autograft. Although PES treated nerves regenerated farther than the negative controls (**$p<0.01$), nerves treated with CES one week prior to grafting (blue) had significantly longer axon extension compared to animals treated with either PES (*$p<0.05$) or the no-ES controls (**$p<0.01$).

(Figure 5.3A). Although PES was significantly improved from the no-ES controls at 12 and 13 weeks of regeneration ($p<0.05$), they did not compare to the increased regenerative potential of the CES treated animals.

Von Frey behavioral outcomes were corroborated by quantification of footpad reinnervation at 14 weeks post-nerve transection and autograft repair. Intraepidermal nerve fiber (IENF) density was determined by counting the number of nerves crossing the dermal-epidermal junction. Significantly more nerves crossing the dermal-epidermal junction were identified in the CES cohort (10.1 ± 0.3 nerves/mm) compared to PES alone (4.8 ± 0.8 nerves/mm; $p<0.001$), or the no-ES cohort (1.6 ± 0.8 nerves/mm; $p<0.001$) (Figure 5.3B). PES animals had improved regeneration into the skin compared to their No-ES counterparts ($p<0.001$).

5.5.3 CES improves motor reinnervation outcomes in a nerve autograft model

Motor outcomes included performance on the horizontal ladder test, toe-spread gait analysis, nerve conduction testing, and gastrocnemius muscle weight. Performance on the horizontal ladder test is a measure of the motor reinnervation and dexterity of the injured limb. A score of 0-6 was awarded for each foot-placement on the ladder rungs, with a higher score indicating a more accurate placement. Performance on the horizontal ladder was similar between all three cohorts at six weeks of nerve regeneration (CES, 2.0 ± 0.2; PES, 2.1 ± 0.2; no-ES, 2.1 ± 0.1). By seven weeks, however, foot placement scores for animals treated with CES improved to 3.8 ± 0.3, significantly higher than PES (2.0 ± 0.2, $p<0.001$) or no-ES (2.2 ± 0.2, $p<0.001$). Foot placement scores remained significantly higher in animals treated with

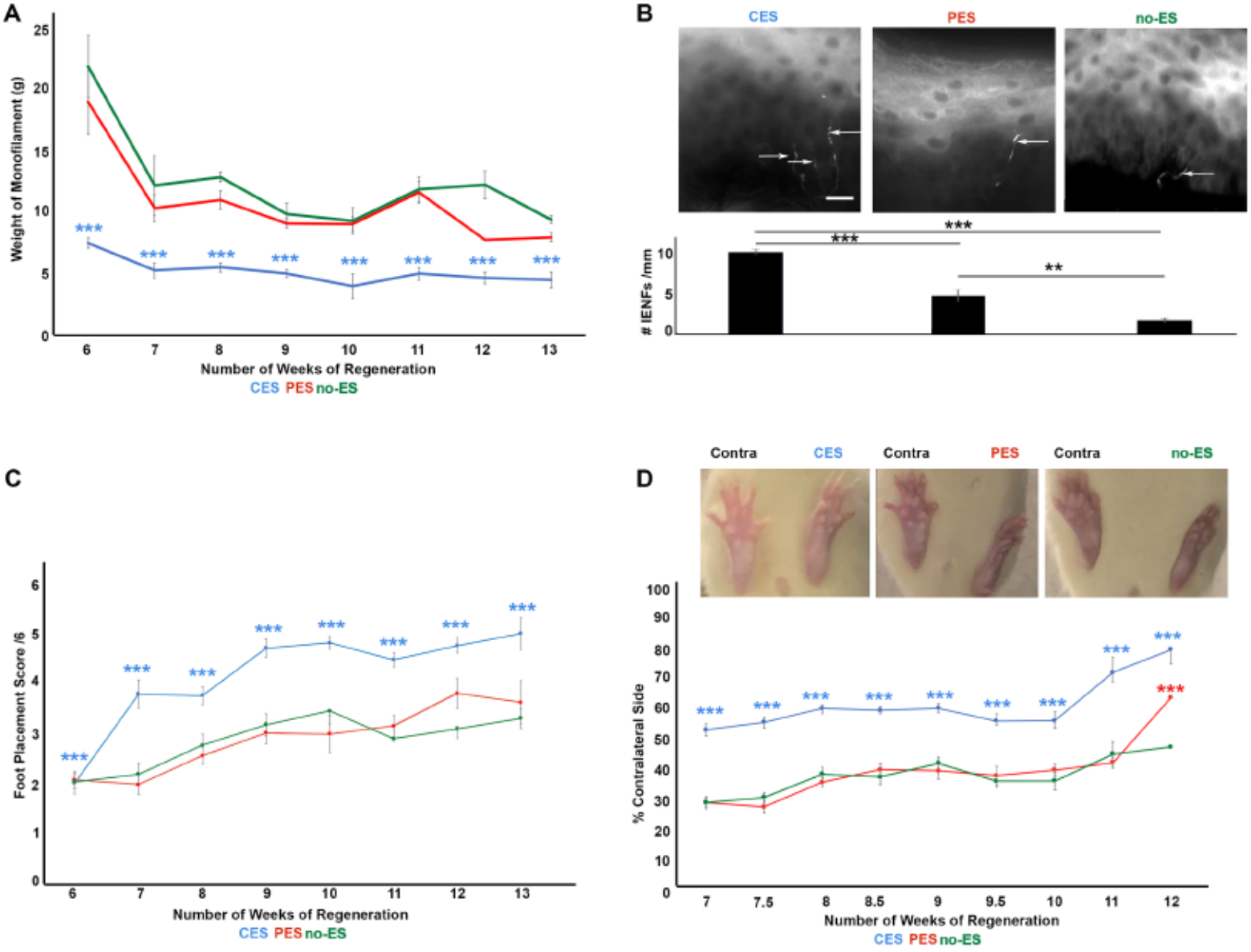

A
Weight of Monofilament (g)
Number of Weeks of Regeneration
CES PES no-ES
B
CES
PES
no-ES
IENFs /mm
C
Foot Placement Score /6
Number of Weeks of Regeneration
CES PES no-ES
D
Contra
CES
Contra
PES
Contra
no-ES
% Contralateral Side
Number of Weeks of Regeneration
CES PES no-ES

Figure 5.3: CES improves functional recovery in a 0.5 cm neve autograft reconstruction

A) Line graph depicts results of von Frey filament testing at 6-13 weeks of regeneration. At all timepoints, animals treated with CES (blue) had significantly improved sensory recovery when compared to animals treated with PES (red), or no-stimulation negative controls (green) (***$p<0.001$). (B) Footpad biopsy specimens were collected at 14 weeks of regeneration and processed for intraepidermal nerve fiber innervation. Significantly more PGP9.5 labelled axons were counted crossing the derma-epidermal junction in specimens obtained from CES-treated animals when compared to PES (**$p<0.01$), or negative controls (***$p<0.001$). (C) Line graph depicts average foot placement scores at 6.5, 7, 7.5, and 8 weeks of regeneration. Animals treated with CES (blue) had significantly higher foot placement scores at all timepoints compared to PES (red, ***$p<0.001$), ***$p<0.001$), or negative controls (green, ***$p<0.001$). (D) Representative photographs of toe-spread width (in % of contralateral side) of animals treated with CES, PES, or negative controls. Line graph of toe-spread at 6-13 weeks of regeneration. Wider toe-spread, indicating improved reinnervation of the intrinsic muscles of the foot, in animals treated with CES (blue) was significantly improved compared to animals treated with PES (red ***$p<0.001$), or no stimulation (green, ***$p<0.001$).

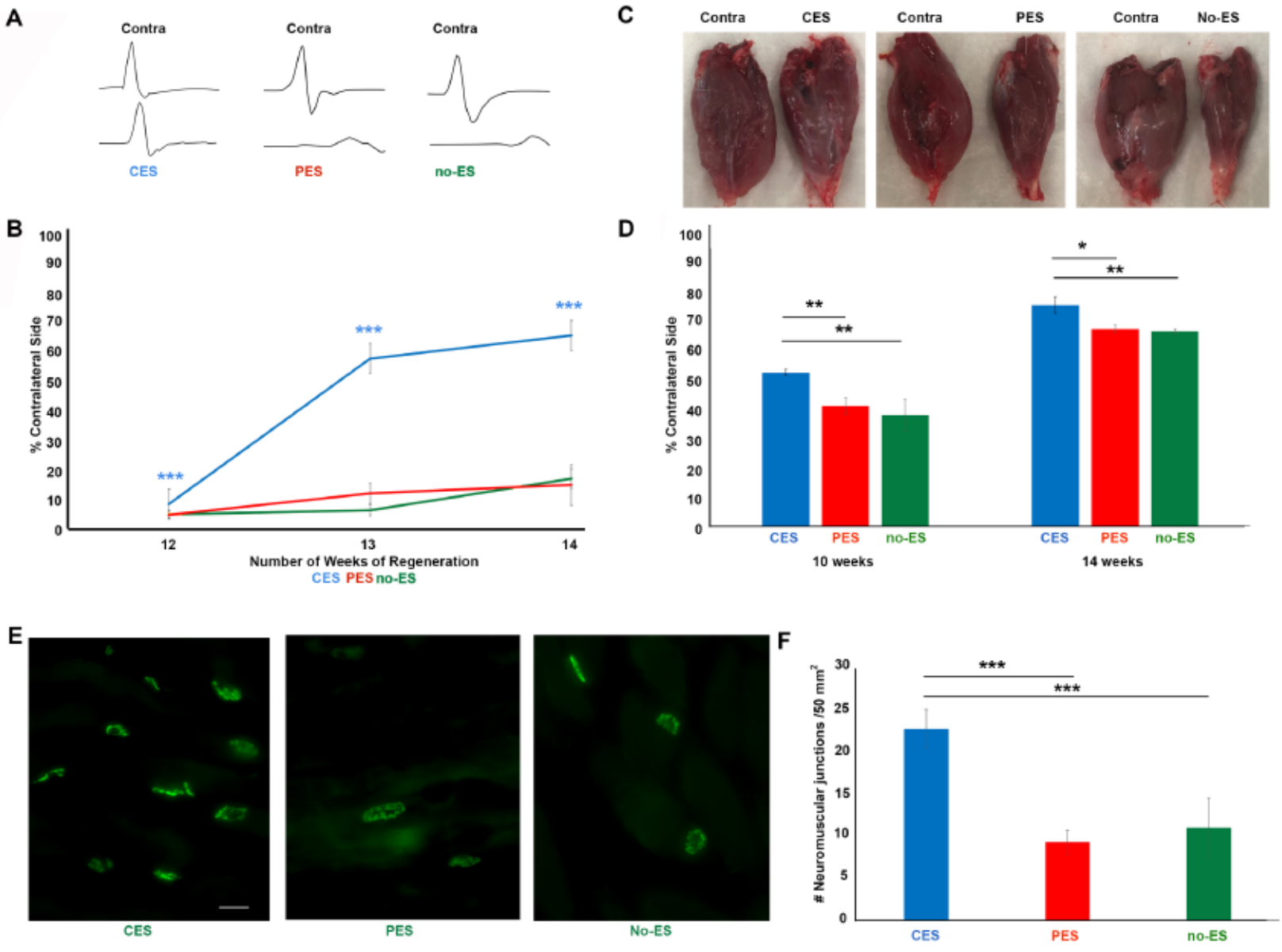
CES when compared to PES or no-ES at eight (3.8 ± 0.2, 2.6 ± 0.2, 2.8 ± 0.2, $p<0.001$), nine (4.8 ± 0.1, 3.0 ± 0.4, 3.2 ± 0.3, $p<0.001$), ten (4.9 ± 0.2, 3.0 ± 0.3, 3.5 ± 0.2, $p<0.001$), eleven (4.5 ± 0.3, 3.2 ± 0.4, 2.9 ± 0.2; $p<0.001$), twelve (4.8 ± 0.4, 3.8 ± 0.4, 3.1 ± 0.1, $p<0.001$), and thirteen (5.0 ± 0.1, 3.7 ± 0.7, 3.3 ± 0.2, $p<0.001$) weeks of nerve regeneration (Figure 5.3C).

Gait analysis to evaluate toe-spread as a measure of the reinnervation to intrinsic muscles of the foot was performed at 8-14 weeks following nerve injury and autograft repair surgery. At six weeks of nerve regeneration, a wider toe spread, indicating greater reinnervation of the intrinsic muscles of the foot, was identified in animals treated with CES (54.7 ± 2.1% of contralateral uninjured control toe-spread) when compared to PES (29.9 ± 1.5% of contralateral, $p<0.001$) or no-ES negative control animals (29.8 ± 2.1% of contralateral, $p<0.001$). Animals in the CES cohort had a consistently a wider toe spread, which was represented as a percentage of their contralateral paw, when compared to PES or no-ES at eight (62.1 ± 1.1%, 36.7 ± 1.6%, 39.5 ± 2.4%, $p<0.001$), nine (62.1 ± 1.5%, 40.6 ± 2.6%, 43.3 ± 2.1%, $p<0.001$), ten (57.8 ± 2.5%, 41.0 ± 3.2%, 37.2 ± 2.2% , $p< 0.001$), eleven (74.4 ± 5.0%, 43.5 ± 2.2%, 46.5 ± 3.0%, $p<0.001$), twelve (82.2 ± 3.1%, 65.7 ± 1.8%, 48.9 ± 4.2%, $p<0.001$), and thirteen (79.7 ± 5.5, 48.9 ±, 4.6, 45.0 ± 3.8, $p<0.001$) weeks of nerve regeneration (Figure 5.3D).

Nerve conduction studies were performed at 10, 12, and 14 weeks of nerve regeneration. At 10 weeks of regeneration, CMAP amplitude, expressed as a percentage of the uninjured contralateral limb, was poor in all cohorts: CES (9.7 ± 1.6%), PES (6.1 ± 1.5%), and no-ES

(6.2 ± 1.1%). By 12 weeks of regeneration, however, animals treated with CES had a significantly improved CMAP amplitude (58.6 ± 8.7%) when compared to animals treated with PES (13.2 ± 3.4%, $p<0.001$) or without ES (7.6 ± 1.8%, $p<0.001$). Similarly, at 14 weeks of regeneration, CES-treated animals had a significantly greater CMAP amplitude (66.3 ± 16.8%) than PES (16.0 ± 6.9%, $p<0.001$) or no-ES (18.3 ± 3.3%, $p<0.001$) treated animals (Figure 5.4 A, B).

The gastrocnemius muscles of the injured and contralateral uninjured lower limbs were weighed to assess loss of muscle bulk. Results were analyzed as the injured weight as a percentage of the uninjured limb to account for differences in animal size. At 10 weeks of regeneration, the weight of the gastrocnemius muscle on the injured side, represented as a percentage of the contralateral uninjured control, was significantly greater in the CES cohort (22.8 ± 2.3%) than in animals treated with PES (9.3 ± 1.5%, $p<0.01$) or no-ES (11.0 ± 3.6%, $p<0.05$). Similarly, at 14 weeks of regeneration, CES-treated animals had significantly greater muscle weight (79.8 ± 3.0%) than PES (71.2 ± 1.3%, $p<0.05$) or no-ES controls (70.3 ± 1.0%, $p<0.01$) (Figure 5.4 C, D). It was observed that there were significantly more innervated neuromuscular junctions (AchRs) in the CES animal at 14 weeks compared to the PES and no-ES cohorts. Collectively, CES had superior decreased gastrocnemius atrophy, increased NMJ innervation and improved CMAPs compared to the PES and no-ES groups.


A
Contra
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CES
PES
no-ES
B
% Contralateral Side
Number of Weeks of Regeneration
12
13
14
CES PES no-ES
C
Contra CES
Contra PES
Contra No-ES
D
% Contralateral Side
CES
PES
no-ES
10 weeks
14 weeks
E
CES
PES
No-ES
F
Neuromuscular junctions /50 mm²
CES
PES
no-ES

Figure 5.4: CES improves motor recovery in a 0.5 cm neve autograft reconstruction

(A) Representative compound muscle action potential (CMAP) amplitudes identified on nerve conduction studies at 14 weeks of regeneration (B). Line graph representing CMAPs (in % of contralateral side) of each animal were recorded at 10, 12, and 14 weeks post autograft repair surgery. CMAP amplitudes of the injured limb were normalized to the contralateral control. At 12 and 14 weeks of regeneration, animals treated with CES (blue) had significantly greater CMAP amplitude recovery compared to the PES (red, ***$p<0.001$) or no-electrical stimulation (green, ***$p<0.001$) cohorts. (C) Representative photographs of injured (right) and contralateral control gastrocnemius muscle (left) are shown above a histogram displaying muscle weight. (D) Gastrocnemius muscles were harvested at 10 and 14 weeks of regeneration, and weight of the injured muscle was normalized to the contralateral control side. Animals treated with CES had significantly greater muscle weight recovery in the CES (blue) animals compared to the PES (red, *$p<0.01$) and negative controls (green, $p<0.01$) at 10 weeks and 14 weeks. (E) Representative photomicrographs of neuromuscular junctions labelled with alpha-bungarotoxin obtained from the gastrocnemius muscle of animals treated with CES, PES, or no electrical stimulation. All NMJs were confirmed to be innervated by NF200 (data not shown). Animals treated with CES had significantly more innervated acetylcholine receptor compared to the other cohorts (***$p<0.001$).

5.6 Discussion

CES is a clinically feasible means of effectively mimicking the traditional crush conditioning lesion, causing upregulation of regeneration associated genes and conferring an increased length of axon regeneration when compared to negative controls (Senger et al., 2017). We further demonstrated CES significantly improves sensory and motor recovery as assessed with behavioral, electrophysiological, and immunohistochemical analysis (Senger et al., 2019). Clinical translation of CES necessitates a direct comparison to the current 'best practice management' strategies. PES remains the only perioperative technique to improve nerve regeneration that has been successfully translated to, ad integrated in clinical practice.

5.6.1 CES has a greater pro-regenerative effect than PES

Successful functional recovery following peripheral nerve injury is directly related to the time required for regenerating motor or sensory axons to reach their target muscle or sensory receptor. After nerve injury, axons regenerate across the site of surgical coaptation in a 'staggered' distribution rather than as a unified front. This process is termed 'staggered regeneration' and results in a temporary stall in axon extension, delaying end target reinnervation. Delivery of one hour of PES immediately following surgical nerve repair improves the efficiency of staggered regeneration, decreasing this delay and thereby accelerating time to reinnervation (Brushart et al., 2002). PES has long been established as an effective and safe technique to enhance peripheral nerve regeneration. Pioneered by Nix and Hopf in the 1980s (Nix & Hopf, 1983), PES has since been thoroughly investigated and shown to improve regeneration and reinnervation in animal (Al-Majed, Neumann, Brushart, & Gordon, 2000; Al-Majed, Tam, & Gordon, 2004; Geremia, Gordon, Brushart, Al-Majed,

& Verge, 2007) and human models of peripheral nerve injury(Barber et al., 2018; Tessa Gordon, Brushart, Amirjani, & Chan, 2007; Wong et al., 2015).

The regenerative effects of PES are limited, as this technique enhances regeneration only at the site of surgical coaptation, and does not affect the rate of axon extension along the distal stump (Brushart et al., 2002). A conditioning lesion, by contrast accelerates the intrinsic rate of nerve regeneration. These differences in mechanism underline our results suggesting that nerves treated with CES prior to transection and repair have significantly greater regeneration and functional recovery compared to nerves treated with PES. The differences between these two techniques may be particularly important in a human model of peripheral nerve injury rather than a rodent, given differences in size. If PES accelerates regeneration only at the size of coaptation for a proximal nerve injury, regenerating axons have a great distance to regenerate which will proceed at the baseline rate of 1 mm/day, as PES has no effects distal to the site of injury. By contrast, CES accelerates the rate of nerve regeneration and therefore has the potential to significantly decrease the amount of time necessary for end target reinnervation, thereby improving functional recovery.

5.6.2 CES enhances regeneration and reinnervation in a nerve autograft model

The nerve gap is a common challenge for the peripheral nerve surgeon. Primary repair under tension precludes regeneration and worsens outcomes, therefore reconstruction alternatives are required to bridge the gap. Nerve autografting is the 'gold standard' technique for overcoming a nerve gap (Huang et al., 2009) due to the relative technical simplicity and

availability of donor nerves. In addition to traumatic injuries of the upper and lower limbs, nerve grafts are an important tool in the treatment of facial paralysis and for oncologist reconstruction. Though PES is well studied in animal and human models of primary coaptation, the effects of electrical stimulation on promoting regeneration through a graft and two sites of coaptation are poorly understood. Our findings suggest that while PES may be beneficial in early regeneration, it has no effect on reinnervation outcomes. These findings are in concordance with Witzel et al, who delivered electrical stimulation (1 hour, 20Hz) to the sciatic nerve at the time of nerve grafting (1 cm graft). When compared to non-stimulated control grafts, they found that animals receiving PES had a significant increase in regenerating axons within the grafted nerve with greater arborization at 5 and 7 days post-grafting, but no significant difference in length or speed of regeneration (Witzel et al., 2016). In keeping with our findings, Huang et al (2009) found that 1 hour of electrical stimulation delivered at the time of nerve grafting (1 cm) accelerated regeneration across the nerve gap; while PES improved initial motor recovery based on gait analysis and nerve conduction studies, final outcomes did not differ between animals who received PES and those that did not (Huang et al., 2009). PES was, however, shown to improve outcomes when a long nerve gap was reconstructed using a longitudinal scaffold (Huang, Lu et al. 2010). Regeneration outcomes with allograft or nerve conduits have poorer outcomes than autograft repair; therefore, the effects of PES on enhancing outcomes in these models may be more apparent.

