EFFECTS OF ELECTRICAL STIMULATION AND TESTOSTERONE ON REGENERATION-ASSOCIATED GENE EXPRESSION AND FUNCTIONAL RECOVERY IN A RAT MODEL OF SCIATIC NERVE CRUSH INJURY

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Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Doctor of Philosophy
in the Medical Neuroscience Program,
Indiana University

April 2014

Accepted by the Graduate Faculty, Indiana University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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DEDICATION

I dedicate this dissertation to my parents and fiancé, for they gave me the courage and support to exceed my goals. To all of my fellow graduate students who have experienced failure, good things come with time.

ACKNOWLEDGMENTS

First and foremost, I would like to thank my advisor Dr. Kathryn J. Jones for her support and guidance. I was fortunate that she took me under her wing when I came to her looking for a new opportunity two years ago. She was supportive of my interest in teaching gross anatomy and trusted me to pioneer a research project while pursuing my teaching interests. Prior to working with Dr. Jones, I was a member of Dr. Cynthia Hingtgen's laboratory. It wasn't until I left the laboratory that I realized how much I learned from her. She pushed me to be an independent scientist and taught me to critically analyze results, for which I am extremely thankful.

Next, I would like to thank my current and past committee members, including my chair Dr. Xiao-Ming Xu and Dr.'s Jinhui Chen, Dale Sengelaub, Fletcher White, and Gerry Oxford. Their guidance, support, and encouragement to think outside of the box improved my dissertation project and have made me a better scientist.

I owe a special thank you to my coworkers, Kathryn McMillan, Richard Batka, Deborah Olmstead, Whitney Miller, and Dr.'s Melissa Haulcomb and Todd Brown. Kate and Dick devoted countless hours to this project and were invaluable. Dr. Haulcomb was a fantastic mentor who taught me more than she will ever realize and I consider her a great friend. Dr. Brown was a vital asset for this project including being the encyclopedia resource of gonadal steroid research. He is a fellow Buckeye and Cleveland Browns fan which was an added bonus that made me feel at home in the laboratory.

I owe a great amount of gratitude to Sarah Richards and the Sengelaub laboratory at IU Bloomington for all of the dendritic analysis. In addition, I would like to thank Dr.'s Grant Nicol, Michael Vasko, Neilia Gracias, Ramy Habashy, and Djane Duarte for mentoring and training me during my earlier graduate years. I appreciate Dr. Chris Boehm for lending a hand in helping me write and submit animal protocols and Nancy Meiring for going above and beyond her work duties to make my life in LARC less stressful. To these individuals I am extremely indebted.

I would like to extend a special thank you to the staff in the Stark Neurosciences Research Institute and Departments of Anatomy and Cell Biology and Pharmacology and Toxicology. They were extremely helpful in all of the day-to-day tasks that are often unappreciated and for them I am grateful.

Additionally, I wish to express appreciation to my fellow graduate students Chao Li and Kyle Martin and Dr.'s Karl Koehler, Kristin Hollister, Jennifer Speth, and Brent Penque. It was always nice to have friends who understood the stress of graduate school and could make you laugh when your experiments failed.

Lastly, I would like to thank my friends and family back home and my fiancé Jerry Hunter. Although my friends are not scientists, they would listen to my graduate woes and were extremely supportive. My parents are my greatest fans and have encouraged me to dream big. My fiancé Jerry is my best friend, my rock, and has supported me through two graduate degrees. Since he is also a graduate student, he understands the stress that graduate school may bring and would always turn the seemingly negative events into a positive.

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EFFECTS OF ELECTRICAL STIMULATION AND TESTOSTERONE ON REGENERATION-ASSOCIATED GENE EXPRESSION AND FUNCTIONAL RECOVERY IN A RAT MODEL OF SCIATIC NERVE CRUSH INJURY

Although peripheral motoneurons are phenotypically endowed with robust regenerative capacity, functional recovery is often suboptimal following peripheral nerve injury (PNI). Research to date indicates that the greatest success in achieving full functional recovery will require the use of a combinatorial approach that can simultaneously target different aspects of the post-injury response. In general, the concept of a combinatorial approach to neural repair has been established in the scientific literature but has yet to be successfully applied in the clinical situation. Emerging evidence from animal studies supports the use of electrical stimulation (ES) and testosterone as one type of combinatorial treatment after crush injury to the facial nerve (CN VII). With the facial nerve injury model, we have previously demonstrated that ES and testosterone target different stages of the regeneration process and enhance functional recovery after facial nerve crush injury. What is currently unknown, but critical to determine, is the impact of a combinatorial treatment strategy of ES and testosterone on functional recovery after crush injury to the sciatic nerve, a mixed sensory and motor spinal nerve which is one of the most serious PNI clinical problems. The results of the present study indicate that either treatment alone or in combination positively impact motor recovery. With regard to molecular effects,

single and combinatorial treatments differentially alter the expression of regeneration-associated genes following sciatic nerve crush injury relative to facial nerve injury. Thus, our data indicate that not all injuries equally respond to treatment. Furthermore, the results support the importance of treatment strategy development in an injury-dependent manner and based upon the functional characteristics of spinal vs. cranial nerves.

Xiao-Ming Xu, M.D.,Ph.D., Chair

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α_I–tubulin	alpha I tubulin
ACh	acetylcholine
AChR	acetylcholine receptor
adcyap1	pituitary adenylate cyclase-activating peptide
ALS	amyotrophic lateral sclerosis
α-motoneurons	alpha motoneurons
ANS	autonomic nervous system
AR	androgen receptor
atat1	alpha I-tubulin
Ax	axotomy
Aα fibers	A alpha fibers
Aβ fibers	A beta fibers
Aδ fibers	A delta fibers
b2mg	beta-2 microglobulin
βII–tubulin	beta II tubulin
bdnf	brain derived neurotrophic factor
BHRP	horseradish peroxide conjugated to cholera toxin B subunit

BOS	base of support
C	contralateral
cAMP	cyclic adenosine monophosphate
CE	coefficients of error
CN VII	cranial nerve VII
CNS	central nervous system
crmp2	collapsin response mediator protein 2
CT	computed tomography
Ct	critical threshold
DH	dorsal horn
DHT	dihydrotestosterone
dpysl2	collapsin response mediator protein 2
DRG	dorsal root ganglion
E	experimental
EIA	enzyme immunoassay
EMG	electromyography
ES	electrical stimulation

FASt	automated functional assessment station
gap43	43-kiloDalton growth-associated protein
gapdh	glyceraldehyde-3-phosphate dehydrogenase
gdnf	glial cell derived neurotrophic factor
gfap	glial fibrillary-acidic protein
gusb	glucuronidase, beta
HIV	human immunodeficiency virus
hprt1	hypoxanthine phosphoribosyltransferase 1
HRP	horseradish peroxidase
HSP	heat shock proteins
I	ipsilateral
IL-1β	interleukin-1 beta
iNOS	nitric oxide synthase
ITS	intermediate toe spread
LG	lateral head of gastrocnemius
LIF	leukemia inhibitory factor
LSD	Fisher's least significant difference

MCP-1	macrophage chemoattractant protein 1
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
N	normal
NGF	nerve growth factor
NMJ	neuromuscular junction
nrn1	neuritin
NT-3	neurotrophin-3
NT-4/5	neurotrophin-4/5
oazi	ornithine decarboxylase antizyme 1
p0	myelin associated gene
pacap	pituitary adenylate cyclase-activating peptide
par-3	Schwann cell polarity gene
PEG	polyethylene glycol
PFI	peroneal functional index
PL	manual print length
PNI	peripheral nerve injury

PNS	peripheral nervous system
polr2a	polymerase (RNA) II (DNA directed) polypeptide A
qRT-PCR	quantitative real time PCR
RH	right hind
rRNA	ribosomal ribonucleic acid
sdha	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)
SEM	standard error of the mean
SFI	sciatic functional index
TA	tibialis anterior
TFI	posterior tibial functional index
TLR	toll-like receptors
TNF-α	tumor necrosis factor alpha
T	testosterone
TP	testosterone propionate
trkB	tyrosine kinase receptor B
TS	toe spread
tubb2b	beta II-tubulin

VH	ventral horn
WldS	Wallerian degeneration mouse model
Ywhaz	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta

“Research is what I'm doing when I don't know what I'm doing.”

Wernher von Braun

Rocket scientist

CHAPTER 1: INTRODUCTION

Peripheral nerve injuries (PNI) affect nearly 3% of trauma patients and are caused by physical trauma, such as motor vehicle accidents, fractures, and damage incurred during surgery, or through disease, including multiple sclerosis and amyotrophic lateral sclerosis (ALS; Kline et al., 1998; Noble et al., 1998; Genain et al., 1999; Marcuzzo et al., 2011). It is well-established that robust regeneration occurs following damage to peripheral nerves (Lieberman, 1971); however, functional recovery following PNI is often suboptimal, particularly when nerves are transected and require surgical repair (Höke & Brushart, 2010). A major limiting factor in successful recovery from PNI in humans appears to be related to the large size of human peripheral nerves, long distances over which injured nerves need to regenerate, and the specificity of motor-sensory reinnervation related to functional outcomes associated with activities of daily living (Scheib & Höke, 2013).

PNI frequently results in sensory and/or motor deficits and, in severe cases, paralysis that negatively impacts quality of life. Additionally, individuals with a PNI typically undergo long-term rehabilitation and have an increased risk for additional medical complications. The ability of a nerve to regenerate and achieve complete functional recovery is dependent on a multitude of factors: type and location of injury, age of individual, and regeneration distance to target (Birch & Raji, 1991; Perry et al., 1992; Fu & Gordon, 1995a, 1995b) and, if surgical repair is required, enough intact nerve or availability of nerve grafts. Currently,

there are no non-surgical treatment strategies in clinical use for PNI, thus finding a non-surgical therapy that enhances functional recovery offers immediate translational opportunity in the clinical setting.

Our laboratory has extensively studied the effects of the combination of the gonadal steroid testosterone and electrical stimulation (ES) on properties of regeneration and functional recovery utilizing several models of nerve injury, including extratemporal and intratemporal facial nerve, recurrent laryngeal nerve, and sciatic nerve injuries. These models have allowed us to investigate treatment effects in motor vs. mixed (i.e. motor and sensory) nerves, cranial vs. spinal nerves, and proximal vs. distal injuries. From these studies, we have discovered that ES and testosterone propionate (TP) target two different aspects of the regeneration process: delay time before sprout formation and regeneration rate, respectively (Kujawa et al., 1991; Sharma et al., 2009). Likewise, we have shown that ES and TP differentially enhance regeneration-associated genes after facial nerve crush injury which further supports the concept that ES and TP may be working through separate, yet interconnected, mechanisms (Sharma et al., 2010a). Interestingly, the combination of ES and TP significantly improves functional recovery compared to either ES or TP alone after facial nerve crush injury, but not after recurrent laryngeal nerve crush injury (Sharma et al., 2010b; Monaco et al., 2013). These findings have led to the idea that not all injuries equally respond to treatment. Additionally, location of injury and whether the injured nerve carries motor and/or sensory information are important factors in determining the most appropriate therapeutic interventions.

Although the combination of ES and TP has shown promise as a treatment after proximal nerve injury, as observed with the facial nerve injury model, the question remains as to whether ES and TP improve functional recovery after distal injury to a spinal nerve, such as the sciatic nerve. The **central hypothesis** of this dissertation is that ES and TP will additively improve regeneration, functional recovery, and the increased expression of regeneration-associated genes in a spinal injury model, comparable to improvements observed in the facial nerve injury model. The central hypothesis was tested by the following specific aims:

Specific Aim 1: Evaluate whether ES and/or TP alter the expression of regeneration-associated genes after sciatic nerve crush injury. *The working hypothesis for this aim was that the combination of ES and TP will differentially enhance the molecular response following sciatic nerve crush injury.* Real time PCR was used in Aim 1 to examine changes in several regeneration-associated genes in order to elucidate the molecular profile following injury to the sciatic nerve. The results demonstrate that the two treatments differentially enhance the expression of regeneration-associated genes after sciatic nerve injury in a manner distinct from that observed after facial nerve injury.

Specific Aim 2: Determine if ES and/or TP improve axon regeneration and functional recovery after sciatic nerve crush injury. *The working hypothesis for this aim was the combination of ES and TP will accelerate axon regeneration*

leading to an enhanced functional recovery after sciatic nerve crush injury compared to either treatment alone. It has been well-established that the combination of ES and TP enhances functional recovery after facial nerve crush axotomy more effectively than either treatment alone. In contrast, the combination of ES and TP provides no benefit in functional recovery after recurrent laryngeal injury. Experiments performed in Aim 2 specifically investigated functional recovery of rats in a different injury model, sciatic nerve crush, by employing several motor behavior tests, electromyography, and examining anatomical correlates. The results of the present study indicate that either treatment alone or in combination positively impact motor recovery.

CHAPTER 2: LITERATURE REVIEW

A. The mammalian nervous system: a general overview

Neurons work collectively to execute sensory, motor, and secretory functions and process information through four functional units: (1) dendrites, (2) cell body, (3) axon, and (4) presynaptic axon terminal. Dendrites extend from the neuron as branched, tree-like projections and play a critical role in receiving incoming signals through receptors that line their surface. Upon stimulation, neurotransmitters, i.e proteins or chemical compounds, bind to their receptors on the dendrites. The cell nucleus and other organelles contained within the soma, or cell body, are vital components that carry out normal housekeeping functions, including protein synthesis and processing. Incoming signals from dendrites accumulate at the axon hillock. In order for a message to be carried down the axon and converted into a response, the incoming signals must reach a critical threshold. Only if threshold is achieved will the signal be propagated down the axon in the form of an action potential. Once the action potential reaches the final portion of the neuron, the presynaptic axon terminal, a cascade of events leads to the release of neurotransmitters from the terminal. Next, the neurotransmitters cross a small gap, or synapse, toward a nearby target. Lastly, the neurotransmitters bind to their receptors and communicate their signal to adjacent cells (Kiernan & Rajakumar, 2013).

Neurons are characterized by their morphology, size, and function and based upon these criteria, can be classified into three general categories: (1)

sensory neurons, (2) motoneurons, and (3) interneurons. The connections among these three classes of neurons are intricate, as well as necessary for all aspects of the mammalian nervous system to communicate. Interneurons are enclosed entirely within the central nervous system (CNS) and connect sensory neuron terminals to motoneurons (Kiernan & Rajakumar, 2013). This literature review will briefly describe sensory neurons as their input on motoneurons is of extreme importance; however the work presented in this dissertation will focus primarily on motoneurons.

The nervous system is a complex, sophisticated system comprised of a network of neurons that is responsible for receiving, comprehending, and sending information from all parts of the body. In mammals, the nervous system is divided into three functionally distinct, yet connected, entities: (1) CNS, (2) peripheral nervous system (PNS), and (3) autonomic nervous system (ANS).

1. Central nervous system (CNS)

The CNS is the largest part of the nervous system and is formed by two main organs, the spinal cord and brain. A highly selective permeability barrier comprised of capillary endothelial cells connected by tight junctions known as the blood-brain barrier anatomically divides the CNS from the PNS and protects the CNS from potentially harmful chemicals and pathogens, while regulating transport of essential nutrients and molecules that are necessary to maintain a stable environment. Although neurons are considered to be the main component of the nervous system, non-neuronal cells exceed the number of neurons in the

nervous system. Neuroglia, often referred to as glia, in Greek means “nerve glue;” though this simple definition undervalues their function. As an ancillary cell of the nervous system, glia maintain neuronal life by providing support, myelin, nutrients, and protection for neurons in both the CNS and PNS. The chief types of glia in the CNS are oligodendrocytes, ependymal cells, microglia, and astrocytes; whereas, in the PNS, glial cells include satellite cells in autonomic and sensory ganglia, enteric glial cells, and Schwann cells.

2. Peripheral nervous system (PNS)

The PNS communicates to the CNS through bundles of axons called nerves. Primary afferent or sensory neurons are located within the PNS and detect several types of modalities, such as proprioception, touch, temperature, and pain, through specialized receptors. Sensory neurons convert external signals from the environment into internal electrical signals and have a unique pseudounipolar morphology that distinguishes them from other neuron types. These particular sensory neurons have a cell body that gives rise to one axon that bifurcates and sends a long process to the periphery while a second, shorter process terminates in the spinal cord. The cell bodies of pseudounipolar sensory neurons are grouped within a distinct structure called a dorsal root ganglion (DRG). The axon diameter of a sensory axon signifies the type of modality it transmits. For example, sensory neurons that carry pain and temperature information are small in size and are defined as either unmyelinated C or lightly myelinated Aδ fibers. Sensory axons that are myelinated and have a thicker

diameter detect cutaneous and subcutaneous mechanical stimuli and are referred to as Aα or Aβ fibers (Kiernan & Rajakumar, 2013).

After a sensory signal is interpreted by the CNS, a motor response is delivered to the PNS through motoneurons. A distinguishing characteristic of a somatic motoneuron is that their cell bodies and dendrites are located entirely within the CNS. Two types of motoneurons, upper and lower motoneurons, relay efferent signals from the CNS to skeletal muscle to elicit movement. Upper motoneurons are essential in regulating lower motoneurons, but remain entirely within the CNS and do not directly synapse on the target musculature. The cell body of an upper motoneuron is located in the motor cortex of the cerebral cortex or brain stem. Additionally, axons from upper motoneurons bundle together to form a tract and connect to lower motoneurons in the brainstem or spinal cord. In contrast, lower motoneurons act as a link between the upper motoneurons and skeletal muscle. Particular classes of lower motoneurons, known as alpha-motoneurons (α-motoneurons), send their axons from the brain stem or spinal cord to the periphery as a nerve and innervate the extrafusal fibers of skeletal muscle. It is important to note that extrafusal muscle fibers are located outside the muscle spindle, encompass the majority of the muscle belly, and are involved in skeletal muscle contraction. An α-motoneuron and all of the muscle fibers it innervates is referred to as a motor unit. Importantly, interneurons, descending tracts from the forebrain and brainstem, and some sensory neurons activate α-motoneurons and can trigger a variety of responses, such as withdrawal reflexes or muscle contraction (Kiernan & Rajakumar, 2013).

In the mid 1800's, it was discovered that an α-motoneuron did not directly contact muscle, but rather is separated from the muscle interface by a small gap referred to as the neuromuscular junction (NMJ) (Hughes et al., 2006). The NMJ is a specialized synapse that allows electrical signals from the α-motoneuron to be easily transmitted to the skeletal muscle via the neurotransmitter acetylcholine (ACh). The structure of a NMJ has three main components: (1) presynaptic nerve terminal, (2) synaptic cleft, and (3) postsynaptic motor endplate (Hughes et al., 2006). Synaptic vesicles filled with ACh are located within the presynaptic axon terminal of an α-motoneuron. The second component of the NMJ connects the presynaptic terminal to the motor endplate, which is characterized by the presence of nicotinic ACh receptors (AChR). When an action potential reaches the end of the nerve terminal, it causes the release of ACh into the synaptic cleft. Next, ACh traverses the cleft to the postsynaptic motor endplate where it binds to the AChR. Binding of ACh to its receptor triggers a cascade of events that ultimately leads to muscle contraction and movement (Hughes et al., 2006).

Lastly, the CNS and PNS are structurally connected by a third portion of the nervous system, known as the ANS. The ANS is functionally distinct from the CNS and PNS and is vital in regulating and controlling visceral or involuntary functions of internal organs, such as heart rate, blood pressure, digestion, respiratory rate, and reproduction (Kiernan & Rajakumar, 2013).

3. Regenerative capabilities of the CNS vs. PNS

The first estimations of the rates of nerve regeneration were based on clinical observation. Tinel estimated regeneration rate to be 1-2 mm/day using the “Tinel’s sign,” where he tapped the tip of regenerating axons to elicit paresthesias or tingling (Tinel, 1916). Other reports using this technique estimated nerve regeneration to occur at a rate of 2-5 mm/day, depending on the nerve tested (Dustin, 1917). Sunderland reported a case of a 23-year-old male who had a laceration of the sciatic nerve at the buttock level (Sunderland et al., 1993). Over the next 7 years, Sunderland used Tinel’s sign with electromyography (EMG) recordings to estimate the rate of sciatic nerve regeneration and found the rates to be 2.6 mm/day in the thigh, 1.2 mm/day in the proximal ¾ of the leg, and 0.9 mm/day in the distal ¼ of the leg. Using these rates, Sunderland calculated that motor axons regenerated a total distance of 70 cm and sensory axons regenerated 90 cm, which indicates sensory axons regenerate at a faster rate than motor axons. Various methods in rabbit, rat, and hamster confirm regeneration is the fastest after crush injury (3.5-4.6 mm/day), followed by transection and suture (3.2-3.5 mm/day) of the nerve. The slowest rate of regeneration is after nerve graft repair (1.5 mm/day; Gutmann & Guttmann, 1942; McQuarrie et al., 1977; Forman et al., 1979; Kanje et al., 1988; Kujawa et al., 1991; Holmquist et al., 1993; Sharma et al., 2009). Taken together, the aforementioned studies indicate that nerve regeneration is much slower in humans compared to what has been observed in rodent studies. A major limiting factor in successful recovery from peripheral nerve injury (PNI) in humans

appears to be related to the large size of human peripheral nerves, long distances over which injured nerves need to regenerate, and the specificity of motor-sensory reinnervation related to functional outcomes associated with activities of daily living (Scheib & Höke, 2013).

Following injury to an axon, the somata of adult mammalian neurons respond by undergoing chromatolysis and substantially increasing RNA and protein synthesis (Lieberman, 1971). Of particular interest, the inherent capacity of a neuron to respond to injury is markedly reduced in the CNS compared to the PNS. It was first thought that the inability of axons in the CNS to regenerate was due to CNS neurons lacking intrinsic growth capacity to regenerate. In the 1980's, Aguayo and colleagues demonstrated CNS neurons could in fact regenerate when provided with a PNS graft; however, axon elongation was arrested if axons were redirected from the PNS graft back to the CNS (Richardson et al., 1980; David & Aguayo, 1981; Benfey & Aguayo, 1982; Richardson et al., 1984). Their results provided a substantial body of evidence that not only disproved the theory that CNS neurons lack inherent regenerative capability but also that a permissive environment is critical for axon elongation. It was later determined that the lag in CNS neuron growth was due to the presence of the degenerated nerve that produced an inhibitory milieu which ultimately stunted axon regeneration (Filbin, 2003; Vargas & Barres, 2007; Huebner & Strittmatter, 2009). The aforementioned studies reiterate the importance of clearing remnants of the degenerated nerve in order to have a permissive environment in favor of axon regeneration.

Augustus Volney Waller was the first to describe in the mid 1800's the detailed process of regressive changes in severed nerves (Augustus volney waller (1816-1870) wallerian degeneration, 1969). After transecting the glossopharyngeal nerve of the frog, Waller observed diminished movement of tongue muscles and loss of sensation. Additionally, he noticed alterations in the nerve tubes within the papillae having an appearance he described as "curdling" or coagulated. Upon further inspection, he discovered after two weeks many of the single tubules disappeared post-injury (Waller, 1850). It was not until years later that Waller received praise for his incredible work describing the process that would take his name, "Wallerian degeneration."

Wallerian degeneration is a cascade of events that leads to the removal of axonal debris and is essential in order to clear the path for an injured nerve to regenerate back to its target. Much of our understanding on the process of Wallerian degeneration has come from studies utilizing the Wallerian degeneration (*WldS*) mouse model which was initially termed *Ola* (Lunn et al., 1989; Perry et al., 1990; Glass et al., 1993). Lunn and colleagues reported that *WldS* mouse nerves degenerated more slowly than wild-type mouse nerves by several weeks, suggesting the *WldS* gene delayed the process of Wallerian degeneration (Lunn et al., 1989).

PNI elicits a multitude of changes in the axon, including the onset of Wallerian degeneration. Wallerian degeneration occurs in several stages: acute axon degeneration, latency in distal axon, and granular fragmentation (Wang et al., 2012). These phases are followed by axon regeneration, successful target

reinnervation, and functional recovery. As early as 5-30 minutes after PNI, both proximal and distal axonal segments surrounding the injury site begin to fragment and undergo degeneration that continues to occur over several hours (Lubińska, 1977; Kerschensteiner et al., 2005; Brushart, 2011; Wang et al., 2012). An influx of extracellular calcium initiates the process of acute axon degeneration and increases the number of injured axons that undergo acute axon degeneration (Knöferle et al., 2010). In addition, calcium influx leads to axoplasmic organelles from anterograde and retrograde transport to pool at the terminals of transected axon ends (Griffin et al., 1977). Subsequently, calcium activates the protease calpain which cleaves neurofilaments and microtubule-associated proteins resulting in dissembly of the axon (Zimmerman & Schlaepfer, 1982; Fischer et al., 1991). During this process, the blood-nerve barrier degenerates allowing proteins, hormones, and ions from the blood to enter the nerve and trigger focal edema (Gaudet et al., 2011; Weerasuriya & Mizisin, 2011).

Despite the rapid process of degeneration in the proximal stump, degeneration in the distal stump exhibits a period of quiescence after acute axon degeneration. The latent period before the onset of fragmentation in the distal axon depends on fiber diameter, intermodal length, temperature, and species (Lubińska, 1977; Tsao et al., 1999; Wang et al., 2012) and can last anywhere from 25.6 to 45.0 hours in the rat phrenic nerve (Lubińska, 1977), or up to several days in humans (Chaudhry & Cornblath, 1992). The end of this latent period is defined by the onset of cytoskeletal breakdown.

After the end of the latent period, the distal axon begins to undergo a final stage of granular fragmentation. Axon destruction proceeds at a rate of 46 mm/day for large axons to a rapid rate of 250 mm/day for small axons (Lubińska, 1977). Depending on the severity of injury, degeneration can occur in an anterograde or retrograde manner. Recently, Beirowski and colleagues reported axons degenerated anterogradely after a transection injury, but retrogradely after a crush injury (Beirowski et al., 2005).

Products of axon fragmentation prompt myelin breakdown and clearance. Since the myelin debris is inhibitory, it must be removed rapidly by Schwann cells and macrophages in order to clear the way for an axon to regenerate (McKerracher et al., 1994; Mukhopadhyay et al., 1994; Shen et al., 1998). One cell that is responsible for myelin phagocytosis is the Schwann cell (Stoll et al., 1989; Liu et al., 1995). Once Schwann cells are no longer associated with an axon, they stop producing myelin and dedifferentiate, in a mechanism dependent on the ubiquitin-proteasome system (White et al., 1989; Lee et al., 2009). This process of dedifferentiation drives the Schwann cell to proliferate and upregulate regeneration-associated genes, such as *glial cell line-derived neurotrophic factor* (*GDNF*) and *growth-associated protein* (*Gap-43*; Mehta et al., 1993; Murinson et al., 2005; Xu et al., 2013). Lee et al. (2006) reported necrotic neuronal cells stimulated the upregulation of several inflammatory factors in Schwann cells, such as leukemia inhibitory factor (LIF), macrophage chemoattractant protein-1 (MCP-1), tumor necrosis factor alpha (TNF-α), and nitric oxide synthase (iNOS). NOS is an enzyme that converts L-arginine to nitric oxide. The product nitric

oxide is important in the Wallerian degeneration process by participating in the breakdown of myelin and subsequent nerve regeneration (Levy et al., 2001; Keilhoff et al., 2002).

Macrophages assist in the removal of myelin debris by facilitating myelin breakdown and removing myelin debris through phagocytosis (Liu, 1974; Perry et al., 1987; Stoll et al., 1989). *In vitro* studies show two substances released from Schwann cells, LIF and MCP-1, attract macrophages (Tofaris et al., 2002), while another substance produced by Schwann cells, TNF-α, augments the injury response by increasing expression of MCP-1 and interleukin-1β (IL-1β; Subang & Richardson, 2001; Shamash et al., 2002). After macrophages are recruited to the denervated nerve, they must be activated before assisting in the breakdown of myelin. Shamash and colleagues report that both TNFα and IL-1β augment myelin phagocytosis by macrophages *in vitro* (Shamash et al., 2002).

B. Peripheral nervous system injury

1. Statement of the problem

PNI were first classified by Seddon (1942) into three broad categories: (1) neurotmesis (herein defined as transection), (2) axonotmesis, and (3) neurapraxia. The most severe injury is neurotmesis, where the epineurium is cut into two. In an axonotmesis injury the enclosed nerve fibers are severed similar to that observed with transection, but the epineurium is spared. Neurapraxia is

the least severe injury type and is described as the presence of a short-lived paralysis without true regeneration.

PNI frequently results in sensory and/or motor deficits and, in severe cases, nerve paralysis that negatively impact quality of life. Additionally, individuals with a PNI typically undergo long-term rehabilitation and have an increased risk for additional medical complications. The ability of a nerve to regenerate and achieve complete functional recovery is dependent on a multitude of factors: type and location of injury, age of individual, and regeneration distance to target (Birch & Raji, 1991; Perry et al., 1992; Fu & Gordon, 1995a, 1995b) and, if surgical repair is required, enough intact nerve or availability of appropriate grafting materials. Currently, there are no non-surgical treatment strategies in clinical use for PNI, thus finding a non-surgical therapy that enhances functional recovery offers immediate translational opportunity in the clinical setting.

2. Anatomy of the peripheral nerve

Named after German physiologist Theodor Schwann, Schwann cells are indispensable cells and play numerous roles in the PNS. Two types of Schwann cells, myelinating and non-myelinating, ensheath and insulate all axons in the periphery by wrapping them with a substance known as myelin. Myelinating Schwann cells form myelin sheaths around axons in a 1:1 Schwann cell to axon ratio, while a non-myelinating Schwann cell encases several axons into a group known as a Remak bundle. A nerve and its accompanying Schwann cell are

surrounded by a continuous tube known as the Schwann cell basal lamina. This specialized tube consists of the matrix proteins laminin and collagen, and has an essential role in the uninjured nerve by acting as a supporting structure to promote and guide nerve regeneration (Brushart, 2011). It is important to note that the Schwann cell basal lamina remains intact throughout Wallerian degeneration.

The interaction between a nerve and Schwann cell is necessary for basal lamina formation (Bunge et al., 1982). The Schwann cell basal lamina plays a key structural role at the neuromuscular junction (NMJ) by capping the presynaptic nerve terminal portion of the NMJ and providing a means of cell adhesion amongst the nerve and NMJ components. Within the past decade, the importance of Schwann cells during development and maintenance of NMJ has been investigated (Feng & Ko, 2008; Zuo & Bishop, 2008). During development of the NMJ, multiple alpha-motoneurons (α-motoneurons) send axonal branches to the same target musculature. Interestingly, within the first several postnatal weeks all but one of the axonal branches withdraws from its target and eventually disappears. Bishop and colleagues recently used time-lapse imaging of fluorescently labeled axons and serial electron microscopy to examine the phenomenon of synapse elimination at the NMJ (Bishop et al., 2004). Their work revealed that α-motoneurons disintegrate and shed membrane-bound remnants referred to as axosomes that are subsequently engulfed by surrounding glia. It has been postulated that the mixing of axonal and glial cytoplasm could signal the Schwann cell to migrate away or undergo apoptosis leading to synapse

elimination (Nakao et al., 1997). Although additional studies are warranted to better support whether glia signal synapse elimination of α- motoneurons, Schwann cells are likely to play an essential role in maintaining NMJ structure and function after injury (Koirala et al., 2000; Court et al., 2008).

Several layers of connective tissue encase a peripheral nerve to prevent the nerve from potential injury. The outermost layer of nerve sheath, the epineurium, consists of collagen and elastin fibers that act together to protect the nerve fibers and form a supporting wall. Within the epineurium are nerve fibers enclosed in a bundle or funiculi by perineurium (Sunderland, 1965). Layers of flattened cells and collagen fibers give the perineurium tensile strength to further protect the nerve fibers (Thomas, 1963; Sunderland, 1965). Each nerve fiber and its accompanying Schwann cells within the nerve bundle are ensheathed by a thin, delicate layer of connective tissue called the endoneurium that functions in cushioning the nerve fiber. Lastly, between the Schwann cell layer and endoneurium is an additional delicate sheath referred to as the neurilemma or inner endoneurium (Sunderland, 1965). Together, these three sheaths form a protective defense barrier and allow the nerve to efficiently transmit electrical signals.

3. Current status of peripheral nerve injury (PNI) and repair

a. Therapeutic rationale

Typically, the severity of injury determines the treatment regimen that will be administered. For minor injuries, physical therapy, splints, or administration of analgesic or other medications may be possible; though more severe injuries that do not respond to therapy and medication may improve with surgical intervention. Recently, the outcomes of neurolysis surgery, or cutting of the epineurium, were examined in a retrospective study. The results indicated that half of the patients that undergone neurolysis surgery were considered to have an excellent outcome (Maalla et al., 2013). Decompression, suture, and nerve grafting also have been utilized as surgical interventions and have had some success. Despite this success, functional recovery was not seen up to 18 months after surgical repair (Kline et al., 1998; Kim et al., 2004). These reports indicate mixed nerves are capable of regeneration over long distances and the ability for a nerve to regenerate after injury is not purely limited by distance.

Although surgery is the standard treatment for PNI, only half of individuals with PNI regain functional recovery (Kallio & Vastamäki, 1993). Suboptimal results observed with surgical intervention stress the need for additional non-surgical approaches for the treatment of PNI. Finding a novel therapy that enhances functional recovery would not only repair sensory and motor deficits resulting from injury, but would significantly reduce the time that an individual would undergo rehabilitation.

Successful functional recovery following nerve injury is dependent upon a sequence of events, each of which can be manipulated with treatment to promote successful target reinnervation. In the first phase following injury, the intrinsic growth state must be activated and maintained. Following injury, neurons undergo molecular changes, such as chromatolysis and an increase in RNA and protein synthesis, to prepare for regeneration (Lieberman, 1971). Treatments that stimulate the intrinsic growth state of a neuron by increasing the expression of regeneration-associated genes produce a regenerative neuronal phenotype and promote axon elongation across the injury site (Lieberman, 1971; Sharma et al., 2010a). There are two general phases of the regeneration process that can be targeted to decrease the time to functional recovery: (1) time before sprout formation and (2) axon regrowth to the appropriate target (Kujawa et al., 1993). The time from the start of the Wallerian degeneration process until the daughter axon sprouts is defined as the time before sprout formation (Oblinger & Lasek, 1984). During Wallerian degeneration, the distal axon stump dissembles and is removed. By removing the inhibitory myelin debris and creating a permissive environment, the nerve can regenerate towards its target (McKerracher et al., 1994). Treatments that either decrease the time before sprout formation or accelerate axon regrowth enhance functional recovery and decrease the time individuals will need for rehabilitation services. In humans, regeneration rates are relatively slow and an injured nerve likely will have to regenerate over a long distance. For successful target reinnervation and functional recovery, it is of extreme importance to guide the regenerating motor nerve to the correct distal

stump path to prevent synkinesis or involuntary muscle actions accompanying voluntary movements (Al-Majed et al., 2000b). One method of achieving appropriate target reinnervation is utilizing treatments that increase regeneration specificity. Most importantly, the target musculature must remain viable and intact during the slow regeneration process to preserve the ability for the establishment of new functional neuromuscular junctions (Fischer et al., 2004; Hughes et al., 2006; Marcuzzo et al., 2011).

b. Surgical approaches

The standard surgical care for transection injuries when epineurium reattachment is necessary is nylon suture. Unfortunately, only approximately 50% of individuals regain functional recovery with nylon suture treatment (Kallio & Vastamäki, 1993). The low success rate appears to be due to the difficulty of aligning the fascicles in the right orientation prior to suture. Additionally, the success of surgical repair after nerve injury is dependent upon several factors, including the nature of the injury, type and timing of repair, requirement for a nerve graft, location of injury (i.e., how far the injury is from target muscle or skin), and age of patient (Omer, 1974; Navarro et al., 1988; Birch & Raji, 1991; Kawabuchi et al., 1998; Kim et al., 2004; Ruijs et al., 2005; Höke, 2006). A nerve graft is required for transection injuries that have stumps that cannot be reattached with primary surgical care without causing excessive tension. Two general types of nerve grafts, each with their own potential advantages, are in clinical use, autografts and allografts. Autografts utilize an individual's own nerve

tissue and are considered the standard nerve graft material. The most common source for an autograft is the sural nerve, due to its size, ease of harvest, and moderate dispensability (Lee & Wolfe, 2000); although other sources include the anterior branch of the medial antebrachial cutaneous nerve, lateral femoral cutaneous nerve, and the superficial radial sensory nerve (Sunderland, 1991). While an autograft is a safe and fast healing option, making it the standard choice for nerve graft repair, harvesting the autograft tissue creates a second surgical site which takes time to heal and may cause discomfort. The second category of graft, allograft, utilizes donor tissue. Although a second surgical site is not created, an allograft takes longer to incorporate into the recipient's body (Squintani et al., 2013).

Axon fusion with fibrin glue or polyethylene glycol (PEG) also has been explored as a therapy for PNI. Earlier studies found fibrin glue alone to be inferior to microsuture when examining electrophysiological properties of the nerve and fibrin glue was less reliable in keeping the proximal and distal stumps connected (Moy et al., 1988; Maragh et al., 1990; Sames et al., 1997). In contrast, recent reports demonstrate fibrin glue alone significantly accelerated motor recovery, axonal regeneration, and nerve conduction properties compared to microsuture (Ornelas et al., 2006a; Ornelas et al., 2006b; Barbizan et al., 2013). Although mixed results utilizing fibrin glue as a treatment after PNI have been observed, it is relatively quick and easy to use and still remains an attractive alternative to microsuture. PEG has been utilized as a method to fuse the plasmalemmas of proximal and distal stumps of severed axons. Several years ago, Bittner and

coworkers discovered PEG could fuse severed crayfish and earthworm axons (Bittner et al., 1986; Krause & Bittner, 1990). The ground breaking *in vitro* results stimulated Bittner and colleagues to investigate the use of PEG as a treatment to reconnect severed mammalian axons *in vivo* (Stavisky et al., 2005; Britt et al., 2010). The aforementioned studies reveal that additional treatments in combination with surgical repair may be necessary to obtain full functional recovery.

c. Non-surgical approaches

Non-surgical approaches are an attractive form of treatment after PNI since they are less invasive. Although no non-surgical treatment is approved in the clinical setting, several non-surgical therapies have shown promise in *in vitro* and *in vivo* studies. ES, gonadal steroids, trophic factors, and exercise are a few non-surgical therapies that are being actively investigated. Utilizing a non-surgical therapy in combination with surgical repair may enhance functional recovery.

i. Electrical stimulation (ES)

Electrical stimulation (ES) is currently being investigated as a therapeutic approach following injury and has been utilized in the clinical setting as a therapy to enhance nerve regeneration following PNI (Gordon et al., 2009; Gordon et al., 2010). Concurrent with carpal tunnel release surgery, brief, low frequency ES (20

Hz) for 1 hour has been used clinically as a method to treat carpal tunnel syndrome (Gordon et al., 2010). The results from this study demonstrated a significant increase in motor unit number and sensory nerve conduction values in the stimulated group at 6-8 months compared to the unstimulated control group. *In vitro* studies suggest ES can promote neurite outgrowth, orientation, and sprouting (Patel & Poo, 1982; Manivannan & Terakawa, 1994; Matthew & Rebecca Kuntz, 2009). Moreover, various forms of ES have shown promising results in the clinic for a variety of neurobiological disorders, including Bell's palsy, chronic pain, PNI, and multiple sclerosis (Gordon et al., 2010; Buchmuller et al., 2012; Happe & Bunten, 2012; Heller et al., 2013).

The effect of ES on the morphological properties of neurons has been extensively studied *in vitro*. In 1979, Jaffe and Poo discovered that electrical fields increase neurite outgrowth of chick dorsal root ganglion (DRG) explants (Jaffe & Poo, 1979). Later reports confirmed these results in *Xenopus laevis*, but also revealed that electrical fields alter neurite orientation and branching in a way that is dependent upon duration, amplitude, and frequency of the stimulus pulse (Patel & Poo, 1982; Patel & Poo, 1984; McCaig, 1990). From these studies, it was concluded that ES alters neurite morphology; however the question of whether these changes have functional benefit remains to be tested.

The capability of ES to enhance neurite outgrowth and orientation led scientists to investigate whether ES could promote cell survival and axon regeneration after injury. Several elegant experiments completed by Borgens and coworkers laid the foundation for ES as a potential therapy following injury. In the

late 1980's, they established that ES enhanced neuronal regeneration and behavioral recovery across a spinal cord lesion in adult guinea pigs (Borgens et al., 1986; Borgens et al., 1987). A decade later, they utilized naturally injured, neurologically intact paraplegic dogs as a model of spinal cord injury and discovered that oscillating electrical field stimulation significantly improved neurological recovery (Borgens et al., 1999). Another group examined whether ES would promote cell survival (Morimoto et al., 2002). In this study, Morimoto et al. observed that ES increased retinal ganglion cell survival if ES was applied at 20 Hz for 2 hours after optic nerve transection. These innovative findings stimulated others to explore the beneficial effects of ES after PNI.

Currently, ES has been hypothesized to enhance neurite outgrowth via an increase in intracellular Ca^{2+}, followed by an increase in the second messenger cyclic adenosine monophosphate (cAMP; **Fig. 1**). In the early 1990's, Garyantes and Regehr stimulated cultured superior cervical ganglion neurons at 10 Hz for up to 1 hour. This brief, low frequency stimulation was enough to elicit a rise in intracellular Ca^{2+} but was not sufficient to increase neurite outgrowth (Garyantes & Regehr, 1992). Others experimented using a frequency of 10 Hz and found contradictory results to those observed by Garyantes and Regehr. Lin and coworkers stimulated DRG neurons at 10 Hz but increased the time of stimulus delivery to 3 days (Lin et al., 1993). They concluded that extending the delivery of the stimulus from 1 hour to several days increased neurite outgrowth of the cultures which supported earlier *in vitro* findings. Okazaki and colleagues later experimented with several different ES paradigms, including 10-120 minute

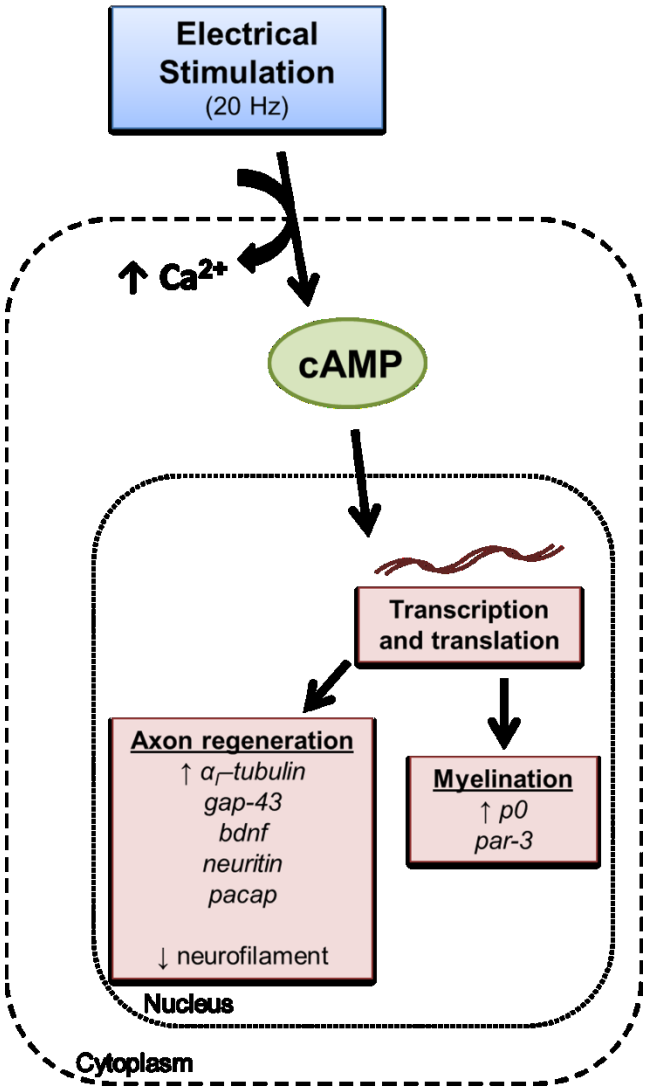

Fig. 1: Schematic of the proposed mechanism by which a brief, low frequency electrical stimulation alters regeneration-associated gene expression in motoneurons.

Electrical stimulation increases intracellular calcium and cAMP levels that in turn alters the expression of several genes involved in promoting axon regeneration and myelination.

stimulation period and 10, 20, and 50 Hz frequencies (Okazaki et al., 2008). Their results showed a minimum of 30 minutes of ES was necessary to promote survival of retinal ganglion cells and a 20 Hz stimulation frequency was most effective. Of note, both 10 Hz and 20 Hz stimulation frequencies have been shown to increase levels of cAMP which is thought to mediate neuron survival and axon growth (Udina et al., 2008; Corredor et al., 2012). The use of a 20 Hz stimulation frequency to elicit neuroregenerative effects has been confirmed by our laboratory, as well as others (Ahlborn et al., 2007; Hetzler et al., 2008; Alrashdan et al., 2010; Wan et al., 2010).

Following a rise in intracelulluar Ca^{2+}, ES enhances the expression of regeneration-associated genes. Al-Majed et al. examined the effects of a 20 Hz ES on regeneration-associated genes expression utilizing *in situ* hybridization and a model of femoral nerve transection with suture repair (Al-Majed et al., 2000a). They observed a significant increase in *gap-43* and *α_I–tubulin* mRNA expression 2 days post-axotomy, followed by a significant increase in *brain-derived neurotrophic factor (bdnf)* and *trkB* mRNA expression 7 days post-axotomy in motoneurons (Al-Majed et al., 2000a; Al-Majed et al., 2004). These data suggest that ES upregulates *gap-43* and *α_I–tubulin* prior to activating BDNF signaling following injury. A few studies suggest that ES triggers BDNF signaling in a manner dependent on both Ca^{2+} and extracellular signal-regulated kinase (Erk) activation (Wenjin et al., 2011; Yan et al., 2013). In addition, Al-Majed and coworkers found that the 20 Hz ES paradigm significantly decreased the cytoskeletal medium-molecular-weight neurofilament protein 2 days post-

axotomy (Al-Majed et al., 2004). This result is not surprising since neurofilament has been shown to interfere with the axonal transport by other cytoskeletal proteins, actin and tubulin, and is associated with enhanced regeneration (Bisby & Tetzlaff, 1992). Lin and coworkers did not observe an increase *gap-43* expression following ES treatment; however, this could be due to utilization of an insufficient ES frequency (10 Hz) that does not stimulate neurite outgrowth (Lin et al., 1993). Recently, we confirmed that a 20 Hz ES stimulation after facial nerve injury enhances early expression of *α_I–tubulin*, *gap-43*, and *bdnf* mRNA (Sharma et al., 2010a). In addition, we found significant increases in *neuritin* and *pituitary adenylate cyclase-activating peptide (pacap)* mRNA. Neuritin, also known as candidate plasticity gene 15, has been shown to enhance neurite extension and arborization (Naeve et al., 1997; Javaherian & Cline, 2005; Marron et al., 2005), while PACAP has been reported to increase after spinal cord injury (Tsuchikawa et al., 2012) and promote axonal sprouting after facial nerve transection (Suarez et al., 2006). In addition to enhancing regeneration-associated genes expression, ES recently has been shown to induce the expression of the myelin associated gene *p0*, Schwann cell polarity gene *par-3*, and stimulate myelination (Wan et al., 2010; Yang et al., 2012). In 2002, Brushart et al. demonstrated that ES promotes the onset of motor axon regeneration, but not the speed of regeneration (Brushart et al., 2002). We later confirmed this finding after facial nerve axotomy in rats (Sharma et al., 2009). Recently, the ability of ES to enhance axonal regeneration was reported to signal through androgen receptors (Thompson et al., 2013). Collectively, these data support the ability of ES to increase

expression of regeneration-associated genes early after injury and decrease the delay to sprout formation in a way that is dependent on androgen receptor signaling.

Although accumulating evidence has shown that ES enhances neurite outgrowth and promotes survival of neurons following injury, the protocol by which ES should be administered is controversial. An ES protocol of 1 hour to 2 weeks was applied after femoral nerve transection with immediate repair in rats (Al-Majed et al., 2000b). The results from this study demonstrated that ES reduced the time it took for motoneurons to regenerate by 5 weeks and enhanced preferential motor reinnervation, which is a key feature of motor axon regeneration and the tendency of regenerating motor axons in a mixed nerve to selectively reinnervate muscle. In support of the results reported by Al-Majed and colleagues, ES increases the number of regenerated axons across suture gaps to their appropriate target (Brushart et al., 2002; Vivó et al., 2008). We have shown that 30 minute daily administration of a 20 Hz ES frequency starting immediately after injury shortened functional recovery by nearly a week compared to unstimulated rats (Lal et al., 2008). Recently, we demonstrated that a one-time, 30 minute ES treatment at 20 Hz immediately after facial nerve crush injury is just as effective as daily ES treatments for up to 1 week (Foecking et al., 2012). Analogous results have been observed in rodents after sciatic nerve injury (Hamilton et al., 2011; Singh et al., 2012). Together, these data confirm that short- and long-term ES are equally effective in motoneuron reinnervation of their appropriate targets and promote the onset of axonal regeneration.

The current consensus in the field is that a 20 Hz stimulus frequency delivered for 30 minutes to 1 hour will be sufficient to promote functional recovery in the clinic. First, this frequency is physiologically relevant to the normal slow firing patterns of motoneurons. As discussed, this ES protocol was shown to be optimal *in vitro*, and has had success *in vivo* in both rodents and humans following PNI. The ES protocol will most likely have to be altered depending on the type of nerve (i.e., motor, sensory, or mixed) and severity of injury. In conclusion, these data support the use of ES as a strategy to improve functional recovery by promoting appropriate target reinnervation and enhancing axonal regeneration.

ii. Gonadal steroids

To date, numerous studies suggest gonadal steroids have profound neuroprotective and neuroregenerative effects (Chowen et al., 1992; Singer et al., 1996; Tanzer & Jones, 1997; Chen et al., 1999). For example, progesterone has been reported to have neuroprotective roles in a variety of experimental models, such as stroke, Alzheimer's disease, and traumatic brain injury (Jayaraman et al., 2012; Si et al., 2013; Wali et al., 2013), as well as the promotion of functional recovery after spinal cord injury (Thomas et al., 1999). Our laboratory has shown previously that estrogen accelerates the rate of facial nerve regeneration following facial nerve crush injury (Tanzer & Jones, 1997) which is supported by a more recent study demonstrating that estrogen and selective estrogen receptor modulators stimulate sciatic nerve regeneration in

mouse models (McMurray et al., 2003). Emerging evidence supports the ability of androgens to enhance regenerative properties, increase muscle mass, and promote functional recovery following nerve injury, as described below (Kujawa et al., 1993; Brown et al., 1999; Sinha-Hikim et al., 2003).

Currently, androgens have been used clinically to treat several disorders, including hypogonadism, muscle wasting in human immunodeficiency virus (HIV), and androgen deficiency making them a highly translational therapeutic intervention following nerve injury (Bhasin et al., 1996; Strawford et al., 1999; Arlt, 2006; Selice et al., 2013). It is well understood that androgens have trophic effects on the developing nervous system which may translate to its use as a therapy following PNI. Androgens are well known for their essential role in male reproductive control and development of male secondary sex characteristics, yet their function is widespread. During adulthood, androgens support sperm production, promote the enlargement of skeletal muscle, and inhibit fat deposition (Bhasin et al., 1996; Arner, 2005; Walker, 2010). In the mammalian CNS, androgens function to maintain synaptic connections and promote neurite outgrowth. For example, androgens are essential for postnatal dendritic growth (Goldstein et al., 1990), as well as maintenance of dendrites in adulthood (Kurz et al., 1986) in motoneurons located in the spinal nucleus of the bulbocavernosus.

The primary and most recognized androgen is testosterone, although other less known androgens, such as dihydrotestosterone (DHT) and androstenedione, have equally important roles in development. Androgens, like

all gonadal steroid hormones, are derived from cholesterol and thus are similar in structure. The gonadal steroids are composed of a central skeleton containing three cyclohexane rings and one cyclopentane ring and a side chain that varies among the different gonadal steroid hormones. The Leydig cells of the testes are the primary site of testosterone production, but considerable levels of testosterone can also be found in the adrenal cortex. Testosterone is present in both females and males, albeit at different levels, and can be prescribed as an exogenous therapeutic if levels fall below normal physiological levels (Selice et al., 2013).

Within the last half-century, we have begun to understand the cellular and molecular mechanisms of testosterone action on target tissues. Testosterone can act directly through its androgen receptor (AR) or through its aromatization to estrogen. In the direct or classical steroid pathway, free testosterone is transported to its target tissue where it easily crosses the cell membrane (**Fig. 2**). Upon entering the cell, testosterone acts as a ligand to the AR to which it binds, stimulating a conformational change in the receptor. In order for the ligand/receptor complex to translocate to the nucleus, it must dimerize. After the receptor complexes dimerize, they enter the nucleus and bind to DNA sites called hormone response elements. This binding has a direct effect on the level of transcription at that site ultimately leading to an increase or decrease in mRNA synthesis (Brann et al., 1995). Although the direct pathway is considered to be the primary mechanism by which testosterone mediates its effects, indirect or nongenomic actions of testosterone recently has become the subject of

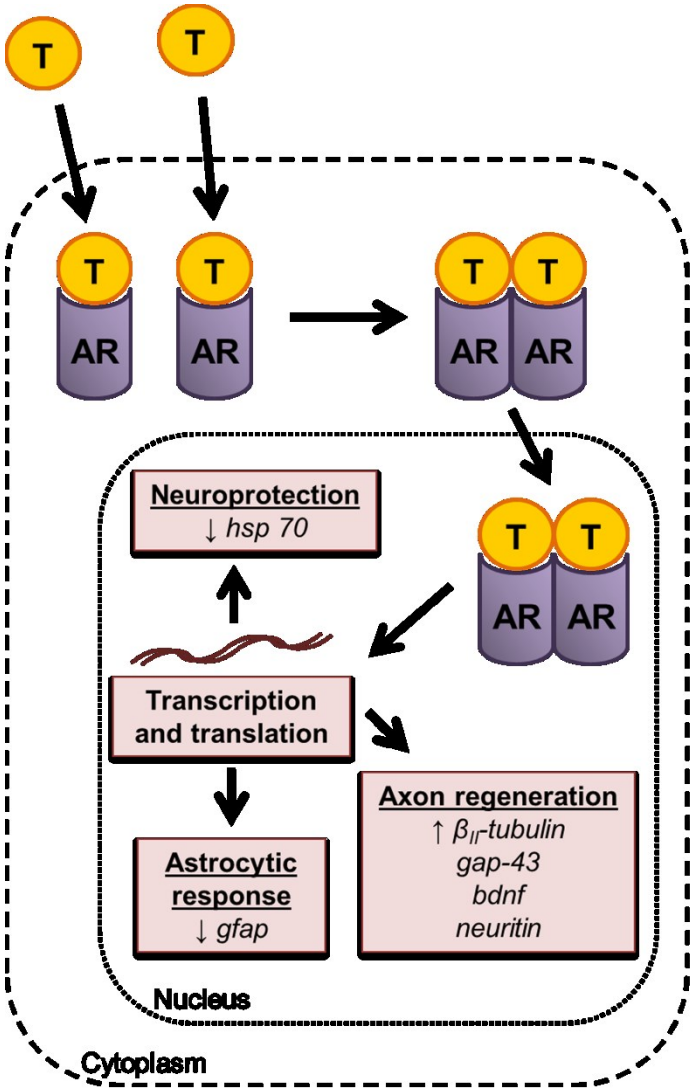

Fig. 2. Schematic of the proposed mechanism by which testosterone (T) alters regeneration-associated gene expression in motoneurons.

Testosterone enters the cell where it is able to bind to its receptor, the androgen receptor (AR), and induce a conformational change. The ligand/receptor complex dimerizes with another ligand/receptor complex and translocates to the nuclues. After the receptor complexes dimerize, they enter the nucleus and bind to DNA sites called hormone response elements. This binding has a direct effect on transcription and translation of genes responsible for neuroprotection, the astrocytic response, and axon regeneration.

increased interest. In indirect mechanisms of action, testosterone binds to membrane or neurotransmitter receptors and rapidly exerts its effect through signaling pathways that do not directly involve an increase in genomic DNA (McEwen, 1991; Falkenstein et al., 2000; Watson & Gametchu, 2003).

Androgens have been shown to act in a variety of tissues due to the widespread distribution of AR. Although AR are present in both males and females, males have a significantly higher concentration of AR (Yu & McGinnis, 2001). Several areas within the brain contain AR, including the preoptic area, arcuate nucleus, amygdala, regions of the hippocampus, ventromedial and dorsomedial nucleus, and medial hypothalamic area (Simerly et al., 1990). AR are also distributed in lower motoneurons of the brainstem and spinal cord and can be observed outside the CNS in muscle tissue, nerves, and DRG (Breedlove & Arnold, 1981; Simerly et al., 1990; Jordan et al., 2002; Luo et al., 2008; Oki et al., 2013). These distinct locations of AR allow gonadal steroids to have selective actions at these sites. For example, the effects of gonadal steroids on neuron ultrastructure have been well studied in the ventromedial nucleus of rats. Estradiol treatment has been shown to increase rough endoplasmic reticulum stacks and dense-cored vesicles, which may reflect increased biosynthesis of a secretory product (Cohen & Pfaff, 1981). Our laboratory reported similar findings and detected alterations in the nucleolus, an increase in cell size, and changes in the nuclear shape from nonspherical to spherical (Jones et al., 1985). Moreover, our laboratory utilized *in situ* hybridization to show an increase in gene transcription in the hypothalamic neurons of rats within 30 minutes (Jones et al.,

1990). This work is supported by more recent literature which suggests estradiol recruits protein synthesis machinery for local protein synthesis in dendrites of newly developing synapses (McCarthy & Milner, 2003).