Unlike PES, animals treated with CES prior to autograft nerve reconstruction had significantly improved regeneration and sensorimotor reinnervation outcomes at all timepoints investigated. CES may therefore be a promising technique to improve outcomes

for patients undergoing elective autograft repair where a large nerve gap is anticipated, such as in oncologic resections, cross-face nerve grafting for facial reanimation, or in the presence of significant soft tissue damage. The decision to perform nerve autograft reconstruction, however, is often not made until intraoperatively when the size of the nerve gap can be adequately assessed. Given the atraumatic nature of electrical stimulation, delivering CES prior to surgery is not anticipated to confer substantial risk to the patient. If it is shown that CES is as well-tolerated and safe as PES, patients with a nerve injury suspected to require grafting could still be treated with CES prior to exploration without increasing the potential risk of injury or discomfort to the patient. Human trials are therefore critical to determining the safety profile and patient acceptance of this technique.

5.7 Conclusions

Conditioning electrical stimulation significantly improves outcomes following peripheral nerve regeneration. Guiding best practice patient management requires a comparison of outcomes of CES with the only perioperative intervention currently used routinely in clinical practice, postoperative electrical stimulation. Our results suggest that CES enhances regeneration and reinnervation outcomes greater than PES. We furthermore show that the combination of CES and PES does not have a synergistic effect as we hypothesized, but rather delivering PES to a conditioned nerve appears to decrease the conditioning effect. Further research to determine the molecular mechanism of CES, and how PES affects these processes is required to explain this relationship. Finally, we suggest that CES may be a clinically feasible preoperative technique to significantly improve regeneration and reinnervation outcomes in autograft nerve regeneration.

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Chapter 6

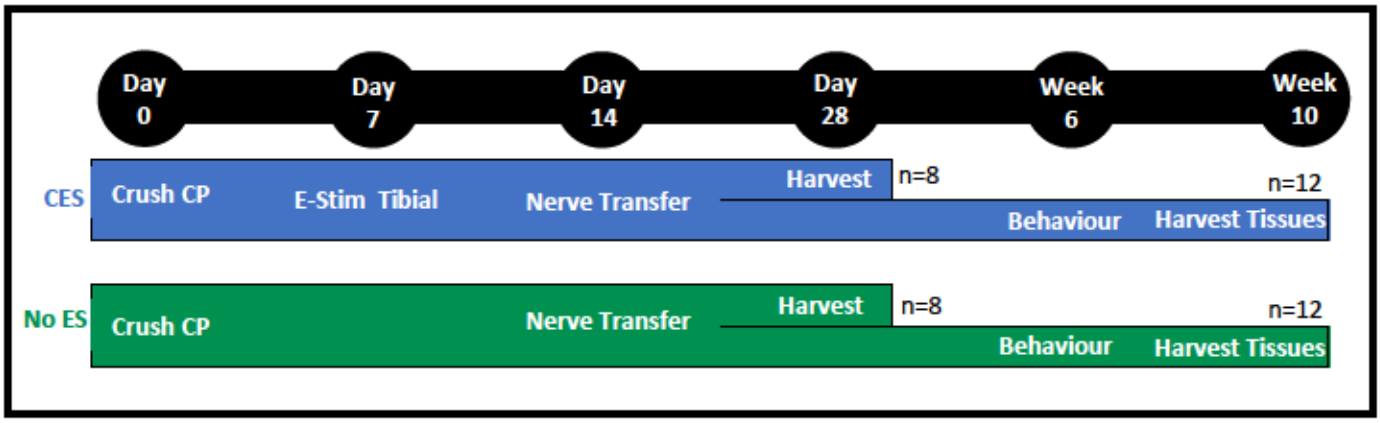
Electrical Stimulation as a Conditioning Strategy for Promoting Peripheral Nerve Regeneration in a Common Fibular Nerve Injury Model

6.1 Preface

A common criticism of the conditioning lesion is its apparent irrelevance to a clinical population. As traumatic nerve injuries are typically unplanned accidents, it is commonly believed to be impossible to intervene prior to injury. Recent trends in peripheral nerve surgery favoring the distal nerve transfer (DNT) have changed the management of proximal and/or severe peripheral nerve injuries. In this surgery, a branch of a non-injured 'donor' nerve is cut and coapted to the distal stump of the injured nerve to supply the target denervated muscle. As DNTs are elective surgery, the timing of transection for the donor nerve is known; therefore, delivery of conditioning to this nerve is clinically feasible.

In Chapter 6, we describe the effects of CES on promoting regeneration in a rodent model of a DNT that is currently used in clinical practice to treat foot drop. The common fibular nerve was crushed in all animals to replicate the clinical scenario of a traumatic nerve injury. CES was delivered to the tibial nerve in half the animals one-week post-injury. A DNT was performed seven days following conditioning, in which the tibial branch to the lateral gastrocnemius was cut and coapted to the distal stump of the common fibular branch to restore tibialis anterior function.

The time-line below outlines the 2 experimental paradigms: a common fibular nerve crush injury with conditioning (blue) or without conditioning (green) of the tibial nerve was followed by DNT and either 1) nerve harvest 2 weeks following to measure the extent of nerve regeneration, or 2) behavior testing from weeks 6-8 followed by harvesting target tissue.



Using a series of kinetic (ankle-tibial angle), kinematic (force analysis), skilled locomotion (horizontal ladder test), electrophysiologic (nerve conduction studies), and immunohistochemical (neuromuscular junction analysis) outcomes, we found that animals treated with CES prior to nerve transfer had significantly greater functional recovery compared to animals treated with a DNT alone.

Finally, we demonstrate that the tibial nerve was not injured by CES, with no dorsiflexion functional deficits at the ankle and no evidence of Wallerian degeneration or macrophage-mediated inflammation in response to the electrical stimulation. This is an important observation given that clinically the CES will need to be delivered to the main nerve, not only the single branch that will be surgically transferred. Injury to the main trunk of the donor nerve with iatrogenic denervation of downstream targets would be unacceptable and therefore an atraumatic conditioning technique is necessary for pre-DNT CES to be clinically accepted.

Electrical Stimulation as a Conditioning Strategy for Promoting Peripheral Nerve Regeneration in a Common Fibular Nerve Injury Model

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6.2 Abstract

Conditioning electrical stimulation (CES), one hour of electrical stimulation delivered to a nerve seven days prior to a nerve injury and repair surgery, accelerates nerve regeneration and promotes functional sensorimotor recovery. Clinical translation of this clinically safe and well-tolerated conditioning paradigm has been impeded by the necessity to treat the nerve prior to injury, which is often an unpredictable event. Recent trends in surgical techniques for treating severe nerve injuries, however, favour the distal nerve transfer (DNT), a surgery in which a branch of an uninjured functioning nerve is transected and re-routed to innervate a denervated nerve stump. The DNT surgery is elective and therefore the time of nerve transection and repair is known; thus, delivering CES to the donor nerve one week prior is clinically feasible.

A common DNT surgery to treat foot drop entails rerouting the tibial nerve's branch to the lateral gastrocnemius muscle to the denervated common fibular nerve stump in attempt to reinnervate the tibialis anterior muscle and thus allow foot dorsiflexion during the heel-rise stage of the step cycle. To replicate the clinical scenario of this traumatic nerve injury, we crushed the common fibular (peroneal) nerve in our adult rat animal model. CES was delivered to the uninjured tibial nerve branch in half the animals one week after the common peroneal nerve injury. Seven days following CES, we performed the DNT. Animals treated with CES prior to nerve transfer had significantly improved motor reinnervation and functional recovery, as evidenced by a series of kinetic, kinematic, skilled locomotion, electrophysiologic, and immunohistochemical outcomes. We further demonstrated that the distal tibial nerve was not injured by CES, as there were no functional deficits in the

remaining tibial-innervated muscles. Immunohistochemical analysis of the conditioning site and the distal nerve had no evidence of Wallerian degeneration or macrophage-mediated inflammation. In summary, the conditioned DNT from the tibial branch to lateral gastrocnemius muscle significantly improved functional recovery of the common fibular nerve and its muscle targets without causing a deficit to the distal tibial nerve.

6.3 Introduction

Common fibular (common peroneal) nerve injury results in foot drop due to denervation of the tibialis anterior muscle impeding active dorsiflexion. This injury significantly impacts all aspects of a patient's life, often precluding professional and recreational activities (Nath, Lyons et al. 2008). Historically, the only treatment options were the lifelong use of an ankle-foot orthotic (AFO) or surgical tendon transfer. More recently, reinnervating the distal stump of the degenerated common peroneal nerve with a branch of the tibial nerve in a distal nerve transfer (DNT) has gained popularity as the treatment of choice for this patient population (Giuffre, Bishop, Spinner, Levy, & Shin, 2012; Nath, Lyons, & Paizi, 2008). Clinical outcomes suggest cortical relearning from DNT nerves innervating disparate motor pools occur spontaneously although training further improves functional outcomes (Anastakis, Malessy, Chen, Davis, & Mikulis, 2008; Brown, Shah, & Mackinnon, 2009). Overall however, outcomes following DNT are often poor despite a shorter distance to regenerate. Successful outcomes remain critically dependent on i) the speed of nerve regrowth and ii) preferential motor reinnervation (Brushart, 1988)).

Our previous work has shown that conditioning electrical stimulation (CES) greatly improves the regenerative potential of both the common fibular and tibial nerves (Senger et al., 2019; Senger, Chan, Verge, & Webber, 2017). In fact, we demonstrated that CES increases the sensorimotor functional recovery beyond that of the gold-standard regeneration paradigm, the conditioning nerve lesion. In these studies, the healthy uninjured common fibular or tibial nerves underwent CES one week prior to a nerve cut and microsurgical repair; thus, the CES was performed prior to nerve injury. DNT surgery is the ideal model to

recapitulate our CES studies as, similar to our common fibular and tibial nerve studies, the electrical stimulation is performed on an uninjured healthy nerve branch. One week later, the CES-treated donor tibial nerve branch will be cut and coapted to the dennervated distal nerve stump of the injured common fibular nerve. We hypothesized that delivering one hour of CES to the donor nerve one week prior to DNT will significantly improve regeneration and reinnervation outcomes, resulting in improved foot dorsiflexion and sensorimotor recovery.

6.4 Methods

All animal procedures followed the ARRIVE guidelines. Adult male Sprague Dawley rats (200 g, Charles River) were placed under the care of the Health Sciences Laboratory Animal Services (HSLAS) at the University of Alberta. Sixteen animals were used to analyze the length of nerve regeneration (n=8/cohort of CES and unconditioned/no-electrical stimulation, no-ES), and 24 animals were used for behavioural testing to investigate motor recovery (n=12/cohort of CES and unconditioned). All animals underwent denervation of the common fibular nerve; one week later half of the animals were treated with CES. DNT surgery was performed in all animals 14 days following common fibular nerve denervation and the animals were placed back into their home cages until nerve growth or behaviour was analyzed 2 weeks and 2 months later, respectively. In a separate experiment in which the above methods were replicated, 9 animals were used to investigate the potential inflammatory and injury response induced by CES compared to a positive control crush injury, or a negative control unconditioned nerve (no-ES; n=3/cohort).

6.4.1 Denervation surgery: Animals were anesthetized with inhalational isoflurane (2% titrated at 1-2 L/min to maintain a surgical anesthetic plane) and injected subcutaneously with 0.01 mg/Kg of buprenorphine for analgesia. Under 3.5x loupe magnification, the common fibular nerve was identified through an incision along the lateral aspect of the leg, and the nerve was crushed at the level of the fibular neck. The skin was closed with 4-0 Vicryl suture (Ethicon Inc., Somervile, NJ) and the animal was returned to its cage. Another dose of buprenorphine was delivered to each animal 18 hours after surgery.

6.4.2 Conditioning of tibial nerve: CES to the tibial nerve was delivered as previously described (Senger et al., 2019). Briefly, animals were anesthetized as above and the posterior mid-thigh skin was cut to identify the tibial nerve deep to the biceps femoris muscle, distal to the site of the sciatic nerve trifurcation. A stainless-steel cathode wire with ends bared of insulation was wrapped around the tibial nerve at the level of the bifurcation of the gastrocnemius head, and the anodal wire was placed into the belly of the tibialis anterior muscle. These wires were connected to an SD-9 stimulator (Grass Instruments Co., Quincy, MA) and a continuous train of biphasic electrical stimulation at 20 Hz of 0.1 ms duration was delivered to the nerve, with the voltage titrated to maintain a visible twitch in the lower limb flexors. After one hour of stimulation, the wires were removed and the skin was repaired with 4-0 Vicryl suture. Our previous studies of CES in the common fibular and tibial nerves demonstrated similar results for both the sham-ES (in which the skin was cut and the unconnected wires were placed alongside the nerve) and unconditioned negative controls (Senger et al., 2019; Senger et al., 2017). Thus the act of preparing the animal for electrical

stimulation was not responsible for the improved regeneration seen for the CES cohorts and therefore only unconditioned (no-ES) animals were included herein as negative controls.

6.4.3 Distal nerve transfer: Animals were anesthetized and analgesia was provided as described above. The skin was incised at the posterior midthigh, and using a Leica operating microscope, the sciatic nerve was identified, and its tibial and common fibular nerve branches were isolated. The tibial nerve was dissected distally and the branch of the tibial nerve innervating the lateral head of the gastrocnemius muscle was identified by a palpable twitch using a nerve stimulator. The branch to the gastrocnemius muscle was transected as distally as possible to maximize the length of nerve available for transfer. The site of common fibular nerve crush (performed 2 weeks prior) was identified and the nerve was re-transected distal to the first injury site. The proximal end of the transected tibial branch was aligned with the transected distal stump of the common fibular nerve and Evicel fibrin sealant (Ethicon) was applied to the coaptation site to maintain the nerve coaptation. Once the Evicel solidified, the hamstrings were re-suspended and the skin was closed with 4-0 Vicryl suture.

6.4.4 Evaluation of injury response: In a separate cohort, 9 animals were anesthetized and provided with analgesia as above prior to skin opening at the mid-thigh level to expose the tibial nerve. The tibial nerve was treated with a) CES as described above (n=3), b) a positive control conditioning crush injury delivered by a non-toothed thin hemostat for ten seconds (n=3), and c) a negative control cohort in which the animals received no conditioning surgery (n=3). On day 7, the tibial nerves were harvested and macrophage immunostaining as well

as morphological observation of the NF200 labeled axons was performed at the conditioning site.

6.4.5 Tissue analysis: Animals were euthanized at 7 days (evaluation of injury response), 14 days (evaluation of length of regeneration), or 10 weeks of regeneration (evaluation of functional outcomes) by inhalational carbon dioxide and exsanguination by left cardiac ventricle puncture. Nerve tissue was collected by identifying and harvesting the site of the conditioning (to evaluate for an injury response) or of the DNT (to evaluate length of axon extension). Once harvested, the nerve was fixed in Zamboni's fixative (paraformaldehyde, picric acid, NaOH, American MasterTech Scientific, Lodi CA) for immunofluorescence to determine axon integrity. Tissue was fixed for four hours, rinsed in 0.01 M phosphate buffered saline (PBS) (Thermo Fisher Scientific, Waltham MA) five times, post-fixed in 30% sucrose solution overnight at 4°C, then frozen in Optimum Cutting Temperature (OCT) (Sakura Finetek, Torrance CA) using indirect exposure to liquid nitrogen. Using a crytostat (Leica), the nerves were cut longitudinally into 12 µm. sections and thaw-mounted on Superfrost Plus microscope slides (Thermo Fisher Scientific) and stored at -80°C until processing.

At 10 weeks of regeneration, the entire tibialis anterior muscles were harvested bilaterally and both muscles were weighed to determine the percentage of muscle loss on the affected side compared to the uninjured contralateral control (n=normalized wet weight of the tibialis anterior muscle/animal). Muscles from the injured side were fixed in Zamboni's solution overnight, rinsed five times with PBS, sunk in 30% sucrose overnight and frozen in OCT.

Twenty micron (20 μm) sections were cut in the cryostat and stored at -80°C for future neuromuscular junction innervation analysis.

6.4.6 Immunofluorescence: Experimenter was blinded to all treatment conditions for all analyses. Slides were warmed to room temperature and underwent twenty minutes of antigen retrieval in a 60°C citrate buffer (10mM sodium citrate, 0.05% Tween- 20, pH. 6.0), then cooled to room temperature. Slides were washed three times for five minutes each in 0.01 M PBS, permeabilized with 0.1% Triton-100x (Thermo Fisher Scientific) for 10 minutes, and blocked in 10% normal goat serum (MP Biomedicals, Santa Ana CA) and 3% bovine serum albumin (BSA) (Sigma-Aldrich, St Louis MO) in 0.01 M PBS for 90 minutes. Primary antibody mouse anti-neurofilament 200 (1:500, Sigma-Aldrich) or CD68 (1:50) was diluted in 0.01 M PBS and 3% BSA and applied to muscle and nerve sections. The following day, the slides were washed three times for five minutes each with 0.01 M PBS. Secondary antibody Cy3-conjugated goat anti-mouse (1:500, Sigma-Aldrich) or Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad CA) was applied for one hour to the muscle and nerve sections respectively. Conjugated anti-α-bungarotoxin (1:1000) was used to stain the acetylcholine receptors (AchRs) in the muscle sections. All slides were mounted onto coverslips with 50% glycerol in 0.01 M PBS.

In the cohort of animals used to analyze the length of nerve regeneration, the histomorphology of the NF200 labeled axons were evaluated to differentiate regenerating and degenerating axons (n=average length of regeneration/animal). The length of axon extension into the distal stump of the common fibular nerve in DNT nerve sections was measured from the site of nerve coaptation between the tibial and common fibular branches.

The distal-most site of regeneration was defined as the presence of a minimum of 10 regenerating axons.

In the cohort of animals used to evaluate for an injury response, nerves treated with CES 7 days prior were compared to positive-control conditioning crush lesions, and negative-control unconditioned nerves. Distal to the site of conditioning, the histomorphology of the NF200-labeled axons were observed for evidence of Wallerian degeneration, and to assess for CD68-positive macrophage infiltration (n=average CD68-positive cells/animal).
Muscle sections were evaluated for neuromuscular junction (NMJ) innervation. Six muscle sections per animal were assessed. The number of innervated endplates, as evidenced by co-staining with NF-200 (axons) and alpha-bungarotoxin (acetylcholine receptors, AChRs) was quantified by averaging the total number of NMJs in 10 separate tissue sections per animal (n=average number of innervated NMJs/animal).

6.4.7 Behavioural outcomes: Behavioural testing was performed weekly from 6-10 weeks of regeneration. Gait analysis and the horizontal ladder tests were performed in a blinded fashion. The horizontal ladder test was performed by placing animals in an elevated ladder with plexiglass walls and metal ladder rungs placed in variable positions with each testing session. Animals were filmed crossing the ladder three times per testing session, and videos were analyzed frame-by-frame and each individual foot placement on a ladder rung was graded on a scale of 0-6 as described by Metz and Whishaw, where 0 is a total miss and 6 is scored as a correct placement (Metz & Whishaw, 2009). The average score for each attempt on the ladder was averaged for the uninjured limb (n=average score/animal).

Kinetic and kinematic data were collected simultaneously, and the methods were modified from previous studies (Allen et al., 2012; McNeill, Wu, Rabey, Schmitt, & Guilak, 2014; Schmitt, Zumwalt, & Hamrick, 2010; Zumwalt, Hamrick, & Schmitt, 2006). Briefly, rats were placed in a custom-built force plate runway (60 cm x 15 cm) composed of three transparent acrylic sides, with a back wall and floor made of wood. The runway was equipped with a pressure plate modified to collect single foot contacts embedded flush with the runway at midpoint. Animals explored the runway for 30 minutes weekly (7, 8, 9, and 10 weeks of regeneration). When the rat's limb struck the isolated section on the floor where the platform was located, ground reaction forces (GRFs) were collected using Bioanalysis with NetForce software (Advanced Mechanical Technology Inc., Watertown, MA). Two orthogonal components of GRFs were collected: vertical (out of the floor – weight borne by the limb during locomotion), braking/propulsion (direction of travel). The number of trials varied per animal since each rat was never forced to walk on the plate, and instead it was encouraged to explore the runway on its own. This, along with keeping the room quiet, minimized stress of the rats to obtain locomotion under normal conditions. For each testing session, three trials were performed and the number of contacts for each hindlimb (right and left) per animal were analyzed (n = 72 total contacts for each CES and no-ES/week). Force-plate data were imported into Matlab (Mathworks, Natick, MA) filtered with a 25 Hz low-pass filter and normalized to body weight and velocity. This normalization allows GRFs to be described by dimensionless terms that are less sensitive to differences in an animal weight and changes in stance time between trials (Allen et al., 2012).

Animals were filmed from two separate cameras at 120 frames/second to permit calculation of velocity of movement and kinematic data. Only videos where the animal was moving comfortably and steadily (e.g., without hesitating or stopping on or immediately after the force plate) were selected. Videos were converted to AVIs using MPEG Streamclip (Squared 5, Rome, Italy) and digitized using DLT Viewer with MatLAB (Mathworks, Natick, MA) and processed using MatLAB scripts written for this project. Stance and stride times were collected and at least three trials for each limb per animal were analyzed (minimum of 36 strides per animal/week). Stance and stride times were used to calculate limb duty factor (stance time of limb/stride time of limb). Rats walk with a symmetrical gait (left and right duty factors are similar for all limbs) and deviation from this pattern can easily be identified. The final duty factor was calculated by taking the left hindlimb duty factor minus the right hindlimb duty factor.

Kinematic data of the ankles during stance phase were collected using the stills from the videos, segments of the leg and foot from both hindlimbs were defined as follows: the proximal lower third of the tibia, lateral malleolus, calcaneus, and fifth metatarsal head (Varejao et al., 2002). The sagittal ankle angle was calculated as the difference between the foot angle and leg angle (θ ankle = θ foot – θ leg -90°). If θ ankle was positive, the foot was considered dorsiflexed, if negative, the foot was considered plantar flexed. Angles were collected at four different points during stance phase: 1) Initial contact (IC): at the time of initial contact (IC) of the measured limb (during normal walking, the ankle should be plantar flexed). 2) Initial contact-opposite toe off (OT): as the opposite limb toes off, ankle is usually also found at plantarflexion but going towards dorsiflexion. 3) Opposite heel-rise (HR):

maximum stance phase, where during a normal stride, the measured ankle should be in dorsiflexion. 4) Toe-off (TO): moment in the stance phase where dorsiflexion is replaced once again by plantarflexion.

6.4.8 Statistical analysis: Results are presented as mean ± standard error of the mean. Groups were compared using Student's t-tests followed by a Dunnett's post-hoc analysis. Statistical significance was accepted with a level of $p<0.05$. All statistical analyses were performed using STATA 14 (StataCorp LP, Collagen Station, Texas).

6.5 Results

6.5.1 CES enhances length of regeneration through a DNT

To mimic a foot drop injury, the common fibular nerve was crushed prior to its bifurcation into the superficial and deep fibular branches (Day 0, n=16) (Figure 6.1). One week later, half of the animals underwent tibial nerve CES and on day 14, all animals underwent DNT surgery in which the tibial nerve branch to the lateral head of the gastrocnemius muscle was transected and coapted to the distal stump of the common fibular nerve (Figure 6.1C). Nerve regeneration at 2 weeks following DNT was measured (Figure 6.2). In CES animals, new

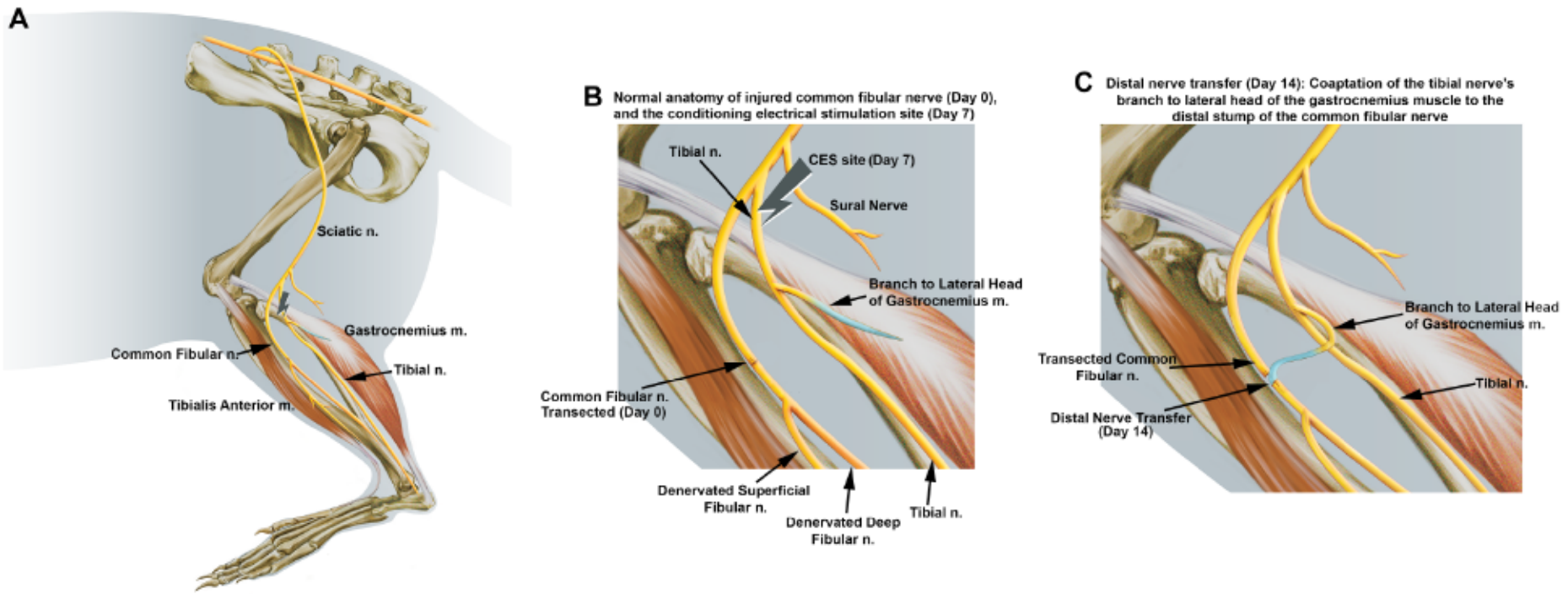

A
Sciatic n.
Gastrocnemius m.
Common Fibular n.
Tibial n.
Tibialis Anterior m.
B
Normal anatomy of injured common fibular nerve (Day 0), and the conditioning electrical stimulation site (Day 7)
Tibial n.
CES site (Day 7)
Sural Nerve
Branch to Lateral Head of Gastrocnemius m.
Common Fibular n. Transected (Day 0)
Denervated Superficial Fibular n.
Denervated Deep Fibular n.
Tibial n.
C
Distal nerve transfer (Day 14): Coaptation of the tibial nerve's branch to lateral head of the gastrocnemius muscle to the distal stump of the common fibular nerve
Branch to Lateral Head of Gastrocnemius m.
Transected Common Fibular n.
Tibial n.
Distal Nerve Transfer (Day 14)

Figure 6.1: Diagrammatic Representation of DNT to mimic Foot Drop Repair Surgery

(A) Cartoon diagrams to show the normal anatomy of the sciatic nerve and its three main nerve branches at its trifurcation site. The tibial nerve innervates the gastrocnemius muscle whereas the common fibular nerve innervates the tibialis anterior muscle which is responsible for ankle dorsiflexion of the toes during the step cycle. B) For the nerve length and the behavioral analyses, all animals first received common fibular nerve transection on Day 0 and one week later, half of those animals received CES (depicted as a lightning bolt). C) All animals underwent DNT surgery on day 14 in which the tibial branch to the lateral head of the gastrocnemius muscle (depicted as the blue nerve) was relocated to the distal stump of the common fibular nerve.

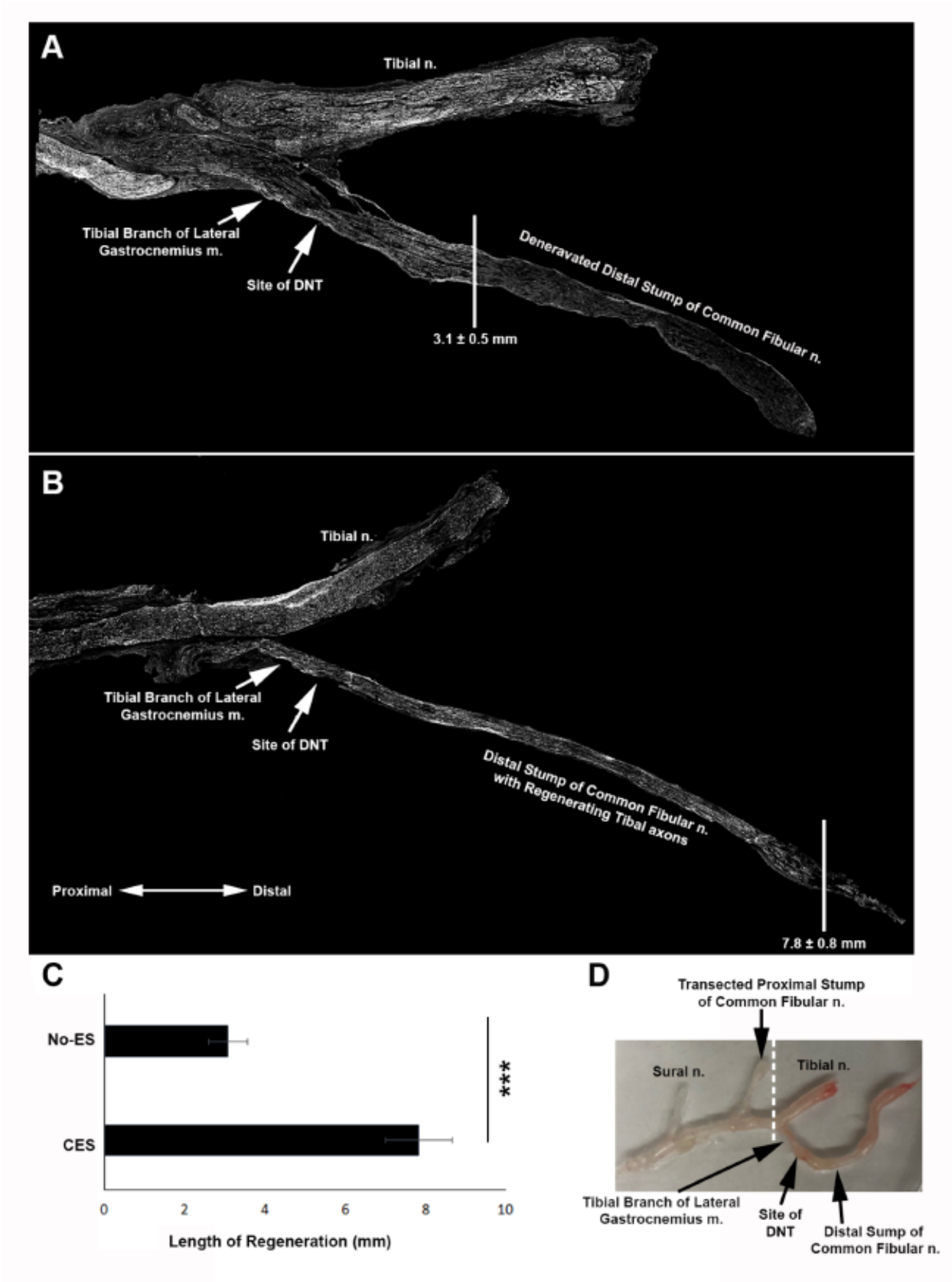

A
Tibial n.
Tibial Branch of Lateral Gastrocnemius m.
Site of DNT
3.1 ± 0.5 mm
Deneravated Distal Stump of Common Fibular n.
B
Tibial n.
Tibial Branch of Lateral Gastrocnemius m.
Site of DNT
Distal Stump of Common Fibular n. with Regenerating Tibal axons
Proximal
Distal
7.8 ± 0.8 mm
C
No-ES
CES
***
0
2
4
6
8
10
Length of Regeneration (mm)
D
Transected Proximal Stump of Common Fibular n.
Sural n.
Tibial n.
Tibial Branch of Lateral Gastrocnemius m.
Site of DNT
Distal Sump of Common Fibular n.

Figure 6.2: CES to the uninjured tibial nerve accelerated axon regeneration into the distal stump of the common fibular nerve following DNT surgery.

A-B) NF200 immunolabeling of the nerve at the surgery site two weeks post-DNT. The representative tissue sections depict the tibial nerve branch in which the nerve to the lateral head of the gastrocnemius muscle was transferred to the distal stump of the common fibular nerve. The 'site of the DNT' is identified morphologically by observing each tissue section (operator is blinded to tissue identity) and by the identification of the tibial nerve branch. The extent of regeneration into the common fibular nerve distal stump was measured at 0.5 mm intervals with a minimum of 10 axons required at each site. Using z-stacks and sewing the microscopy images together with ImageJ, the length of the regeneration into the common fibular nerve stump is shown for both the no-ES (A) and the CES (B) cohorts. (C) A histogram illustrating that the no-ES tibial nerve had 3.1 ± 0.49 mm nerve growth into the common fibular nerve stump whereas the CES treated donor tibial nerve regenerated on average 7.8 ± 0.8 mm. Student's t-test, n=average length of regeneration/animal, ***$p<0.001$. (D) Dissection of the sciatic nerve and its trifurcation depicts the successful coaptation of the tibial nerve branch to the common fibular nerve stump. Dotted white line represents the approximate location from which the tissue sections (A, B) are shown.

axonal growth from the coaptation site extended 7.8 ± 0.8 mm into the common fibular nerve stump, which was significantly longer than the 3.1 ± 0.5 mm of axonal extension in the no-ES nerve transfers ($p<0.001$) (Figure 6.2A-C). Dissection of the sciatic nerve trifurcation and a successful DNT demonstrates the effectiveness of the surgical intervention (Figure 6.2D).

5.5.2 CES enhances functional recovery

Animals from both cohorts started the functional trials during week 7 of regeneration at a slow walking speed (CES 52.1 ± 6.9 cm/s; no-ES 50.5 ± 5.8 cm/s) but were trotting by the end of week 10 (CES 65.0 ± 2.9 cm/s; no-ES 61.7 ± 3.3 cm/s) (Figure 6.3A). Both cohorts had a similar continual increase in velocity with time, however the CES group had a significantly greater increase in speed at 8 (CES 56.5 ± 5.2 cm/s; no-ES 54.2 ± 3.9 cm/s; $p=0.03$), 9 (CES 62.3 ± 3.9 cm/s; no-ES 58.2 ± 4.1 cm/s; $p<0.001$), and 10 ($p<0.001$) weeks of regeneration.

Rats typically walk with a balanced, symmetrical gait with similar vertical peak forces between the two hindlimbs. Vertical peak forces are exerted by the floor onto the animal, indicating the weight borne by the limb during locomotion. In all cohorts at week 7 of regeneration, there was an increased vertical peak force in the contralateral (left) hindlimb (Figure 6.3B; CES 0.81 ± 0.03; no-ES 0.82 ± 0.03) and a concomitant decreased vertical peak force in the injured hindlimb (CES 0.60 ± 0.05; no-ES 0.59 ± 0.05). This indicates that animals in both cohorts were initially bearing less weight on their injured hindlimb compared to their contralateral hindlimb ($p<0.001$). At weeks 8-10 in the CES animals, there were significant shifts in vertical forces towards the right (injured) hindlimb compared to the no-

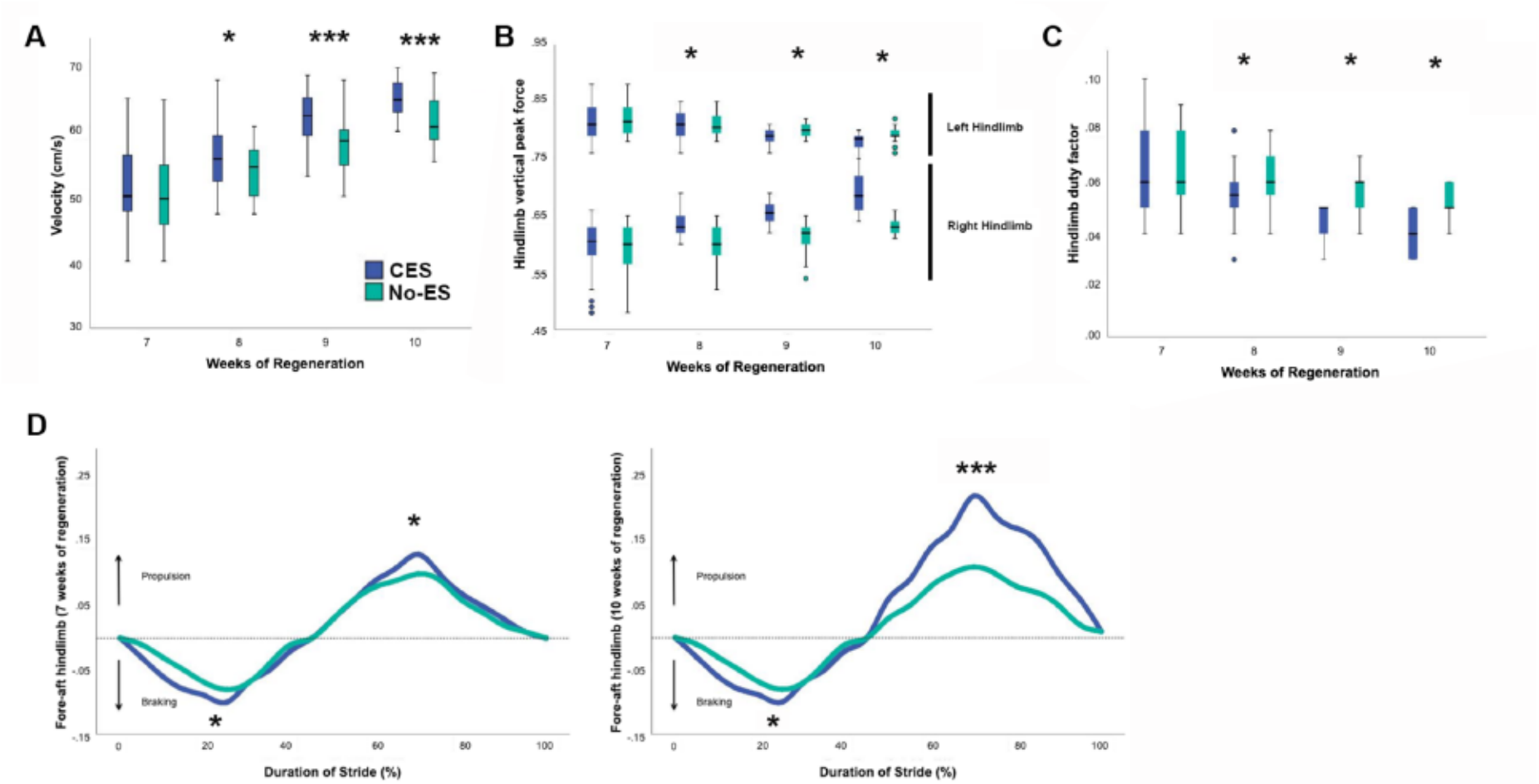

A
Velocity (cm/s)
Weeks of Regeneration
CES
No-ES
B
Hindlimb vertical peak force
Weeks of Regeneration
Left Hindlimb
Right Hindlimb
C
Hindlimb duty factor
Weeks of Regeneration
D
Fore-aft hindlimb (7 weeks of regeneration)
Propulsion
Braking
Duration of Stride (%)
Fore-aft hindlimb (10 weeks of regeneration)
Propulsion
Braking
Duration of Stride (%)

Figure 6.3: CES improves force equalization following DNT surgery

A) Boxplots representing the average walking speed of the animals at weeks 7-10. B) Boxplots of vertical (Fz) peak forces (largest vertical force achieved) for each limb. The force data normalized by body mass (n = 72 contacts/cohort/week). Forces on the contralateral limb were higher for both CES and no-ES cohorts throughout weeks 7 to 10 of regeneration which was similar to the vertical peak force of both limbs from an uninjured animal (normal gait would have both sides exhibiting similar ground reaction forces, data not shown). The injured hindlimb vertical peak force was similarly low at 7 weeks in the CES and no-ES cohorts, however the CES animals started to equalize their hindlimb forces on both sides at a significantly higher rate than the no-ES group at weeks 8-10 ($p<0.05$). C) Boxplots of hindlimb duty factor demonstrated that throughout the weeks of regeneration (n = 36 strides/cohort/week), the animals spent more time on the contralateral (left) side (significantly above zero, representing an uninjured animal's duty factor). The CES group started to balance their gait by week 8 of regeneration and significantly increased the time spent on their injured (right) limb compared to the no-ES group (week 8-10) ($p<0.01$). D) Fore-aft (Fx) forces normalized by body mass (n = 72 contacts/cohort/week). Braking forces of the CES animals were significantly improved at both weeks 7 and 10 compared to the no-ES cohorts ($p<0.05$). Propulsive forces were significantly higher in the CES group at week 7 ($p<0.05$) which improved well beyond the no-ES cohort at week 10 ($p<0.001$).

ES animals (week 8: CES 0.63 ± 0.02; no-ES 0.6 ± 0.04, p=0.007; week 9: CES 0.65 ± 0.02; no-ES 0.62 ± 0.02, p=0.004: week 10: CES 0.69 ± 0.03; no-ES 0.63 ± 0.01, p<0.001). As more weight was borne on the injured limb with each week of recovery, especially in the CES group, a small reduction of forces on the contralateral (left) hindlimb was observed (week 9: CES 0.79 ± 0.01; no-ES 0.80 ± 0.01, p=0.04; week 10: CES 0.78 ± 0.01; no-ES 0.79 ± 0.01, p=0.04).

Duty factor represents the percentage of time a hindlimb is in contact with the ground. A normal symmetrical gait is represented by a value of zero (left duty factor – right duty factor), while a positive duty factor indicates the animal is spending more time on his uninjured hindlimb and less on his injured hindlimb. At 7 weeks, all animals had a high positive hindlimb duty factor (CES 0.064 ± 0.016; no-ES 0.065 ± 0.015). Similarly to the weekly improvement of vertical ground reaction forces in CES animals, the CES cohort had a significant decrease in duty factor in weeks 8-10 (week 8: 0.056 ± 0.012, p=0.01; week 9: 0.046 ± 0.005, p<0.001; week 10: 0.041 ± 0.008, p<0.001) compared to the no-ES group (week 8: 0.063 ± 0.012; week 9: 0.058 ± 0.009; week 10: 0.051 ± 0.007) (Figure 6.3 C). As the CES group increased motion of the injured ankle, so did the opposite toe-off moment of the left limb, indicating less time spent on the contralateral limb with increased joint motion (data not shown).

Braking forces are the forces opposite the direction of travel (negative value) and thus slow the forward translation of the centre of mass when a limb initially contacts the ground. Later in the stride cycle, the limb generates propulsive forces (positive value) to translate the centre

of mass forward. At week 7 of regeneration, both cohorts had reduced braking (CES -0.11 ± 0.038; no-ES -0.07 ± 0.035) and propulsive (CES 0.13 ± 0.042; no-ES 0.1 ± 0.023) forces of the hindlimbs, however CES had significantly higher braking/repulsive (fore-aft) forces compared to no-ES controls ($p<0.05$) (Figure 6.3D). The CES group showed almost complete recovery of the normal braking (CES -0.12 ± 0.041; no-ES -0.075 ± 0.03, $p=0.03$) and propulsion (CES 0.22 ± 0.031; no-ES 0.11 ± 0.037, $p<0.001$) forces by week 10 of regeneration in hindlimbs.