Several reports have investigated the neuroprotective role of heat shock proteins (HSP) in the motor neuron disease spinal and bulbar muscular atrophy and after injury to the optic, facial, and sciatic nerves (Kalmar et al., 2002; Katsuno et al., 2005; Tetzlaff et al., 2007; Nagashima et al., 2011; Wang et al., 2013). HSP are stress-induced, molecular chaperones that are critical in maintaining correct assembly, folding, and intracellular transport of proteins. A characteristic of the heat shock response is a rapid upregulation of HSP and the folding of nonnative proteins after injury (Morimoto & Santoro, 1998). Recent literature suggests androgens may exert their neuroprotective effects by modulating HSP following injury. In 1990, Sanchez and colleagues established that unbound steroid receptors complex with HSP 90 and HSP 70 (Sanchez et al., 1990). It was later determined that HSP are bound to AR in the cytoplasm and are released in the presence of a synthetic androgen (Veldscholte et al., 1992). This work is supported by more recent findings in our laboratory demonstrating that AR protein localizes to the nucleus following the administration of a form of testosterone, testosterone propionate (TP; Tetzlaff et al., 2007). Our laboratory also has shown that levels of *hsp 70* mRNA and protein increase dramatically following facial nerve transection in hamsters and are significantly reduced after testosterone treatment (Jones et al., 2000; Tetzlaff et al., 2007). Additionally, our laboratory has reported testosterone significantly

augments the ribosomal response 18 hours after injury and shortens the interval between rRNA transcription and processing in the presence of gonadal steroids (Kinderman & Jones, 1993). From this work, it has been hypothesized that testosterone treatment may reduce the normal stress response to allow for a more rapid neuroregenerative response by making available pre-existing HSP 70.

It has been hypothesized that testosterone drives the regenerative response by altering expression of regeneration-associated genes, including *bdnf*, *gap-43*, *neuritin*, and *β_{II}-tubulin* (Sharma et al., 2010a; Verhovshek et al., 2010). Previous studies indicate that BDNF promotes axonal sprouting, motoneuron survival, and functional recovery after injury (Serpe et al., 2005; Sasaki et al., 2009). Moreover, GAP-43 is a crucial component of the presynaptic terminal and necessary in growth cone guidance (Shen et al., 2002). Conversely, tubulin has a rather different role in the regeneration process as it is the main component of microtubules and functions in maintaining cell structure and intracellular transport (Hoffman & Lasek, 1975). McQuarrie et al. found that if a lesion precedes peripheral nerve injury, known as a conditioning lesion, tubulin levels are increased in the axonal shaft and axonal outgrowth is accelerated (McQuarrie, 1986). Recently, we reported that administration of testosterone following injury causes a delayed (2-7 days post-axotomy) upregulation of several regeneration-associated genes, including *bdnf*, *neuritin*, and *β_{II}-tubulin* mRNA in rat facial motoneurons (Sharma et al., 2010a), which is consistent with previous results from our laboratory (Jones & Oblinger, 1994; Jones et al., 1999;

Brown et al., 2001; Storer et al., 2002; DeLucia et al., 2007; Fargo et al., 2008). In contrast to our earlier results, we did not see an upregulation in *gap-43* following testosterone treatment (Jones et al., 1997a; Sharma et al., 2010a). Interestingly, from these studies we found testosterone had no effect on mRNA levels of a different subclass of tubulin, *α_I–tubulin*. In accordance with existing literature, testosterone regulates the expression of BDNF in both spinal motoneurons and muscle, as well as the BDNF receptor, trkB, in spinal motoneurons (Osborne et al., 2007; Verhovshek et al., 2010; Verhovshek & Sengelaub, 2013). Since BDNF regulates AR expression in motoneurons after injury, it is likely that BDNF and AR augment one another and have a feed-forward mechanism (Al-Shamma & Arnold, 1997; Yang & Arnold, 2000).

Since gonadal steroids have been established to play a role in CNS plasticity, our laboratory investigated their effects on motoneurons after injury. After transection of the facial nerve, we saw an 81% reduction in the percent somal membrane covered by synaptic profiles and a 26% decrease in the average synaptic length of axosomatic synapses (Jones et al., 1997b). Exposure to TP immediately after nerve transection reduced both of these measurements to 48% and 16%, respectively. These results indicate that treatment of TP following axotomy attenuated the synaptic stripping that occurred 5 days after injury. In support of these data, we found axotomy alone increased *glial fibrillary-acidic protein* (*gfap*) expression in the facial motor nucleus after facial nerve transection, which was attenuated with treatment of TP (Jones et al., 1997c; Coers et al., 2002). Taken together, these observations indicate TP attenuates

the initial response of motoneurons to injury allowing for an accelerated regeneration response and that is able to diminish the astrocytic response after axotomy.

Seminal studies conducted in the early 1980's, examined the effect of androgens on axonal regeneration. The first of several elegant papers published by the Yu laboratory investigated the effect of TP and DHT on axonal regeneration following transection of the hypoglossal nerve in female rats (Yu & Srinivasan, 1981). Their results demonstrated that as early as two weeks after transection axotomy of the right hypoglossal nerve there was a greater number of HRP-labeled neurons in the right hypoglossal nucleus of rats injected with TP or DHT compared to vehicle-injected rats. Similar results were observed at three weeks post-axotomy but not four weeks post-axotomy for TP treated animals compared to control animals. In contrast, the DHT treated animals showed fewer labeled HRP-labeled motoneurons three and four weeks post-axotomy compared to control animals. The following year, Yu observed that TP treatment following transection axotomy of the hypoglossal nerve also increased the number of HRP-labeled neurons that reached the tongue in male rats compared to female rats (Amy Yu, 1982; Yu, 1982). Furthermore, similar results were observed with TP treatment following crush axotomy of the hypoglossal nerve (Yu & Yu, 1983). Taken together, these data suggest that TP promotes axonal regeneration, but it is unclear if TP shortens the time before sprout formation or accelerates the rate of axonal regeneration.

In 1989, a study conducted by Kujawa and colleagues examined the effect of TP on functional recovery following facial nerve crush injury in male hamsters (Kujawa et al., 1989). In addition to assessing functional recovery, they also tested varying doses and administration of TP, i.e., capsule implants vs. injection. From these experiments, they had several unique discoveries. First, functional recovery was accelerated in all groups treated with TP, albeit at different levels. Moreover, they found that increasing the dose and frequency of TP injections directly altered functional recovery; however, TP-filled Silastic capsules elicited the most enhanced effects on functional recovery. Lastly, there was no difference in functional recovery when comparing gonadally intact to gonadectomized male hamsters, suggesting endogenous levels of testosterone was not sufficient to enhance functional recovery.

At this point in time, it was known that TP promoted axon regeneration from the observations reported by our laboratory and others; however, the mechanism by which TP elicited its effects was unknown. Several subsequent papers from our laboratory examined the effects of TP on regeneration rate following facial nerve injury. Using linear regression analysis, we determined that TP significantly increased regeneration rate after facial nerve injury in intact female hamsters by ~10% but had a more enhanced effect in males (~30%; Kujawa et al., 1991). Additionally, we confirmed our previous finding that endogenous testosterone was not sufficient to alter regeneration rate. To test whether these observations were AR dependent, we gave daily injections of flutamide, a known antiandrogen (Kujawa et al., 1995). In this study, we found

that flutamide completely abolished the ability of TP to enhance regeneration rate which suggests that TP acts through an AR dependent mechanism. Interestingly, we discovered that there is a critical interval of 6 hours after injury in which TP must be administered to significantly enhance regeneration and augment functional recovery (Kujawa & Jones, 1990; Tanzer & Jones, 2004). Although metabolites of testosterone, including DHT and estradiol, increase the rate of regeneration following injury, the effect is not as pronounced as seen with TP treatment (Tanzer & Jones, 1997; Sharma et al., 2009).

The evidence we have accumulated in the last few decades supporting the neurotherapeutic role of testosterone after facial nerve injury has been confirmed in other injury models. TP has been shown to accelerate functional recovery after sciatic nerve crush injury (Brown et al., 1999). In a similar model, Swallow and colleagues observed enhanced anatomical recovery with TP, but they did not see behavioral recovery following treatment with TP (Swallow et al., 1999). It was later determined that this discrepancy stemmed from differences in administration of TP and provided further evidence that TP is most effective when delivered through subcutaneous capsule implants that elevate serum levels of TP to supraphysiological levels. Enhanced functional recovery with TP treatment also has been reported following recurrent laryngeal nerve crush injury and hypoglossal nerve injury (Yu & Srinivasan, 1981; Yu & Yu, 1983; Brown et al., 2013; Monaco et al., 2013). In summary, we and others have established that androgens significantly accelerate functional recovery if administered at a supraphysiological level and in the propionated form.

iii. Growth factors

The utilization of growth factors to promote peripheral nerve regeneration has been investigated with limited success. Neurotrophin-3 (NT-3) has been observed to enhance neurite outgrowth *in vitro* (Avila et al., 1993); however, NT-3 failed to enhance functional recovery after transection of either the lingual or sciatic nerves (Young et al., 2001; Robinson et al., 2004). Similarly, neither BDNF nor nerve growth factor (NGF) enhanced peripheral nerve regeneration after nerve transection in rats and was inferior compared to no treatment (Young et al., 2001; Boyd & Gordon, 2002). In contrast, glial cell-derived neurotrophic factor (GDNF) delivered by fibrin glue or collagen gel at the repair site has been demonstrated to enhance functional recovery and partially recover contractile muscle force, muscle mass, and the number of estimated motor units after nerve transection and repair (Chen et al., 2001; Wood et al., 2013), but the overexpression of GDNF impaired motor axon growth and target reinnervation (Tannemaat et al., 2008). In 2001, Yin and colleagues discovered that NT-4/5 treatment delivered at the repair site in fibrin glue after sciatic nerve transection and repair significantly improved the number of regenerated axons, axon diameter, and myelin thickness (Yin et al., 2001). English et al. later demonstrated that NT-4/5 enhances the early reinnervation of the distal stump (English et al., 2005).

The limited success of growth factors to lead to functional recovery is supported by modest changes in mRNA and protein levels following nerve injury. Omura and coworkers investigated the expression of several growth factors in

nerve and muscle after various types of PNI (Omura et al., 2005). Their results demonstrated that *bdnf* mRNA and protein levels increased with nerve transection in the sciatic nerve and soleus muscle as early as 7 days post-axotomy. In addition, *nt-3* mRNA and protein levels showed no difference in either the sciatic nerve or soleus muscle in any of the tested PNI, whereas *nt-4/5* mRNA decreased as early as 4 days post-axotomy after sciatic nerve transection. One mechanism by which GDNF could mediate its effects on enhancing peripheral nerve regeneration is through upregulating GAP-43 (Chen et al., 2001). In support of the results reported by Chen et al., Storer and colleagues discovered application of GDNF to the injury site following hemisection to the rubrospinal tract significantly upregulated *gap-43* 7 days post injury (Storer et al., 2003). Additionally, Storer et al. observed increases in β_{II}-*tubulin* 1 day post injury, whereas there was no effect of BDNF treatment on β_{II}-*tubulin* or *gap-43*. The aforementioned results suggest growth factors alone are less promising than other non-surgical options in improving functional recovery following PNI.

iv. Exercise

Over the past decade, literature describing the effect of exercise on axonal regeneration and functional recovery has increased considerably. Several reports demonstrate exercise positively impacts axonal regeneration and functional recovery of injured peripheral nerves by increasing axon elongation (Sabatier et al., 2008), enhancing functional recovery (Cobianchi et al., 2010), and increasing

muscle reinnervation (Udina et al., 2011). Moreover, treadmill training within the first two weeks following PNI promoted motor axon regeneration without disrupting regeneration specificity (English et al., 2009). Treadmill training programs significantly reduced neuropathic pain symptoms after chronic constriction injury of the sciatic nerve in rats and mice (Cobianchi et al., 2010; Chen et al., 2012). Recently, Wood and colleagues investigated sex differences in treadmill training in mice following transection injury with allograft repair of the common fibular nerve (Wood et al., 2012). Interestingly, Wood et al. discovered continuous treadmill training significantly enhanced axon regeneration in males, whereas in female's interval training significantly enhanced axon regeneration.

Some studies have begun to elucidate the mechanisms underlying the effect of exercise on axon regeneration. Gómez-Pinilla et al. established that exercise significantly increased *bdnf* mRNA and protein in lumbar motoneurons and the soleus muscle after 5 days of training in uninjured rats (Gómez-Pinilla et al., 2001), as well as mRNA levels of the BDNF receptor, *trkB*, in the lumbar spinal cord after 3 days of exercise (Gómez-Pinilla et al., 2002). In contrast, *nt-3* mRNA expression was initially downregulated in the spinal cord after 1 day of exercise training but was upregulated after 5 days of continuous exercise training in uninjured rats (Gómez-Pinilla et al., 2001). In the soleus muscle, Gómez-Pinilla and coworkers observed *nt-3* mRNA levels to be significantly upregulated after both 1 and 5 days of exercise training. Additionally, Gómez-Pinilla observed that exercise significantly increased both mRNA and protein levels of *gap-43* and the synaptic mediator *synapsin 1* in lumbar spinal cord (Gómez-Pinilla et al., 2002).

Similarly, Molteni et al. reported increased neurite outgrowth of cultured lumbar DRG neurons from exercised animals which was associated with significantly higher levels of mRNA of the neurotrophins *bdnf* and *nt-3*, *synapsin 1*, and *gap-43* (Molteni et al., 2004). Interestingly, Wood and coworkers demonstrated that castration eliminated the ability of exercise training to enhance axon regeneration in male rats (Wood et al., 2012). Conversely, Wood et al. treated female rats with the aromatase inhibitor, anastrozole, to demonstrate that axon regeneration is significantly enhanced without increasing serum testosterone levels (Wood et al., 2012). The results presented by Wood and colleagues suggest axon regeneration is enhanced in a sex-dependent manner. Similar findings were later confirmed in male and female rats utilizing the androgen receptor blocker flutamide (Thompson et al., 2013). Together, these results suggest exercise is a potential therapy which enhances axon regeneration of both sensory and motor neurons and that androgen signaling is likely involved.

d. Therapeutic outcomes after PNI

The basal lamina acts as a conduit for axon regeneration and is necessary for proper regeneration. In a crush injury axotomy, the basal lamina is left intact resulting in a quicker recovery than observed with transection. Furthermore, a faster recovery time after crush injury is undoubtedly due to lack of surgical intervention and a faster axonal regeneration rate, as described above (McQuarrie et al., 1977; Bittner et al., 2012). Despite an accelerated recovery seen with crush axotomy, problems can still arise after both crush axotomy and

transection. For example, injury to a mixed nerve can lead to axonal regeneration from the proximal stump to inappropriate distal pathways resulting in failed functional connections. Moreover, the presence of a nerve in the incorrect pathway could inhibit proper axons from entering appropriate, specific pathways. This conundrum has been largely investigated as a potential mechanism underlying poor functional recovery. In 1988, Brushart labeled sensory neurons and motoneurons and quantified the number of neurons that reinnervated sensory and motor branches of the rat femoral nerve after lesion and repair. Brushart further demonstrated that, when motoneurons are given equal access to either a motor or cutaneous pathway, they preferentially reinnervated the motor pathway to the quadriceps muscle (Brushart, 1988). Interestingly, this same outcome was apparent even if the repair was intentionally misaligned. Aberrant nerve regeneration of motor axons to incorrect muscle groups can lead to synkinesis which is a common characteristic seen in individuals with cranial nerve injuries (Bodénez et al., 2010).

C. Rodent models of PNI

Animal models offer a valuable therapeutic approach to investigate PNI. Unlike humans, rodents typically have full functional recovery from crush (Hetzler et al., 2008) but not transection injuries (Deumens et al., 2007). Our laboratory and others have explored the use of animal models of the following cranial nerves: optic nerve (II; Wu et al., 2014), oculomotor (III; Fernandez et al., 2003), trochlear (IV; Fukuoka et al., 1999), trigeminal (V; Okubo et al., 2013), abducens

(VI; Pásaro et al., 1985), facial (VII; Kujawa et al., 1989), vestibulocochlear (VIII; Shimamura et al., 2002), glossopharyngeal (IX; Geran & Travers, 2011), vagus (X; Halum et al., 2013; Monaco et al., 2013), and hypoglossal (XII; Murakami & Yoshida, 2012). Injury of the facial nerve is commonly associated with the human disorder Bell's palsy, which is characterized by ipsilateral paralysis of the muscles of facial expression (Bodénez et al., 2010). Although the facial nerve has a "sensory root," the neuronal cell bodies are anatomically separate from the motor root making facial nerve axotomy one of the most widely used animal models to study motoneuron degeneration and regeneration (Moran & Graeber, 2004). Approximately two months following facial nerve injury, there is approximately a 40% motoneuron loss observed in adult mice (Serpe et al., 2000) but virtually all motoneurons survive after axotomy in adult hamsters (LaVelle & LaVelle, 1984). A benefit to interspecies differences in motoneuron death allows one to easily study mechanisms of nerve regeneration following injury. Commonly utilized models of facial nerve injury include extratemporal transection and crush, intratemporal crush, and intracranial transection of the facial nerve, all of which are considered proximal nerve injury models (Mattsson et al., 1999; Jones et al., 2001; Sharma et al., 2009). Similar to the facial nerve, the recurrent laryngeal nerve is classified as a cranial nerve as it is a branch of the vagus nerve. The recurrent laryngeal nerve is commonly injured during thyroidectomy procedures, thus leading to unilateral vocal fold paralysis and a loss of sensation to the inferior portion of the larynx (Rosenthal et al., 2007). A benefit to utilizing animal models of recurrent laryngeal nerve injury is that

functional recovery is easily quantifiable; however, mechanism is difficult to study as the cell bodies of the recurrent laryngeal nerve are not easily accessible.

In addition to using animal models of cranial nerve injuries, our laboratory and others have investigated animal models of spinal nerve injury, such as the median, ulnar, femoral, and sciatic nerves (Brown et al., 1999; Wang et al., 2008; Gordon et al., 2009). A benefit to utilizing animal models of spinal nerves is that they are characterized as mixed nerves and have both motor and sensory components, which is advantageous when studying preferential motor reinnervation and treatments that enhance specificity of axons to their appropriate targets. Due to its large size and accessibility in the rat, the sciatic nerve is an ideal spinal nerve to study PNI. Furthermore, the sciatic nerve is the longest nerve in the human and rodent body and can be utilized to explore treatment effects on proximal vs. distal injuries. Unlike cranial nerve injuries, there is virtually no motoneurons loss after spinal nerve injury in adult rodents which allows the motoneuron cell bodies to be easily studied after injury (Schmalbruch, 1984; Pollin et al., 1991). In addition, the timeline for functional recovery following sciatic nerve crush injury has been well documented (Forman & Berenberg, 1978; Alberghina et al., 1985; Brown et al., 1999; Lago & Navarro, 2006); however, the functional recovery timeline after sciatic nerve transection is more variable (Hare et al., 1992; Ijkema-Paassen et al., 2002; Hamilton et al., 2011). Previously, we have reported work completed on a proximal, mid-thigh sciatic injury (Brown et al., 1999) and this dissertation investigates the effects of ES and TP on a distal-thigh sciatic injury. The strength of animal models of PNI

has allowed us and others to investigate treatment effects in motor vs. mixed nerves, cranial vs. spinal nerves, and proximal vs. distal injuries to provide insight on how to appropriately guide therapeutic intervention in clinical situations.

D. Sciatic nerve injury and repair

1. Clinical relevance

Sciatica is observed in an estimated 40% of adults at some point throughout their lives, and is characterized as pain/weakness of the hip and lower extremity resulting from pathologies affecting the sciatic nerve. In this disorder, pain may extend from the gluteal region, down the posterior and lateral thigh and leg, and into the lateral aspect of the foot (Ergun & Lakadamyali, 2010). Individuals with sciatica may experience weakness to muscles in the posterior compartment of the thigh or all muscles of the leg and foot. This may result in the inability of patients to perform any one of the following actions: extend the thigh, flex the leg, plantarflex or dorsiflex the foot, flex or extend the toes, and invert or evert the foot. Typically, individuals with sciatica develop a distinguishing gait abnormality called footdrop. This is caused by a loss of motor innervation to the tibialis anterior muscle in the anterior leg, which leads to the inability to dorsiflex the foot. As a result, an individual with footdrop will alter their gait to prevent dragging their toes on the ground.

2. Anatomy of the human and rat sciatic nerve

The sciatic nerve is a major nerve of the sacral plexus and is the longest and largest nerve in the human and rodent body. Axons from spinal levels L4-S3 come together to form the sciatic nerve and stay together in a common sheath before dividing into the tibial and common fibular divisions. These divisions remain together as the sciatic nerve exits the pelvis via the greater sciatic foramen and as it emerges at the inferior border of the piriformis muscle. The sciatic nerve travels down the posterior thigh deep to semitendinosus and long head of bicpes femoris where it supplies innervation to both of these muscles. At the superior border of the popliteal fossa, the sciatic nerve divides into two independent nerves the tibial and common fibular nerves, which innervate muscles of the leg and foot. The path of the tibial nerve runs vertically through the center of the fossa where it gives rise to the medial sural cutaneous nerve before continuing into the proximal leg and passing between the medial and lateral heads of the gastrocnemius muscle. In contrast, the common fibular nerve gives off a lateral sural cutaneous branch in the popliteal fossa region before passing superficial to the lateral head of the gastrocnemius muscle. The route of the common fibular nerve continues laterally around the neck of the fibula toward the lateral compartment followed by the anterior compartment of the leg. It passes through the fibularis longus muscle and further divides into the superifical and deep fibular nerves. The superficial fibular nerve supplies innervation to the lateral compartment leg muscles, while the deep fibular nerve supplies innervation to the anterior compartment leg muscles, including tibialis anterior

muscle, and muscles of the anterior foot. The medial and lateral sural cutaneous nerves join in the posterior leg to form the sural nerve and provide cutaneous innervation to the lateral foot (Kim et al., 2004; Ergun & Lakadamyali, 2010; Brushart, 2011).

3. Etiology of sciatic nerve damage

Despite an elaborate protective sheath, trauma, disease, and complications during surgery can lead to injury of the sciatic nerve. In a retrospective study completed by Kim and colleagues, injections were the leading cause of sciatic nerve injuries at the level of the buttocks, and gunshot wounds constituted roughly a third of all sciatic injuries reported at the level of the thigh (Kim et al., 2004). Other reported methods of injury include, but are not limited to: fracture or dislocation of the femur, hip arthroplasty, iatrogenic, laceration, compression, stretch injury, tumors of the sciatic nerve, piriformis syndrome, and pregnancy (Kline et al., 1998; Kim et al., 2004; Ergun & Lakadamyali, 2010).

Clinical evaluation through neurological examination is the first step in determining the severity of sciatic nerve injury. Loss of motor and sensory function is quantified using electrophysiological methods, such as compound muscle action potentials, nerve conduction velocities, electromyography, and sensory nerve action potentials (Masakado et al., 2008; Derr et al., 2009). Additionally, radiological assessment with CT scans and MRI can be used to determine underlying traumatic injuries.

E. Combinatorial approaches

For researchers to successfully improve functional recovery after PNI a combinatorial approach should be considered because it has the ability to target and activate multiple pathways and mechanisms. PEG has demonstrated promise alone and in conjunction with the antioxidants melatonin and methylene blue *in vitro* and *in vivo* (Stavisky et al., 2005; Britt et al., 2010; Spaeth et al., 2012). Bittner et al. also have reported positive motor recovery with microsuture, hypotonic Ca^{2+}, methylene blue, and PEG following complete transection of the sciatic nerve near that of sham controls (Bittner et al., 2012). In contrast, a more recent study suggested seeding Schwann cells that overexpress GDNF into an allograft, but failed to promote regeneration into the distal stump thus preventing functional regeneration (Santosa et al., 2013).

Promising results have been observed utilizing the combinatorial treatment of ES and exercise following axotomy. Asensio-Pinilla and colleagues demonstrated a 1 hour, 20 Hz ES paradigm and treadmill running for 4 weeks significantly improved muscle reinnervation following sciatic nerve transection with repair compared to either treatment alone (Asensio-Pinilla et al., 2009). A more recent study suggested ES enhanced motor and sensory reinnervation, whereas the combination of ES and treadmill training decreased nociceptive responses to mechanical and thermal stimuli (Cobianchi et al., 2013). Taken together, these data indicate the combination of ES and exercise as a potential therapeutic intervention.

As previously mentioned, ES and TP target different stages of the regeneration process: delay time before sprout formation occurs and regeneration rate, respectively (Kujawa et al., 1993; Sharma et al., 2009). The ability of these two treatments to target different aspects of the regeneration process and enhance functional recovery provides a logical rationale for combinatorial treatment application following PNI. In support of a combinatorial approach, Sharma and colleagues demonstrated that ES and TP together acted synergistically to accelerate recovery of the vibrissae movement in rats after a facial nerve crush injury (Sharma et al., 2010b), which supported an earlier study (Hetzler et al., 2008). Interestingly, the presence of the steroid prednisone, which is an anti-inflammatory utilized to decrease edema associated with nerve injury, had no additional benefit. Also, we have determined that ES and TP treatment after a facial nerve injury increases *neuritin*, *pacap*, and *bdnf* mRNA expression in rat in an additive manner (Sharma et al., 2010a). In contrast, the combination of ES and TP had no additional benefit in functional recovery than either ES or TP alone after injury to the recurrent laryngeal nerve (Monaco et al., 2013). This is likely due to the fact that the recurrent laryngeal nerve injury was a more distal injury when compared to the facial nerve injury model used in these studies. It remains to be determined whether these same effects occur after a spinal nerve injury, such as a sciatic nerve injury, and if ES and TP will have differential effects on proximal vs. distal sciatic nerve injuries.

This literature review was modified from a forthcoming book chapter in the 3rd edition of *Sunderland's Nerves and Nerve Injuries*: Chapter 81: Cellular Aspects of Nerve Injury and Regeneration published by Elsevier

Meadows RM, Sengelaub DR, and Jones KJ. Cellular Aspects of Nerve Injury and Regeneration. *Sunderland's Nerves and Nerve Injuries*, 3rd edition. (in press)

CHAPTER 3: MATERIALS AND METHODS

A. Animals and sciatic nerve injury paradigm

All surgical procedures were reviewed and approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee and were conducted in accordance with National Institute of Health guidelines. Adult male Wistar rats (175-199 g) were purchased from Harlan (Indianapolis, IN) and used for all experiments. All animals were maintained with a 12/12 h light/dark cycle with standard feed and water *ad libitum*. No animals displayed a ≥15% weight loss throughout the study. 7 animals were removed from the study due to autonomy. For behavioral tests, uninjured animals were used as baseline throughout the training and testing period. A total of 178 (Aim 1 – 145; Aim 2 – 33) male Wistar rats were used in this study.

Surgeries were completed in a sterile, aseptic environment. Rats were anesthetized with 3% isoflurane gas with 1.5 L/min oxygen. Surgical site was shaven and cleaned. An incision approximately 2 cm in length was made on the dorsal surface of the hip and the skin was retracted to expose the underlying musculature. Using blunt dissection, the heads of biceps femoris were separated to expose the sciatic nerve. The right sciatic nerve was crushed approximately 2 mm proximal to its trifurcation with Dumont forceps (#3). Two successive 30-second crushes from alternating directions were completed while leaving the epineurium intact. Visible loss of motor function in the right hind foot and loss of electrical muscle activity were used as confirmation to ensure a successful crush.

After injury (Ax), rats were divided into four experimental groups: 1) No treatment (Ax only), animals receiving axotomy, with implantation of blank capsules not containing testosterone propionate (TP), 2) Electrical Stimulation (Ax + ES), animals receiving axotomy and ES treatment, 3) Ax + TP, animals receiving axotomy and TP treatment, 4) Ax + ES + TP, animals receiving axotomy and ES and TP treatments. Animals receiving both ES and TP treatments, ES was administered followed by delivery of TP. After injury and treatment, the heads of biceps femoris were sutured together and the overlying skin closed with wound clips as well as coated with triple antibiotic ointment. Immediately after surgery, 0.025 mg/kg of buprenorphine was administered. Wound clips were removed 7-10 days post-axotomy.