The ability to dorsiflex the foot during a step cycle (i.e., ankle angle) was measured by subtracting the leg angle (horizontal plane in relationship to the tibia) from the foot angle (horizontal plane in relationship to calcaneus) (Figure 6.4A) (Varejao et al., 2002). At all time points measured, the contralateral limbs demonstrated the normal dorsiflexion and plantar flexion motions within the step cycle (Figure 6.4B). At week 7 of regeneration, dorsiflexion (usually peaking during heel-rise, HR) was not achieved by either cohort (CES -11.6 ± 2.32°; no-ES -14.7 ± 2.88°) (Figure 6.4C). However, at week 8, there was less plantar flexion, indicating increased range of motion at the ankle, in the CES cohort (-4.9 ± 2.29°) compared to the no-ES group (-13.8 ± 2.71°) ($p=0.01$). At weeks 9 (CES 4.36 ± 2.94°; no-ES -6.36 ± 3.4°) and 10 (CES 10.5 ± 4.1°; no-ES -3.93 ± 2.07°) of regeneration, the CES animals had almost normal heel-rise ($p<0.001$). Screenshots of no-ES animals at week 10 following DNT surgery confirmed the absence of both toe-off (Figure 6.4D), dorsiflexion during heel-rise (E), and these animals had curled toes immediately before foot contact (F); the CES group had normal toe-off (G), improved dorsiflexion during heel-rise (H), and an absence of toe-curl immediately prior foot contact (I).

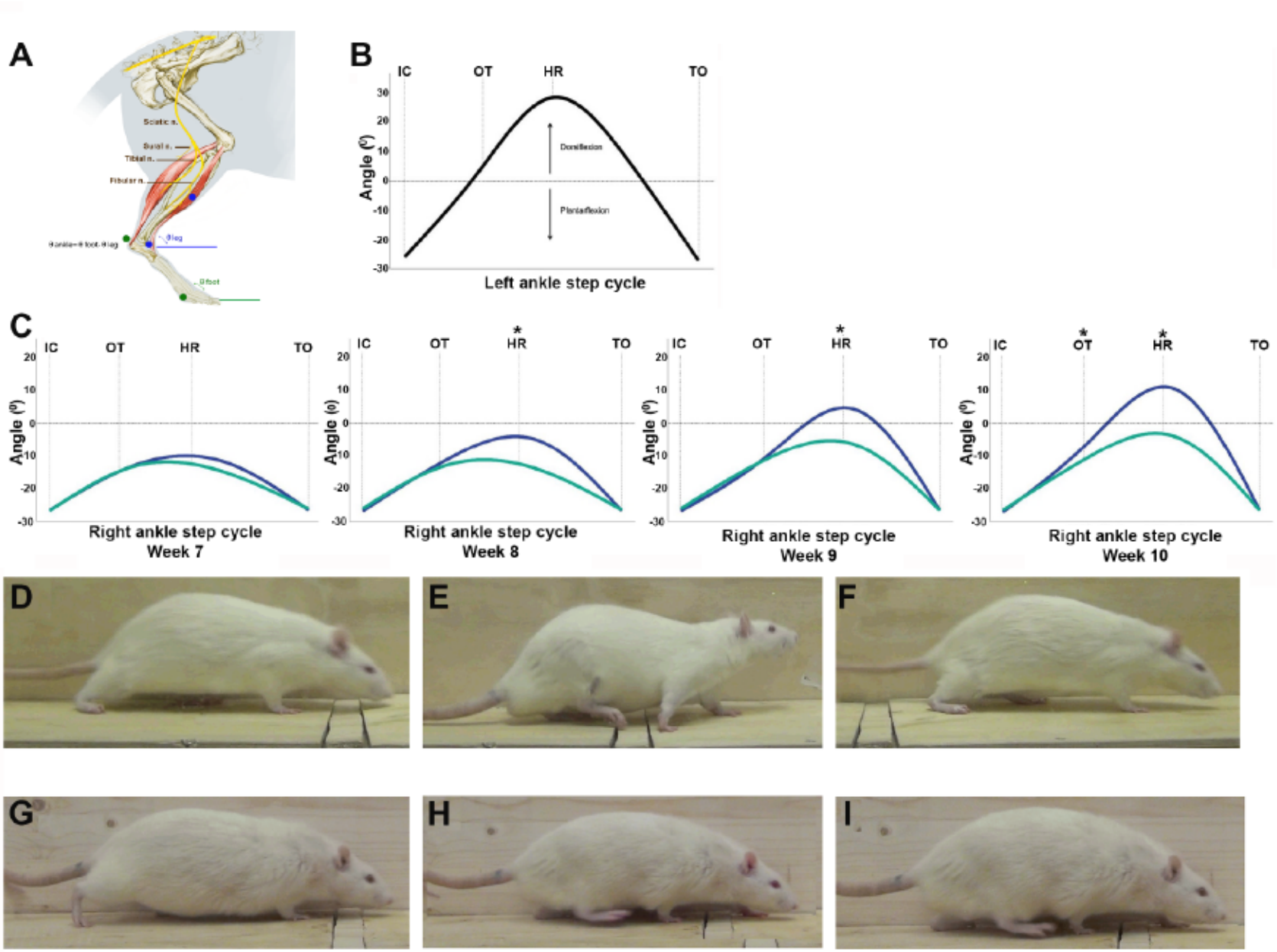

A
B
IC
OT
HR
TO
Angle (°)
Dorsiflexion
Plantaflexion
Left ankle step cycle
C
Right ankle step cycle
Week 7
Right ankle step cycle
Week 8
Right ankle step cycle
Week 9
Right ankle step cycle
Week 10
D
E
F
G
H
I

Figure 6.4: CES improves dorsiflexion

(A) Diagrammatic representation of the rodent skeleton depicts the angles measured between the tibia and horizontal plane (θ leg), and the foot and the horizontal plane (θ foot) to calculate the ankle angle (θ ankle = θ foot - θ leg – 90º). A positive number of this calculation represents dorsiflexion at heel-rise the moment in stance phase where (in an uninjured limb) the ankle angle changes from plantar flexion to dorsiflexion. (B) The normal step cycle of the contralateral uninjured limb in all animals at all times tested is represented. (C) Both groups show no sign of dorsiflexion (positive angle values) at week 7. Both groups start to recover normal dorsiflexion by week 8, however the CES group have almost full range by week 10 ($p < 0.001$). IC = initial contact; OT = opposite toe-off; HR = heel-rise; TO = toe-off; * $p \leq 0.05$. (D-I) Screenshots of no-ES (D-F) and CES (G-I) animals following DNT surgery at week 10. The no-ES animal demonstrates an absence of normal toe-off (D), plantar flexion at heel-rise (E), and toe-curling during foot contact (F). The CES animal showed significantly improved toe-off (G), dorsiflexion at heel-rise (H) and a lack of toe-curling immediately before foot contact (I).

At six weeks of regeneration, animals in the CES and no-CES cohorts had similarly poor performance on the horizontal ladder (2.5 ± 0.3 au; 2.3 ± 0.2 au, respectively), however, by 7 weeks, the CES animals had significantly improved foot placement scores (3.5 ± 0.1) compared to no-ES animals (2.6 ± 0.2 au, $p<0.001$) (Figure 6.5A). CES animals continued to improve at 8 (4.6 ± 3.5 au), 9 (4.7 ± 0.1 au), and 10 (4.9 ± 0.1 au) weeks of regeneration compared to no-ES controls (3.5 ± 0.2, 3.5 ± 0.1, and 3.5 ± 0.2 respectively, $p<0.001$ for all timepoints). At 10 weeks of regeneration, the tibialis anterior muscle of animals treated with CES weighed 80.1 ± 5.4% of their contralateral uninjured limb (Figure 6.5B, C). This was significantly greater than the no-ES animals (36.5 ± 8.0% of contralateral, $p<0.01$). Immunohistochemical analysis of tibialis anterior muscle sections were labeled with NF200 to demonstrate the nerves (red) innervating the alpha-bungarotoxin stained acetylcholine receptors (green). We confirmed that at 10 weeks of regeneration there were significantly more reinnervated neuromuscular junctions in the animals treated with CES prior to transfer (23.3 ± 1.9 innervated NMJ /50 mm^2 tissue section) compared to no-ES animals (11.8 ± 3.1 innervated NMJ/50 mm^2 tissue section, $p<0.001$) (Figure 6.5 D-F).

5.5.3 CES does not evoke an inflammatory response or injure the distal tibial nerve

Supporting our theory that CES is non-inflammatory and non-injurious, distal nerve branches of the CES-treated tibial nerve (ie: distal tibial nerve not used for DNT) did not display axonal injury. Histomorphological evaluation of the tibial nerve distal to the lateral head of the gastrocnemius muscle used for DNT at 10 days post-CES confirmed intact axons with no indication of Wallerian degeneration (Figure 6.6A-B). Positive control sections of a nerve crushed 10 days prior demonstrated significant Wallerian degeneration at this time point (E-

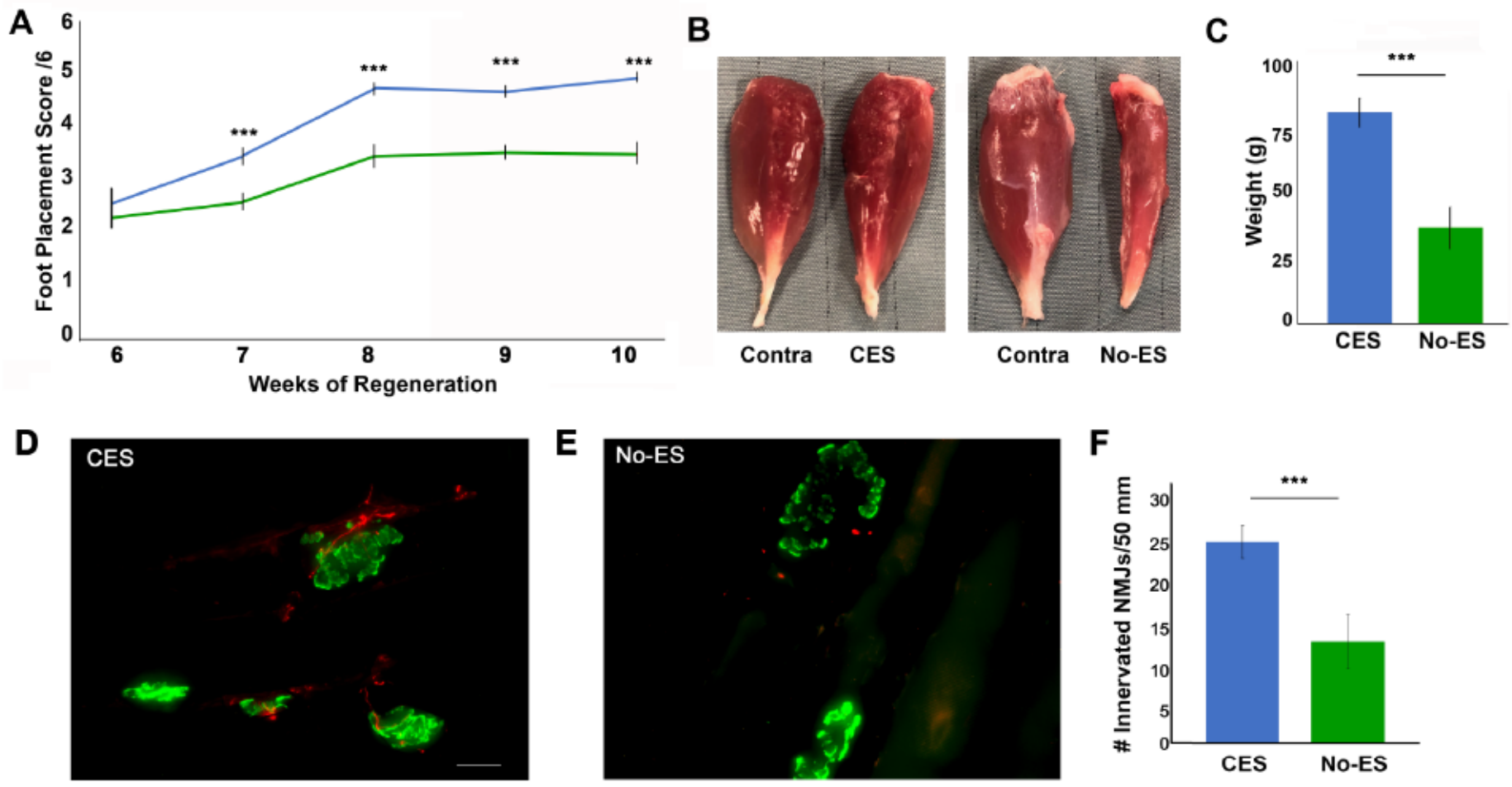
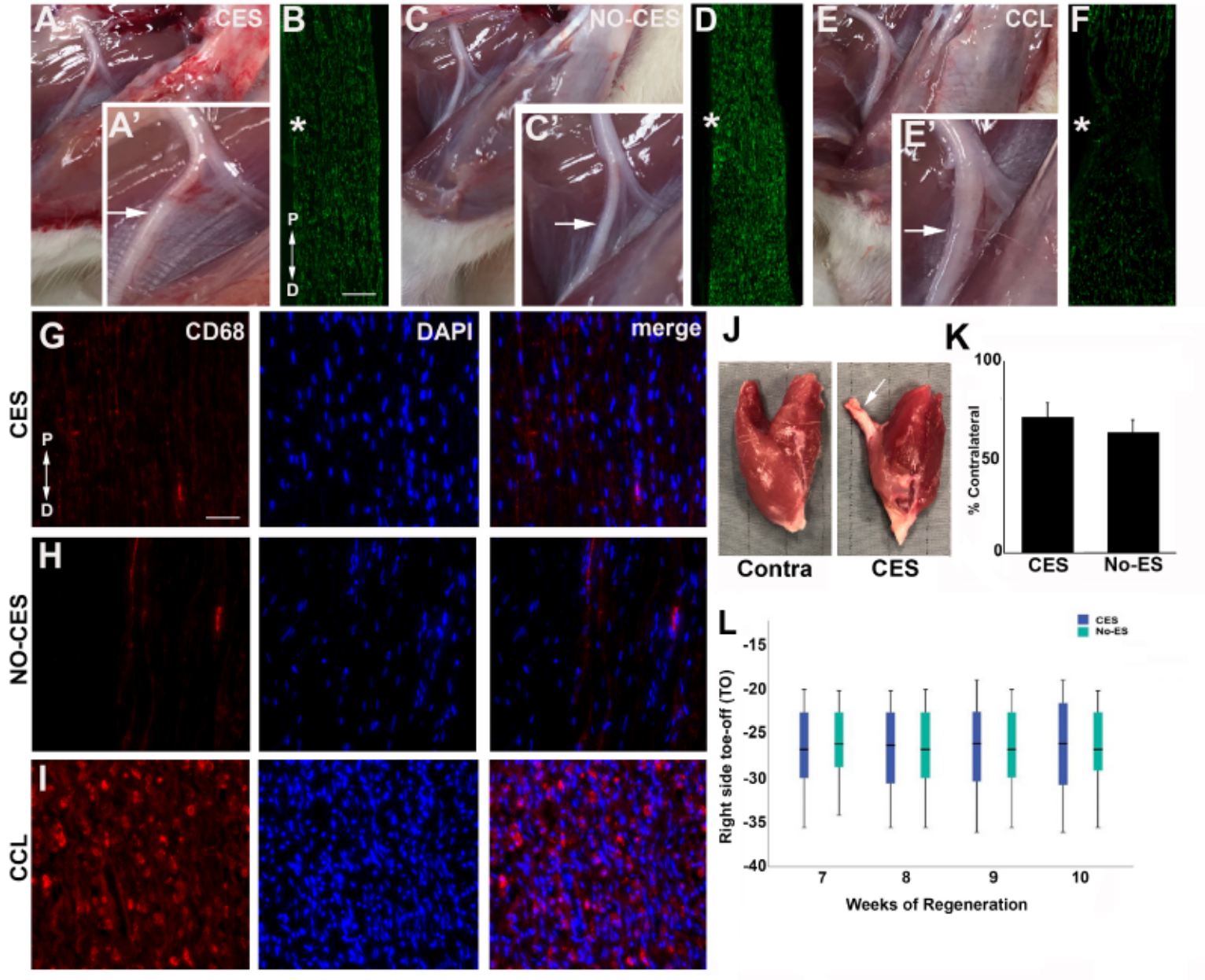

A
Foot Placement Score /6
Weeks of Regeneration
***
B
Contra
CES
Contra
No-ES
C
Weight (g)
CES
No-ES
D
CES
E
No-ES
F
Innervated NMJs/50 mm
CES
No-ES

Figure 6.5: CES promotes muscle reinnervation and functional recovery

(A) Line graph represents foot placement scores on the horizontal ladder test at 6-10 weeks of regeneration following DNT surgery. From week 7 onward, animals in the CES cohort (blue) had significantly higher foot placement scores than no-ES animals (green, ***$p<0.001$). (B) Representative photographs of the entire tibialis anterior muscles from both limbs from a CES and a no-ES animals. (C) Muscle weights were normalized to their contralateral limb to determine that at 10 weeks, animals treated with CES had significantly greater muscle weight suggesting decreased muscle atrophy (***$p<0.001$). (D, E) Representative photomicrographs of CES (D) and no-ES (E) tibialis anterior muscle sections used to quantify the number of neuromuscular junctions. Innervating nerves were immunolabeled with NF200 (red) and the acetylcholine receptors were stained with alpha-bungarotoxin (green). (F) Animals treated with CES prior to DNT had significantly more innervated neuromuscular junctions compared to the animals that did not receive pre-DNT conditioning (*** $p<0.001$). A-F) n= average per animal. Scale bar is 20 µm.


A
CES
A'
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*
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NO-CES
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CES
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100
50
0
% Contralateral
CES
No-ES
L
CES
No-ES
-15
-20
-25
-30
-35
-40
Right side toe-off (TO)
7
8
9
10
Weeks of Regeneration

Figure 6.6: CES does not evoke an inflammatory response or injury to the nerve

Photographs of tibial nerves and the corresponding immunolabeled tibial nerve sections treated three days prior with CES (A, B), no-ES (C, D) or with a conditioning crush lesion (CCL) (E, F). The crush lesion produced localized swelling and site of injury (E', arrow) and immunohistochemical analysis with NF200 (green) showed evidence of Wallerian degeneration distal to the crush site (F, asterisk). By contrast, CES and no-ES nerves have no swelling (A, A'; C, C', arrows) and the nerve sections revealed there was no injury site or Wallerian degeneration (B, D, asterisk), respectively. (G-I) Representative immunohistochemical staining of nerve sections showed resident macrophages (CD68 labeled cells, red) only at the site of CES (G) which was similar to no-ES negative controls (H) whereas there was a significant macrophage presence at the injury site of the crush injured animals (I). J) Representative photograph of the entire gastrocnemius muscles 10 weeks post-DNT shows denervation of the lateral head in the CES animal but normal muscle bulk of the medial uninjured head. K) Gastrocnemius muscle weights were similar between animals that received CES and without ES, suggesting no injury was sustained to the tibial nerve by the electrical stimulation. L) TO is the moment where stance phase moves into stride phase. At this point, the plantar flexors (innervated by the tibial nerve distal to the branch used for DNT) must activate to bring the ankle from dorsiflexion to plantarflexion (negative values). As can be seen from the boxplot, there were no significant differences between the CES and no-ES cohorts suggesting that the remaining tibial nerve was not impacted by CES.

F); however, CES-treated nerves closely resembled unconditioned negative controls (C-D). As expected, CD68 labeling demonstrated there was a significant presence of macrophages at the crush site of the CCL nerves (I). Animals treated with CES had low expression of CD68-positive cells, comparable to nerves that received no-ES (G, H).

On gross examination of all of the gastrocnemius muscles two months following DNT, the denervated lateral head was atrophied whereas the medial head appeared healthy with a comparable muscle bulk (Figure 6.6J). Animals treated with (70.0 ± 7.4%) or without CES (62.6 ± 6.3%) prior to nerve transfer had no significant difference in the weight of their gastrocnemius muscle at 10 weeks of regeneration ($p=0.42$) suggesting the medial head may have compensated for the loss of the lateral head (K). To show further support that CES does not injure the distal tibial nerve, our functional assays indicated that there were no differences in ankle plantarflexion (which involves tibial nerve innervation) of the injured hindlimb at any point between animals treated with CES and animals without CES (Figure 6.6L).

6.6 Discussion

Primary coaptation following a traumatic common fibular nerve injury is often not possible. DNTs shorten the distance between surgical coaptation and the target muscle endplate, resulting in earlier reinnervation of the tibialis anterior muscle. In the only published rodent study investigating lower limb DNT for foot drop, Kemp et al., (2010) showed that electrophysiological and skilled locomotor recovery outcomes were comparable in animals treated with a nerve transfer and the gold-standard primary coaptation. Despite these promising results, animals treated with a DNT had significant impairment in the amount of

force that the animal exerted on the ground during locomotion (ground reaction force), as well as in ankle angle kinematic measures, compared to animals treated with a direct repair (Kemp, Alant, Walsh, Webb, & Midha, 2010). A technique to augment the regeneration through a DNT is therefore needed to improve functional recovery.