B. Thin film disk receiver implant

Immediately prior to the sciatic nerve crush injury, a sterilized, thin disk receiver (RedRock Laboratories, St. Louis, MI) was implanted subcutaneously on the posterior surface of the rat. The circular receiver device was less than the size of a penny (3 mm x 18 mm) and was covered with a silastic coating to make it flexible and soft. The receiver was attached to a silastic cuff (2 mm I.D., 8 mm length) by two, thin, aluminum chloride or platinum insulated multi-conductor wires (4-5 in). The cuff was gently wrapped around the sciatic nerve, proximal to the injury site, and sutured shut. The implant was used to elicit ES (see below) immediately after injury and to stimulate the nerve for electromyography (EMG) recordings. The device was remained in the animal for the length of the study.

C. Electrical stimulation (ES)

The Automated Functional Assessment Station (FASt; Red Rock Laboratories, St. Louis, MO, USA) was used to deliver ES to all animals receiving ES treatment. ES was delivered through either wire hooks or a nerve cuff. For animals that did not undergo behavioral tests, two aluminum chloride or platinum coated wires were bent into hooks and placed around the sciatic nerve just proximal to the injury site. The anode and cathode connector pins were attached to the leads of a continuous ES box (Red Rock Laboratories, St. Louis, MO, USA). For animals that underwent behavior analysis, a silastic cuff housing aluminum chloride or platinum electrodes was inserted around the nerve. The wires were connected to a subcutaneously implanted disk receiver. A wireless transducer with 10 settings, 1-10, was placed above the disk and turned to a pre-determined setting known to elicit a right hind twitch. The transducer was connected to a continuous ES box. Immediately after axotomy, the continuous ES box was turned on and rats were stimulated at 20 Hz (200 μsec pulses, 1000 μA amplitude) for a continuous 30 minutes.

D. Hormone administration

TP was administered through subcutaneous capsule implants. The capsules were made out of silastic tubing (0.062 in. (1.57 mm) I.D. by 0.095 in (2.41 mm) O.D.) and were 16 mm in total length, 10 mm of which contained 100% crystalline TP (Sigma-Aldrich, St. Louis, MO, USA). Wooden plugs sealed both ends of the capsule. As a control, animals receiving axotomy, but no

treatment were implanted with blank capsules. Capsules were equilibrated in physiological saline for 30-60 minutes prior to implantation. Immediately following injury or ES, a subcutaneous pocket was formed by making an incision on the dorsal surface of the animal between the scapulae. Two TP capsules were implanted in the pocket and the wound site was closed with wound clips. Wound clips were removed 7-10 days post-axotomy. Capsules were left in the animal for the length of the study.

E. Molecular techniques

1. Enzyme immunoassay

Serum was separated from whole blood by spinning whole blood samples at 4°C for 15 minutes at 1000 x g. Serum samples were diluted with enzyme immunoassay (EIA) buffer and ran in triplicate. An EIA kit was utilized to confirm testosterone levels from rats according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, USA).

2. Quantitative real time PCR (qRT-PCR)

Rats were euthanized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) followed by bilateral pneumothorax at 6 hours, 1, 2, 7, and 21 days post-axotomy. The lumbar region of the spinal column was removed and the ends of the column were cleaned of bone debris. A 10 mL syringe containing ice-cold saline solution fitted with a blunt-ended needle (16 G)

was used to eject the spinal cord, from the caudal end, into a 100 mm size petri dish containing ice-cold saline solution. The spinal cord was placed ventral side up on a petri dish on top of dry ice and trimmed with a razor blade to isolate a 1 cm section of the lumbar enlargement. The ventral and dorsal horns of the spinal cord were isolated by making a sagittal cut down the central sulcus, turning the two tissue pieces until the flat portion was facing up, and making a final sagittal cut to separate the ventral horns, lateral side of tissue half, from the dorsal horns, medial side of tissue half (**Fig. 3**). All four quadrants were kept at -80^{0}C. To use as a control, non-lumbar spinal cord was removed and kept at -80^{0}C.

Spinal cord tissue was homogenized with Lysing Matrix D (MP Biochemical, Santa Ana, CA, USA) and lysis buffer (PureLink® RNA Mini Kit Cat# 12183025; Ambion, Carlsbad, CA, USA) containing 1% 2-mercaptoethanol (Gibco, Carlsbad, CA, USA) using a fast prep 24 homogoenizer (MP Biochemical). RNA was extracted from the homogenized tissue sample with 30 µL of RNase-Free Water according to the manufactures instructions, with the exception of incubating the column for 10 minutes instead of 1 minute during the extraction step with RNase-Free Water (PureLink® RNA Mini Kit). DNA was removed from the RNA samples using the PureLink® DNase Set according to the manufactures instructions (Cat# 12185010; Invitrogen, Carlsbad, CA). 100 ng of RNA was converted to cDNA using SuperScript® VILO™ Master Mix according to manufactures instructions (Cat# 11755250; Invitrogen). Rat-specific Taqman® probes were purchased to amplify cDNA of candidate genes, (Applied

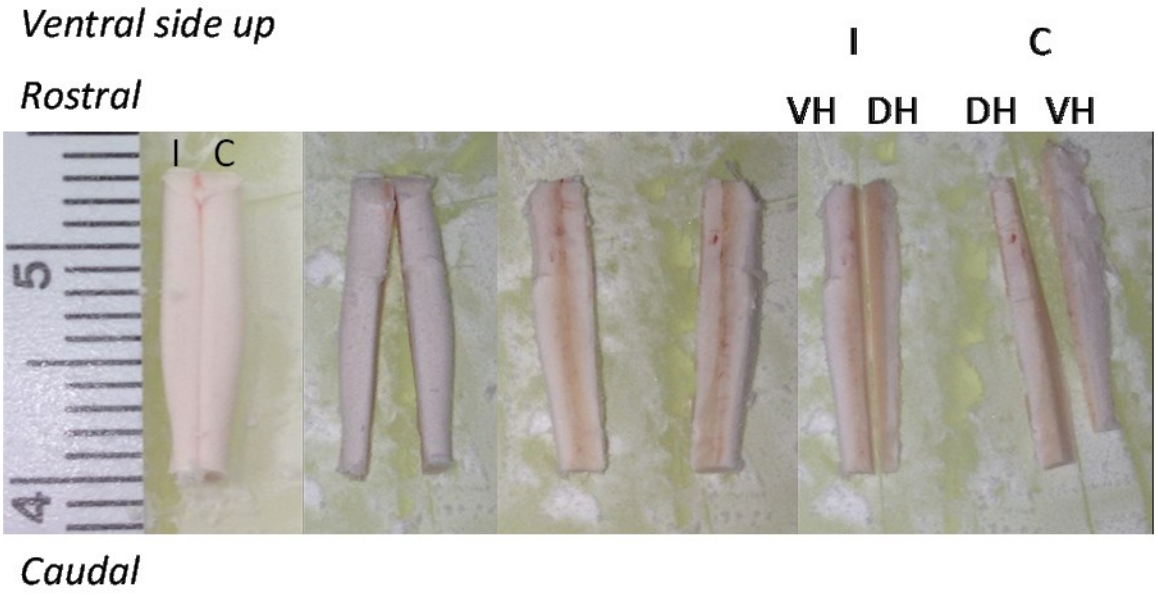

Fig. 3: Image depicting the quadrant sectioning process of the lumbar portion of the spinal cord.

I is ipsilateral, C is contralateral, VH is ventral horn, and DH is dorsal horn.

Biosystems, Carlsbad, CA, USA; **Table 1** and **Table 2**). qRT-PCR was carried out in triplicate using Taqman® Gene Expression Master Mix (Applied Biosystems). The cycling parameters were as follows: UDG optimization at 50°C for 2 minutes, AmpliTaq Gold activation at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and combined annealing/extension at 60°C for 1 minute. geNorm software was used to determine the most optimal reference genes for this study out of 8 candidate housekeeping genes (**Table 1**) (Bangaru et al., 2012). Relative mRNA expression levels acquired from PCR amplification were analyzed using the ΔCt method, as previously published by our laboratory (Haulcomb et al., 2014), against two housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) and hypoxanthine phosphoribosyl transferase 1 (*hprt1*), which were determined using geNorm software (Bangaru et al., 2012). ΔCt is the difference between the threshold cycle level for the gene of interest and the threshold cycle for the housekeeping gene within each sample, which is calculated for the control and axotomized sides separately. The geometric Ct mean of both *gapdh* and *hprt1* was used as the threshold cycle for the housekeeping gene. Relative mRNA expression is expressed as the relative quantity mRNA of either the injured or uninjured ventral horn of the lumbar spinal cord by $2^{-\Delta Ct}$ ± the standard error of the mean (SEM). The average percent change mRNA expression was calculated using the the ΔΔCt method, as previously published by our laboratory (Fargo et al., 2008; Mesnard et al., 2010) against *gapdh* and *hprt1*. The ΔΔCt value was obtained by calculating the difference between the axotomized ΔCt and the control ΔCt. All data is

Gene Name	Taqman® Abbreviation	Taqman® ID	Accession Number
glucuronidase, beta	*gusb*	Rn00566655_m1	NM_017015.2
glyceraldehyde-3-phosphate dehydrogenase	*gapdh*	Rn01775763_g1	NM_017008.3
hypoxanthine phosphoribosyltransferase 1	*hprt1*	Rn01527840_m1	NM_012583.2
beta-2 microglobulin	*b2mg*	Rn00560865_m1	NM_012512.2
polymerase (RNA) II (DNA directed) polypeptide A	*polr2a*	Rn01752026_m1	XM_002727723.1
succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	*sdha*	Rn00590475_m1	NM_130428.1
ornithine decarboxylase antizyme 1	*oazi*	Rn01408148_g1	NM_139081.1
tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta	*ywhaz*	Rn00755072_m1	NM_013011.3

Table 1: Candidate reference genes.

Gene Name	Taqman® Abbreviation	Taqman® ID	Accession Number
Cytoskeletal			
αI–tubulin	*atat1*	Rn00821045_g1	NM_022298.1
βII-tubulin	*tubb2b*	Rn01435337_g1	NM_001013886.2
Growth cone guidance			
43-kiloDalton growth-associated protein	*gap43*	Rn01474579_m1	NM_017195.3
collapsin response mediator protein 2	*dpysl2*	Rn01534654_m1	NM_001105717.2
Growth factors and neurotrophic			
brain derived neurotrophic factor	*bdnf*	Rn02531967_s1	NM_012513.3
glial cell derived neurotrophic factor	*gdnf*	Rn00569510_m1	NM_019139.1
pituitary adenylate cyclase-activating peptide	*adcyap1*	Rn00566438_m1	NM_016989.2
Plasticity			
neuritin	*nrn1*	Rn00584304_m1	NM_053346.1

Table 2: List of regeneration-associated genes.

expressed as the average percent change in mRNA expression, by $(2^{-\Delta\Delta Ct} - 1) \times 100$, of the axotomized samples relative to the control samples ± SEM. Ct values of 39 or higher were excluded from analysis. Outliers were determined and removed utilizing Grubbs' Test (Graph Pad, La Jolla, CA, USA). Primer efficiencies are noted in **Table 3**.

F. Behavioral tests

1. Automatic foot misplacement apparatus

The automatic foot misplacement apparatus consisted of a corridor with an arrival box at one end (Bioseb, Vitrolles cedex, France). The corridor was a flat ladder, with alternating rungs removed, on which the rat could move freely from one end toward the arrival box. Rats were trained to traverse the walkway five times per week for two consecutive weeks prior to injury. During the testing period, rats traversed the walkway up to three times per session until a run without rearing and minimal stopping was achieved. Baseline data was acquired 7, 5, and 3 days before injury. Sciatic nerve crush injury always occurred on the first day of the week. Following injury, rats were tested at 2 and 4 days post-axotomy or day 3 and 5 of the week, respectively. Testing continued in succession on day 1, 3, and 5 for each of the following 8 consecutive weeks. Data from the runs was gathered using the provided Locotronic software and is expressed as the total number of rear leg errors ± SEM. It should be noted, due

Gene Name	Primer Efficiency
α_I–tubulin	1.935
β_{II}-tubulin	2.0065
43-kiloDalton growth-associated protein	2.081
collapsin response mediator protein 2	2.053
brain derived neurotrophic factor	2.393
glial cell derived neurotrophic factor	2.962
pituitary adenylate cyclase-activating peptide	1.942
neuritin	2.031
glyceraldehyde-3-phosphate dehydrogenase	1.9695
hypoxanthine phosphoribosyltransferase 1	1.979

Table 3: Gene primer efficiencies.

to altering the software settings, 6 rats did not start the test until 21 days post-axotomy.

2. Grip strength

Hind limb strength was measured using a grip strength test with a rod and bar apparatus attached to a senor. To complete the bar task, rats were held by their torso and allowed to grab the bar with their hind legs. After successful contact of both hind paws with the bar, the rat was gently pulled in a horizontal direction by the base of their tail until they released the bar. After each pull, the sensor was reset and the rat was allowed to grip the bar. This process was repeated five consecutive times and the results were recorded. The grid test was used as a second grip strength measure. Similar to the bar, animals were allowed to grab the grid with their hind paws. Once the rat successfully made contact with the grid with both hind paws, the rat was gently pulled by the tail in a parallel direction with the surface of the grid. After each grasp, the sensor was reset and five consecutive trials were recorded. Animals were tested on the first day of each week for 8 consecutive weeks. Baseline data was recorded exactly one week prior to injury and the morning of surgery day. Results for both the bar and grid were expressed as an average of the five trials and as the maximum grip strength within the five trials for each animal. Each group contained an *n* of 3-6.

3. CatWalk XT© apparatus

The CatWalk apparatus (Noldus Information Technology, Wageningen, The Netherlands) consists of an enclosed walkway (8 cm wide) on a glass plate, on which the rat could move freely from one end to the other. We made the following modifications to the existing apparatus: on the departure end of the apparatus, was a plexiglass ledge while at the arrival end was a cage in which the rat could enter after completing a run. Since the glass plate was larger than the enclosed walkway, the rat could leave the arrival cage and traverse the glass plate in the opposite direction to the departure plexiglass ledge. One passage from departure ledge down the runway back to the departure ledge will be referred to as 1 run.

All data acquisition was completed in a dark and silent room. The following parameters were used during data acquisition: length of the runway was approximately 90 cm, camera gain was 19.02, and intensity threshold was 0.1. No food restrictions took place during the study. The behavioral training consisted of placing the animals in the arrival cage and coaxing them to loop in a unidirectional manner. Fruit Loops or Apple Jacks were placed along the path in order to encourage the animal to loop. Training occurred on days 1-5 for two consecutive weeks prior to injury at which time 5-20 runs were acquired per training session. Baseline data (10-20 runs per training session) was acquired 7, 5, and 3 days prior to injury. During data acquisition, if the rats stopped or displayed uneven speed they were encouraged to move as previously published (Batka et al., 2014). The glass runway was cleaned with Sparkle™ glass cleaner

after the completion of all runs for a particular animal, or as needed to remove debris.

Following sciatic nerve crush injury, rats were tested on a similar schedule to that described in the automatic foot misplacement apparatus section. Testing lasted for 8 consecutive weeks starting at 2 days post-axotomy and 10 runs were acquired during each testing session. Runs in which the animal displayed behavioral anomalies (i.e. constant sniffing, turning, rearing) in the middle of the run were wholly-discarded. Within runs chosen to be classified, partial footprints and rearing as entering or exiting the runway were not classified. Of the classified runs, only runs which met the following criteria set in the data acquisition profile were exported: 10 minimum consecutive steps per run, average speed range from 1.0-150.0 cm/second, maximum allowed speed variation of 55%, and fully classified runs. Of the exported runs, only runs with an 'other statistics maximum variation' of ≤ 60% were analyzed. All exported run statistics were combined for each animal to create trial statistics; trial statistics were analyzed and compared across groups at various time points.

The following trial statistics have been shown to change following injury and were thus chosen for analysis: print length, print width, print area, swing, swing speed, stride length, step cycle, duty cycle, max contact max intensity, sciatic functional index (SFI), peroneal functional index (PFI), posterior tibial functional index (TFI), base of support (BOS), regularity index, and cadence (Bain et al., 1989; Hamers et al., 2001; Vrinten & Hamers, 2003; Koopmans et al., 2005; Hendriks et al., 2006; Deumens et al., 2007; Bozkurt et al., 2008). Formulas for

SFI, TFI, and PFI can be found in **Fig. 4**. For explanations of each parameter, see **Table 4**. Each group contained an *n* of 4-6.

3. Electromyography (EMG) recordings

All rats were anesthetized with isofluorane and the FASt (Red Rock Laboratories) was used for all EMG measurements. Before injury, baseline EMG values were determined, whereas functional recovery was monitored by taking recordings on day 1 and 5 of each week beginning immediately after injury for 8 consecutive weeks. Cathodic, monophasic electrical impulses (5000 μA, 0 second delay, 0.3 second burst width, 200 μs pulse duration, and 0 Hz) were generated using a single-channel isolated pulse stimulator (Model 2100, A-M Systems Inc., Carlsborg, WA, USA) and delivered to the sciatic nerve proximal to injury via a silastic cuff. Stimulation of the nerve occurred wirelessly by placing the transducer just above the subcutaneous disk implant. A total of 9 trials (settings 1-9 on the transducer) with an inter-trial delay of 10 seconds were acquired for each animal. Stimulus amplitude ranged from 5001-5009, which was equivalent to a voltage output of 0.408-8.16 V from the transducer. Recordings were taken from tibialis anterior and lateral head of gastrocnemius muscles by placing 30 G needles into the belly of each muscle. Anode and cathode connector pins were attached to the needle probes, while a ground connector pin was attached to a 25 G needle placed subcutaneously on the back of the animal. Measurements were achieved using custom data acquisition software (RRL V.1.3, Red Rock Laboratories). Data was exported for analysis using MATLAB

A

$$SFI = -38.3 \times \left[\frac{PL_E - PL_N}{PL_N}\right] + 109.5 \times \left[\frac{TS_E - TS_N}{TS_N}\right] + 13.3 \times \left[\frac{ITS_E - ITS_N}{ITS_N}\right] - 8.8$$

B

$$TFI = -37.2 \times \left[\frac{PL_E - PL_N}{PL_N}\right] + 104.4 \times \left[\frac{TS_E - TS_N}{TS_N}\right] + 45.6 \times \left[\frac{ITS_E - ITS_N}{ITS_N}\right] - 8.8$$

C

$$PFI = 174.9 \times \left[\frac{PL_E - PL_N}{PL_N}\right] + 80.3 \times \left[\frac{TS_E - TS_N}{TS_N}\right] - 13.4$$

D

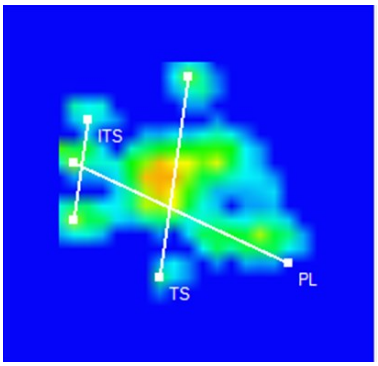

Fig. 4: Formulas to calculate rat sciatic functional index (SFI), posterior tibial functional index (TFI), and peroneal functional index (PFI).

PL is Manual Print Length, TS is Toe Spread, ITS is Intermediate Toe Spread, and the subscripts E and N indicate the Experimental and Normal contralateral hind paws, respectively. Formulas taken from Bain et al. (1989).

CatWalk Parameter	Definition
Paw-related parameters	
Print length	length (horizontal direction) of the complete print
Print width	the width (vertical direction) of the complete print
Print area	surface area of the complete print
Swing	duration in seconds of no contact of a paw with the glass plate
Swing speed	speed (cm/s) of the paw during Swing
Stride length	distance (cm) between successive placements of the same paw
Step cycle	time in seconds between two consecutive initial contacts of the same paw [Stand + Swing]
Duty cycle	expresses Stand as a percentage of Step Cycle Duty cycle = (Stand)/[(Stand) + (Swing)] * 100
Max contact max intensity	maximum intensity at max contact of a paw
Non paw-related parameters	
Sciatic functional index (SFI)	measure for the functional recovery of the sciatic nerve which innervates the hind paws
Peroneal functional index (PFI)	measure for the functional recovery of the peroneal nerve which innervates the hind paws
Posterial tibial functional index (TFI)	measure for the functional recovery of the tibial nerve which is a branch of the sciatic nerve
Base of support (BOS) front paws	average width between the front paws
BOS hind paws	average width between the hind paws
Regularity Index	expresses the number of normal step sequence patterns and total paw placements
Cadence	expressed in steps per second

Table 4: Definitions of CatWalk parameters/variables.

software (The Mathworks Inc., Natick, MA, USA) and is expressed as the EMG amplitude (mV) ± SEM. 3 animals were removed from EMG analysis due to malfunctioning wireless electrodes. Each group contained an *n* of 4-6 animals.

G. Histological and histochemical processing

Eight weeks or 56 days post-axotomy after injury, animals were anesthetized with isofluorane, and the right anterior tibialis muscle was exposed and injected with horseradish peroxidase conjugated to the cholera toxin B subunit (BHRP; 0.5 µl, 0.2%; List Biological, Inc.). BHRP labeling permits population-level quantitative analysis of motoneuron somal and dendritic morphologies (Kurz et al., 1986; Goldstein & Sengelaub, 1990). Forty-eight hours after BHRP injection, a period that ensures optimal labeling of motoneurons (Kurz et al., 1986; Goldstein & Sengelaub, 1990), animals were weighed, anesthetized with Ketamine/Xylazine (i.p.), and perfused intracardially with saline followed by cold fixative (4% paraformaldehyde). Tibialis Anterior and lateral head of gastrocnemius muscles were extracted and weighed.

1. Motoneuron number and morphology

The tibialis anterior muscle is innervated by motoneurons located in column 4 of the lateral motor column in the L3 spinal segment (Nicolopoulos-Stournaras & Iles, 1983). Following perfusion, the lumbar portion of the spinal cord of each animal was removed, postfixed 5 hours in 1% paraformaldehyde/

1.25% glutaraldehyde, and then transferred to sucrose phosphate buffer (10% w/v, pH 7.4) overnight for cryoprotection. Spinal cords were then embedded in gelatin, frozen, and sectioned transversely at 40 μm; all sections were collected into four alternate series. One series was stained with thionin for use in cell counts. For visualization of BHRP, the three remaining series were immediately reacted using a modified tetramethylbenzidine protocol (Mesulam, 1982), mounted on gelatin-coated slides, and counterstained with thionin.

2. Motoneuron counts

To assess potential motoneuron loss after nerve crush, counts of motoneurons in the tibialis motor pool were performed. Motoneurons innervating the tibialis anterior muscles do not form a discrete nucleus, but instead are contained within the large continuous populations of motoneurons located within the lateral motor column. Thus, to identify the appropriate area within the lateral motor column for motoneuron counts in the unreacted series, we used a method similar to that of Little et al. (2009). Briefly, for each animal the range of sections in which motoneurons labeled with BHRP after injection into the tibialis anterior muscle were present in the reacted series was identified, and then motoneuron counts were performed in the appropriate matching sections in the unreacted series. For each animal, estimates of the total number of motoneurons in the left and right lateral motor columns were obtained using the optical disector method as previously described (Little et al., 2009). Counts were made using a video-based morphometry system (Stereo Investigator; MBF Bioscience, Williston, VT,

USA) at 937.5X under brightfield illumination. Motoneurons are easily recognizable as large, darkly staining, multipolar cells. A counting frame (110 μm X 80 μm) was moved systematically throughout an area of each ventral horn (approximately 300 μm X 300 μm, defined by the actual distribution of BHRP-labeled somata from all of the animals used in the study) in each section within the identified range. Only motoneurons in which there was a clear nucleus and nucleolus were counted, provided they did not contact the forbidden lines of the counting frame; motoneuron nucleoli were counted as they appeared while focusing through the z axis, and nucleoli in the first focal plane (i.e., "tops") were excluded to avoid double counting. The length of the dissector was approximately 16 μm, which was adequate for visualizing nucleoli in multiple focal planes. Motoneuron counts were derived from a mean of 15.0 sections spaced 480 μm apart and distributed uniformly through the entire rostrocaudal extent of the tibialis motoneuron pool range. This sampling scheme produced average estimated coefficients of error (CE) of 0.051 for normal animals and 0.046 for crush animals. Cell counts for each animal were corrected for the proportion of sections sampled and reported as per side and as a ratio [(right side/left side) X 100].

Using similar methods, the number of BHRP-labeled motoneurons was assessed in all sections of the reacted series through the entire rostrocaudal extent of their distribution for all animals. Counts of labeled tibialis motoneurons were made under brightfield illumination, where somata could be visualized and cytoplasmic inclusion of BHRP reaction product confirmed.

3. Soma volume

To assess potential changes in motoneuron morphology after nerve crush, soma volumes were measured. The volume of tibialis motoneuron somata was assessed in at least one set of alternate sections (160 μm apart) using the Nucleator method (Gundersen, 1988). A set of 4 rays emanating from a point randomly chosen within each BHRP-labeled motoneuron soma was drawn and oriented randomly. Soma volumes of an average of 27.3 motoneurons were measured for each animal using Stereo Investigator at a final magnification of 780X. Average estimated coefficients of error (CEs) were 0.022 for normal animals and 0.028 for crush animals. Soma volumes within each animal were then averaged for statistical analysis.

4. Dendritic length

To assess potential changes in motoneuron morphology after nerve crush, dendritic lengths and distributions were measured. For each animal, dendritic lengths in a single representative set of alternate sections were measured under darkfield illumination. Beginning with the first section in which BHRP-labeled fibers were present, labeling through the entire rostrocaudal extent of the tibialis motoneuron dendritic field was assessed in every third section (480 μm apart) in three dimensions using a computer-based morphometry system (Neurolucida; MBF Bioscience, Williston, VT, USA) at a final magnification of 250X. No attempt was made to identify BHRP-labeled fibers as either dendrites or axons. Average dendritic length per labeled motoneuron was estimated by summing the

measured dendritic lengths of the series of sections, multiplying by three to correct for sampling, then dividing by the total number of labeled motoneurons in that series. This method does not attempt to assess the actual total dendritic length of labeled motoneurons (Kurz et al., 1991), but has been shown to be a sensitive and reliable indicator of changes in dendritic morphology in normal development (Goldstein & Sengelaub, 1990; Goldstein et al., 1993; Goldstein & Sengelaub, 1994), after changes in dendritic interactions (Goldstein et al., 1993), afferent input (Kalb, 1994; Hebbeler et al., 2002; Hebbeler & Sengelaub, 2003), and after injury (Little et al., 2009; Byers et al., 2012).

5. Dendritic distribution

To assess potential redistributions of dendrites across treatment groups, for each animal the composite dendritic arbor created in the length analysis was divided using a set of axes oriented radially around the center of the collective labeled somata. These axes divided the spinal cord into twelve bins of 30° each. The portion of each animal's dendritic arbor per labeled motoneuron contained within each location was then determined. This method provides a sensitive measure of dendritic redistribution in response to changes in dendritic interactions (Goldstein et al., 1993), afferent input (Hebbeler et al., 2002; Hebbeler & Sengelaub, 2003), and injury (Byers et al., 2012).

6. Dendritic extent

The comparability of BHRP labeling across groups was assessed by quantifying both the rostrocaudal and the radial extent of tibialis motoneuron dendritic arbors. The rostrocaudal extent of the dendritic arbor was determined by recording the rostrocaudal distance spanned by tibialis motoneuron dendrites for each animal. The maximal radial extent of the arbor in the transverse plane was also measured for each animal, using the same radial axes and resultant 30° bins used for the dendritic distribution analysis. For each bin, the linear distance between the center of the tibialis motor pool and the most distal BHRP-filled process was measured. Radial dendritic extent is independent of overall dendritic length and reflects the maximal linear distance (in the transverse plane) of BHRP transport to the most distal dendritic processes.

H. Statistical analysis

All data were expressed as mean ± SEM. For molecular and behavioral analyses, all data were analyzed by t-tests or analyses of variance (one way, two way, or repeated measures as appropriate) followed by post hoc analyses using either Student-Newman-Keuls or Holm-Šidák with significance at $P \leq 0.05$ (SigmaPlot, version 12.3).

For behavioral tests, animals were subjected to the tests in a randomized order. All statistical tests for the CatWalk behavioral test were performed at a two-sided 5% significant level using SAS, version 9.3 (SAS Institute, Cary, North Carolina, USA). Statistical significance was evaluated using linear mixed effects

models including an interaction term for time as a qualitative variable and treatment group. Each model included random intercepts and slopes for speed and weight for each animal. Post hoc pairwise multiple comparisons were performed within time points using the Holm-Šidák method when group differences were detected.

For histological analyses, all data were analyzed by t-tests or analyses of variance (one way, two way, or repeated measures as appropriate) followed by post hoc analyses using Fisher's least significant difference (LSD).

I. Figure preparation

Digital light micrographs were obtained using an MDS 290 digital camera system (Eastman Kodak Company, Rochester, NY, USA). Brightness and contrast of these images were adjusted in Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

CHAPTER 4: RESULTS

A. Validation experiments

1. Verification of sciatic nerve crush axotomy

We validated our model of sciatic nerve crush axotomy by measuring electromyography (EMG) activity of two muscles that are innervated by branches of the sciatic nerve — tibialis anterior (TA) and lateral head of gastrocnemius (LG). The EMG amplitudes of both the TA and LG muscles (22.89 ± 1.76 mV and 18.21 ± 1.90 mV, respectively) were significantly reduced in the rats following axotomy (0.80 ± 0.13 mV and 0.98 ± 0.18 mV, respectively; $P < 0.001$; **Fig. 5**). Thus, we achieved successful crush axotomy of the sciatic nerve.

2. Serum testosterone propionate (TP) levels are supraphysiological by 6 hours post-axotomy

We have reported previously in hamster and rat that testosterone propionate (TP) capsule implants significantly elevate serum levels of testosterone (Kujawa et al., 1989; Kinderman & Jones, 1993; Brown et al., 2001; Tanzer & Jones, 2004; Hetzler et al., 2008). Serum samples were obtained and tested for the presence of systemic TP at 6 hours, 1, 2, 7, and 21 days post-axotomy to confirm TP diffused out of the Silastic capsules into the blood where it could exert its effects. Statistical analysis revealed an overall main effect between treatment groups ($F_{3,69} = 63.55$; $P < 0.001$), time ($F_{4,69} = 13.79$; $P <$

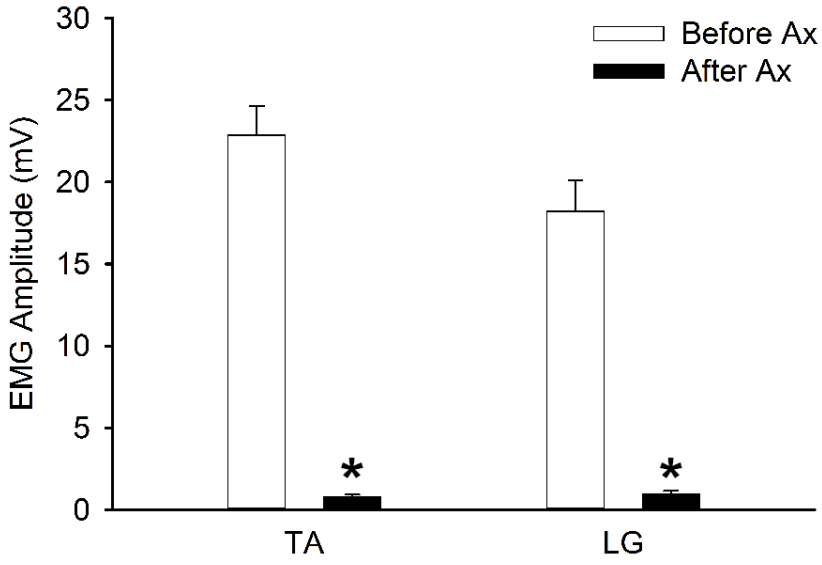

Fig. 5: Validation of sciatic nerve crush axotomy.

Data is represented as EMG amplitude (mV) ± SEM. A Student's t-test was utilized for statistical analysis to compare EMG amplitude before axotomy (Ax) to after Ax for both tibialis anterior (TA) and lateral head of gastrocnemius (LG) muscles. * represents a significant difference between before Ax relative to after Ax, at $P < 0.05$. For each experimental group, n = 23-24 animals/time-point.

0.001) as well as an interaction between treatment x time ($F_{12,69}$ = 6.60; *P* < 0.001; **Fig. 6**). At 6 hours post-axotomy, systemic administration of TP in both the TP only (Ax + TP; 31.6 ± 3.8 ng/mL) and the combined treatment (Ax + ES + TP; 21.8 ± 3.7 ng/mL) groups were significantly increased compared to injury alone (Ax only; 2.5 ± 1.8 ng/mL; *P*< 0.001) and electrical stimulation (ES) treatment (1.3 ± 0.8 ng/mL; *P* < 0.001). TP-treated animals (15.6 ± 3.1 ng/mL) and animals treated with ES plus TP (17.1 ± 2.8 ng/mL) had significantly elevated levels of testosterone at 1 day post-axotomy compared to both injury alone (2.1 ± 0.7 ng/mL; *P* < 0.001) and ES only animals (2.4 ± 0.8 ng/mL; *P* < 0.001). At 2 days post-axotomy, TP-treated animals (10.6 ± 1.4 ng/mL) and ES plus TP-treated animals (15.1 ± 1.7 ng/mL) had significantly elevated levels of testosterone relative to both injury alone (1.0 ± 0.3 ng/mL; *P* = 0.006 and *P* < 0.001, respectively) and ES-treated animals (1.3 ± 0.04 ng/mL; *P* = 0.007 and *P* < 0.001, respectively). By 7 days post-axotomy, administration of TP alone was significantly higher than ES-treated animals (11.2 ± 0.7 ng/mL and 2.5 ± 0.7 ng/mL, respectively; *P* = 0.026), but there was no significant increase relative to injury alone (3.7 ± 0.9 ng/mL) or the combined treatment (7.3 ± 2.7 ng/mL). At 21 days post-axotomy, TP-treated animals (7.9 ± 1.0 ng/mL) had a significantly enhanced serum level of TP compared to injury alone (2.3 ± 0.4 ng/mL; *P* = 0.05) and ES only (2.7 ± 0.6 ng/mL; *P* = 0.031); however, the combined treatment (8.0 ± 2.0 ng/mL) was not significantly different from any of the other groups.

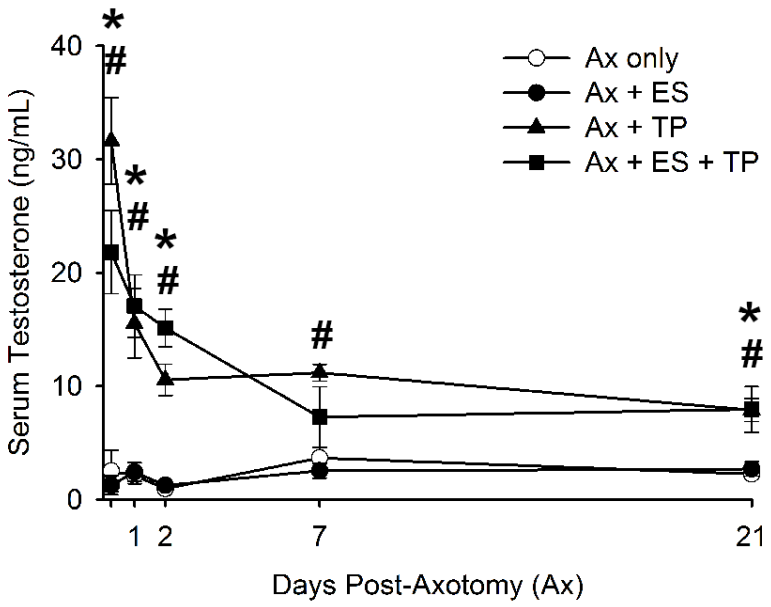

Fig. 6: Serum TP levels post-axotomy.

Shown is a time-course of the serum testosterone level at 6 hours, 1, 2, 7, and 21 days post-axotomy (Ax). Control animals, which were axotomized but left untreated, and animals receiving Ax + ES are compared to animals receiving Ax + TP and Ax + ES + TP. Data is represented as serum testosterone concentration (ng/mL) ± SEM. A two-way ANOVA (treatment x time) with a Student-Newman-Keuls multiple comparison post hoc test was utilized for statistical analysis among all experimental groups. * represents a significant difference between Ax + TP or Ax + ES + TP compared to Ax only, at $P < 0.05$. # represents a significant difference between Ax + TP or Ax + ES + TP relative to Ax + ES, at $P < 0.05$. For each experimental group, n = 6-7 animals/time-point.

3. Expression of *βII-tubulin* mRNA increases with axotomy

We have demonstrated previously that axotomy alone significantly increased the mRNA expression of *β_{II}-tubulin* in facial and sciatic motoneurons (Jones & Oblinger, 1994; Brown et al., 2001); however, this data was obtained utilizing a sensitive technique, *in situ* hybridization, and localized *β_{II}-tubulin* mRNA to the cell bodies of motoneurons. In the current study, mRNA was extracted from the injured and uninjured ventral horns of the lumbar spinal cord which contains several uninjured cell types, in addition to the cell bodies of the injured motoneurons. We examined *β_{II}-tubulin* mRNA expression in two different sciatic nerve crush injury models — distal and proximal — to confirm the mRNA samples from the injured cells were not diluted by mRNA from uninjured cells. We found that there was an upregulation in *β_{II}-tubulin* mRNA at 7 days post-axotomy after both distal and proximal sciatic nerve crush injuries (**Fig. 7**). In addition, there was a statistically significant increase in the expression of *β_{II}-tubulin* mRNA with the proximal sciatic nerve crush, compared to the distal sciatic nerve crush (50.4 ± 15.0% and 13.2 ± 12.1%, respectively; $P = 0.05$; **Fig. 7**). Thus, we conclude that our method of sectioning the spinal cord allowed us to detect differences in gene expression.

4. Contralateral effect of regeneration-associated gene expression

Previously, we have reported changes in mRNA as a percent ratio of injured (axotomy) to uninjured (control) facial nuclei (Haulcomb et al., 2014). Unlike the right and left facial nuclei, there is significant cross-talk among right

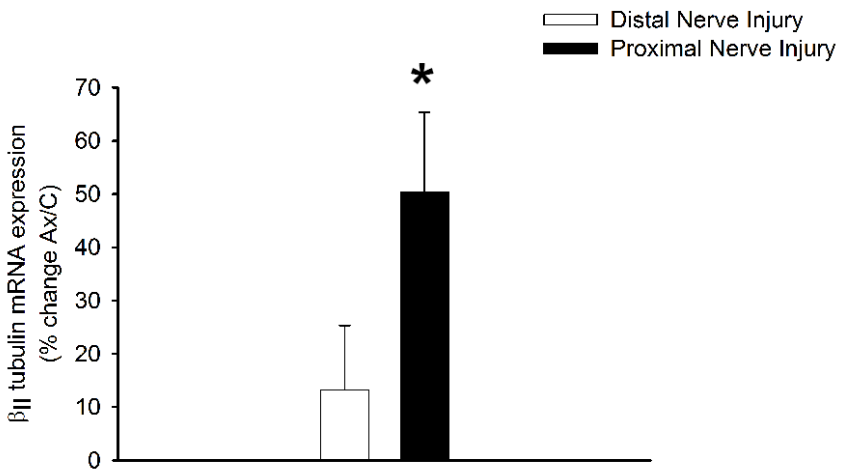

Fig. 7: Distal vs. proximal injury *β_{II}-tubulin* mRNA expression.

Shown is the average percent of mRNA expression of *β_{II}–tubulin* in the axotomized ventral horn of the spinal cord (Ax) relative to the non-axotomized control (C) ventral horn of the spinal cord at 7 days post-axotomy. Two injury paradigms were examined: distal and proximal nerve injuries. Data is represented as average percent of *β_{II}–tubulin* mRNA expression ± SEM. A Student's t-test was utilized for statistical analysis to compare *β_{II}–tubulin* mRNA following distal and proximal nerve injuries. * represents a significant difference between distal and proximal nerve injuries, at $P = 0.05$. For each experimental group, n = 5-6 animals/injury.

and left sides of the spinal cord (Kiernan & Rajakumar, 2013). Thus, we examined whether axotomy and/or treatment influenced levels of gene expression in the contralateral ventral horn of the spinal cord and whether the contralateral side could be utilized as an internal control for analysis of regeneration-associated gene expression. This dissertation analyzed the relative mRNA expression of several genes involved in the regeneration process over the time-course of 6 hours, 1, 2, 7, and 21 days post-axotomy (**Table 2**).

a. Cytoskeleton genes: *α_I–tubulin and β_{II}–tubulin*

Statistical analysis revealed an overall main effect for the relative mRNA expression of *α_I–tubulin* between treatment groups ($F_{3,101}$ = 8.05; P < 0.001), time($F_{4,101}$ = 3.78; P = 0.007), as well as an interaction between treatment x time ($F_{12,101}$ = 4.42; P < 0.001; **Fig. 8**). Injury alone significantly decreased *α_I–tubulin* expression at 1 day post-axotomy (13.594 ± 0.508) compared to 6 hours, 2, 7, and 21 days post-axotomy (19.755 ± 1.322, 18.339 ± 1.279, 17.903 ± 1.615, and 17.912 ± 1.033, respectively; P < 0.05). Administration of ES did not alter *α_I–tubulin* mRNA levels at 6 hours, 1, 2, and 7 days post-axotomy (18.192 ± 1.578, 17.068 ± 1.267, 16.014 ± 1.835, and 19.447 ± 1.210, respectively), but *α_I–tubulin* mRNA was significantly downregulated at the later time-point of 21 days post-axotomy with ES treatment (13.270 ± 1.137) relative to both 6 hours and 7 days post-axotomy (P < 0.05; **Fig. 8A**). In contrast to treatment with ES, systemic treatment of TP alone did not alter *α_I–tubulin* mRNA across the time-course of 6 hours, 1, 2, 7, and 21 days post-axotomy (17.843 ± 1.013, 16.983 ± 1.587,

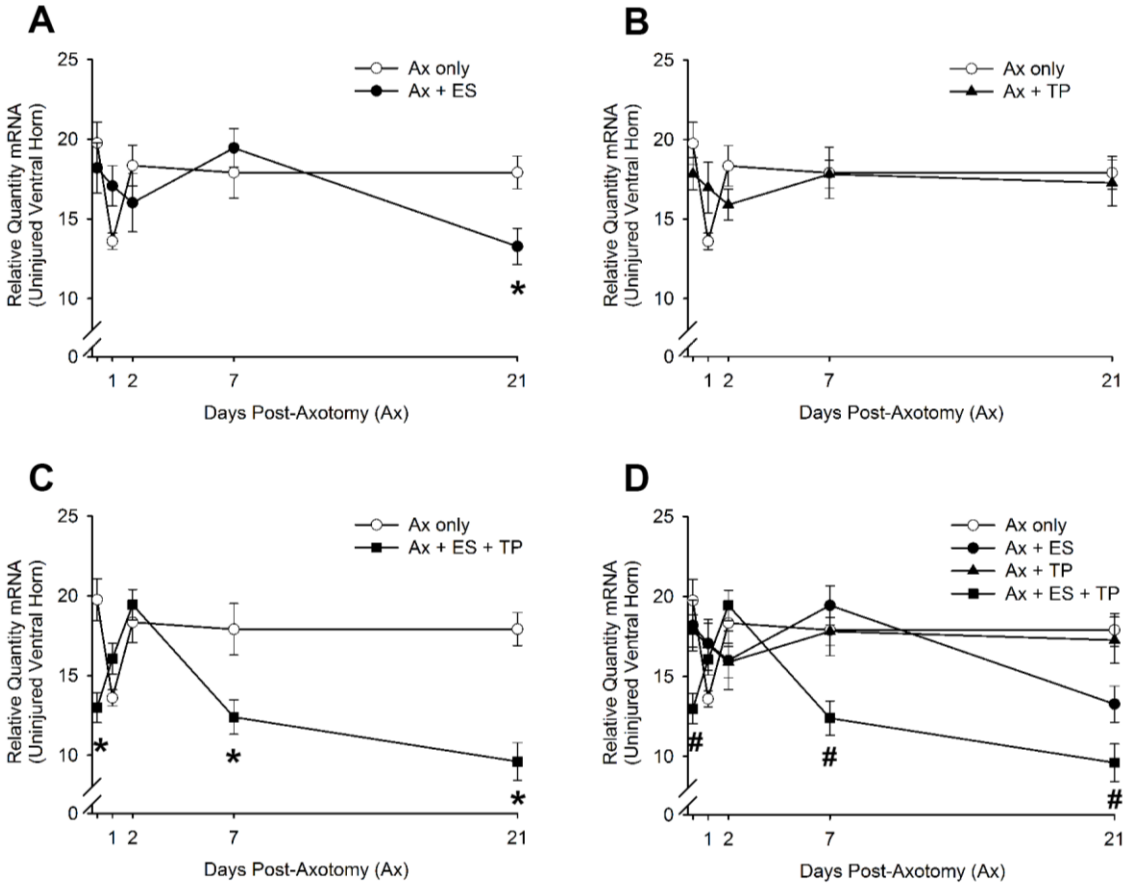

Fig. 8: Contralateral *α$_I$–tubulin* mRNA expression.

Shown is a time-course of the relative mRNA expression levels of *α$_I$–tubulin* in the uninjured ventral horn at 6 hours, 1, 2, 7, and 21 days post-axotomy (Ax). Control animals, which were axotomized but left untreated, are compared to axotomized animals receiving only ES (A), only TP (B), or the combination of both ES and TP (C). (D) Represents the comparison between ES, TP, and the combination of ES and TP. Data is represented as relative *α$_I$–tubulin* mRNA expression ± SEM. A two-way ANOVA (treatment x time) with a Student-Newman-Keuls multiple comparison post hoc test was utilized for statistical analysis among all experimental groups. * represents a significant difference between ES, TP, or ES and TP compared to axotomy alone, at $P < 0.05$. # represents a significant difference between ES, TP, or ES and TP, at $P < 0.05$. For each experimental group, n = 6-7 animals/time-point.

15.903 ± 0.972, 17.818 ± 0.878, and 17.274 ± 1.443, respectively; **Fig. 8B**). The combined treatment maintained levels of *α_I–tubulin* mRNA from 6 hours to 1 day post-axotomy (12.980 ± 0.937 and 16.062 ± 0.971, respectively) before peaking at a significant level at 2 days post-axotomy (19.444 ± 0.949; $P \leq 0.05$; **Fig. 8C**). The combination of ES and TP significantly downregulated the relative mRNA expression of *α_I–tubulin* by 7 and 21 days post-axotomy (12.387 ± 1.077 and 9.607 ± 1.178, respectively; $P < 0.001$) relative to 2 days post-axotomy. Only at the latest time-point of 21 days post-axotomy did ES of the sciatic nerve elicit a significant decrease in the expression of *α_I–tubulin* relative to axotomy alone ($P < 0.05$; **Fig. 8A**). In contrast, treatment with TP did not alter *α_I–tubulin* mRNA relative to axotomy alone at any time-point (**Fig. 8B**). The combined treatment group significantly downregulated the expression of *α_I–tubulin* at 6 hours, 7, and 21 days post-axotomy compared to all other groups ($P < 0.05$; **Fig. 8C** and **Fig. 8D**, respectively).

We found an overall main effect in the relative mRNA expression of *β_{II}–tubulin* between treatment groups ($F_{3,100} = 4.00$; $P = 0.010$), time ($F_{4,101} = 13.95$; $P < 0.001$), as well as an interaction between treatment x time ($F_{12,100} = 3.48$; $P < 0.001$; **Fig. 9**). Axotomy alone significantly enhanced the relative expression of *β_{II}–tubulin* mRNA at 7 days post-axotomy (0.949 ± 0.117) relative to 6 hours and 1 day post-axotomy (0.626 ± 0.021 and 0.596 ± 0.087, respectively; $P < 0.05$), but not to 2 and 21 days post-axotomy (0.720 ± 0.031 and 1.013 ± 0.132, respectively; **Fig. 9**). Similar to injury alone, ES of the sciatic nerve significantly peaked *β_{II}–tubulin* mRNA expression at 7 days post-axotomy (1.155 ± 0.129)

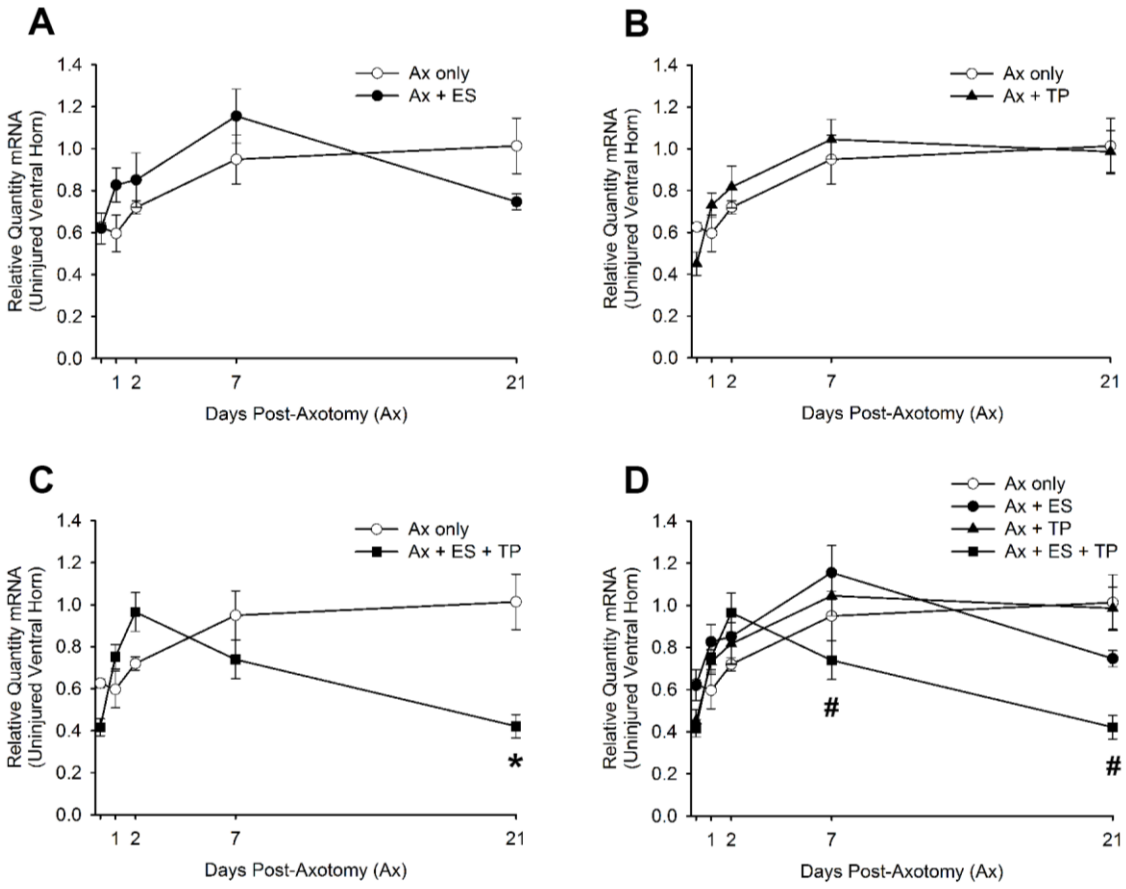

Fig. 9: Contralateral *β_{II}–tubulin* mRNA expression.

Shown is a time-course of the relative mRNA expression levels of *β_{II}–tubulin* in the uninjured ventral horn at 6 hours, 1, 2, 7, and 21 days post-axotomy (Ax). Control animals, which were axotomized but left untreated, are compared to axotomized animals receiving only ES (A), only TP (B), or the combination of both ES and TP (C). (D) Represents the comparison between ES, TP, and the combination of ES and TP. Data is represented as relative *β_{II}–tubulin* mRNA expression ± SEM. A two-way ANOVA (treatment x time) with a Student-Newman-Keuls multiple comparison post hoc test was utilized for statistical analysis among all experimental groups. * represents a significant difference between ES, TP, or ES and TP compared to axotomy alone, at $P < 0.05$. # represents a significant difference between ES, TP, or ES and TP, at $P < 0.05$. For each experimental group, n = 5-7 animals/time-point.

compared to 6 hours, 1, 2, and 21 days post-axotomy (0.620 ± 0.073, 0.827 ± 0.081, 0.851 ± 0.129, and 0.747 ± 0.039, respectively; $P < 0.05$; **Fig. 9A**). TP-treated animals demonstrated a significant, steady increase in relative mRNA expression of *β_{II}–tubulin* at the early time-points of 6 hours, 1, and 2 days post-axotomy (0.450 ± 0.056, 0.731 ± 0.058, and 0.817 ± 0.100, respectively; $P < 0.05$) until it peaked at 7 days post-axotomy (1.045 ± 0.095) relative to 6 hours and 1 day post-axotomy ($P < 0.05$; **Fig. 9B**). At the latest time-point of 21 days post-axotomy (0.986 ± 0.100), *β_{II}–tubulin* remained significantly upregulated with TP treatment compared to 6 hours post-axotomy ($P < 0.001$). Administration of ES and TP demonstrated a significant increase in *β_{II}–tubulin* mRNA expression from 6 hours to 1 day post-axotomy (0.416 ± 0.041 and 0.752 ± 0.058, respectively; $P < 0.05$; **Fig. 9C**). *β_{II}–tubulin* levels remained significantly elevated in animals treated with both ES and TP at 2 and 7 days post-axotomy (0.965 ± 0.093 and 0.739 ± 0.091, respectively; $P < 0.05$) before significantly decreasing at 21 days post-axotomy (0.421 ± 0.056; $P < 0.05$). No differences in *β_{II}–tubulin* mRNA expression, with respect to injury alone, were observed in ES- or TP-treated animals (**Fig. 9A** and **Fig. 9B**, respectively). The combined treatment of ES plus TP demonstrated a significantly lower *β_{II}–tubulin* expression at 7 days post-axotomy compared to either treatment alone ($P < 0.05$; **Fig. 9D**). At the latest time-point of 21 days post-axotomy, ES and TP demonstrated a significant decrease in *β_{II}–tubulin* expression compared to all other groups ($P < 0.05$).

b. Axonal growth genes: *gap-43 and crmp2*

When investigating the relative mRNA expression of *43-kiloDalton growth-associated protein* (*gap-43*) in the contralateral ventral horn we observed a main effect of post-operative time ($F_{4,99}$ = 4.14; *P* = 0.004) and an interaction between treatment x time ($F_{12,99}$ = 2.53; *P* = 0.006; **Fig. 10**); although, there was no overall effect between treatment groups ($F_{3,99}$ = 0.91; *P* = 0.44; **Fig. 10**). Axotomy alone did not alter the relative level of *gap-43* expression throughout the time course of 6 hours, 1, 2, 7, or 21 days post-axotomy (0.313 ± 0.019, 0.363 ± 0.030, 0.377 ± 0.030, 0.304 ± 0.026, and 0.365 ± 0.045, respectively; **Fig. 10**). In animals receiving ES treatment, the value of *gap-43* mRNA expression remained steady at 6 hours, 1, 2, and 7 days post-axotomy (0.321 ± 0.045, 0.323 ± 0.039, 0.352 ± 0.042, and 0.388 ± 0.050, respectively), but significantly declined at the latest time-point of 21 days post-axotomy (0.237 ± 0.017; *P* < 0.05; **Fig. 10A**). Similar to axotomy alone, administration of TP had no effect on the relative mRNA expression of *gap-43* at 6 hours, 1, 2, 7, or 21 days post-axotomy (0.388 ± 0.040, 0.290 ± 0.028, 0.357 ± 0.022, 0.386 ± 0.036, and 0.321 ± 0.023, respectively; **Fig. 10B**). Unlike animals treated with TP, treatment with the combination of ES plus TP significantly upregulated the mRNA expression of *gap-43* at 1 and 2 days post-axotomy compared to 6 hours-axotomy (0.364 ± 0.054, 0.462 ± 0.075, and 0.217 ± 0.024, respectively; *P* < 0.05; **Fig. 10C**). The combination treatment demonstrated a significant decrease in *gap-43* mRNA at both 7 and 21 days post-axotomy (0.315 ± 0.041 and 0.213 ± 0.018, respectively) relative to 2 days post-axotomy (*P* < 0.05). In animals receiving ES treatment, the value of *gap-43*

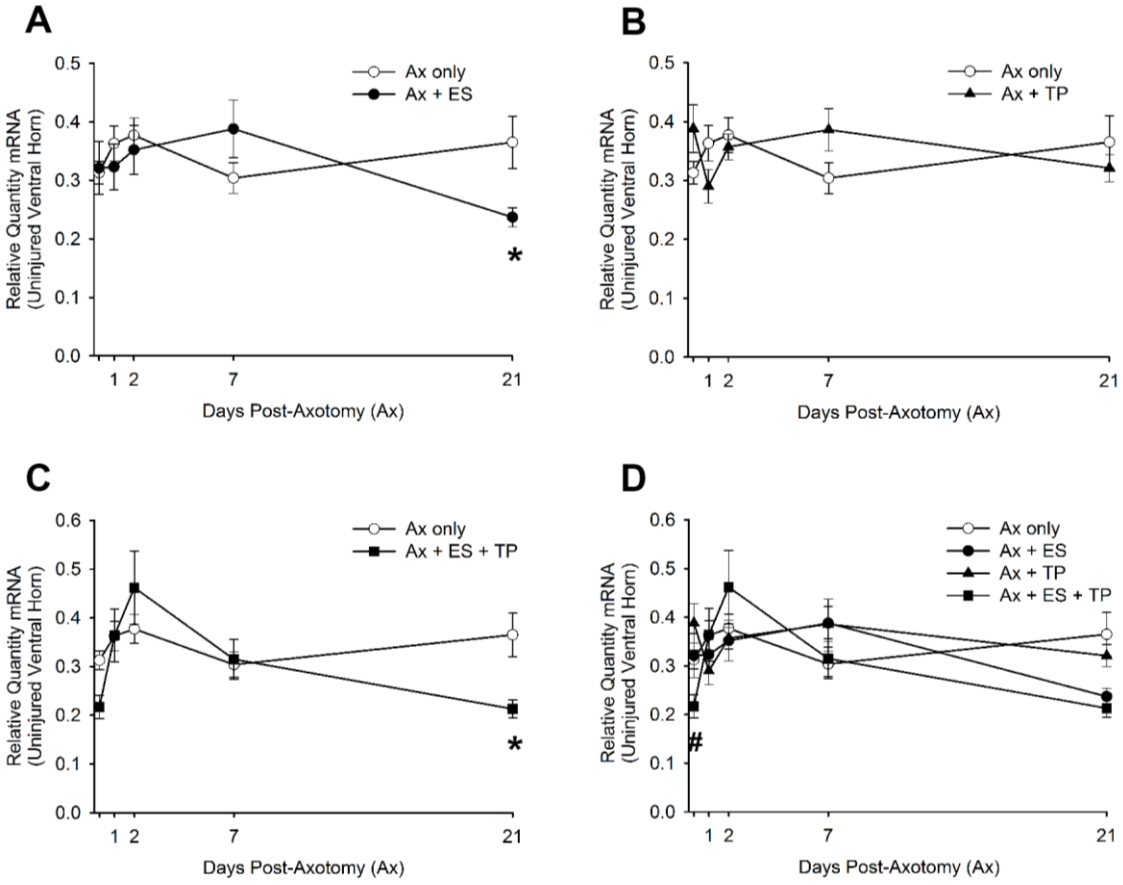

Fig. 10: Contralateral *gap-43* mRNA expression.