Reported clinical outcomes of a tibial to common fibular nerve transfer are limited. Only six studies have evaluated foot drop outcomes following DNT (Curran, DeSerres, Morhart, Olson, & Chan, 2018; Flores, Martins, & Siqueira, 2013; Giuffre et al., 2012; Leclere, Badur, Mathys, & Vogelin, 2015; Nath et al., 2008; Strazar, White, & Bain, 2011). These studies are small, ranging from one (Leclere et al., 2015) to 16 patients (Curran et al., 2018). The percentage of patients obtaining at least a Medical Research Council (MRC) grade 3 (movement against gravity) is highly variable, between 20% (Flores et al., 2013) and 78% (Nath et al., 2008). Predictors for good outcome remain poorly understood. These inconsistencies have led some researchers to suggest that DNT is a poor choice for patient treatment(Flores et al., 2013); however, alternative treatment options for these patients are limited. Ankle-foot orthotics are often poorly tolerated due to discomfort and difficulties in mobility, and posterior tibial tendon transfers confer significant long-term complications such as arthritis, flat footedness, and hindfoot valgus (Giuffre et al., 2012). A strategy to improve regeneration and reinnervation through a lower-limb DNT is therefore of significant clinical importance.

In the normal gait cycle, dorsiflexion peaks at heel-strike during stance phase. Contraction of the tibialis anterior muscle ensures toe clearance during swing phase and slows the descent

of the foot to prevent foot slap at heel strike (Nordin & Frankel, 2001; Perry, 1992). Denervation of the tibialis anterior muscle results in a functional loss of dorsiflexion. Footdrop is characterized by the toe, rather than the heel, contacting the floor, followed by the lateral ridge of the foot to the heel. Loss of normal function during swing phase will also result in increased knee and hip flexion, called steppage gait. The loss of normal function of the tibialis anterior muscle therefore results in an asymmetric unstable gait pattern (Nordin & Frankel, 2001; Wiszomirska, Blazkiewicz, Kaczmarczyk, Brzuszkiewicz-Kuzmicka, & Wit, 2017). These changes are compensated for by a 100- 200% increase in the force generated by the flexor digitorum and hallucis, tibialis posterior, and semitendinosus muscles (Blazkiewicz & Wit, 2019). Furthermore, patients with foot drop have a near 50% decrease in walking speed (Wiszomirska et al., 2017). These characteristic gait patterns of foot drop are similar to that observed in rodents; our results suggest animals developed a decrease in speed, a decrease of dorsiflexion during both stance and swing phases, resulting in a similar unstable steppage gait. These results are in keeping with previous the description of common fibular nerve injury in rodents (Kemp et al., 2010) demonstrating that the rat may be an adequate model for mimicking human foot drop and testing perioperative interventions to enhance outcomes.

The conditioning lesion is well described as one of the most effective techniques for accelerating the rate of nerve regeneration and improving outcome (Senger, Verge, Chan, & Webber, 2018). Traditionally a conditioning lesion was delivered by crushing the nerve one week prior to injury; however, our laboratory has recently identified electrical stimulation as a less invasive, more effective technique for inducing a conditioning effect with even greater

sensorimotor recovery (Senger et al., 2019; Senger et al., 2017). CES is well suited for clinical translation to a DNT population. As these surgeries are elective, percutaneous electrical stimulation could be delivered to the donor nerve one week prior to scheduled DNT to enhance the regenerative potential through the nerve transfer. Clinical translation of CES to a DNT patient population requires evidence that CES does not cause axonal injury. Given the small caliber of the nerve branch to the lateral gastrocnemius muscle, conditioning would most likely be delivered to the main nerve (as in our rodent model using the tibial nerve) to ensure electrode proximity. As such, it is imperative to demonstrate that CES does not damage the remaining non-transferred branches of the nerve. In this context, we compared nerve sections treated with CES to positive-control crush injury and unconditioned nerve sections to evaluate for macrophage infiltration and Wallerian degeneration. Our results suggest that CES does not cause axonal degeneration, nor does it mount a macrophage-mediated inflammatory response (Figure 6.6). These results are corroborated by functional outcomes that show no functional deficits in the tibial nerve motor distribution; at no point was the plantar flexion angles of the injured limb affected. Gastrocnemius muscle weight was comparable between the two cohorts, indicating similar innervation to the medial head following transfer.

CES to the donor nerve significantly improves rodent functional recovery following DNT to restore common fibular nerve function. Animals treated with CES were found to have significant improvements in skilled locomotion, kinematic and kinetic assessment, electrophysiologic evaluation, and nerve and muscle tissue analysis, when compared to animals who did not receive pre-DNT conditioning (Figures 6.3-5). Even more significantly,

CES delivers a conditioning effect without causing axonal degeneration or macrophage infiltration, and without causing functional deficits in the motor endplates of the donor nerve (Figure 6.6). Importantly, CES is well-tolerated clinically and unlike in our animal model, can be performed percutaneously. As such, CES is a clinically feasible method of significantly improving outcomes for patients undergoing DNT.

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Chapter 7

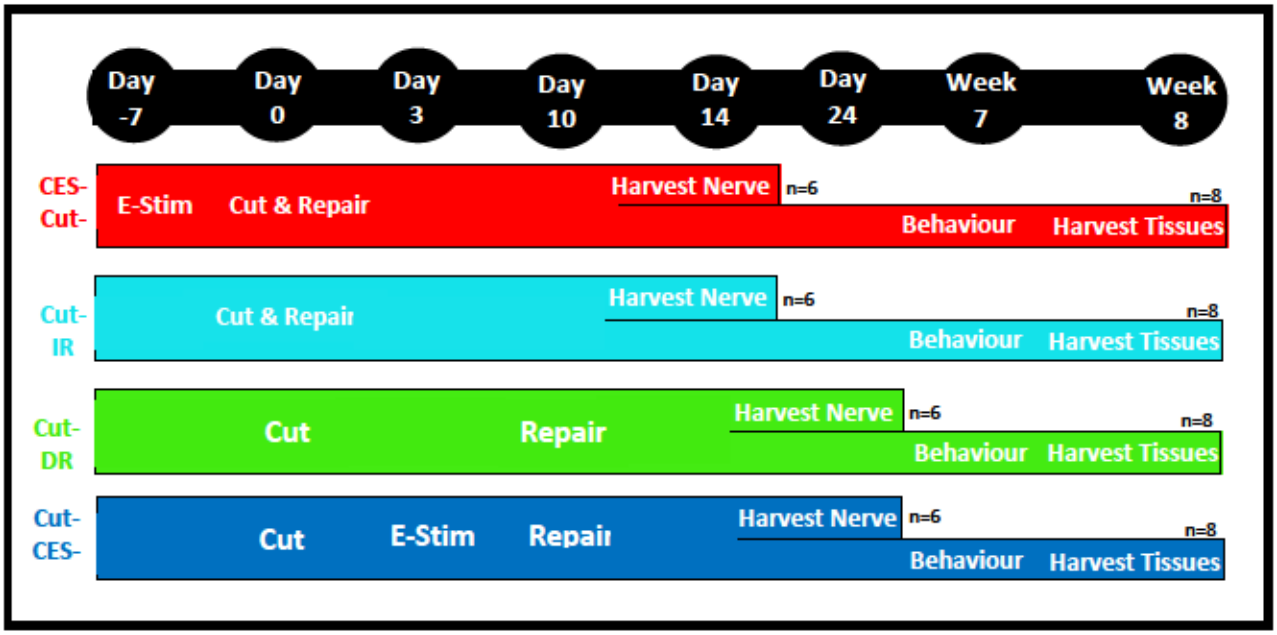
Post-injury Electrical Stimulation Enhances Regeneration, Reinnervation and Functional Recovery Following Nerve Laceration

7.1 Preface

In Chapters 2 and 3 we established pre-injury electrical stimulation as an effective technique for conditioning the nerve to improve regeneration and reinnervation outcomes, and subsequently showed the pro-regenerative effects of CES are greater than PES (Chapter 4). In Chapters 5 and 6 we showed that this technique can be used to improve outcomes in peripheral nerve reconstruction surgery where the timing of surgery is scheduled, such as in a nerve autograft repair or a distal nerve transfer. Both of these models of nerve reconstruction are amenable to conditioning, as these surgeries are elective and therefore conditioning seven days prior can be arranged. The majority of patients with an acute nerve transection, however, do not require complex reconstruction but rather are amenable to primary repair. Current dogma suggests that this injury pattern should be repaired immediately; therefore, these patients are not suitable candidates for receiving pre-injury conditioning therapy.

The aim of Chapter 7 was to determine if the pro-regenerative effects of CES could be harnessed to enhance outcomes in a pre-cut nerve requiring primary repair. The timeline below describes the four treatment groups studied: i) **CES-Cut-IR** (red): our positive control in this study, animals received CES seven days prior to a nerve transection and repair similar to Chapters 2-5; ii) **Cut-IR** (blue): in keeping with current clinical practices, the nerve was cut and immediately repaired to mimic the patient that presents for repair immediately following injury, iii) **Cut-DR** (green): to simulate patients who must be referred to a peripheral nerve surgeon from a primary care center prior to repair, the nerve is cut and the repair is performed 10 days post-injury, and iv) **Cut-CES-DR** (indigo): our test group, the

nerve is cut, treated with CES three days later, and repaired after a 7-day conditioning intervals. For all four cohorts, nerve lengths were collected after two weeks of regeneration, and behaviour testing was performed between 7-8 weeks of regeneration.



Our results suggest that a transected tibial treated with CES, then repaired one week later has significantly improved regeneration and sensorimotor reinnervation compared to either an immediate or a delayed repair. Despite differences in timing, delivery of electrical stimulation seven days prior to repair of a transected nerve had similar outcomes as the traditional conditioning paradigm in which CES is delivered prior to injury and repair.

Post-injury Electrical Stimulation Enhances Regeneration, Reinnervation and Functional Recovery Following Nerve Laceration

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7.2 Abstract

Background: Traumatic nerve injuries are common and outcomes in severe cases with current management protocols are poor. Conditioning electrical stimulation (CES) prior to nerve injury significantly improves regeneration, reinnervation and functional recovery. It is, however, impossible to perform CES prior to accidental nerve lacerations. Clinical research has shown that the results of delay of nerve repair by 2 weeks are equivalent to immediate repair. As this delayed time to surgery would permit sufficient time for instituting CES, we designed a clinically relevant animal model to study post-injury electrical stimulation. In this paradigm, the injured tibial nerve was stimulated 3 days following transection, and nerve coaptation was delayed for 1 week to allow for the conditioning effect.

Methods: Fifty-six male Sprague Dawley rats were randomly divided into four cohorts: i) CES one week prior to nerve transection & immediate coaptation (positive control, CES-cut-IR), ii) nerve transection & immediate coaptation, no conditioning (negative control #1, cut-IR), iii) nerve transection and delayed repair one week following, no conditioning (negative control #2, cut-DR), and iv) nerve transection, CES three days later, repair one week post-CES (experimental cohort; cut-CES-DR). Length of nerve regeneration was assessed 2 weeks post-repair (n=6) and physiological and behavioural testing were performed 7-8 weeks post-coaptation (n=8).

Results: Animals in the experimental cohort had regenerative and reinnervation outcomes that were similar to the CES-cut-IR cohort, and significantly greater than the unconditioned cohorts. The length of axonal extension two weeks following coaptation was similar in both

cohorts of animals treated with electrical stimulation (12.4 ± 0.8 mm in pre-injury CES vs. 12.5 ± 1.0 mm in post-injury CES), and significantly longer than in non-conditioned animals that were repaired immediately (6.9 ± 0.7 mm, $p<0.001$) or 10-days following injury (7.2 ± 0.5 mm, $p<0.001$). Compared to non-conditioned cohorts, both CES cohorts had significantly enhanced physiological and functional sensory (von Frey filaments) and motor (toe spread width, horizontal ladder, nerve conduction testing, muscle weight) reinnervation outcomes.

Conclusion: CES following nerve injury, prior to nerve repair significantly improves regeneration and sensorimotor reinnervation. These findings are of significant clinical importance, as our results suggest that immediate repair following nerve injury is inferior to post-injury CES followed by delayed nerve coaptation.

7.3 Introduction

Forty years of research has identified conditioning as one of the most effective methods of accelerating axonal extension and improving functional outcomes in animal models of peripheral nerve injury. This technique traditionally requires that the nerve be crushed one week prior to definitive nerve injury and primary repair. Clinical translation of a conditioning crush lesion (CCL) remains, however, impossible given ethical and technical barriers: the injurious nature of a crush, and the challenge of predicting the time of nerve injury. Our laboratory has recently demonstrated that conditioning electrical stimulation (CES) confers comparable rates of axon extension, and significantly greater motor and sensory reinnervation outcomes compared to a CCL (Senger et al., 2019; Senger, Chan, Verge, & Webber, 2017); however, unlike a crush, electrical stimulation is non-injurious and therefore has less inherent obstacles to clinical translation. The remaining challenge in delivering a conditioning effect to a human patient population is that of timing; it is unknown when a traumatic peripheral nerve injury will occur; therefore, it is impossible to intervene one week prior. This is a particular challenge in patients with an acute nerve transection injury pattern amenable to primary repair, as current dogma dictates repair should be performed as an urgent priority and therefore preoperative conditioning is not feasible. We therefore sought to determine whether we could re-create the pro-regenerative effects of conditioning in a transected nerve.

7.4 Methods

7.4.1 Animals*:* All experiments were approved by the Animal Research Ethics Board at the University of Alberta. Healthy adult male Sprague Dawley rats (Charles River laboratory)

were placed under the care of Health Sciences Laboratory Animal Services at the University of Alberta. Two animals were housed per cage, with alternating 12-hour on/off light cycles and *ad libitum* access to rat chow and water.

Animals were equally divided into four cohorts: a) CES 1 week prior to transection and immediate repair (**CES-cut-IR**, positive control); b) cut with immediate repair, no electrical stimulation (**cut-IR,** negative control #1); c) delayed repair, no electrical stimulation (**cut-DR**, negative control #2); and d) nerve transection, CES, then delayed repair (**cut-CES-DR**, experimental cohort) (Figure 7.1). Twenty-four (n=6/cohort) animals were used for nerve regeneration studies (culled 14 days following nerve repair), and 32 animals (n=8/cohort) for behavioural testing (culled 8 weeks following nerve repair).

7.4.2 Nerve transection & immediate repair (cohorts a, b): All animals were anesthetized with inhalational isoflurane (2% titrated to maintain a surgical anesthetic plane) and received 0.01mg/Kg of subcutaneous buprenorphine. An incision was made along the lateral thigh and the hamstrings were released to visualize the sciatic nerve. The tibial branch was identified, isolated, and transected 1 cm distal to the trifurcation point of the sciatic nerve. The proximal and distal stump were reapproximated and coaptation was performed with 9-0 silk suture under 3.5x loupe magnification.

7.4.3 Nerve transection & delayed repair (cohorts c, d): Identification and transection of the tibial nerve was performed as above. Proximal and distal nerve stumps were sutured into

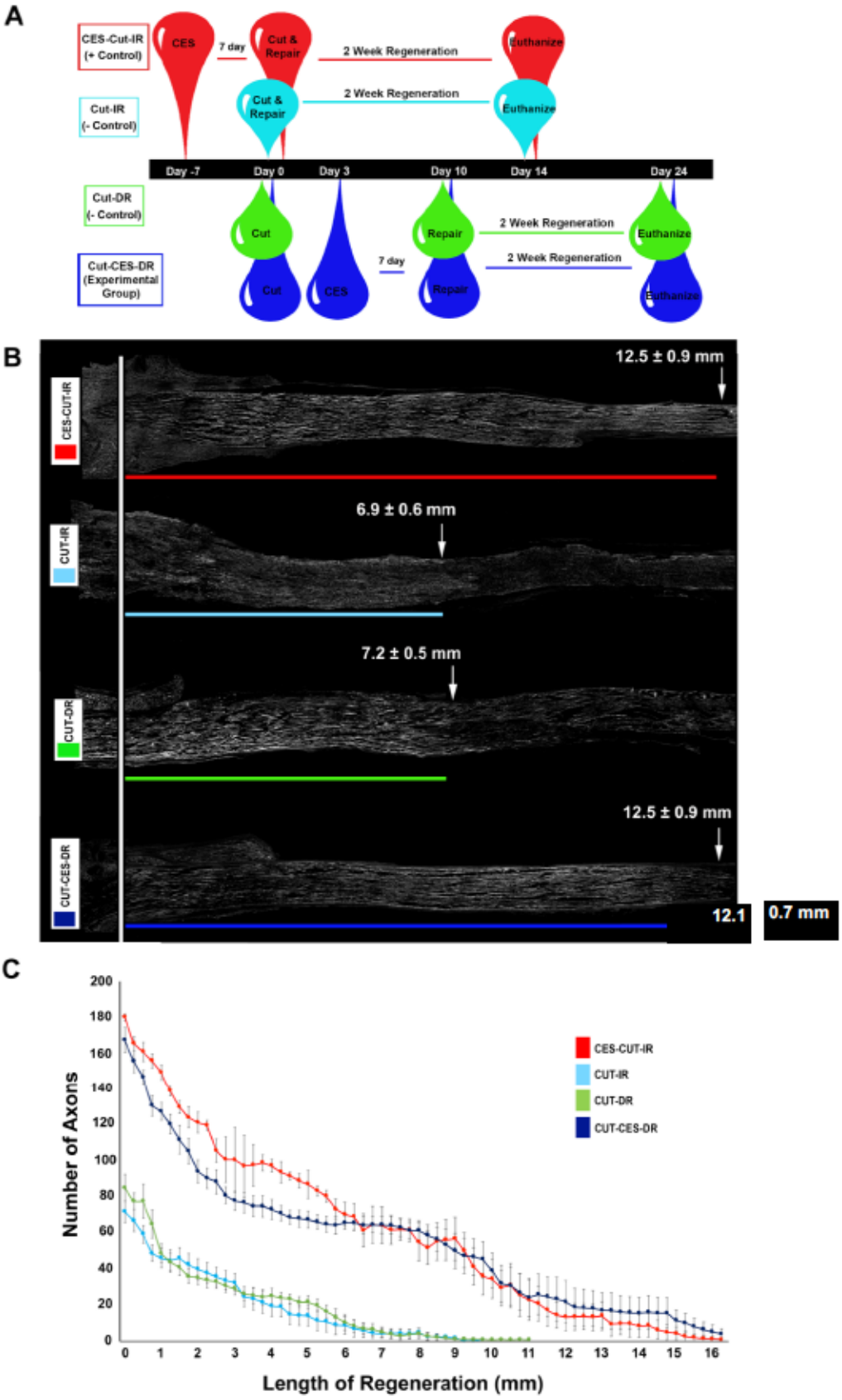
A
CES-Cut-IR
(+ Control)
CES
7 day
Cut &
Repair
2 Week Regeneration
Euthanize
Cut-IR
(- Control)
Cut &
Repair
2 Week Regeneration
Euthanize
Day -7
Day 0
Day 3
Day 10
Day 14
Day 24
Cut-DR
(- Control)
Cut
Repair
2 Week Regeneration
Euthanize
Cut-CES-DR
(Experimental
Group)
Cut
CES
7 day
Repair
2 Week Regeneration
Euthanize
B
CES-CUT-IR
12.5 ± 0.9 mm
CUT-IR
6.9 ± 0.6 mm
CUT-DR
7.2 ± 0.5 mm
CUT-CES-DR
12.5 ± 0.9 mm
12.1
0.7 mm
C
Number of Axons
Length of Regeneration (mm)
CES-CUT-IR
CUT-IR
CUT-DR
CUT-CES-DR

Figure 7.1: Post-injury CES promotes nerve regeneration

(A) Timeline depicts the four treatment paradigms compared: i) CES-Cut-IR: the tibial nerve received one hour of conditioning electrical stimulation (CES) seven days prior to tibial nerve cut and immediate repair (positive control, red), ii) Cut-IR: the tibial nerve was cut and immediately repaired (negative control, light blue), iii) Cut-DR: the tibial nerve was cut, and delayed repair was performed ten days later (negative control, green), and iv) Cut-CES-DR: the tibial nerve was cut, CES was delivered three days later, and delayed repair was performed seven days after conditioning (experimental group, dark blue). Nerve tissues were harvested after two weeks of regeneration. (B) Representative photomicrographs of NF200 labeled longitudinal tibial nerve sections after fourteen days of regeneration. Solid white line depicts the site of coaptation, and arrow depicts the distal-most point of regeneration, defined as the most distal site along the nerve where a minimum of 10 axons were counted. (C) Line graph depicts the average length of axonal regeneration (x-axis) and number of regenerating axons at 250 μm intervals (y-axis). Injured tibial nerves conditioned with electrical stimulation prior to repair (indigo) had a significantly longer length of regeneration compared to transected tibial nerves treated immediately (light blue, ***$p<0.001$) or in a delayed fashion (green, ***$p<0.001$) and comparable to the nerves treated with true CES prior to transection (red).

regional muscles to prevent retraction and facilitate identification. The hamstring muscles were resuspended and skin closed with 3-0 Vicryl. At the designated timepoint, the animal was anesthetized, and the two ends of the tibial nerve were identified. Ends of the tibial nerve were debrided and a primary coaptation was performed with 9-0 silk suture under 3.5x loupe magnification.

7.4.4 Conditioning electrical stimulation: Under general anesthetic, animals in cohort (a) were treated with a longitudinal incision over the lateral aspect of the leg, and blunt dissection identified the tibial nerve as it emerged between the two heads of the gastrocnemius muscle. Among animals in cohort (d), CES was delivered to the sciatic nerve. A stainless-steel wire bared at the ends was wrapped around the nerve (anode) and a second was inserted into the hamstring muscle (cathode). Wires were connected to an SD-9 stimulator (Grass Instruments Co., Quincy, MA). A continuous train of biphasic pulses at 20Hz of 0.1 ms duration was delivered over one hour, with voltage titrated to maintain a visible twitch in the ankle. Skin was closed with 3-0 Vicryl at the completion of the stimulation.