Shown is a time-course of the relative mRNA expression levels of *43-kiloDalton growth-associated protein* (*gap-43*) in the uninjured ventral horn at 6 hours, 1, 2, 7, and 21 days post-axotomy (Ax). Control animals, which were axotomized but left untreated, are compared to axotomized animals receiving only ES (A), only TP (B), or the combination of both ES and TP (C). (D) Represents the comparison between ES, TP, and the combination of ES and TP. Data is represented as relative *gap-43* mRNA expression ± SEM. A two-way ANOVA (treatment x time) with a Student-Newman-Keuls multiple comparison post hoc test was utilized for statistical analysis among all experimental groups.
* represents a significant difference between ES, TP, or ES and TP compared to axotomy alone, at $P < 0.05$. # represents a significant difference between ES, TP, or ES and TP, at $P < 0.05$. For each experimental group, n = 5-6 animals/time-point.

expression was significantly lower at 21 days post-axotomy compared to injury alone (P = 0.05; **Fig. 10A**). Unlike ES-treated animals, TP-treated animals were similar to axotomized, untreated animals at all time-points (**Fig. 10B**). In contrast to TP treatment, the combination treatment of ES and TP was significantly downregulated at 6 hours post-axotomy compared to TP-treated animals and at 21 days post-axotomy relative to axotomy alone ($P \leq 0.05$; **Fig. 10D** and **Fig. 10C**, respectively).

Overall, we observed a main effect between treatment groups ($F_{3,100}$ = 6.68; $P < 0.001$), time ($F_{4,100}$ = 5.40; $P < 0.001$), as well as an interaction between treatment x time ($F_{12,100}$ = 4.47; $P < 0.001$; **Fig. 11**) when examining the relative mRNA expression of *collapsin response mediator protein 2* (*crmp2*). In axotomized, untreated animals there was a significant decline in *crmp2* mRNA expression at 1 day post-axotomy compared to 6 hours post-axotomy (1.134 ± 0.050 and 1.556 ± 0.077, respectively; $P < 0.05$; **Fig. 11**). By 2 days post-axotomy (1.553 ± 0.136), *crmp2* levels returned to levels similar to that of 6 hours post-axotomy ($P < 0.05$) where they were maintained throughout the rest of the time-course of 7 and 21 days post-axotomy (1.425 ± 0.107 and 1.520 ± 0.060, respectively). Treatment with ES did not alter *crmp2* expression at the earlier time-points of 6 hours, 1, 2, and 7 days post-axotomy (1.518 ± 0.140, 1.355 ± 0.093, 1.310 ± 0.147, and 1.516 ± 0.107, respectively), but did significantly downregulate *crmp2* expression at 21 days post-axotomy (1.061 ± 0.114) relative to 6 hours and 7 days post-axotomy ($P < 0.05$; **Fig. 11A**). In contrast to treatment with ES, administration of TP did not alter the relative mRNA expression of

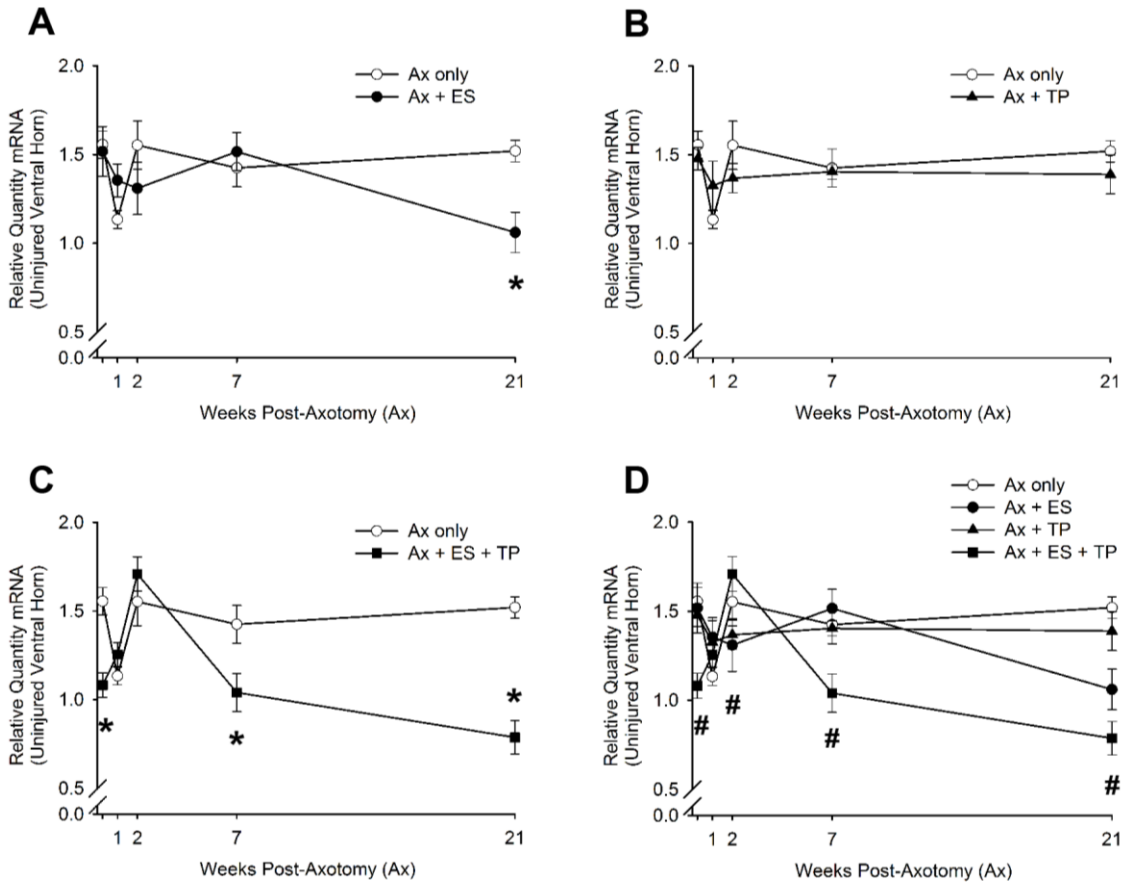

Fig. 11: Contralateral *crmp2* mRNA expression.

Shown is a time-course of the relative mRNA expression levels of *collapsin response mediator protein 2* (*crmp2*) in the uninjured ventral horn at 6 hours, 1, 2, 7, and 21 days post-axotomy (Ax). Control animals, which were axotomized but left untreated, are compared to axotomized animals receiving only ES (A), only TP (B), or the combination of both ES and TP (C). (D) Represents the comparison between ES, TP, and the combination of ES and TP. Data is represented as relative *crmp2* mRNA expression ± SEM. A two-way ANOVA (treatment x time) with a Student-Newman-Keuls multiple comparison post hoc test was utilized for statistical analysis among all experimental groups.
* represents a significant difference between ES, TP, or ES and TP compared to axotomy alone, at $P < 0.05$. # represents a significant difference between ES, TP, or ES and TP, at $P < 0.05$. For each experimental group, $n = 6$ animals/time-point.

crmp2 throughout the entire time-course of 6 hours, 1, 2, 7, and 21 days (1.478 ± 0.065, 1.326 ± 0.138, 1.368 ± 0.083, 1.404 ± 0.042, and 1.389 ± 0.109, respectively; **Fig. 11B**). The combination of ES and TP significantly elevated *crmp2* levels at 2 days post-axotomy compared to 6 hours and 1, 7, and 21 days post-axotomy (1.709 ± 0.097, 1.082 ± 0.070, 1.255 ± 0.066, 1.040 ± 0.108, and 0.787 ± 0.095, respectively; $P < 0.05$; **Fig. 11C**). *Crmp2* expression was similar between systemic TP treatment compared to axotomy alone throughout the entire time-course (**Fig. 11B**); however, animals treated with ES demonstrated a significant decrease in *crmp2* mRNA at the latest time-point of 21 days post-axotomy compared to injury alone and TP-treated animals ($P < 0.05$; **Fig. 11A** and **Fig. 11D**, respectively). Animals treated with the combination of ES and TP demonstrated a significant downregulation in the mRNA expression of *crmp2* at 6 hours and 7 days post-axotomy compared to all groups ($P < 0.05$) and at 21 days post-axotomy relative to axotomy alone and TP treatment ($P < 0.001$; **Fig. 11D**). In contrast, the combination treatment significantly elevated *crmp2* mRNA expression at 2 days post-axotomy compared to either treatment alone ($P < 0.05$; **Fig. 11D**).

c. Growth factor and neurotrophic genes: *bdnf, gdnf, and pacap*

We found an overall main effect in the relative mRNA expression of *brain derived neurotrophic factor* (*bdnf*) between treatment groups ($F_{3,98} = 10.09$; $P < 0.001$), time ($F_{4,98} = 4.29$; $P = 0.003$), as well as an interaction between treatment x time ($F_{12,98} = 3.18$; $P < 0.001$; **Fig. 12**). The time-course of *bdnf* expression at 6

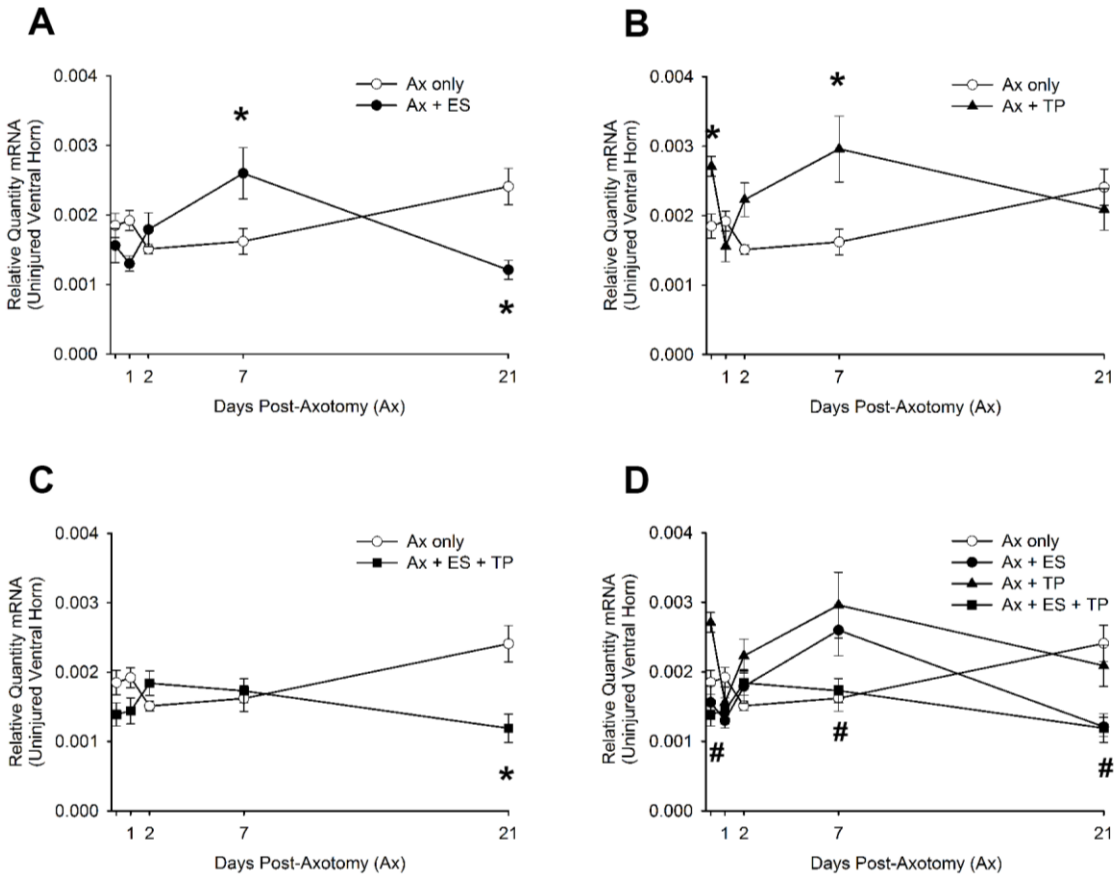

Fig. 12: Contralateral *bdnf* mRNA expression.

Shown is a time-course of the relative mRNA expression levels of *brain derived neurotrophic factor* (*bdnf*) in the uninjured ventral horn at 6 hours, 1, 2, 7, and 21 days post-axotomy (Ax). Control animals, which were axotomized but left untreated, are compared to axotomized animals receiving only ES (A), only TP (B), or the combination of both ES and TP (C). (D) Represents the comparison between ES, TP, and the combination of ES and TP. Data is represented as relative *bdnf* mRNA expression ± SEM. A two-way ANOVA (treatment x time) with a Student-Newman-Keuls multiple comparison post hoc test was utilized for statistical analysis among all experimental groups. * represents a significant difference between ES, TP, or ES and TP compared to axotomy alone, at $P < 0.05$. # represents a significant difference between ES, TP, or ES and TP, at $P < 0.05$. For each experimental group, n = 5-7 animals/time-point.

hours, 1, 2, 7, and 21 days post-axotomy was not altered by axotomy alone (0.00185 ± 0.00017, 0.00192 ± 0.00015, 0.00151 ± 0.000007, 0.00162 ± 0.00019, and 0.00241 ± 0.00026, respectively; **Fig. 12**). In contrast to axotomy alone, ES of the sciatic nerve significantly upregulated *bdnf* expression at 7 days post-axotomy compared to 6 hours, 1, 2, and 21 days post-axotomy (0.00260 ± 0.00037, 0.00156 ± 0.00025, 0.00130 ± 0.00011, 0.00179 ± 0.00024, and 0.00121 ± 0.00014, respectively; **Fig. 12A**). We observed a significant downregulation in the relative mRNA expression of *bdnf* in TP-treated animals at 1 day post-axotomy compared to 6 hours post-axotomy (0.00156 ± 0.00022 and 0.00271 ± 0.00014, respectively; $P < 0.05$; **Fig. 12B**). With TP treatment, *bdnf* levels were maintained at 2 days post-axotomy (0.00223 ± 0.00024) but peaked to a significant level at 7 days post-axotomy (0.00296 ± 0.00047) compared to 1 day post-axotomy ($P < 0.001$) and the latest time-point of 21 days post-axotomy (0.00209 ± 0.00030; $P < 0.05$). Similar to axotomized animals, *bdnf* expression in animals treated with the combination of ES and TP remained unaltered throughout the entire time-course of 6 hours, 1, 2, 7, and 21 days post-axotomy (0.00139 ± 0.00017, 0.00144 ± 0.00019, 0.00184 ± 0.00018, 0.00173 ± 0.00018, and 0.00119 ± 0.00021, respectively; **Fig. 12C**). In animals treated with ES there was a significant upregulation in *bdnf* expression at 7 days post-axotomy relative to injury alone ($P < 0.05$; **Fig. 12A**). In contrast, a significant downregulation in *bdnf* expression was observed at 21 days post-axotomy compared to injury alone and TP-treated animals ($P < 0.05$; **Fig. 12D**). Administration of TP significantly increased *bdnf* levels at 6 hours post-axotomy compared to all other groups ($P <$

0.05) and at 7 days post-axotomy relative to injury alone and the combination treatment ($P < 0.05$; **Fig. 12B** and **Fig. 12D**). Animals treated with the combination of ES plus TP demonstrated a significant downregulation in *bdnf* mRNA expression at 21 days post-axotomy compared to injury alone and TP-treated animals ($P < 0.05$; **Fig. 12C** and **Fig. 12D**, respectively).

The expression of *glial cell derived neurotrophic factor* (*gdnf*) mRNA following axotomy led to a main effect of treatment group ($F_{3,96} = 4.77$; $P = 0.004$); although, there was no overall effect of post-operative time ($F_{4,96} = 1.96$; $P = 0.107$) or interaction between group x post-operative time ($F_{12,96} = 0.760$; $P = 0.690$; **Fig. 13**). There was a significant difference in *gdnf* expression in the combined treatment group compared to injury alone and either treatment alone ($P < 0.05$).

Evaluation of the relative mRNA expression of *pituitary adenylate cyclase-activating peptide* (*pacap*) after axotomy revealed a main effect of post-operative time ($F_{4,94} = 2.68$; $P = 0.036$) and treatment x time ($F_{12,94} = 2.074$; $P = 0.026$) but not treatment ($F_{3,94} = 2.361$; $P = 0.076$; **Fig. 14**). The time-course of *pacap* expression at 6 hours, 1, 2, 7, and 21 days post-axotomy was not altered with axotomy (0.0225 ± 0.0027, 0.0424 ± 0.0087, 0.0281 ± 0.0074, 0.0268 ± 0.0062, and 0.0251 ± 0.0031, respectively; **Fig. 14**), treatment with ES (0.0307 ± 0.0079, 0.0227 ± 0.0009, 00.0266 ± 0.0006, 0.0305 ± 0.0046, and 0.0136 ± 0.0013, respectively; **Fig. 14A**), or the combined treatment (0.0164 ± 0.0022, 0.0212 ± 0.0012, 0.0239 ± 0.0030, 0.0342 ± 0.0052, and 0.0156 ± 0.0014, respectively; **Fig. 14C**). In contrast, administration of TP significantly decreased *pacap*

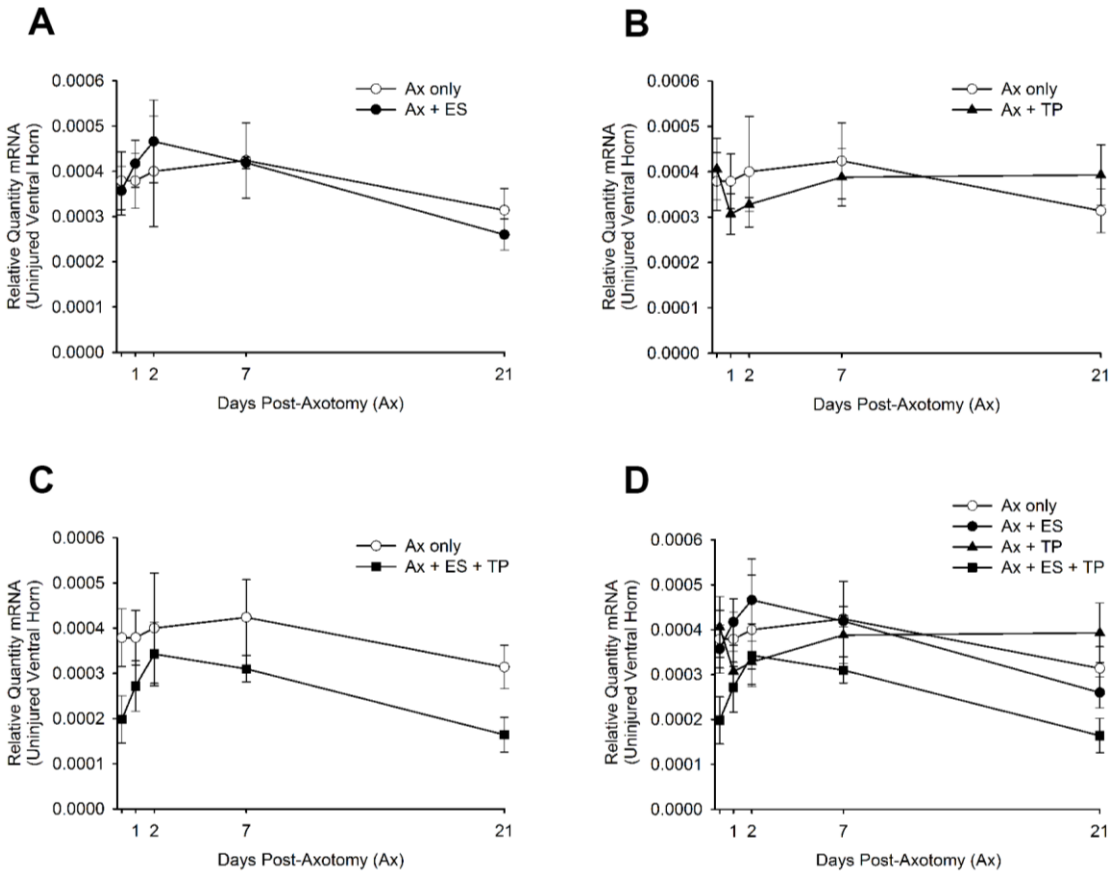

Fig. 13: Contralateral *gdnf* mRNA expression.

Shown is a time-course of the relative mRNA expression levels of *glial cell derived neurotrophic factor* (*gdnf*) in the uninjured ventral horn at 6 hours, 1, 2, 7, and 21 days post-axotomy (Ax). Control animals, which were axotomized but left untreated, are compared to axotomized animals receiving only ES (A), only TP (B), or the combination of both ES and TP (C). (D) Represents the comparison between ES, TP, and the combination of ES and TP. Data is represented as relative *gdnf* mRNA expression ± SEM. A two-way ANOVA (treatment x time) with a Student-Newman-Keuls multiple comparison post hoc test was utilized for statistical analysis among all experimental groups. * represents a significant difference between ES, TP, or ES and TP compared to axotomy alone, at $P < 0.05$. # represents a significant difference between ES, TP, or ES and TP, at $P < 0.05$. For each experimental group, n = 5-6 animals/time-point.

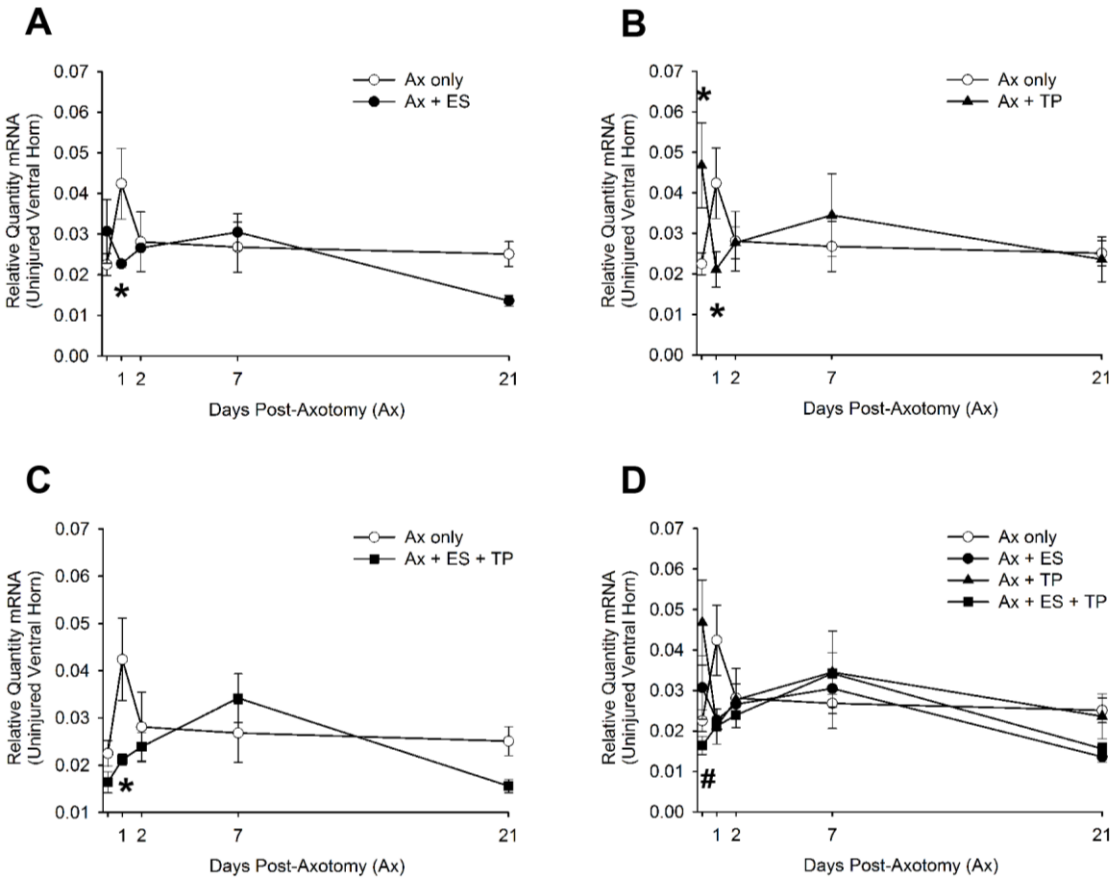

Fig. 14: Contralateral *pacap* mRNA expression.

Shown is a time-course of the relative mRNA expression levels of *pituitary adenylate cyclase-activating peptide* (*pacap*) in the uninjured ventral horn at 6 hours, 1, 2, 7, and 21 days post-axotomy (Ax). Control animals, which were axotomized but left untreated, are compared to axotomized animals receiving only ES (A), only TP (B), or the combination of both ES and TP (C). (D) Represents the comparison between ES, TP, and the combination of ES and TP. Data is represented as relative *pacap* mRNA expression ± SEM. A two-way ANOVA (treatment x time) with a Student-Newman-Keuls multiple comparison post hoc test was utilized for statistical analysis among all experimental groups. * represents a significant difference between ES, TP, or ES and TP compared to axotomy alone, at $P < 0.05$. # represents a significant difference between ES, TP, or ES and TP, at $P < 0.05$. For each experimental group, n = 5-6 animals/time-point.

expression at 1, 2, and 21 days post-axotomy relative to 6 hours post-axotomy (0.0211 ± 0.0043, 0.0277 ± 0.0039, 0.0236 ± 0.0055, and 0.0468 ± 0.0105, respectively; $P < 0.05$) but *pacap* expression at 6 hours post-axotomy was similar to 7 days post-axotomy (0.0345 ± 0.0102; **Fig. 14B**). ES treatment alone significantly downregulated *pacap* mRNA expression compared to injury alone ($P < 0.05$; **Fig. 14A**). Relative mRNA expression of *pacap* was significantly higher at 6 hours post-axotomy in TP-treated animals compared to all other groups ($P < 0.05$; **Fig. 14B**). At 2 days post-axotomy, TP treatment significantly downregulated *pacap* mRNA compared to axotomy alone ($P < 0.05$). Similar to TP treatment, the combined treatment also significantly downregulated *pacap* mRNA at 1 day post-axotomy compared to axotomy alone ($P < 0.05$; **Fig. 14C**).

d. Plasticity gene: *neuritin*

The extended time course did not reveal any differences between treatment groups after axotomy for *neuritin* ($F_{3,100} = 1.18$; $P = 0.320$) or an interaction between treatment x post-operative time ($F_{12,100} = 0.99$; $P = 0.466$; **Fig. 15**). However, analysis did reveal a main effect of post-operative time ($F_{4,100} = 3.06$; $P = 0.020$; **Fig. 15**).

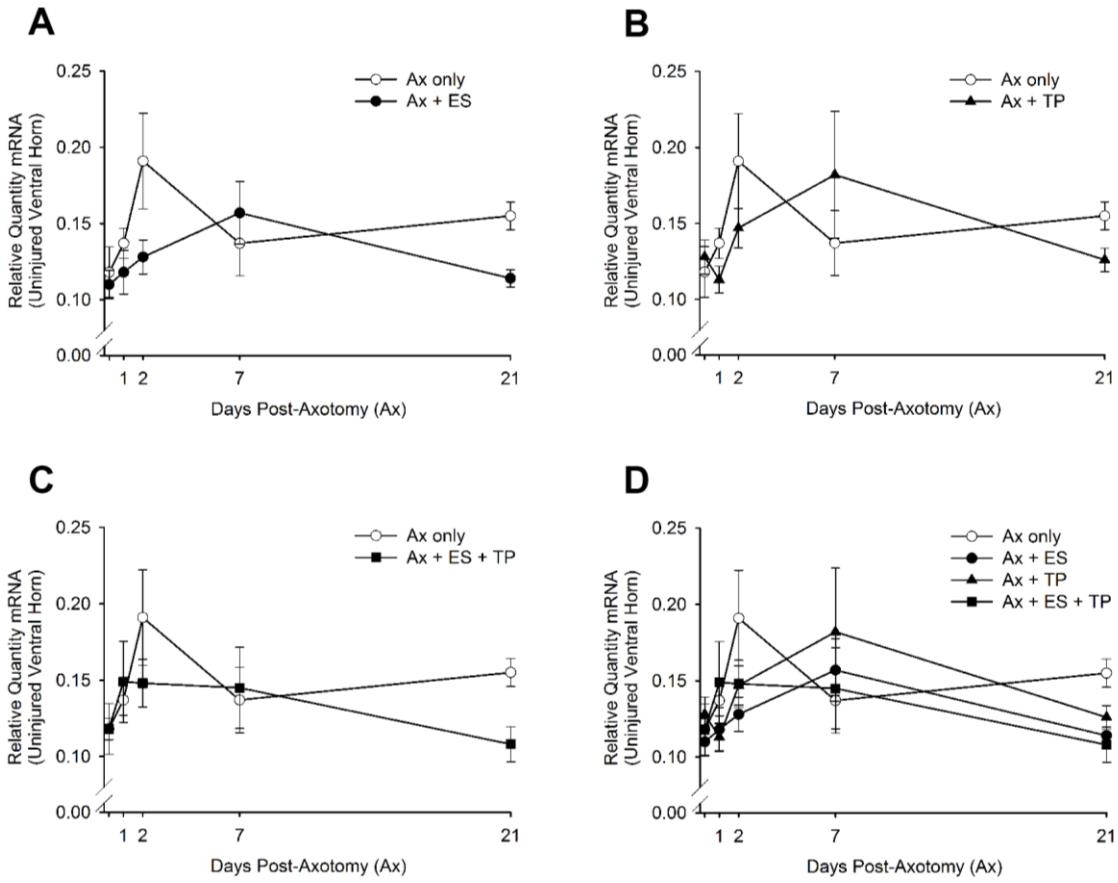

Fig. 15: Contralateral *neuritin* mRNA expression.

Shown is a time-course of the relative mRNA expression levels of *neuritin* in the uninjured ventral horn at 6 hours, 1, 2, 7, and 21 days post-axotomy (Ax). Control animals, which were axotomized but left untreated, are compared to axotomized animals receiving only ES (A), only TP (B), or the combination of both ES and TP (C). (D) Represents the comparison between ES, TP, and the combination of ES and TP. Data is represented as relative *neuritin* mRNA expression ± SEM. A two-way ANOVA (treatment x time) with a Student-Newman-Keuls multiple comparison post hoc test was utilized for statistical analysis among all experimental groups. * represents a significant difference between ES, TP, or ES and TP compared to axotomy alone, at $P < 0.05$. # represents a significant difference between ES, TP, or ES and TP, at $P < 0.05$. For each experimental group, n = 4-7 animals/time-point.

e. Summary

In this study, we characterized the molecular effects of ES and TP on the expression of 8 genes associated with axonal regeneration on the contralateral side of the lumbar ventral horn following sciatic nerve crush axotomy. We found the expression of 3 out of 8 regeneration-associated genes was altered following axotomy. Moreover, our results indicate that ES and/or TP treatment modified the expression of 6 out of 8 genes following axotomy. Our results demonstrate that ES and TP alter gene expression in very distinct patterns when administered alone, and have a combined suppressive effect when administered together (**Table 5**). We have categorized the genes into three groups: (1) the individual treatments altered expression relative to axotomy alone (i.e. differential), (2) the combined treatment altered expression relative to both of the individual treatments (i.e. additive), (3) treatment did not alter gene expression (i.e. no effect; **Table 6**). In summary, ES and TP differentially altered the expression of *gap-43*, *bdnf*, and *pacap*, whereas the combination of both ES and TP exhibited an additive downregulation in the expression of α_I*-tubulin*, β_{II}*-tubulin*, and *crmp2*. In addition, neither treatment altered the expression of *gdnf* and *neuritin*.

Gene	Early (6 hours-2 days)	Middle (7 days)	Late (21 days)
ES			
gap-43	-	-	↓
crmp2	-	-	↓
bdnf	-	↑	↓
pacap	↓	-	-
TP			
bdnf	↑	↑	-
pacap	↑ , ↓	-	-
ES + TP			
α_I–tubulin	↓	↓	↓
β_{II}–tubulin	-	-	↓
gap-43	-	-	↓
crmp2	↓	↓	↓
bdnf	-	-	↓
pacap	↓	-	-

Table 5: Summary of contralateral mRNA expression.

Change in Expression	Sciatic Nerve Crush Injury
Differential	*gap-43*, *bdnf*, and *pacap*
Additive	α_I-*tubulin*, β_{II}-*tubulin*, and *crmp2*
No change	g*dnf* and *neuritin*

Table 6: Differential and additive effects of ES and TP on contralateral gene expression.

B. Effects of ES and TP on regeneration-associated gene expression

Since we found a significant change in mRNA expression with either treatment and/or time in 6 out of 8 genes, we did not use the contralateral side as an internal control. Therefore, all data is expressed as the relative mRNA expression from the ipsilateral ventral horn of the lumbar spinal cord. Experiments were carried out over the time-course of 6 hours, 1, 2, 7, and 21 days post-axotomy.

a. Cytoskeleton genes: *α_I–tubulin and β_{II}–tubulin*

Microtubules are comprised of two members of the tubulin family, *α_I–tubulin* and *β_{II}–tubulin*. In order for the axon to elongate, the cytoskeletal architecture must remodel. These subunits provide cytoskeleton support and have been demonstrated to increase following injury (Jones & Oblinger, 1994; Brown et al., 2001; Sharma et al., 2010a). In addition, *α_I–tubulin* and *β_{II}–tubulin* are differentially regulated by ES and TP following cranial and spinal nerve injury (Brown et al., 2001; Al-Majed et al., 2004; Sharma et al., 2010a). Although it is known that ES elevates *α_I–tubulin* and that TP elevates *β_{II}–tubulin* following spinal nerve injury, it remains to be determined whether the combination of ES and TP has an additive effect on tubulin expression.

A two-way ANOVA for the relative mRNA expression of ipsilateral ventral horn *α_I–tubulin* revealed no differences across time ($F_{3,101}$ = 2.02; P = 0.116) or between treatment groups ($F_{4,101}$ = 1.81; P = 0.134; **Fig. 16**). In contrast,

statistical analysis revealed a significant interaction between treatment x time ($F_{12,101}$ = 3.67; P < 0.001; **Fig. 16**). Axotomy alone significantly decreased *α$_I$–tubulin* expression at 1 day post-axotomy relative to 6 hours post-axotomy (14.758 ± 0.536 and 21.922 ± 2.002, respectively; P < 0.05; **Fig. 16**). For the remaining time-points of 2, 7, and 21 days post-axotomy *α$_I$–tubulin* levels were maintained in axotomized animals (18.902 ± 2.874, 21.189 ± 2.458, and 18.489 ± 1.953, respectively). ES of the sciatic nerve maintained *α$_I$–tubulin* mRNA values at 6 hours, 1, 2, and 7 days post-axotomy (20.627 ± 2.352, 17.080 ± 1.185, 18.219 ± 1.587, and 22.629 ± 2.127, respectively; **Fig. 16A**); however, a significant decline in *α$_I$–tubulin* mRNA was observed at the latest time-point of 21 days post-axotomy (15.488 ± 1.413) compared to 7 days post-axotomy (P < 0.05; **Fig. 16A**). Unlike that observed with axotomy alone and ES treatment, *α$_I$–tubulin* mRNA expression was unaffected by TP treatment at 6 hours, 1, 2, 7, and 21 days post-axotomy (18.797 ± 2.471, 17.784 ± 1.747, 20.385 ± 1.052, 18.757 ± 2.358, and 22.813 ± 0.956, respectively; **Fig. 16B**). There was a transient upregulation of *α$_I$–tubulin* with the combination treatment of ES and TP at 1 and 2 days post-axotomy relative to 6 hours post-axotomy (21.985 ± 1.665, 21.401 ± 1.778, and 13.848 ± 0.388, respectively; P < 0.05; **Fig. 16C**). Levels of *α$_I$–tubulin* started to decline in animals treated with ES and TP at 7 and 21 days post-axotomy (16.727 ± 1.115 and 11.170 ± 1.295, respectively) but were only significantly lower at the latest time-point relative to 1 and 2 days post-axotomy (P < 0.001). ES and TP alone had no effect on expression levels of *α$_I$–tubulin*

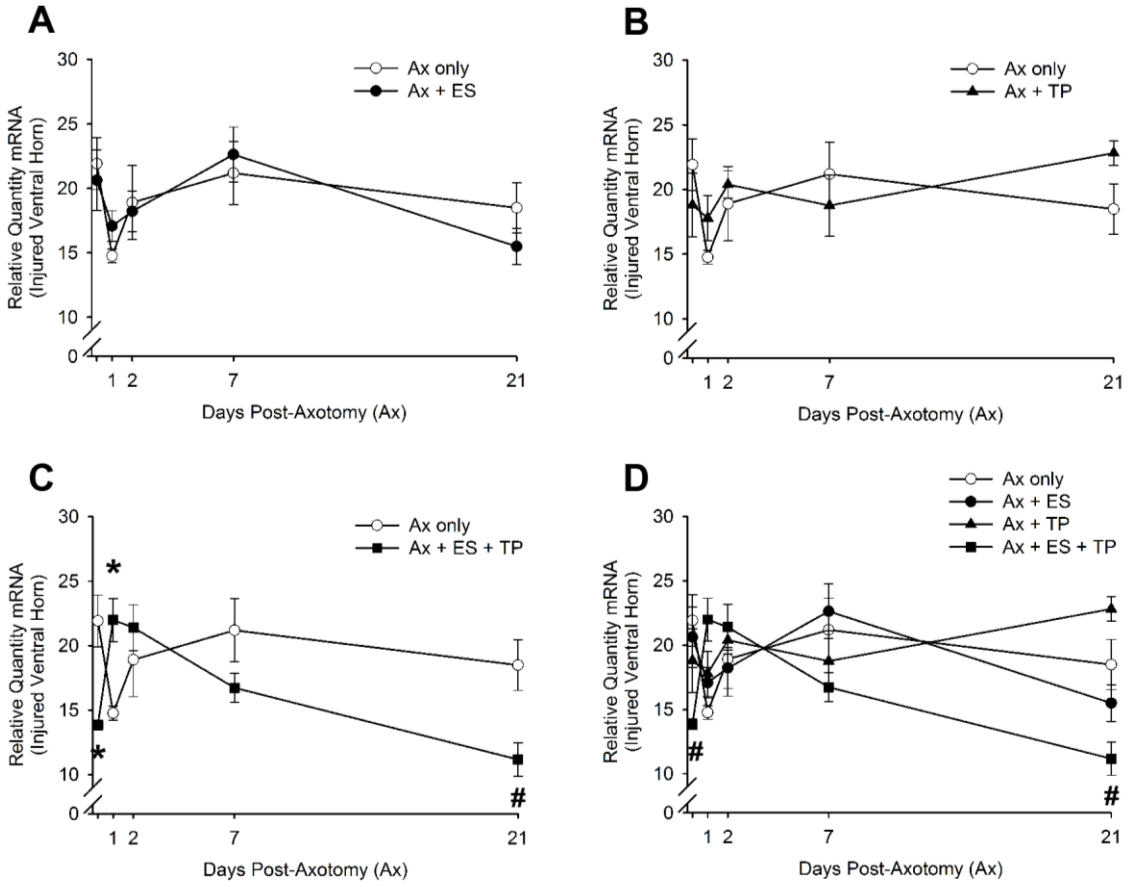

Fig. 16: Ipsilateral *α$_I$–tubulin* mRNA expression.

Shown is a time-course of the relative mRNA expression levels of *α$_I$–tubulin* in the injured ventral horn at 6 hours, 1, 2, 7, and 21 days post-axotomy (Ax). Control (C) animals, which were axotomized but left untreated, are compared to axotomized animals receiving only ES (A), only TP (B), or the combination of both ES and TP (C). (D) Represents the comparison between ES, TP, and the combination of ES and TP. Data is represented as relative *α$_I$–tubulin* mRNA expression ± SEM. A two-way ANOVA (treatment x time) with a Student-Newman-Keuls multiple comparison post hoc test was utilized for statistical analysis among all experimental groups. * represents a significant difference between ES, TP, or ES and TP compared to axotomy alone, at $P < 0.05$. # represents a significant difference between ES, TP, or ES and TP, at $P < 0.05$. For each experimental group, n = 6-7 animals/time-point.

throughout the studied time-course compared to axotomy alone (**Fig. 16A** and **Fig. 16B**, respectively). In contrast, the combined treatment significantly downregulated *α_I–tubulin* at 6 hours post-axotomy relative to axotomy alone and ES treatment ($P < 0.05$; **Fig. 16C** and **Fig. 16D**, respectively). At 1 day post-axotomy, the combined treatment of ES plus TP significantly elevated *α_I–tubulin* expression relative to axotomy alone ($P < 0.05$; **Fig. 16C**). By 21 days post-axotomy, *α_I–tubulin* levels were significantly lower in animals that received the combined treatment relative to axotomy alone and TP treatment ($P < 0.05$; Fig. **Fig. 16C** and **Fig. 16D**, respectively).

In the current study, the extended time course of the relative mRNA expression of *β_{II}–tubulin* revealed a main effect of time ($F_{4,100} = 11.81$; $P < 0.001$) and treatment x time ($F_{12,100} = 2.76$; $P = 0.003$), but there was no statistical interaction between treatment groups ($F_{3,100} = 1.43$; $P = 0.240$; **Fig. 17**). In axotomized, untreated animals *β_{II}–tubulin* expression was maintained at 6 hours, 1, 2, and 21 days post-axotomy (0.723 ± 0.061, 0.587 ± 0.060, 0.778 ± 0.125, and 0.827 ± 0.087, respectively) but peaked at 7 days post-axotomy (1.052 ± 0.134) relative to 1 day post-axotomy ($P < 0.05$; **Fig. 17**). Treatment with ES significantly upregulated *β_{II}–tubulin* expression at 7 days post-axotomy relative to 6 hours, 1, 2, and 21 days post-axotomy (1.181 ± 0.135, 0.697 ± 0.074, 0.751 ± 0.022, 0.758 ± 0.074, and 0.710 ± 0.073, respectively; $P < 0.05$; **Fig. 17A**). Systemic administration of TP sustained *β_{II}–tubulin* levels at the earlier time-points of 6 hours, 1, and 2 days post-axotomy (0.518 ± 0.081, 0.687 ± 0.062, and 0.832 ± 0.085, respectively; **Fig. 17B**). By 7 and 21 days post-axotomy (1.020 ±

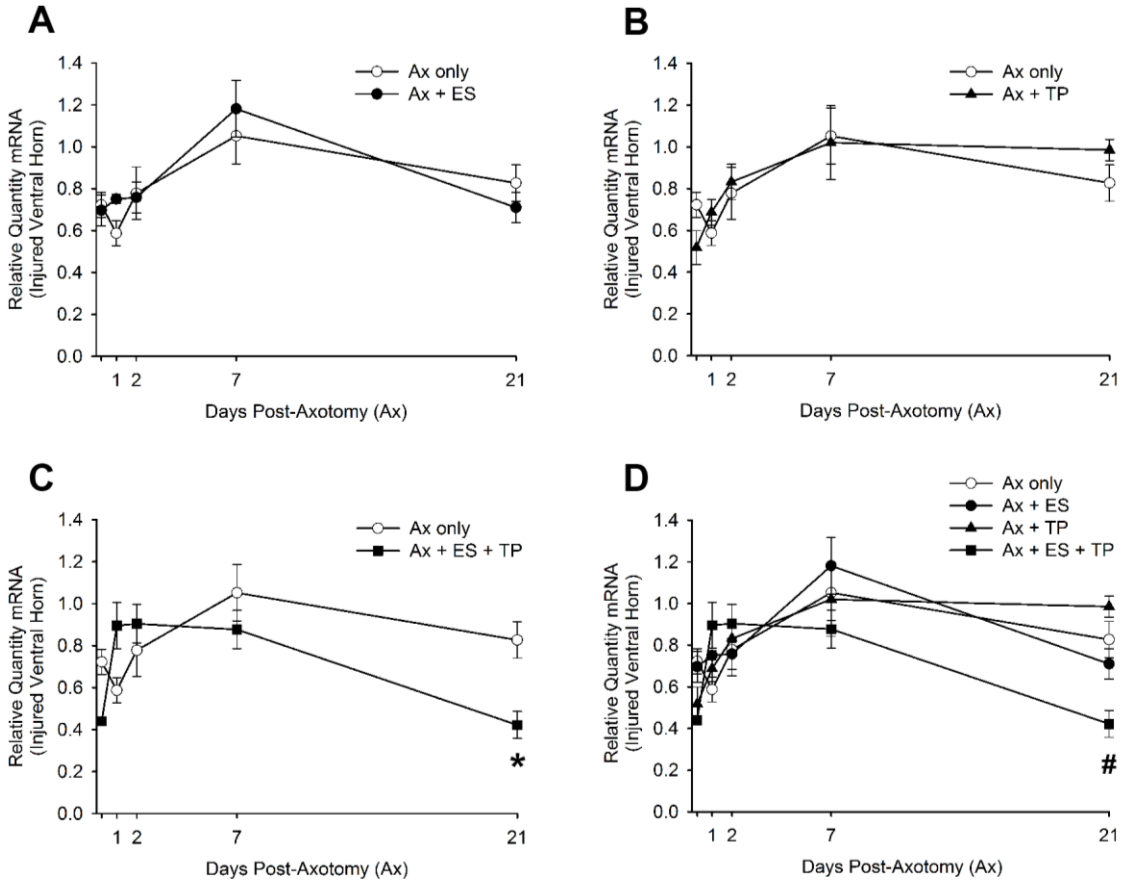

Fig. 17: Ipsilateral *β_{II}–tubulin* mRNA expression.

Shown is a time-course of the relative mRNA expression levels of *β_{II}–tubulin* in the injured ventral horn at 6 hours, 1, 2, 7, and 21 days post-axotomy (Ax). Control (C) animals, which were axotomized but left untreated, are compared to axotomized animals receiving only ES (A), only TP (B), or the combination of both ES and TP (C). (D) Represents the comparison between ES, TP, and the combination of ES and TP. Data is represented as relative *β_{II}–tubulin* mRNA expression ± SEM. A two-way ANOVA (treatment x time) with a Student-Newman-Keuls multiple comparison post hoc test was utilized for statistical analysis among all experimental groups. * represents a significant difference between ES, TP, or ES and TP compared to axotomy alone, at $P < 0.05$. # represents a significant difference between ES, TP, or ES and TP, at $P < 0.05$. For each experimental group, n = 5-7 animals/time-point.

0.177 and 0.985 ± 0.051), *β_{II}–tubulin* expression was significantly elevated relative to 6 hours post-axotomy in TP-treated animals ($P < 0.05$). The combination treatment of ES and TP significantly elevated relative *β_{II}–tubulin* mRNA expression at 1, 2, and 7 days post-axotomy compared to 6 hours post-axotomy (0.896 ± 0.110, 0.904 ± 0.092, 0.877 ± 0.091, and 0.440 ± 0.012, respectively; $P < 0.05$; **Fig. 17C**). At 21 days post-axotomy (0.422 ± 0.064), administration of both ES and TP together downregulated levels of *β_{II}–tubulin* to levels similar to that observed at 6 hours post-axotomy ($P < 0.05$). Statistical analysis revealed no differences between animals treated with either ES or TP relative to axotomized, untreated animals (**Fig. 17A** and **Fig. 17B**, respectively). In contrast, the combination treatment significantly downregulated *β_{II}–tubulin* compared to all other groups at 21 days post-axtomy ($P < 0.05$; **Fig. 17D**).

b. Axonal growth genes: *gap-43 and crmp2*

Following injury, assembly of the growth cone is essential for axon elongation and synaptogenesis. Both *gap-43* and *crmp2* are present in the growth cone and are important in axonal growth, whereas *gap-43* is important in growth cone guidance (Aigner et al., 1995; Fukata et al., 2002). Molecular studies further demonstrate that *gap-43* increases and is regulated by ES and TP following injury (Tetzlaff et al., 1991; Al-Majed et al., 2004; Sharma et al., 2010a). However, it is unknown whether ES and TP regulate the expression of *crmp2* after nerve injury.

Overall, we observed a main effect with time ($F_{4,101}$ = 10.42; P < 0.001) and treatment x time ($F_{12,101}$ = 2.05; P = 0.027; **Fig. 18**); although, there was no effect of time ($F_{3,101}$ = 1.71; P = 0.169; **Fig. 18**) when examining the relative mRNA expression of *gap-43*. In axotomized animals, there was a peak in *gap-43* expression at 7 days post-axotomy relative to 6 hours, 1, and 2 days post-axotomy (0.598 ± 0.094, 0.352 ± 0.035, 0.235 ± 0.023, and 0.326 ± 0.035, respectively; P < 0.05) but not to 21 days post-axotomy (0.457 ± 0.060; **Fig. 18**). ES of the sciatic nerve significantly upregulated *gap-43* expression at 7 days post-axotomy compared to 6 hours, 1, 2, and 21 days post-axotomy (0.649 ± 0.125, 0.308 ± 0.032, 0.348 ± 0.022, 0.442 ± 0.074, and 0.436 ± 0.108, respectively; P < 0.05; **Fig. 18A**). In contrast, animals treated with TP demonstrated no significant difference throughout the time-course of 6 hours, 1, 2, 7, and 21 days post-axotomy (0.334 ± 0.021, 0.302 ± 0.010, 0.444 ± 0.029, 0.433 ± 0.063, and 0.492 ± 0.036, respectively; **Fig. 18B**). The combination of ES and TP had no effect on *gap-43* expression at the earlier time-points of 6 hours, 1, and 2 days post-axotomy (0.227 ± 0.008, 0.403 ± 0.048, and 0.414 ± 0.048, respectively; **Fig. 18C**). At 7 days post-axotomy (0.467 ± 0.057) relative levels of *gap-43* mRNA peaked relative to 6 hours post-axotomy (P < 0.05), but this effect was absent by 21 days post-axotomy (0.261 ± 0.037). ES-treated animals demonstrated a significantly upregulated *gap-43* expression at 7 days post-axotomy compared to TP-treated animals (P < 0.05; **Fig. 18D**). The combination treatment significantly downregulated *gap-43* expression at the

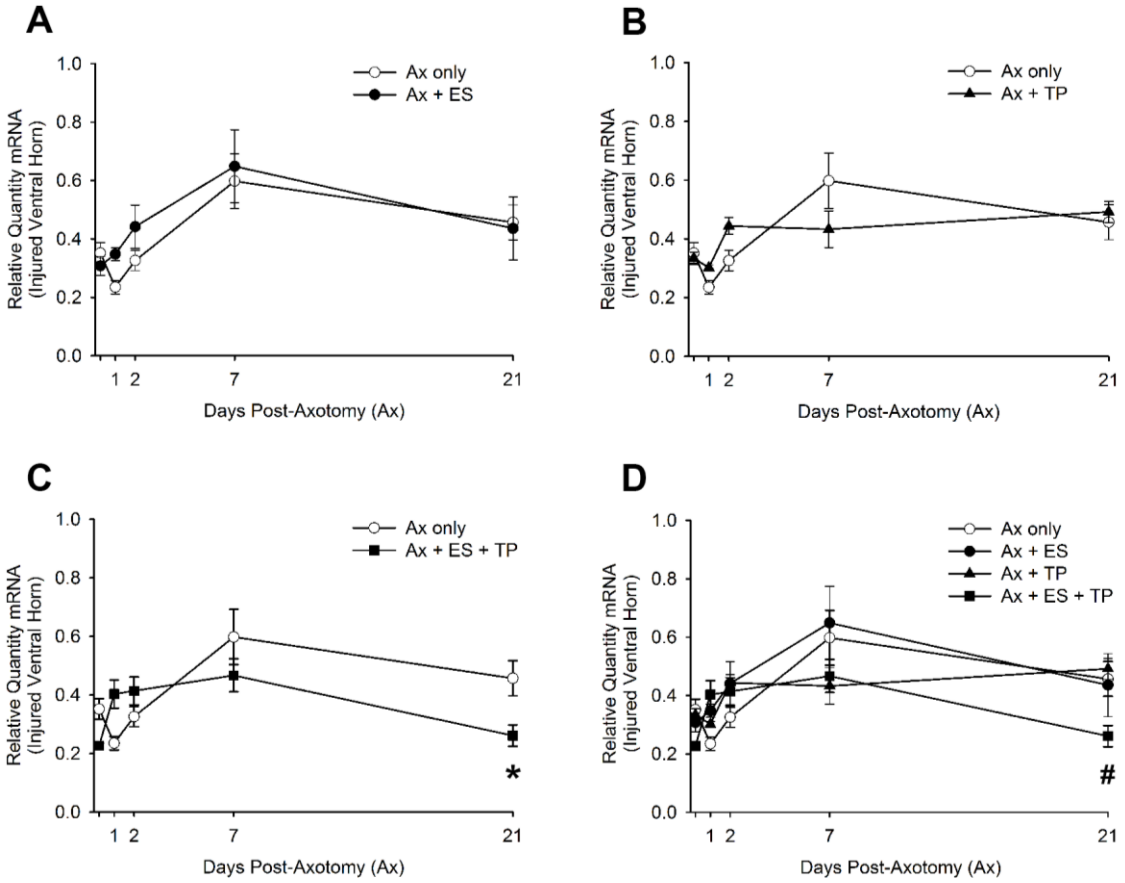

Fig. 18: Ipsilateral *gap-43* mRNA expression.

Shown is a time-course of the relative mRNA expression levels of *43-kiloDalton growth-associated protein* (*gap-43*) in the injured ventral horn at 6 hours, 1, 2, 7, and 21 days post-axotomy (Ax). Control (C) animals, which were axotomized but left untreated, are compared to axotomized animals receiving only ES (A), only TP (B), or the combination of both ES and TP (C). (D) Represents the comparison between ES, TP, and the combination of ES and TP. Data is represented as relative *gap-43* mRNA expression ± SEM. A two-way ANOVA (treatment x time) with a Student-Newman-Keuls multiple comparison post hoc test was utilized for statistical analysis among all experimental groups.
* represents a significant difference between ES, TP, or ES and TP compared to axotomy alone, at $P < 0.05$. # represents a significant difference between ES, TP, or ES and TP, at $P < 0.05$. For each experimental group, n = 6-7 animals/time-point.

latest time-point of 21 days post-axotomy compared to all other groups ($P < 0.05$; **Fig. 18D**).

Statistical analysis did not reveal a main effect of treatment group ($F_{3,101} = 1.80$; $P = 0.153$) or an effect of post-operative time ($F_{4,101} = 1.31$; $P = 0.273$) but did reveal an interaction between treatment x post-operative time ($F_{12,101} = 3.19$; $P < 0.001$; **Fig. 19**). Axotomy alone significantly decreased *crmp2* expression at 1 day post-axotomy relative to 6 hours post-axotomy (1.193 ± 0.062 and 1.932 ± 0.233; $P < 0.05$) but levels were maintained throughout the rest of the time-course at 2, 7, and 21 days post-axotomy (1.504 ± 0.258, 1.714 ± 0.171, and 1.548 ± 0.150, respectively; **Fig. 19**). No significant differences in *crmp2* mRNA were observed at 6 hours, 1, 2, 7, or 21 days post-axotomy in animals treated with either ES (1.842 ± 0.211, 1.480 ± 0.122, 1.536 ± 0.141, 1.815 ± 0.179, and 1.389 ± 0.166, respectively; **Fig. 19A**) or TP (1.474 ± 0.251, 1.498 ± 0.156, 1.704 ± 0.092, 1.533 ± 0.192, and 1.916 ± 0.053, respectively; **Fig. 19B**). The combination treatment of ES plus TP maintained *crmp2* mRNA expression at 6 hours, 1, 2, and 7 days post-axotomy (1.259 ± 0.048, 1.738 ± 0.109, 1.781 ± 0.132, and 1.398 ± 0.113, respectively) but downregulated *crmp2* expression at the later time-point of 21 days post-axotomy (0.908 ± 0.117) relative to 1 and 2 day post-axotomy ($P < 0.05$; **Fig. 19C**). In the combined treatment group, a downregulation in *crmp2* expression was observed at 6 hours relative to axotomy alone and ES treatment ($P < 0.05$) and 21 days post-axotomy relative to all groups ($P < 0.05$; **Fig. 19D**).