7.4.5 Nerve tissue processing: Seven days following nerve repair, 24 animals (n=6/cohort) were euthanized using inhalational carbon dioxide and exsanguination by puncture of the left cardiac ventricle. The tibial nerve was exposed and the site of coaptation was identified. The nerve was dissected from surrounding soft tissue and scar. The resected nerve was placed on a 3 cm length of toothpick to stabilize the repair site, as well as the proximal and distal stumps. Nerve sections were fixed in Zamboni's fixative (paraformaldehyde, picric acid, NaOH, American MasterTech Scientific, Lodi, CA) for four hours, rinsed with 0.01M

phosphate buffered saline (PBS) (Thermo Fisher Scientific, Waltham, MA) five times, post-fixed in 30% sucrose solution (Thermo Fisher Scientific, Waltham, MA) overnight at 4°C, then frozen in Optimum Cutting Temperature (OCT) (Sakura Finetek, Torrance, CA) using indirect exposure to liquid nitrogen. Nerves were cut longitudinally into 12 μm sections and thaw-mounted on Superfrost Plus microscope slides (ThermoFisher Scientific, Waltham, MA) and stored at -80°C until processing.

7.4.6 Tissue analysis: Eight weeks post-nerve coaptation, the gastrocnemius muscles of the injured right and uninjured left limbs were collected and weighed. The weight of the injured muscle was reported as a percentage of the contralateral to control for differences in animal size. The gastrocnemius muscle from the injured limb was fixed, rinsed, sunk, and frozen in OCT as described for nerve sections.

Biopsies from the tibial nerve innervated region of the plantar footpad were collected and fixed in 2% paraformaldehyde, lysine, periodate (PLP) fixative for 16-20 hours. Sections were rinsed five times in Sorensen's phosphate buffer, then cryoprotected at 4°C overnight in 20% glycerol (Thermo Fisher Scientific, Waltham, MA) in 0.1 M Sorensen's phosphate buffer. Muscle and footpads were frozen in OCT, cut into 20 μm sections on Superfrost-Plus microscope slides, and stored at -80°C until processing.

7.4.7 Immunofluorescence: Slides were warmed to room temperature then treated with 20 minutes of antigen retrieval in a 60°C citrate buffer (10 mM sodium citrate, 0.05% Tween-20, pH 6.0; Thermo Fisher Scientific, Waltham, MA). After three washes of five minutes in

0.01 M PBS, slides were blocked in 10% normal goat serum serum (MP Biomedicals, Santa Ana, CA) and 3% bovine serum albumin (BSA) (Sigma-Aldrich, St Louis, MO) in 0.01 M PBS for 90 minutes. Nerve and muscle sections were stained with 1:500 mouse anti-neurofilament-200 (NF200) (Sigma-Aldrich, St Louis, MO), and footpads were labelled with 1:1000 rabbit anti-protein gene product 9.5 (PGP9.5) (Encor Biotechnology Inc, Gainesville, FL); primary antibodies were diluted in a solution of 0.01 M PBS containing 3% BSA. The following day, slides were washed three times for five minutes each in 0.01 M PBS, and the secondary antibodies 1:500 Cy3-conjugated goat anti-mouse (Sigma-Aldrich) or 1:500 Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA) were diluted in 0.01 M PBS containing 3% BSA for 60 minutes at room temperature. Slides were washed three times in 0.01 M PBS. Muscle slides were then stained with 1:5000 conjugated anti-α-bungarotoxin for 20 min. All slides were mounted with a coverslip using 50% glycerol in 0.01 M PBS.

Nerve tissue was assessed under 10x fluorescent microscopy to identify regenerating and degenerating axons. Photomicrographs of the nerve sections were taken and assessed to measure the total length of axonal extension from the site of cut/coaptation, and to quantify the number of regenerating axons at 250 μm intervals.

Fluorescent microscopy was used to image footpad sections at 40x magnification. Consecutive images of the entire dermal-epidermal junction in each section were taken, for a total of 15-20 fields per animal. A z-stack of 1 μm steps was imaged for each field. Using

ImageJ software, the intraepidermal nerve fiber (IENF) density was determined by counting the number of axons stained with PGP9.5 that cross the dermal-epidermal junction.

Muscle tissue was assessed with confocal microscopy to identify acetylcholine receptors (α-bungarotoxin positive) and their innervating axons (NF200 positive). The number of innervated neuromuscular junctions (NMJs) of each cohort was quantified by averaging the total number of NMJs in 10 separate tissue sections per animal.

7.4.8 Behavioural and physiologic outcomes: Animal behaviour testing was performed at 7, 7.5, and 8 weeks following nerve coaptation. All testing was performed by a blinded examiner. Sensory reinnervation was assessed using von Frey filaments; motor testing included gait analysis for toe spread, horizontal ladder testing for motor dexterity, and nerve conduction studies to quantify compound muscle action potential (CMAP).

Von Frey filament testing was performed to assess sensory recovery. Animals were placed individual plexiglass cages with mesh flooring and left to acclimatize for 30 minutes. Calibrated monofilaments (1.4 – 15 g) of increasing weight were applied to the injured and uninjured plantar footpads of the animal within the tibial dermatome. Each monofilament was used to probe the paw five times for 30 seconds each with adequate force to bend the monofilament. Three consecutive paw withdrawals in response to an individual monofilament was recorded as a positive result. Responsiveness to lower-weighted monofilaments indicates greater sensory recovery.

Gait analysis was performed to assess motor reinnervation of the tibial-innervated intrinsic muscles of the foot. Animals were placed in a specially-designed walkway created from plexiglass measuring 48cm in length, with an adjustable mirror underneath to allow for simultaneous visualization of the lateral view of the animal and the plantar surface of the paws. Video was taken of the animals walking the length of the track three times per testing session. The video was analyzed on a frame-by-frame basis and screenshots were taken of individual footsteps at the same point of the gait cycle when the animal was weight-bearing on the injured and uninjured limb. A minimum of 10 images per foot were analyzed per testing session. The distance between the first and last toe was measured in the right (injured) and left (uninjured) foot; toe spread of the injured side is reported as a percentage of the uninjured contralateral limb to control for differences in animal size.

The horizontal ladder test is a measure of animal dexterity and fine motor coordination. Animals were placed at the end of an elevated plexiglass walkway with adjustable ladder rungs; placement of the ladder rungs was changed at each testing session to prevent learning. Animals were filmed crossing the ladder three times per testing session. The video was analyzed on a frame-by-frame basis and each foot placement on the ladder rungs was scored on a scale from 0-6 as described by Metz and Whishaw (Metz & Whishaw, 2009) where 0 was a complete slip, and 6 a correct foot placement. The average score of the injured limb was calculated for each attempt.

Nerve conduction studies (NCS) were performed on the tibial nerves of the injured and uninjured contralateral control limbs at 8 weeks of regeneration to quantify muscle

reinnervation. Animals were anesthesized with inhaled isoflurane and supramaximal electrical stimulation was delivered to the tibial nerve at the knee. A lead was placed in the plantar footpad to record action potentials generated in the intrinsic foot muscles. Lead placement was adjusted to maximize the CMAP amplitude.

7.4.9 Statistical analysis: *Results* are presented as mean ± standard error of the mean. Outcomes of behavioural testing and tissue analysis were compared in individual animals within the four cohorts using a one-way analysis of variance (ANOVA); Bonferroni post hoc test compared significant relationships. Statistical significance was identified as a $p<0.05$. All statistical analyses were performed using STATA 14 (StataCorp LP, Collagen Station, Texas).

7.5 Results

7.5.1 Post-injury CES enhances axonal extension

To determine whether post-injury CES induces a conditioning-like effect, we compared outcomes of four cohorts: a) CES followed one-week later by transection & repair (**CES-cut-IR,** positive control), b) nerve transection and immediate repair, no electrical stimulation (**cut-IR**, negative control), c) nerve transection with a delayed repair (**cut-DR**, negative control), and d) transection, CES, then delayed repair (**cut-CES-DR**). The length of axon extension after 14 days of regeneration in all four cohorts was analyzed by immunofluorescence staining of nerve segments. Both cohorts of animals that had been conditioned with electrical stimulation, either pre- or post-injury, had significantly longer lengths of axonal extension when compared to unconditioned controls. Animals that received

post-injury CES with a delayed nerve repair (CES-cut-DR, 12.1 ± 0.7 mm) had a similar length of axon extension at 14 days post-repair as a nerve conditioned with electrical stimulation one week prior to transection and immediate repair (CES-cut-IR, 12.5 ± 0.9 mm, p= 0.848). This length was significantly longer than the non-electrically stimulated immediate repair (cut-IR, 6.9 ± 0.6 mm, p<0.001) and delayed repair (cut-DR, 7.2 ± 0.5 mm, p<0.001) cohorts (Figure 7.1).

7.5.2 Post-injury ES with delayed repair enhances sensory reinnervation

Sensory reinnervation was assessed at 7, 7.5, and 8 weeks of regeneration using von Frey monofilament testing. At all timepoints, improved sensory reinnervation was identified in the two cohorts that had received electrical stimulation, when compared to the two that did not, supporting our hypothesis that post-injury CES induces a conditioning-like effect. At seven weeks of regeneration, the cut-CES-DR repair cohort required 6.0 ± 0.5 grams of stimulation to elucidate a positive result, which was comparable to animals in the gold-standard CES-cut-IR repair cohort (5.0 ± 0.6 grams, p=0.241). Cut-CES-DR animals required significantly less stimulation, suggesting improved sensory recovery, than animals treated without electrical stimulation repaired immediately (cut-IR, 19.7 ± 2.2 grams, p<0.001) or repaired two weeks following transection (cut-DR, 15.7 ± 2.8 grams, p<0.001). Similarly, at 7.5 and. 8 weeks of regeneration, animals in the cut-CES-DR cohort were responsive to 4.3 ± 0.3 g and 3.3 ± 0.4 grams of stimulation respectively; this was similar to the CES-cut-IR cohort (5.0 ± 0.6 grams, p=0.312; 4.5 ± 0.5 grams, p=0.115), and significantly less than the cut-IR (14.9 ± 2.1 grams, p<0.001; 10.6 ± 1.2 grams, p<0.001) or cut-DR (17 ± 3.3, p< 0.001; 10.3 ± 1.3 grams, p< 0.001) cohorts (Figure 7.2A).

Tissue analysis of footpad biopsies specimens obtained at 8 weeks of regeneration confirmed greater sensory reinnervation in conditioned animals. Though animals in the Cut-CES-DR had less axons crossing the dermal-epidermal junction (12.2 ± 1.1 nerves/mm) than the positive control CES-Cut-IR (16.1 ± 1.0 nerves/mm, $p=0.09$), these animals had significantly greater sensory reinnervation when compared to the Cut-IR (3.8 ± 1.2 nerves/mm, $p<0.001$) or Cut-DR (4.0 ± 0.7 nerves/mm, $p<0.001$) animals (Figure 7.2B, C). These results, in addition to von Frey results, suggest that delivering CES after an injury and prior to nerve repair improves sensory reinnervation greater than an immediate or delayed repair.

7.5.3 Post-injury ES with delayed repair enhances motor reinnervation

Gait analysis and the horizontal ladder test were performed at 7, 7.5, and 8 weeks post-repair to assess functional motor recovery. The width of toe spread was evaluated to quantify reinnervation of the intrinsic muscles of the foot; the toe spread width of the injured limb is reported as the percentage of the contralateral uninjured control. At 7.5 weeks, animals in the CES-Cut-IR cohort had the widest toe spread at 67.5 ± 2.2%; this was significantly greater than all other cohorts ($p<0.001$ for all comparisons). Though not as robust as the CES-Cut-IR cohort, the cut-CES-DR cohort (58.7 ± 1.4%) had significantly improved toe spread width compared to the non-electrically stimulated cut-IR (33.6 ± 1.1%) or cut-DR (33.6 ± 1.9%) cohorts ($p<0.001$). Similar trends were observed at 7.5 and 8 weeks, at which times cut-CES-DR (63.1 ± 1.4% and 73.2 ± 1.55% respectively) continued to showed significant improvement in motor reinnervation compared to the cut-IR (42.2 ± 5.6% and 42.7 ± 3.3%, $p<0.001$) and cut-DR (38.4 ± 2.4% and 43.5 ± 3.5%, $p<0.001$). Interestingly,

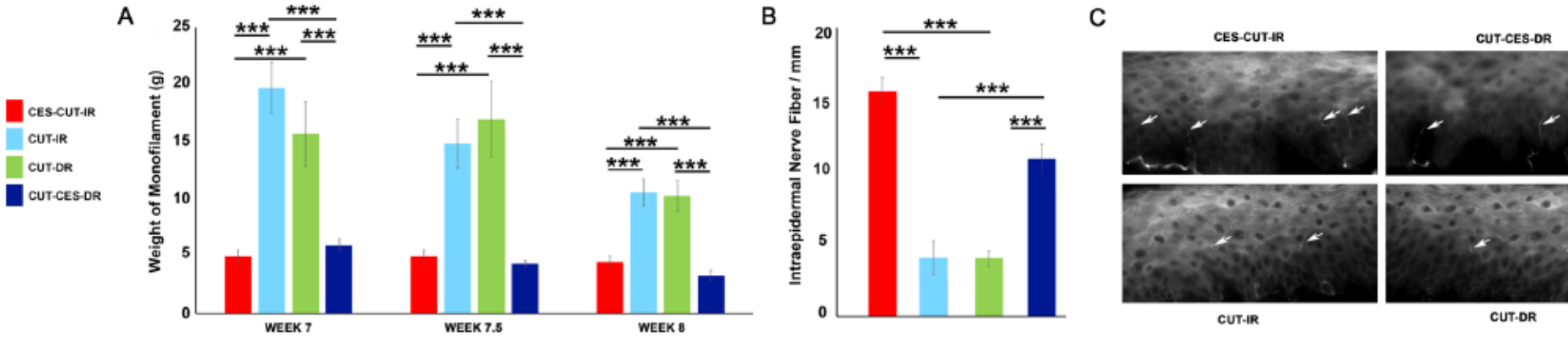

Figure 7.2: Post-injury CES promotes sensory reinnervation. (A) von Frey monofilament testing at 7, 7.5, and 8 weeks of regeneration demonstrates transected tibial nerves treated with CES prior to repair (indigo) had sensory recovery greater than an immediate (light blue, ***p<0.001) or delayed (green, ***p<0.001) repair without electrical stimulation and comparable to a true conditioning lesion (red) at all timepoints. (B) At 8 weeks post-nerve transection and repair, skin biopsies were harvested from plantar footpads and processed for intraepidermal nerve fiber innervation. Animals in the Cut-CES-DR (indigo) had significantly more nerves per µm of epidermis compared to the Cut-IR (light blue, ***p<0.001) or Cut-DR (green, ***p<0.001) cohorts and a comparable number of nerves as the traditional CES animals (red). (C) Representative photomicrographs of footpad biopsy specimens of animals treated with CES-Cut-IR or Cut-CES-DR have a greater number of PGP9.5-stained axons crossing the dermal-epidermal junction than the Cut-IR or Cut-DR cohorts. Scale bar in C is 25 µm.

by 8-weeks the width of toe spread in the cut-CES-DR cohort caught up to gold-standard CES-IR animals ($p=0.278$) (Figure 7.3A, B).

The horizontal ladder test was used to assess motor reinnervation and functional dexterity. Animals with a nerve injury treated with CES prior to repair had significantly improved foot placements scores compared to the non-CES treated cohorts. At 7 weeks, the average foot placement score of cut-CES-DR animals was 4.3 ± 0.1; this was significantly greater than the cut-IR (2.6 ± 0.3, $p<0.01$) and cut-DR (2.7 ± 0.4, $p<0.01$) cohorts, and comparable to the animals conditioned prior to injury (CES-Cut-IR, 4.4 ± 0.1, $p=0.69$). Similarly, at 7.5 and 8 weeks, animals that received post-injury electrical stimulation with delayed primary repair had significantly higher foot placement scores (4.2 ± 0.1 and 5.0 ± 0.1) than animals with an immediate primary repair (2.8 ± 0.2 and 3.6 ± 0.2, $p<0.001$) or delayed primary repair (2.8 ± 0.2 and 3.3 ± 0.2, $p<0.001$), and were comparable to conditioned nerves (4.3 ± 0.1, $p=0.42$; 5.1 ± 0.2, $p=0.81$) (Figure 7.3C).

Nerve conduction studies were performed at eight weeks post-coaptation. The CMAP amplitude was similar between animals who received electrical stimulation either prior to (cut-CES-IR, $21.4 \pm 8.9\%$ of contralateral), or following injury (cut-CES-DR, $16.5 \pm 5.4\%$ of contralateral, $p=0.594$). Post-injury CES animals had significantly improved CMAP amplitudes compared to the cut-IR ($7.3 \pm 1.5\%$ of contralateral, $p<0.05$) and the cut-DR ($4.4 \pm 0.8\%$ of contralateral, $p<0.05$) cohorts (Figure 7.3D, E).

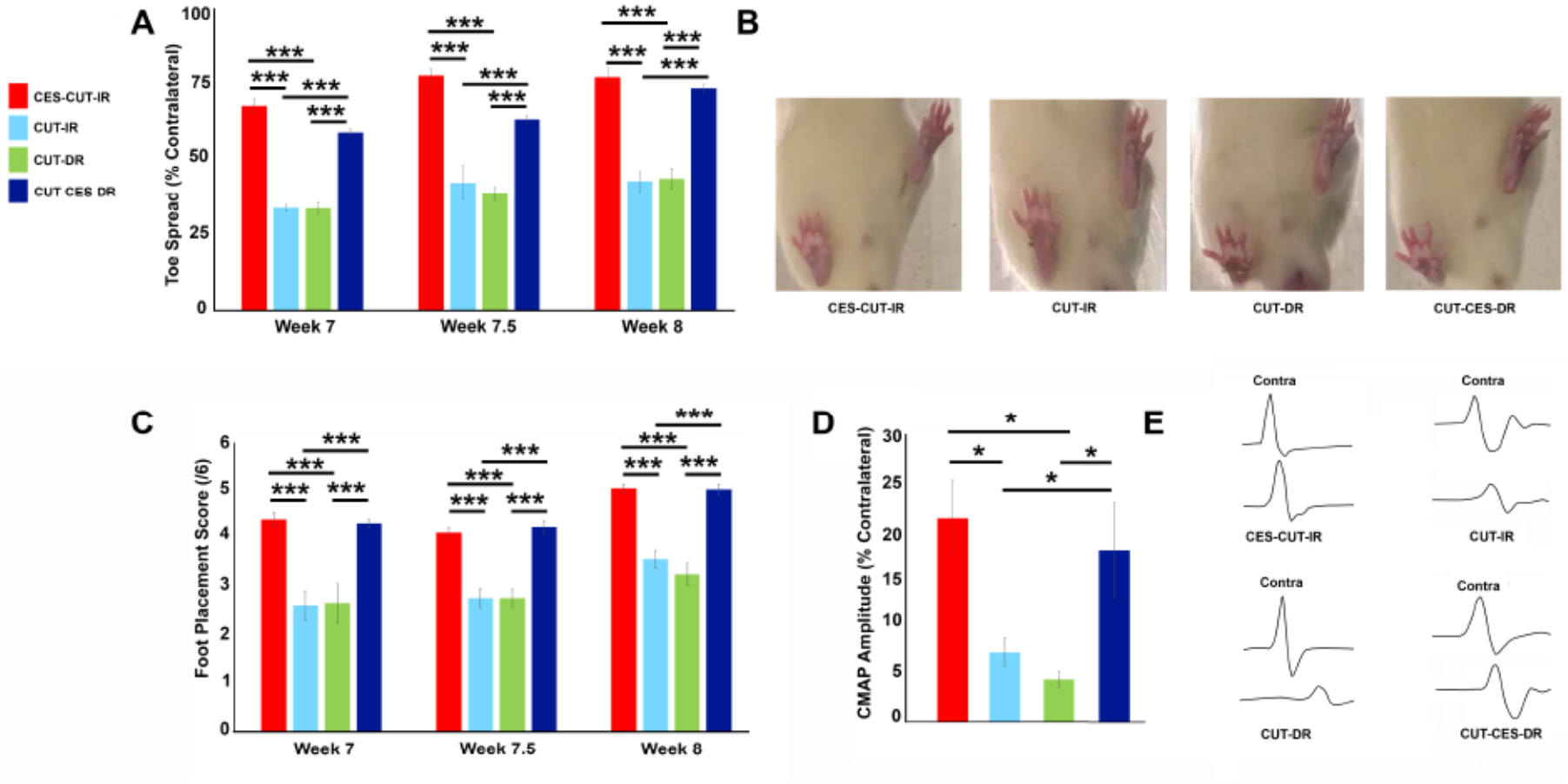
A
B
C
D
E
CES-CUT-IR
CUT-IR
CUT-DR
CUT CES DR
Toe Spread (% Contralateral)
Week 7
Week 7.5
Week 8
CES-CUT-IR
CUT-IR
CUT-DR
CUT-CES-DR
Foot Placement Score (/6)
CMAP Amplitude (% Contralateral)
Contra
CES-CUT-IR
CUT-IR
CUT-DR
CUT-CES-DR

Figure 7.3: Post-injury CES promotes motor reinnervation.

Motor recovery was assessed at 7, 7.5, and 8 weeks following nerve repair. (A) Toe spread was assessed by measuring the distance between the first and fifth toes on the injured foot during normal gait; results were normalized to the contralateral control foot. Histograms at all timepoints demonstrate that animals treated with Cut-CES-DR had significantly wider toe spread compared to non-stimulated Cut-IR (***$p<0.001$) and Cut-DR (***$p<0.001$) animals. (B) Representative photographs of CES-Cut-IR, Cut-IR, Cut-DR, and Cut-CES-DR plantar footpads. (C) Histograms of all timepoints evaluated demonstrate that animals treated with Cut-CES-DR had significantly higher foot placement scores on the horizontal ladder compared to non-stimulated Cut-IR (***$p<0.001$) and Cut-DR (***$p<0.001$) animals, and comparable to the true conditioning electrical stimulation cohort. (D) Compound muscle action potentials (CMAPs) were significantly higher when the transected tibial nerves had been treated with electrical stimulation prior to repair (Cut-CES-DR) compared to Cut-IR (*$p<0.05$) or Cut-DR (*$p<0.05$) animals, and comparable to animals treated with CES prior to injury (CES-Cut-IR). (E) Representative CMAP tracings at 8 weeks of regeneration of CES-Cut-IR, Cut-IR, Cut-DR, and Cut-CES-DR animals.

The gastrocnemius muscle weight of the injured limb, an inverse measure of muscle atrophy, identified greater preservation of muscle weight in animals treated with CES. The injured gastrocnemius muscle of the cut-CES-DR cohort was 53.6 ± 1.7% of the contralateral side, which was significantly greater than 45.8 ± 1.6% in the cut-IR ($p<0.05$) and 48.1 ± 2.8% in the cut-DR ($p<0.05$) cohorts. The cut-CES-DR muscle weight was comparable to the CES-cut-IR positive control cohort (61.8 ± 5.4%, $p=0.1392$) (Figure 7.4A, B). Nine muscle sections per animal were then stained with alpha-bungarotoxin (label acetylcholine receptor) and NF200 (label motor axon) to quantify neuromuscular junction reinnervation. Significantly more innervated NMJs were identified in the cut-CES-DR (14.0 ± 1.0 NMJ/50mm^2) compared the cut-IR (4.3 ± 0.9 NMJ/50mm^2, $p<0.001$) or cut-DR (4.6 ± 0.8 NMJ/50mm^2, $p<0.001$); however, the conditioned CES-cut-IR had significantly more innervated NMJ (19.3 ± 1.3 NMJ/50mm^2) when compared to the post-cut ES cohort ($p<0.05$) (Figure 7.4C, D).

7.6 Discussion

7.6.1 Delayed repair of nerve transections has equivalent outcomes to immediate repair

The timing of traumatic primary nerve injury repair remains an ongoing subject of debate, with no universally accepted algorithm to guide patient management. While it's well established that neurapraxia and axonotmesis usually spontaneously recover, timing to surgical repair of neurotmesis-type injuries repairs poorly defined. Traditional dogma suggests that sharp nerve transections should be repaired within the first couple days of injury, typically in the first 72 hours (Campbell, 2008). Practically, this means that

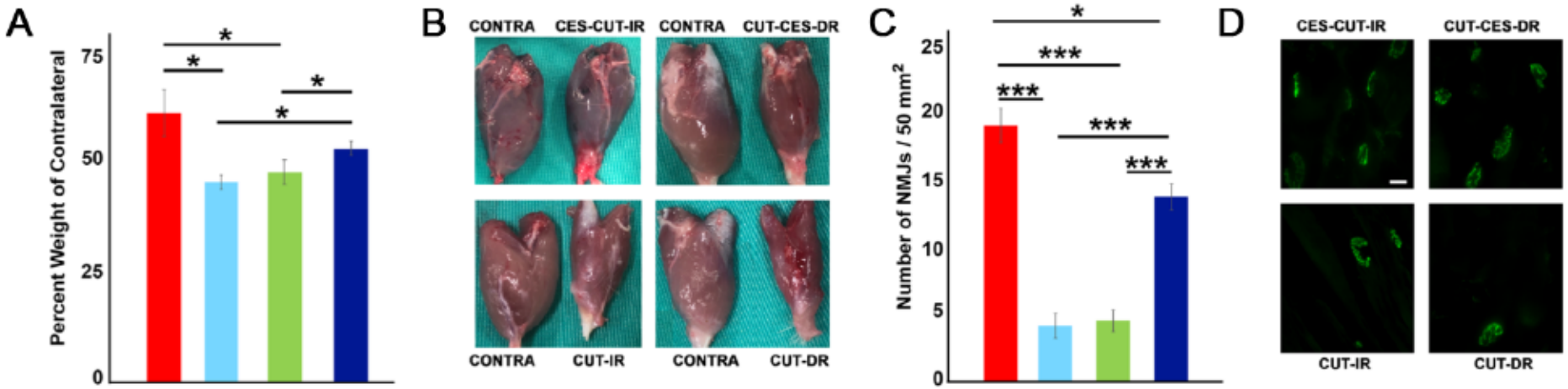

Figure 7.4: Post-injury CES promotes muscle reinnervation. Eight weeks post-nerve repair, the gastrocnemius muscles from the injured and contralateral uninjured limbs were weighed. (A) Histogram demonstrates that transected tibial nerves treated with CES prior to repair (indigo) had significant muscle mass preservation compared to Cut-IR (light blue, *$p<0.05$) or Cut-DR (green, *$p<0.05$) and comparable mass to animals treated with pre-injury CES (red). (B) Representative photographs of gastrocnemius muscles from the injured (right) and contralateral uninjured (left) limbs of animals treated with CES-Cut-IR, Cut-IR, Cut-DR, and Cut-CES-DR. (C) Histogram depicting the quantification of reinnervated neuromuscular junctions in gastrocnemius muscle sections. Animals in the Cut-CES-DR cohort had a greater number of reinnervated neuromuscular junctions compared to Cut-IR (***$p<0.001$) or Cut-DR (***$p<0.001$) animals. (D) Representative photomicrographs of positively stained acetylcholine receptors. Innervation of NMJs was confirmed with NF200. Scale bar in D is 15 µm.

these patients must be booked for surgery as 'urgent', either bumping elective cases the following day or necessitating after-hours surgical management. This in turn confers significant healthcare cost. Such challenges could be overcome with evidence demonstrating that delayed elective repair results in equivalent outcomes as an immediate repair, thus allowing for a scheduled surgical intervention in available time.

A limited number of animal and clinical studies have compared outcomes of immediate and delayed nerve repair. Animal studies have demonstrated similarities in axon numbers, myelin thickness, histomorphology, and tensile strength following rabbit sciatic nerve transections repaired immediately or three days following nerve injury (Piskin et al., 2013). CMAP amplitudes following rat sciatic nerve transection were comparable whether repaired immediately, or at 1 or 4 weeks post-injury (Wu et al., 2013). Clinical studies support these findings of equivalence between immediate and delayed repair: a retrospective study comparing outcomes of peripheral nerve injuries treated on average 3.5 days post-injury found no significant difference in motor or sensory outcomes compared to injuries treated within 24 hours. Furthermore, authors identified no inferior outcomes among patients in whom nerve repair was delayed due to management other trauma. Unfortunately, the specific number of days between injury and repair are not reported; therefore, the true length of delay is unknown (Wang et al., 2017). These findings are echoed in digital nerve transection patient populations, the best studied model for investigating timing to repair. Numerous studies identified no difference in recovery of sensation whether repaired immediately or as late as greater than one year following transection (Bulut et al., 2016; Mermans, Franssen, Serroyen, & Van der Hulst, 2012; Sullivan, 1985).

Our study supports the theory that delayed-repaired nerve transections have equivalent outcomes as those repaired immediately. We identified no difference in the length of nerve regeneration, nor motor and sensory functional recovery, in animals repaired immediately (cut-IR) or 10 days post-injury (cut-DR). Our findings suggest a possible indication for change in clinical practice, in which nerve repair can be performed electively up to 10 days post-injury without increasing the risk of functional deficits. We further demonstrate that the addition of post-injury electrical stimulation delivered one week prior to nerve repair significantly improves nerve regeneration and sensorimotor reinnervation beyond that obtainable with either an immediate or a delayed nerve repair.

7.6.2 Post-injury electrical stimulation enhances regeneration similar to a conditioning lesion

The crush conditioning lesion is well-accepted as one of the most effective methods to significantly enhancing outcomes following nerve injury. In this technique, the nerve is intentionally crushed one week prior to transection and repair. The initial crush injury upregulates pro-regeneration pathways such that regeneration is accelerated following transection and repair. Our lab has previously demonstrated that CES is a clinically feasible method of delivering a conditioning-like effect, with outcomes that supersede those obtainable by a gold-standard conditioning crush lesion (Senger et al., 2019; Senger et al., 2017). The timing of pre-injury CES allows for delivery of electrical stimulation in elective nerve surgeries such as a distal nerve transfer, in which the timing of nerve transection is planned and the donor nerve could be conditioned one week prior to surgery. How to apply this technique to a nerve that is already transected and amenable to primary repair, however,

remains a challenging question. As delivery of conditioning prior to injury is impossible in this clinical scenario given the unpredictable timing of trauma, we evaluated whether the use of electrical stimulation after injury, but prior to repair, would induce a conditioning-like effect.

Our results suggest that post-injury electrical stimulation with repair delayed to 10 days following injury induces a conditioning-like effect in an injured nerve. The length of nerve regeneration at 2 weeks post-coaptation was similar between animals treated with post-injury CES one week before repair (cut-CES-DR 12.1 ± 0.7 mm) and our positive control of nerves conditioned 7 days prior to transection/repair (CES-IR, 12.5 ± 0.8mm). This was significantly longer than animals treated with immediate or delayed repair without electrical stimulation ($p<0.001$). All motor and sensory outcomes in the post-injury CES cohort were significantly greater than the non-electrically stimulated immediate or delayed repair cohorts; however, outcomes following Cut-CES-DR were not quite as robust as true pre-injury conditioning. These results suggest that post-injury CES is creating a significant, though slightly less robust conditioning-like effect than the compared to the traditional conditioning paradigm.

7.6.3 Post-injury electrical stimulation requires clinical investigation

Our results suggest that post-injury electrical stimulation with delayed primary repair significantly enhances nerve regeneration and sensorimotor recovery. Postoperative electrical stimulation is established as safe and well-tolerated by patients (Gordon, Amirjani, Edwards, & Chan, 2010; Wong, Olson, Morhart, & Chan, 2015); therefore, clinical

translation of post-injury CES is anticipated to be well-accepted by patients and clinicians. At our center, patients with peripheral nerve injury are commonly identified in peripheral centers and referred to plastic surgery for definitive repair. To account for this delay in assessing patients, we chose to deliver post-injury CES three days following nerve transection; however, it is likely that outcomes would be comparable if CES was administered earlier.

Despite these exciting results, significant challenges preclude immediate clinical translation. The optimal timing between delivering the electrical stimulation and performing the repair likely differs between rat and human models given inherent differences in nerve length. The traditional conditioning effect requires a retrograde signal from the site of conditioning to the cell body, transcription and translation of regeneration associated genes, and anterograde transportation of protein products to the site of injury, all prior to nerve injury and coaptation (Senger, Verge, Chan, & Webber, 2018). Given that velocity is distance-dependent, the optimal interval between conditioning and definitive repair is likely longer in a human when compared to a rat. Testing on larger primates or human clinical trials are required to assess outcomes with varying conditioning intervals is required to optimize recovery.

A second potential challenge is that of nerve retraction. As immobilization of the lower limb of a rodent is not feasible, the proximal and distal nerve stumps were sutured to nearby muscles to maintain nerve length, allowing for primary repair at ten days post-cut in the two delayed-repair cohorts. Delayed repair in a human clinical setting will likely require immobilization from time of injury to repair to minimize nerve retraction, and more robust

surgical mobilization of the nerve may be required to approximate the proximal and distal nerve stump. Failure to reapproximate the two stumps will result in a nerve gap, and a nerve graft or conduit may be necessary. Outcomes following nerve grafting or use of conduits are inherently poorer than primary coaptation; therefore, a direct comparison of regeneration outcomes of a conditioned graft with to a non-conditioned primary repair is of significant importance.

Our results support a change in clinical management of acutely transected peripheral nerve. Though immediate repair within 72 hours is currently standard of care, we demonstrate that outcomes following primary repair at 10 days are similar to immediate coaptation. We further suggest that post-injury electrical stimulation with repair 7 days following delivers a conditioning-like response, with significant improvement in motor and sensory recovery. Further research to translate this strategy to a human population are necessary to optimize patient outcomes.

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Chapter 8

Discussion

8.1 Overview

In 1973, McQuarrie and Grafstein reported that regeneration following a 'test' cut injury could be improved by nearly 30% if the nerve was crushed two weeks prior (McQuarrie & Grafstein, 1973). This initial crush was named a 'conditioning lesion' as it was hypothesized that the crush 'conditioned' the nerve by initiating the molecular and cellular pathways needed for regeneration such that at the time of the 'test' injury, the locoregional environment would be already primed to support regeneration. As described in Chapter 1, forty decades of research have delineated the molecular mechanisms underlying the conditioning effect and solidified this technique as a highly effective means of improving regeneration and reinnervation outcomes. While these foundational studies have provided valuable insights into the innate mechanisms of nerve regeneration, clinical translation remains impossible due to the injurious nature of a conditioning crush. Attempts to replicate a conditioning effect using a non-injurious, clinically feasible method have included use of ethidium bromide (Hollis et al., 2015), freezing (Bondoux-Jahan & Sebille, 1988), vibration (Dahlin, Necking, Lundstrom, & Lundborg, 1992), and electromagnetic field exposure (Sisken, Kanje, Lundborg, Herbst, & Kurtz, 1989); however, regeneration results have consistently remained inferior to the gold-standard crush conditioning lesion.

A pivotal study by Udina et al. (2008) identified electrical stimulation as a potential technique for inducing a conditioning effect in the central nervous system. Authors found that delivering one hour of 20 Hz conditioning electrical stimulation (CES) to the sciatic nerve prior to culturing the dorsal root ganglia enhanced neurite extension four-times greater than DRGs that received sham control. These pro-regenerative effects translated to an *in*

vitro study where authors found that CES significantly improved regeneration within the spinal cord (Udina et al., 2008). These findings differ from results of Brushart et al (2002), which reported that pre-injury electrical stimulation did not accelerate the rate of axon regeneration when compared to a nerve injury in the non-stimulated contralateral control limb (Brushart et al., 2002). It is, however, possible that no difference was observed because the contralateral limb was inadvertently conditioned by the electrical stimulation. Unilateral crush conditioning has previously been shown to enhance regeneration in the uninjured contralateral limb (Ryoke et al., 2000). To resolve the discrepancy between these two studies, we investigated whether CES promotes regeneration in the peripheral nervous system. This is of particular clinical importance given numerous studies confirming that electrical stimulation is safe and well-tolerated by patients.

To investigate the hypothesis that CES induces a conditioning effect comparable to a CCL, we conducted a pilot study in a rodent model directly comparing regenerative outcomes and gene expression induced by these two forms of conditioning. In Chapter 2, we demonstrate that animals who were treated with CES one week prior to a common peroneal nerve injury and repair had upregulation of regeneration associated genes (RAGs: GAP-43, BDNF, and GFAP) and improved axon extension comparable to animals who were treated with a traditional CCL and greater than negative controls (J.L. Senger, Chan, Verge, & Webber, 2017). These promising preliminary results prompted a more thorough investigation of sensory and motor reinnervation outcomes of CES. To maximize the number of sensory and motor outcomes that could be evaluated, we shifted to a tibial nerve injury model. In Chapter 3, we confirmed our preliminary findings, demonstrating that CES of the tibial nerve

similarly upregulated RAGs and improved axon extension. We further demonstrated that motor and sensory reinnervation outcomes of CES supersede those obtainable by a CCL. This difference was found to be attributable to the injury caused by the crush distal to the site of cut/coaptation through which regenerating axons must regenerate; as CES is noninjurious to axons, regenerating fibers are not delayed at the conditioning site [Figure 3.6] (J. L. Senger et al., 2019).

Having established CES as an effective technique to significantly improve outcomes, we compared the regenerative effects of CES with the only ancillary perioperative technique currently in clinical practice aimed at enhancing outcomes following peripheral nerve injury – postoperative electrical stimulation (PES). In keeping with previous studies, we found PES improved outcomes beyond negative controls; however, CES-treated animals had the best regeneration and reinnervation for all outcomes evaluated (Chapter 4). To lay a strong foundation for future human clinical trials, we investigated the effects of CES on the three most common options for nerve reconstruction: nerve autograft repair (Chapter 5), distal nerve transfer (Chapter 6), and primary repair (Chapter 7). In all clinically-relevant models, nerves treated with CES had significant improvement in regeneration and reinnervation outcomes.

These exciting results establish CES as an effective technique for significantly improving outcomes following peripheral nerve surgeries. Further research is required to determine how CES induces these pro-regenerative effects, whether outcomes can be further improved, and to rectify remaining variables critical to successful human translation.

8.2 Mechanism of action of CES

8.2.1 Inflammation

An important difference between CCL and CES is the perceived extent of injury induced to the nerve itself. It is well accepted that a complete crush injury causes a phenotypic change in native Schwann cells from myelinating to proliferative; these activated Schwann cells secrete chemokines that stimulate neutrophil and macrophage infiltration to assist in the breakdown of neurofilament and thereby evoke Wallerian degeneration (Lindborg, Mack, & Zigmond, 2017). This inflammatory response is critical for mounting a conditioning effect. Nerves conditioned with a crush prior to transection and repair have significantly more macrophages present compared to unconditioned transected controls (Salegio, Pollard, Smith, & Zhou, 2010). Numerous rodent models of macrophage depletion have been described, including a) the injection of liposome-encapsulated clodronate, b) knockout models of the chemokine *Ccl2* or its receptor *Ccr2*, c) delivery of the neutralizing antibody MC-21 against CCR2, or d) pharmacologic agents such as Minocycline or a colony-stimulating factor-1 inhibitor (Zigmond & Echevarria, 2019). These models have established that the crush conditioning effect is eliminated in the absence of macrophages (Kwon et al., 2013; Kwon et al., 2015; Niemi et al., 2013; Salegio, Pollard, Smith, & Zhou, 2011). The importance of a macrophage-mediated inflammatory response is supported by the observation that intrathecal injection of CCL2 cDNA cloned into an adeno-associated viral vector causes a conditioning-like effect in the absence of nerve injury (Niemi, DeFrancesco-Lisowitz, Cregg, Howarth, & Zigmond, 2016).

While the role of macrophages in supporting crush conditioning is well described, the effects of other inflammatory cells remains poorly understood. One potential leukocyte that may play an important role in mediating the relationship between inflammation and regeneration is the neutrophil. Recent work from the Zigmond lab suggests that neutrophils may be the primary phagocytic cell responsible for Wallerian degeneration, not macrophages as previously accepted (Lindborg et al., 2017). Though not previously explored, the role of neutrophils in supporting a crush conditioning effect is of significant interest.

In contrast to a crush lesion, the effects of CES on the nerve and its environment in promoting regeneration are unknown. Our current assumption is that electrical stimulation is a non-injurious technique, as it is currently being used postoperatively with no adverse side effects; however, determining whether the nerve is injured by electrical stimulation, and whether an inflammatory response is induced, is of significant clinical interest. Conditioning the donor nerve prior to DNT necessitates a non-injurious technique to prevent denervation of distal motor and sensory targets beyond the branch selected for transfer. As described in Chapter 7, our preliminary results suggest that CES is non-injurious and does not cause Wallerian degeneration; animals were found to have no tibial nerve deficits on gait analysis, suggesting the nerve distal to the conditioning site was not damaged by delivering CES. The clinical implications of upregulating or altering the inflammatory response are less clear. It is known that inflammation can lead to negative postoperative outcomes, as local inflammation may amplify scar tissue creation which can impair tendon excursion, particularly in patients with concurrent soft tissue injuries. Excessive scar formation may necessitate surgical release of the tendon from surrounding scar (tenolysis) with variable long-term outcomes. Among

patients presenting with a nerve transection, however, the inflammatory process has already been initiated and electrical stimulation may be advantageous in modulating this response. Our preliminary results suggest CES does not induce a macrophage-mediated inflammatory response; however, how CES affects the local environment post-nerve injury when the inflammatory response has already been initiated remains unknown. In a rodent model of demyelination, PES has been shown to change the macrophage phenotype from pro-inflammatory (M1) to pro-repair (M2) (McLean & Verge, 2016). Further investigations are required to determine the effects of CES on the inflammatory microenvironment and the subsequent clinical implications.

If CES is indeed promoting regeneration without creating injury or inflammation, this provides novel insights into current understandings of nerve regeneration. Neuroinflammation is traditionally accepted to be crucial for Wallerian degeneration and the regenerative process (Zigmond & Echevarria, 2019); however, CES allows us to separate the processes of degeneration and regeneration. To determine the roles of macrophages and neutrophils specifically in regeneration, we intend to investigate whether CES maintains its regenerative effects in macrophage-depleted CCR2 knockout animals, or in animals treated with Ly6 which suppresses neutrophils. Identification of a conditioning effect following CES in the absence of these inflammatory cells would strongly suggest its effects are independent of inflammation, and therefore the mechanism of action of CES differs from that of the traditional crush conditioning paradigm.

At the level of the cell body, current dogma suggests that upregulation of RAGs is dependent on injury. Immunohistochemistry, qRT-PCR, and Western blot analysis results suggest that CES upregulates GAP-43, GFAP, pCREB, and BDNF comparably to a crush lesion. Evidence suggesting CES to be non-inflammatory and non-injurious, would suggest that these genes can be upregulated independent of injury. These findings would support our hypothesis that the regenerative processes can be induced by CES without inflammation or injury.

8.2.2 Calcium Signaling

Calcium flux is central to the nerve's response to injury. Injury to the axonal membrane causes an early retrograde calcium wave followed by a delayed retrograde signal mediated by importins and the JUN kinase pathway (Rishal & Fainzilber, 2014). The early calcium wave is attributed to opening of sodium channels causing high levels of intracellular sodium to invert the Na/Ca exchange pump, increasing intracellular calcium. These processes are in keeping with the 'set hypothesis' proposed by Kater and Mills which suggests that a specific level of calcium is required for neuronal survival and growth (Kater & Mills, 1991). At the cell body, the calcium signal is crucial for upregulating immediate early genes such as cAMP and c-fos (Singh et al., 2015). In addition to signaling the cell body of an injury, calcium influx is responsible for sealing the membrane of the proximal stump and the cytoskeletal rearrangement necessary to form the growth cone. Too much calcium, however, is detrimental to the regeneration process; therefore, channel activity is tightly regulated (Rishal & Fainzilber, 2014).

Similar to the injury response, the effects of electrical stimulation appear to be mediated by calcium. Biphasic electrical stimulation delivered to the dorsal root ganglia causes an increase in intracellular calcium concentrations via voltage-dependent calcium channels (Adams, Gupta, & Harkins, 2017). The concentration of calcium influx, however, is less than the threshold to initiate an action potential. The regenerative effects of electrical stimulation are blocked when phosphatidyl-inositol 3-kinase (PI3-K) is inhibited. Intracellular calcium stores do not appear to have a major role in promoting neurite outgrowth after electrical stimulation, suggesting T-type calcium channels are not involved in the mechanism (Singh et al., 2015). These studies suggest that perhaps electrical stimulation induces retrograde electrical signalling from the peripheral nerve to the cell body in a calcium-dependent manner comparable to an injury signal, which prompts the cell body to mount a similar regenerative response. It would be interesting to observe if CES evokes an immediate influx of intracellular calcium and whether the calcium comes from intracellular stores such as the mitochondria, or rather from external sources.

8.2.3 Mitochondrial Transport

Wallerian degeneration and axonal regeneration following peripheral nerve injury necessitate an enormous amount of energy; therefore, the mitochondria are important players in the regenerative process. Though the majority of mitochondria are anchored in regular intervals along the axons, a small proportion are mobile, transported anterogradely and retrogradely along the axon's microtubules by kinesin and dynein respectively. Following nerve injury, anchored mitochondria become mobile, rates of fission are increased, and

transportation is accelerated to the site of injury where they produce the ATP necessary for building new axons (Kiryu-Seo & Kiyama, 2019).

Both PES and crush conditioning influence transportation of mitochondria. A conditioning crush induces an increase in the percentage and total number of mobile mitochondria (Mar et al., 2014). Low frequency (1 Hz) electrical stimulation increases both anterograde and retrograde transportation of mitochondria; however, when delivered at a physiologic frequency (50Hz), only anterograde transportation is accelerated. Additionally, higher frequency conduction results in fission of the mitochondria to increase the number of trafficking organelles (Sajic et al., 2013). It is likely that CES at 20 Hz similarly has effects on mitochondrial transportation and activation.

8.2.4 Molecular Pathways

The molecular pathways underlying the PES and the crush conditioning effect (Chapter 1) are well understood. Numerous overlapping pathways are identified in both models, including the adenylyl cyclase pathway, the BDNF/trkB pathway, and the PI3-K pathway. It is likely that the molecular effects of CES involves similar players. We have collected DRG and nerve samples treated with either electrical stimulation or with a crush at 1 and 3 days following conditioning. These samples have been sent for mass spectrometry to identify similarities and differences in the key proteins responsible for inducing the two conditioning paradigms. Once major pathways have been identified, we intend to perform neurite outgrowth assays of DRGs cultured in the presence of agonists or antagonists to key players within these molecular pathways. Results can be confirmed *in vivo*, either using knockout

models of genes of interest, or using mini-osmotic pumps for local delivery of agonists and antagonists in the presence of CES to determine under which condition RAGs are elevated and enhanced axonal extension is observed.

8.3 Enhancing the effects of CES

The electrical stimulation paradigm chosen for delivering CES, including the frequency, duration, and voltage, were based on the parameters currently being used in both human and rodent models of PES as described in Chapter 1. Similarly, as is discussed later, the time interval between conditioning and nerve surgery, as well as the location of the CES relative to the site of injury and repair, are both based on results obtained in a crush conditioning model. Whether outcomes can be further augmented by adjusting any of these factors will require well-designed studies to systematically evaluate each variable.

Unlike other perioperative techniques currently described to improve nerve regeneration, CES is a preoperative intervention. Accordingly, combining the conditioning effect with a postoperative intervention is feasible and may be a strategy for significantly improving outcomes. As discussed in Chapter 4, the combination of CES with PES had a detrimental effect on regeneration and reinnervation, with the addition of electrical stimulation following nerve repair actually decreasing the pro-regenerative effects of conditioning. The mechanism underlying this relationship will require further investigation, which will lend insight into the effects of electrical stimulation at various times in the regenerative process. How the conditioning effect will be altered by other pro-regenerative postoperative interventions is therefore of considerable interest. A limited number of techniques are currently being

investigated in the laboratory with the potential for patient translation. Postoperative exercise training, acute intermittent hypoxia, and local delivery of tacrolimus or capsaicin are the most promising targets for combining with CES.

8.3.1 Exercise treatment

Post-injury exercise training is well described to improve nerve regeneration following injury. This hypothesis was established based on the supposition that if artificially activating the nerve using electrical stimulation enhances regeneration, naturally activating axonal populations with exercise should have similar effects. Similar to PES, exercise training was reported to reduce the time necessary for staggered regeneration (English, Cucoranu, Mulligan, & Sabatier, 2009) and treadmill training following peripheral nerve transection and repair enhanced the length of axon extension (Sabatier, Redmon, Schwartz, & English, 2008). The optimal exercise training regimen was found to differ based on the sex of the animal, with males benefiting from low-intensity continuous training, and females from high-intensity interval training (Wood et al., 2012). Expression of androgens was essential for the enhancing effects of exercise in both sexes (Thompson, Sengelaub, & English, 2014) When males, but not females, were treated with low-intensity continuous exercise, concentration of serum testosterone and *bdnf/trkB* mRNA in the lumbar spinal cord was identified. It was therefore suggested that continuous treadmill training increased production of androgens in males, which resulted in upregulation of BDNF and TrkB protein. By contrast, high-intensity interval training was hypothesized to inhibit aromatase, which normally converts testosterone to estradiol, and did not appear to significantly upregulate BDNF expression (Wood et al., 2012) however, neuronal BDNF was necessary for the

regenerative effects of exercise in both sexes (Wilhelm et al., 2012). The precise relationship between androgens and BDNF expression requires greater investigation.

It is suggested that following both electrical stimulation and exercise treatment, enhanced regeneration is attributable to autocrine BDNF signalling, in which regenerating axons secrete BDNF that binds to TrkB receptors on adjacent axons to promote growth (English, Wilhelm, & Ward, 2014). The role of androgens and sex differences has not been well studied in the electrical stimulation or the conditioning literature. It has been shown that blocking androgen signaling eliminates the effects of PES (Cafferty et al., 2004), however, the implications of this finding are poorly understood. Should findings in the post-injury exercise literature correspond to electrical stimulation paradigms, perhaps males would benefit greater from the current model of low frequency, continuous lengthy duration stimulation whereas females should receive electrical stimulation at a higher frequency but for repeated short periods of time. Both conditioning and electrical stimulation have predominantly been investigated in sciatic nerve injury models, which are less androgen sensitive compared to other motoneurons (Brown, Khan, & Jones, 1999; Wood et al., 2012). Perhaps an alternative nerve injury model more sensible to androgens, such as the facial nerve, will better delineate differences in the responsiveness of males and females to CES.

Interestingly, pre-injury exercise induces a conditioning-like effect. Animals with voluntary access to a running wheel prior to injury are reported to have increased neurite extension *in vitro* and improved axonal extension following a nerve crush *in vivo* with upregulation of the RAGs (Molteni, Zheng, Ying, Gomez-Pinilla, & Twiss, 2004). How an exercise conditioning

effect compares to the gold-standard crushing technique that traditionally has been the standard for comparison is unknown; a comparison of an exercise conditioning effect with electrical stimulation, as well as determination whether an additive effect can be obtained by combining conditioning exercise with conditioning electrical stimulation, are both therefore of clinical importance.

8.3.2 Acute Intermittent Hypoxia

Exciting new research from the Verge lab has combined motor training with acute intermittent hypoxia as a method of augmenting plasticity after spinal cord injury. The combination of both techniques resulted in upregulation of BDNF and TrkB, the relevance of which is discussed in Chapter 1, as well as the transcriptional activator hypoxia-inducible factor-1a (HIF-1α) and its downstream target vascular endothelial growth factor (VEGF) (Hassan et al., 2018). VEGF is a glycoprotein fundamental to regulating angiogenesis and has a neurotrophic and neuroprotective role within the central and peripheral nervous systems. Given these promising results, investigation of the effects of acute intermittent hypoxia in the peripheral nervous system merits further investigation, particularly given the potential for clinical translation.

8.3.3 Tacrolimus (FK506)

Tacrolimus, or the ligand FK506, is an immunosuppressant used to prevent rejection following allograft transplantation by inhibiting T-cell proliferation. Tacrolimus has a neuroprotective and neurotrophic effect that promotes enhanced regeneration after nerve injury by binding to the immunophilin FKBP-52. FKBP-52 then dissociates from a mature

steroid receptor complex to activate the extracellular signal regulated kinase (ERK) pathway and upregulate expression of c-jun and GAP-43 (Udina et al., 2004). Numerous studies have demonstrated improved outcomes including decreased latency period between injury and initiation of regeneration, accelerated time to end target reinnervation with less muscle atrophy, and accelerated axonal sprouting (Navarro, Udina, Ceballos, & Gold, 2001; Sulaiman, Voda, Gold, & Gordon, 2002; Tung, 2015; Udina et al., 2004). Post-injury tacrolimus doubles the number of regenerating axons after injury and increases the myelin thickness (Tung, 2015). Systemic administration of tacrolimus to enhance nerve regeneration is not safe given the significant side effect profile which includes malignancy, infection, nephrotoxicity, and diabetes. Numerous techniques for local delivery of Tacrolimus to the nerve however have been described, all of which improve nerve regeneration outcomes, including FK506-treated nerve allograft (Shahraki, Mohammadi, & Najafpour, 2015; Yin et al., 2018), dissolving FK506 in fibrin gel (Tajdaran, Shoichet, Gordon, & Borschel, 2015), and controlled-delivery FK506 nerve conduit (Labroo et al., 2016). The effects of Tacrolimus have yet to be directly compared to conditioning or to electrical stimulation.

8.3.4 Maximizing Recovery – Future Directions

The ideal perioperative intervention should be tailored to the type of injury and to the specific patient. Young children may not be suitable candidates for electrical stimulation as it requires percutaneous needle placement, whereas the elderly may not tolerate a vigorous exercise regimen. Similarly, patients with a neurapraxic nerve injury who do not require surgical intervention may be better candidates for minimally invasive interventions to enhance nerve regeneration compared to neurotmetic lesions with a gap requiring bridging with a scaffold

enhanced by growth factors or FK506. In an idyllic future, the peripheral nerve team will have an arsenal of perioperative interventions such that each treatment regimen is tailored specifically to the needs of the individual patient.

Despite the numerous perioperative strategies available to enhance regeneration outcomes, it is likely there is no single intervention that will best serve the needs of all patients with a peripheral nerve injury. Strategies such as CES, PES, and tacrolimus target one specific nerve, exercise can activate all the nerves in a specific limb, and intermittent hypoxia has systemic effects. As described in Chapter 4, the combination of CES and PES not only has no synergistic effect, but rather delivery of PES dampens the conditioning effects. Observations such as these provide insight into the molecular mechanisms of individual and combined therapies. Future research exploring which perioperative technique can be combined to optimize effectiveness will require evaluation in different models of nerve injury, types of nerve involved, surgical treatments planned, and distance of regeneration required.

The combination of post-injury treadmill running and PES has been shown to have a synergistic effect in enhancing the onset of muscle reinnervation; however, long-term motor recovery was similar to either treatment alone (Asensio-Pinilla, Udina, Jaramillo, & Navarro, 2009). How the combination of exercise and CES affects regeneration is an important, clinically relevant question. To lay the foundation for future clinical trials, we are creating an animal model of a common clinical distal nerve transfer to restore elbow flexion. In this DNT of the upper limb first described by Oberlin, an intact branch of the ulnar nerve is

transected and coapted to the distal musculocutaneous nerve as it inserts into the biceps brachii muscle (Oberlin, Ameur, Teboul, Beaulieu, & Vacher, 2002; Oberlin et al., 1994). Once the model is established, we will deliver CES to the ulnar nerve one week prior to nerve transfer and compare outcomes to animals treated with daily reaching tasks exercise to stimulate their ulnar nerve, and animals treated with both CES and a regular exercise paradigm.

8.4 Clinical translation of CES

Our animal data strongly suggests that CES significantly improves regeneration and reinnervation outcomes in numerous common clinical scenarios of nerve repair and reconstruction. Previous experiences with PES suggest electrical stimulation is safe and well-tolerated. Translation of CES to the clinic however is faced with numerous challenges. Two important questions that must be investigated are the location of stimulation, and the timing between stimulation and nerve repair.

8.4.1 Location of stimulation

The optimal location for delivering CES relative to the site of injury and the cell body requires further investigation and is of significant importance for clinical translation of this technique. The CCL literature is divided as to whether conditioning has effects locally or systemically. Some authors suggest that the CCL has improved regenerative outcomes the closer the two lesions are placed on the nerve itself (Bisby & Pollock, 1983; Sjoberg & Kanje, 1990a, 1990b). These studies suggest that CCL induces local changes that are critical to conditioning, indicating that the conditioning lesion needs to be delivered to the site of future

nerve surgery: the proximal stump of the transected nerve planned for primary repair or nerve grafting, or the donor nerve for a DNT at the site where the cut is planned.

Other studies suggest that conditioning only requires a common cell body. Ryoke et al (2000) showed that conditioning the left sciatic nerve significantly decreased the duration of time between injury and the initiation of axonal extension, following a contralateral sciatic nerve injury, suggesting conditioning may have a transganglionic effect, with communication across the spinal column (Ryoke et al., 2000). These findings are supported by recent reports that sciatic nerve transection causes upregulation of RAGs in the contralateral DRGs with enhanced neurite elongation when cultured *in vitro* (Dubovy et al., 2019). Other studies, however, refute this hypothesis, showing that crush conditioning to a single branch of the sciatic nerve followed by transection of the entire nerve enhances regeneration only in the conditioned branch (McQuarrie, 1978).

Whether the optimal location of crush conditioning corresponds to the optimal location for CES is debatable. As previously discussed, the conditioning effect secondary to a crush is highly dependent on an inflammatory response, suggesting that perhaps the site of nerve transection and repair must be within this local inflammatory environment. By contrast, if CES is independent of a localized inflammatory response, it is possible that the optimal site of delivering the electrical stimulation is less restricted to the site of conditioning. Evaluation of outcomes of CES delivered locally and remotely are of significant clinical importance, as the nerve requiring conditioning may not be easily accessible for percutaneous electrical stimulation due to a) proximity to vital structures such as major vessels around the brachial

plexus, b) a small nerve caliber, such as when targeting specific nerve branches for distal nerve transfer, or c) an unpredictable anatomical course such as in traumatic nerve injury with associated soft tissue defects.

8.4.2 Timing of stimulation

The conditioning interval, or the time between delivering a CCL and the subsequent nerve cut and repair, has been well studied in rodent models, concluding in an optimal duration of seven days. A shorter conditioning interval precludes upregulation of RAGs and anterograde transport of their protein products prior to the test lesion (Torigoe, Hashimoto, & Lundborg, 1999; Winderberg, Lundborg, & Dahlin, 2001); by contrast, if the interval is lengthened, the pro-regenerative effects are 'burnt out' by the time the second lesion is delivered (Arntz, Kanje, & Lundborg, 1989). Based on these studies, we similarly chose a conditioning interval of one week between CES and nerve cut and coaptation. Whether the optimal conditioning interval of CES differs from a CCL requires further investigation.

When translating this technique to a human population, determining the optimal conditioning interval is further complicated by the size discrepancy between rodent and human. It is likely the greater distance between the cell body and the conditioning site will alter the ideal conditioning interval. After conditioning is delivered to the nerve, a retrograde signal must travel from the site of conditioning to the cell body to induce transcription and translation of the RAGs. Protein products are then transported in an anterograde manner down the nerve to the site of injury, where the growth cone is being established. As the velocity of retrograde and anterograde transportation is distance-dependent, it is likely that a longer time period

will be required in a human than in a rodent, where the absolute length of the limbs is significantly shorter.

A second variable that for consideration is the site of nerve repair. All of our rodent studies to date have investigated nerve cut/coaptations at a standardized location; however, it is likely that the optimal conditioning interval will be shorter in a proximal injury, where the site of nerve injury and the cell body is closer together, and a longer conditioning interval will be necessary for more distal injuries. To overcome these obstacles, investigation of various conditioning intervals, at several standardized locations along the nerve is necessary in a large animal model to establish a length/time relationship. Ideally, in the future the surgeon will measure the distance between the site of nerve injury and the patient's spine and use this information to calculate the optimal conditioning interval.

8.4.3 Type of nerve involved

Our results suggest that the regenerative potential of the common peroneal nerve differs from that of the tibial nerve. Animals with a tibial nerve injury in the unstimulated negative control cohort (Chapter 3) had a significantly longer length of nerve regeneration when compared to the negative control animals with a common peroneal nerve injury (Chapter 2). Despite this accelerated innate regenerative capacity of the tibial nerve, it was the common peroneal nerve that had a greater improvement in length of regeneration by adding CES. This suggests that the intrinsic rate of nerve regeneration likely differs between specific nerve, and that the conditioning effect may also be variable between different nerves. Clinically, we have observed that patients with injury to specific nerves, such as the radial nerve, tend to recover

faster than when other nerves, such as the ulnar, are involved. While these observations are partially attributable to the distance required for regenerating axons to reach their target endplate, our study suggests inherent differences in nerve regeneration are also responsible. The mechanism underlying differences in rate of regeneration, or responsiveness to conditioning, are poorly understood.

It has been suggested that the regenerative potential of individual nerves may be pre-determined during embryologic development. Franz (2008) suggested that the mature neuron's expression of polysialic acid (PSA) following injury is established during embryogenesis. This is of great importance, as expression of PSA is essential for preferential motor reinnervation, or the specificity of regenerating motor fibers to regenerate down motor branches, and sensory fibers to sensory branches. Motoneurons that do not express PSA do not exhibit preferential motor reinnervation. Interestingly, not only does the level of PSA correspond to the regenerative potential of the nerve, but it also appears to predict the nerve's response to post-injury electrical stimulation: only motoneurons that upregulate PSA were found to benefit from PES, and the pro-regenerative effects of PES were abolished if PSA was removed. Furthermore, authors found that delivery of electrical stimulation increased expression of PSA (Franz, Rutishauser, & Rafuse, 2005). These observations suggest a need to evaluate the effects of CES on promoting PSA expression. Perhaps pre-injury CES upregulates PSA in a naive nerve, such that at the time of injury, expression of PSA is high and therefore preferential motor reinnervation is enhanced. If so, CES may be particularly important for enhancing outcomes of nerves with a poor innate regenerative capacity due to inherently low-levels of PSA expression. This hypothesis is supported by the observation

that both crush conditioning and PES have been shown to significantly improve preferential motor reinnervation (Al-Majed, Neumann, Brushart, & Gordon, 2000; Brushart, 1988).

8.5 Future Clinical Directions

Peripheral nerve injuries are heterogenous in the affected patient population, the extent of functional deficits, and the projected outcome. The potential clinical applications for CES are therefore numerous. CES could be delivered prior to i) decompression surgery for patients with compression neuropathies, ii) distal nerve transfer or graft repair for patients with traumatic brachial plexus injuries, or iii) primary nerve repair for patients with a sharp laceration. Furthermore, CES may be an important tool for improving motor and sensory recovery for patients undergoing oncologic resection of soft tissue tumors with nerve graft reconstruction of deficits. In addition to treating patients with nerve injuries, CES has the potential to significantly improve outcomes for patients with an upper limb amputation undergoing targeted muscle reinnervation (TMR). TMR involves transferring healthy nerves to specific target muscles from which EMG signals are recorded and used to control a motorized prosthesis. This technique allows the user to think about a movement they wish to make, and thereby cause the prosthesis to move accordingly. Robust nerve reinnervation is central to the success of TMR; therefore, these patients are ideal candidates for treatment with CES prior to surgical re-innervation of muscle targets.

Finally, the effect of CES may be extrapolated to improve outcomes for patients undergoing microsurgical free flap breast reconstruction. In many patients, breast reconstruction using abdominal tissue (DIEP flap) is the most effective way of restoring a symmetric,

aesthetically-pleasing breast that responds to weight changes and aging in a similar manner to the native breast. A major drawback of this procedure is the resultant insensate reconstructed breast. Described in the *New York Times* as "the next frontier" in breast reconstruction (Rabin, Jan 29 2017), restoration of breast sensation is of paramount importance. The insensate breast is at high risk of burns and injury (Mohanna, Raveendran, Ross, & Roblin, 2010), as the patient is not aware of tissue trauma; more importantly, it is significantly disadvantageous to the quality of life of these women. Recently, neurotization of the abdominal skin paddle using the intercostal nerves has been described as a strategy to improve sensation in the reconstructed breast and patient quality of life (Cornelissen et al., 2018). Unfortunately, reinnervation outcomes remain incomplete. Delivering percutaneous CES to the donor intercostal nerves one week prior to reconstruction may significantly improve sensation for these women.

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