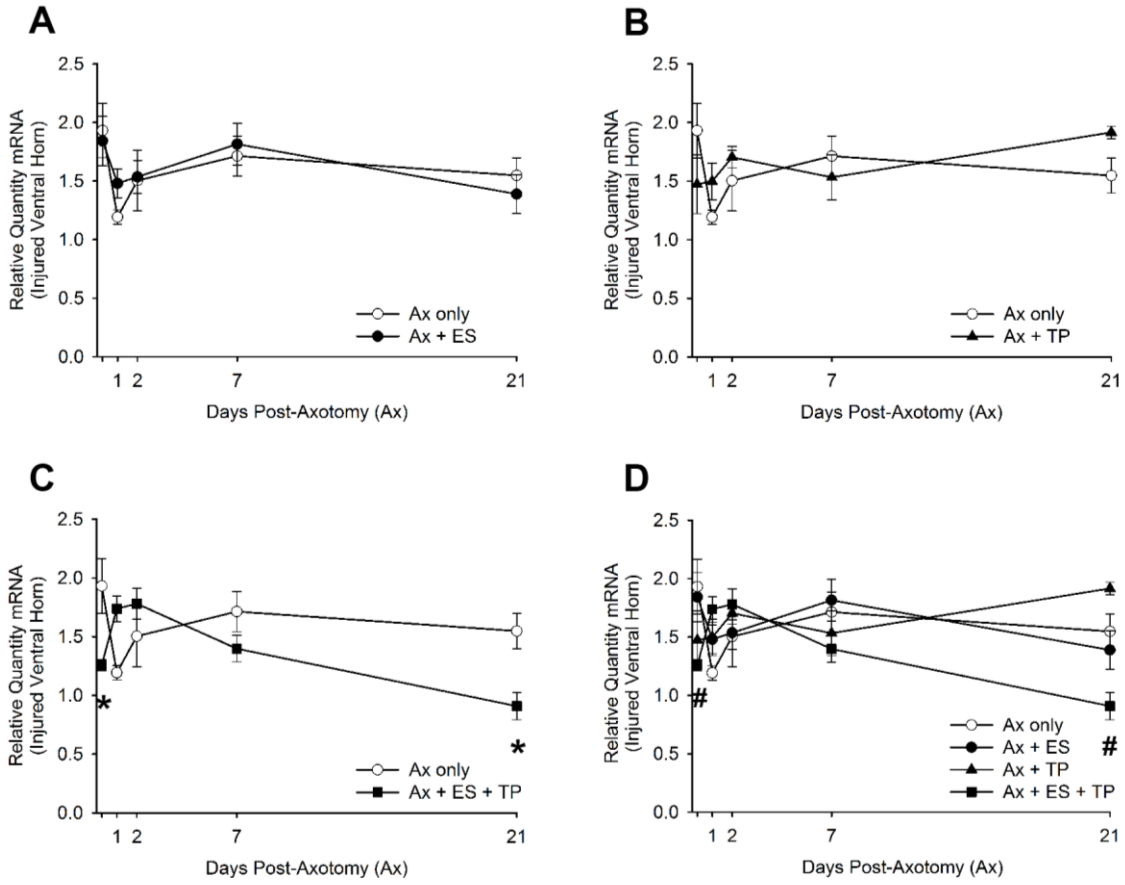

Fig. 19: Ipsilateral *crmp2* mRNA expression.

Shown is a time-course of the relative mRNA expression levels of *collapsin response mediator protein 2* (*crmp2*) in the injured ventral horn at 6 hours, 1, 2, 7, and 21 days post-axotomy (Ax). Control (C) animals, which were axotomized but left untreated, are compared to axotomized animals receiving only ES (A), only TP (B), or the combination of both ES and TP (C). (D) Represents the comparison between ES, TP, and the combination of ES and TP. Data is represented as relative *crmp2* mRNA expression ± SEM. A two-way ANOVA (treatment x time) with a Student-Newman-Keuls multiple comparison post hoc test was utilized for statistical analysis among all experimental groups.
* represents a significant difference between ES, TP, or ES and TP compared to axotomy alone, at $P < 0.05$. # represents a significant difference between ES, TP, or ES and TP, at $P < 0.05$. For each experimental group, n = 6-7 animals/time-point.

c. Growth factor and neurotrophic genes: *bdnf, gdnf, and pacap*

Neurotrophic factors, such as BDNF and GDNF, promote neurite outgrowth and survival of motoneurons following axotomy (Yan et al., 1992; Novikov et al., 1997; Boyd & Gordon, 2003). PACAP is a multifunctional peptide and is important in the regeneration process (Armstrong et al., 2008). Similar to BDNF and GDNF, PACAP has been demonstrated to promote neurite outgrowth and motoneuron survival (Chen & Tzeng, 2005; Suarez et al., 2006). We and others have demonstrated previously that ES and TP differentially regulate the expression of *bdnf* and *pacap* following facial or femoral nerve axotomy (Al-Majed et al., 2000a; Sharma et al., 2010a); although, it has yet to be determined whether the combination of ES and TP alter *bdnf* and *pacap* expression following sciatic nerve axotomy. ES in combination with exercise has been reported to increase the expression of *gdnf* (Cobianchi et al., 2013). However, it is unknown whether ES with administration of systemic TP alter the expression of *gdnf* following sciatic nerve injury.

Statistical analysis revealed a main effect of treatment ($F_{3,97} = 5.93$; $P < 0.001$) and an interaction between treatment x time ($F_{12,97} = 3.26$; $P < 0.001$) but no effect was observed for time ($F_{4,97} = 1.31$; $P = 0.272$; **Fig. 20**) with respect to *bdnf* mRNA expression in the ipsilateral ventral horn of the spinal cord. Injury alone moderately decreased the expression of *bdnf* at 1 day post-axotomy relative to 6 hours post-axotomy (0.00163 ± 0.00017 and 0.00259 ± 0.00054, respectively; $P < 0.05$; **Fig. 20**). By 2 days post-axotomy (0.00169 ± 0.00017), a significant decrease in *bdnf* expression was observed relative to 6 hours post-

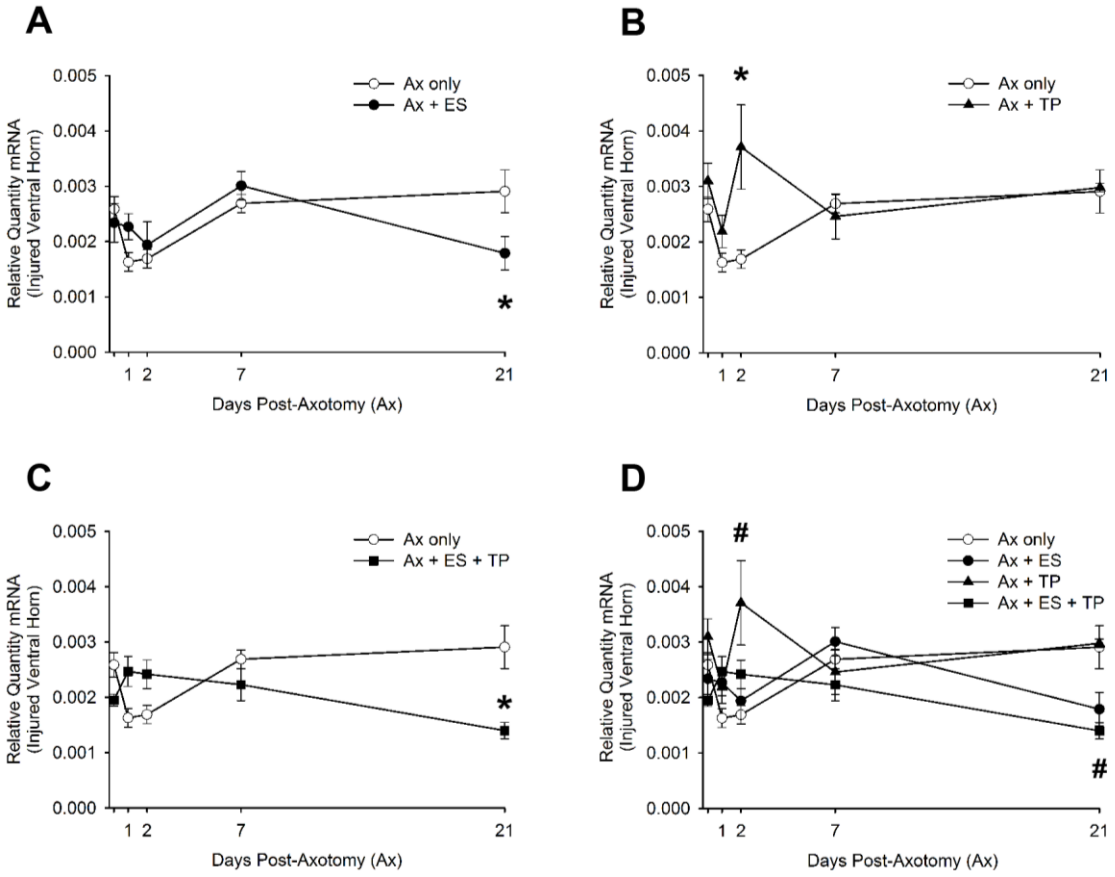

Fig. 20: Ipsilateral *bdnf* mRNA expression.

Shown is a time-course of the relative mRNA expression levels of *brain derived neurotrophic factor* (*bdnf*) in the injured ventral horn at 6 hours, 1, 2, 7, and 21 days post-axotomy (Ax). Control (C) animals, which were axotomized but left untreated, are compared to axotomized animals receiving only ES (A), only TP (B), or the combination of both ES and TP (C). (D) Represents the comparison between ES, TP, and the combination of ES and TP. Data is represented as relative *bdnf* mRNA expression ± SEM. A two-way ANOVA (treatment x time) with a Student-Newman-Keuls multiple comparison post hoc test was utilized for statistical analysis among all experimental groups. * represents a significant difference between ES, TP, or ES and TP compared to axotomy alone, at $P < 0.05$. # represents a significant difference between ES, TP, or ES and TP, at $P < 0.05$. For each experimental group, n = 5-7 animals/time-point.

axotomy ($P < 0.05$). *Bdnf* expression values started to increase by 7 days post-axotomy (0.00269 ± 0.00017) and peaked at 21 days post-axotomy (0.00291 ± 0.00039) to a level that was significantly different relative to 1 and 2 days post-axotomy ($P < 0.05$; **Fig. 20**). In contrast to axotomy alone, ES did not alter *bdnf* expression at 6 hours, 1, 2, 7, or 21 days post-axotomy (0.00234 ± 0.00035, 0.00227 ± 0.00024, 0.00194 ± 0.00042, 0.00301 ± 0.00025, and 0.00179 ± 0.00030, respectively; **Fig. 20A**). TP treatment significantly elevated *bdnf* expression at 2 days post-axotomy relative to 1 and 7 days post-axotomy (0.00371 ± 0.00076, 0.00219 ± 0.00029, and 0.00246 ± 0.00041, respectively) but had no effect at 6 hours and 21 days post-axotomy (0.00310 ± 0.00032 and 0.00298 ± 0.00075, respectively; **Fig. 20B**). Similar to ES treatment, the combination treatment revealed no differences in *bdnf* expression at 6 hours, 1, 2, 7, or 21 days post-axotomy (0.00195 ± 0.00011, 0.00247 ± 0.00027, 0.00242 ± 0.00026, 0.00223 ± 0.00029, and 0.00363 ± 0.00015, respectively; **Fig. 20C**). At the latest time-point of 21 days post-axotomy, there was a significant downregulation in *bdnf* mRNA in animals treated with ES relative to axotomy alone ($P < 0.05$; **Fig. 20A**). Systemic treatment with TP significantly elevated *bdnf* expression at 2 days post-axotomy compared to all groups tested ($P < 0.05$; **Fig. 20B** and **Fig. 20D**). There was a significant downregulation in *bdnf* mRNA at 21 days post-axotomy relative to axotomy alone and TP treatment ($P < 0.05$; **Fig. 20C and Fig. 20D**).

In the current study, the extended time course of the relative mRNA expression of *gdnf* did not reveal a main effect of treatment ($F_{3,97} = 0.92$; $P =$

0.437) or time ($F_{4,97}$ = 2.41; P = 0.055) but did reveal a statistical interaction between treatment x time ($F_{12,97}$ = 2.48; P = 0.007; **Fig. 21**). No differences were observed in the relative mRNA expression of *gdnf* at 6 hours, 1, 2, 7 and 21 days post-axotomy in animals that received axotomy alone (0.000484 ± 0.00008, 0.000297 ± 0.000041, 0.000421 ± 0.000068, 0.000428 ± 0.000072, and 0.000430 ± 0.000069, respectively; **Fig. 21**) or axotomy and TP treatment (0.000484 ± 0.000010, 0.000351 ± 0.000032, 0.000469 ± 0.000048, 0.000480 ± 0.000078, and 0.000486 ± 0.000095, respectively; **Fig. 21B**). Animals treated with ES demonstrated similar levels of *gdnf* expression at 6 hours and 1 day post-axotomy before peaking at 2 days post-axotomy (0.000565 ± 0.000111, 0.000386 ± 0.000054, and 0.000764 ± 0.000256, respectively) compared to 1 day post-axotomy (P < 0.05; **Fig. 21A**). Relative mRNA expression of *gdnf* declined at 7 days post-axotomy and reached a significant low at 21 days-post axotomy (0.000496 ± 0.000083 and 0.000213 ± 0.000053, respectively) relative to 6 hours and 2 days post-axotomy (P < 0.05; **Fig. 21A**). The combination treatment significantly peaked *gdnf* expression at 1 day post-axotomy relative to 6 hours, 7, and 21 days post-axotomy (0.000667 ± 0.000063, 0.000372 ± 0.000055, 0.000323 ± 0.000041, and 0.0002005 ± 0.000056, respectively; P < 0.05), but *gdnf* expression was similar to 2 days post-axotomy (0.000444 ± 0.000033; **Fig. 21C**). Treatment with ES only was significantly different than all groups at 2 days post-axotomy (P < 0.05; **Fig. 21D**) but similar at all other tested time-points. In contrast, TP treatment did not alter *gdnf* expression at any time-point relative to axotomy alone (**Fig. 21B**). The combination of ES and TP

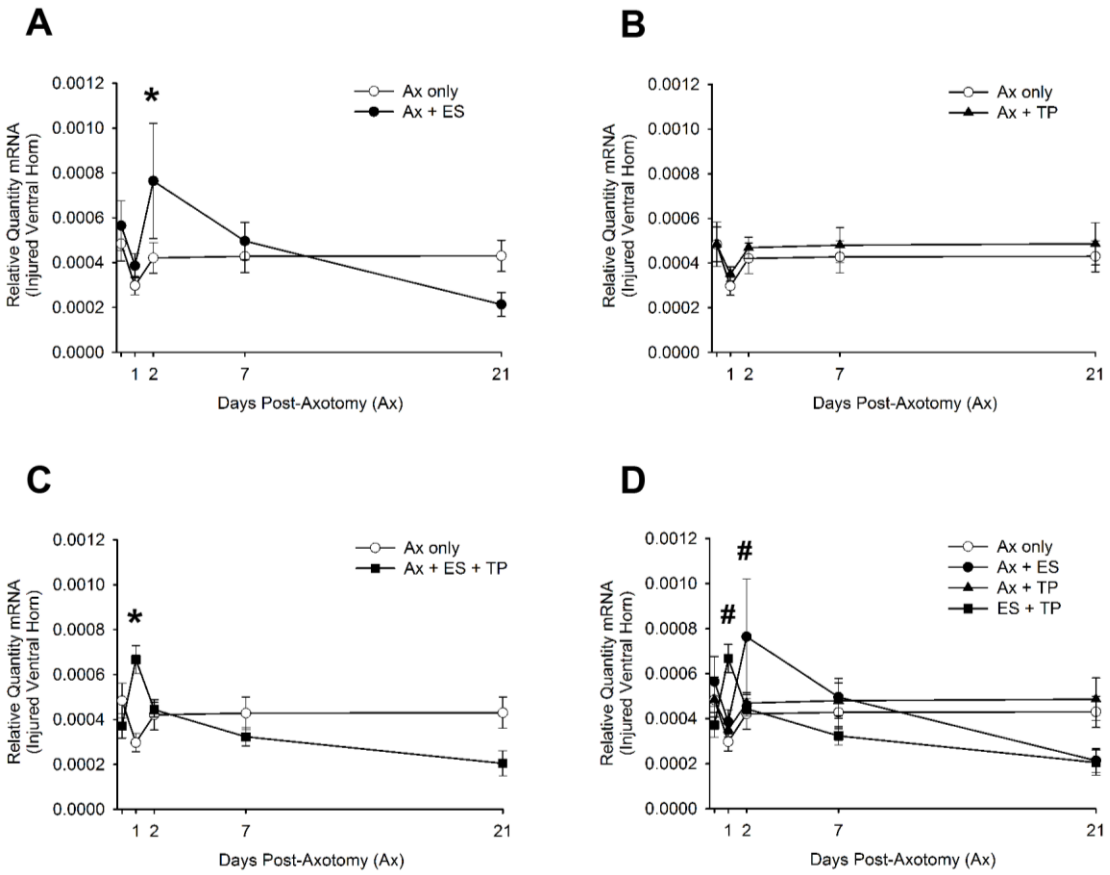

Fig. 21: Ipsilateral *gdnf* mRNA expression.

Shown is a time-course of the relative mRNA expression levels of *glial cell derived neurotrophic factor* (*gdnf*) in the injured ventral horn at 6 hours, 1, 2, 7, and 21 days post-axotomy (Ax). Control (C) animals, which were axotomized but left untreated, are compared to axotomized animals receiving only ES (A), only TP (B), or the combination of both ES and TP (C). (D) Represents the comparison between ES, TP, and the combination of ES and TP. Data is represented as relative *gdnf* mRNA expression ± SEM. A two-way ANOVA (treatment x time) with a Student-Newman-Keuls multiple comparison post hoc test was utilized for statistical analysis among all experimental groups.
* represents a significant difference between ES, TP, or ES and TP compared to axotomy alone, at $P < 0.05$. # represents a significant difference between ES, TP, or ES and TP, at $P < 0.05$. For each experimental group, n = 5-6 animals/time-point.

significantly elevated levels of *gdnf* at 1 day post-axotomy relative to all other groups tested ($P < 0.05$) but was similar at all other time-points (**Fig. 21D**).

When investigating the relative mRNA expression of *pacap* in the ipsilateral ventral horn we observed a main effect of post-operative time ($F_{4,99}$ = 7.30; $P < 0.001$) 0.006; **Fig. 22**); although, there was no overall effect between treatment groups ($F_{3,99} = 1.80$; $P = 0.152$) or treatment x time ($F_{12,99} = 0.79$; P = 0.663; **Fig. 22**).

d. Plasticity gene: *neuritin*

Neuritin was first identified as a candidate plasticity-related gene but since has been shown to promote neurite outgrowth and dendritic arborization (Naeve et al., 1997; Javaherian & Cline, 2005; Marron et al., 2005). Previously, our laboratory has demonstrated that axotomy with either ES or TP increase neuritin levels following facial nerve injury (Fargo et al., 2008; Sharma et al., 2010a). It remains to be determined whether ES and TP alter *neuritin* expression following sciatic nerve injury.

The extended time course did not reveal any differences between treatment groups after axotomy for *neuritin* ($F_{3,101} = 0.532$; $P = 0.662$) or an interaction between treatment x post-operative time ($F_{12,101} = 1.20$; $P = 0.296$; **Fig. 23**). However, analysis did reveal a main effect of post-operative time ($F_{4,101}$ = 4.72; $P = 0.002$; **Fig. 23**).

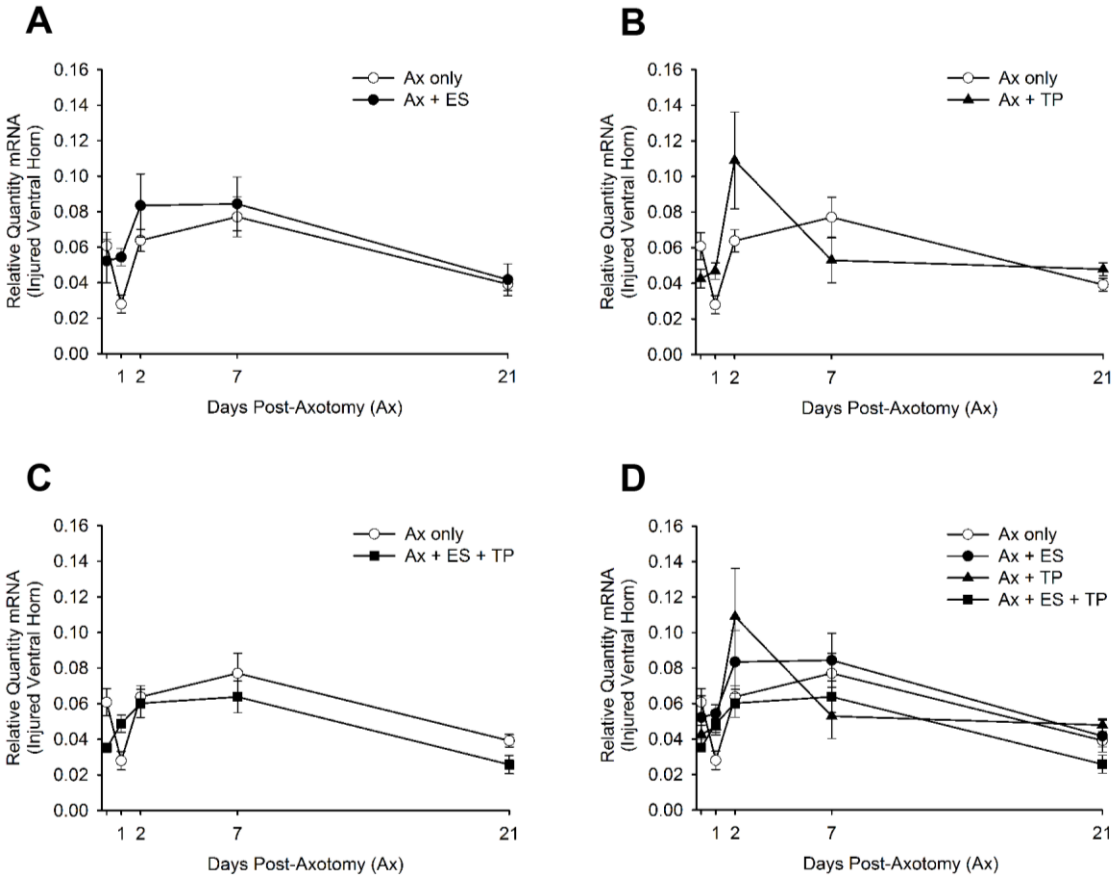

Fig. 22: Ipsilateral *pacap* mRNA expression.

Shown is a time-course of the relative mRNA expression levels of *pituitary adenylate cyclase-activating peptide* (*pacap*)in the injured ventral horn at 6 hours, 1, 2, 7, and 21 days post-axotomy (Ax). Control (C) animals, which were axotomized but left untreated, are compared to axotomized animals receiving only ES (A), only TP (B), or the combination of both ES and TP (C). (D) Represents the comparison between ES, TP, and the combination of ES and TP. Data is represented as relative *pacap* mRNA expression ± SEM. A two-way ANOVA (treatment x time) with a Student-Newman-Keuls multiple comparison post hoc test was utilized for statistical analysis among all experimental groups. * represents a significant difference between ES, TP, or ES and TP compared to axotomy alone, at $P < 0.05$. # represents a significant difference between ES, TP, or ES and TP, at $P < 0.05$. For each experimental group, n = 5-6 animals/time-point.

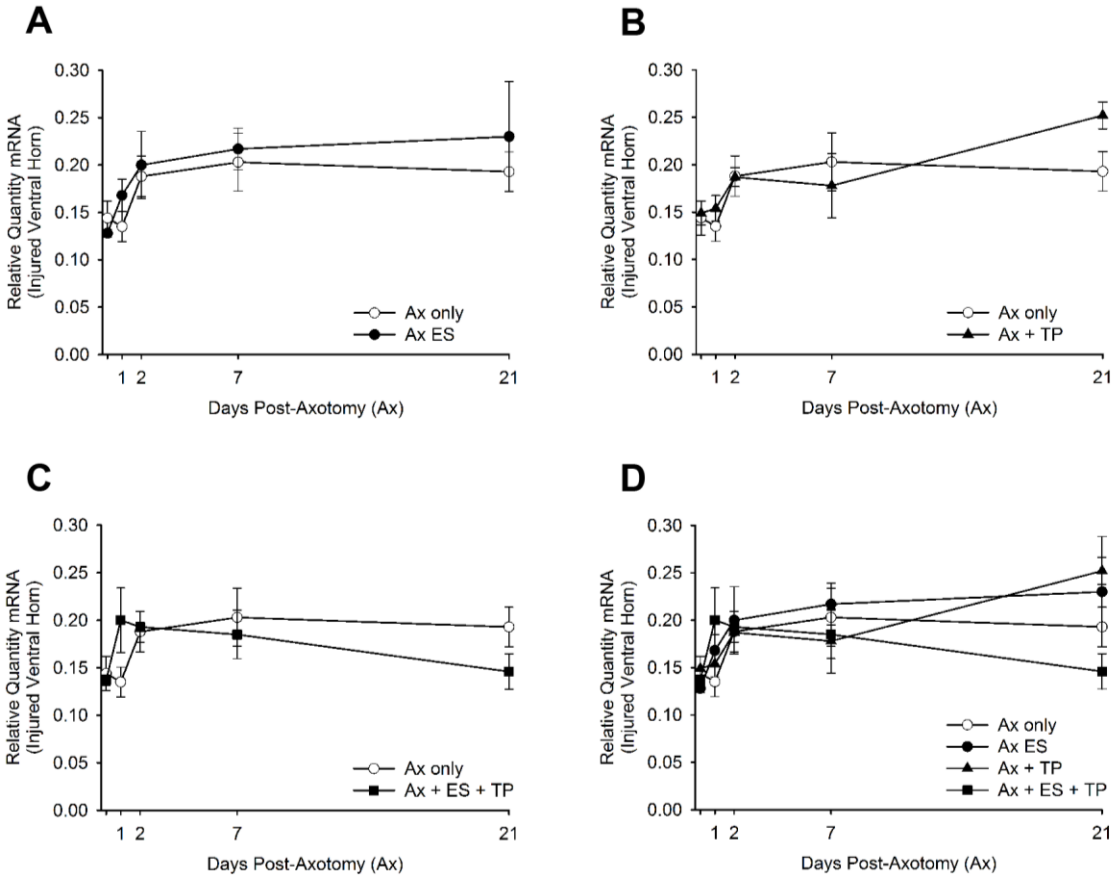

Fig. 23: Ipsilateral *neuritin* mRNA expression.

Shown is a time-course of the relative mRNA expression levels of *neuritin* in the injured ventral horn at 6 hours, 1, 2, 7, and 21 days post-axotomy (Ax). Control (C) animals, which were axotomized but left untreated, are compared to axotomized animals receiving only ES (A), only TP (B), or the combination of both ES and TP (C). (D) Represents the comparison between ES, TP, and the combination of ES and TP. Data is represented as relative *neuritin* mRNA expression ± SEM. A two-way ANOVA (treatment x time) with a Student-Newman-Keuls multiple comparison post hoc test was utilized for statistical analysis among all experimental groups. * represents a significant difference between ES, TP, or ES and TP compared to axotomy alone, at $P < 0.05$. # represents a significant difference between ES, TP, or ES and TP, at $P < 0.05$. For each experimental group, n = 6-7 animals/time-point.

e. Summary

We characterized the molecular effects of ES and TP on the expression of 8 regeneration-associated genes on the ipsilateral side of the lumbar ventral horn following sciatic nerve crush axotomy. Similar to our results from the contralateral analysis, we found that the expression of 3 out of 8 regeneration-associated genes was altered ipsilateral to injury. These genes include *α_I-tubulin, crmp2*, and *gap-43*. Furthermore, our results indicate that ES and/or TP treatment altered the expression of 6 out of 8 genes following axotomy. Our results demonstrate that ES and TP alter gene expression in very distinct patterns when administered alone and have a combined suppressive effect when administered together (**Table 7**). In summary, ES and TP separately altered the expression of *bdnf*, whereas the combination of both ES and TP suppressed the expression of *α_I-tubulin*, *β_{II}-tubulin*, *gap-43*, *crmp2*, and *gdnf* in an additive manner (**Table 8**). In addition, neither treatment altered the expression of *pacap* and *neuritin*.

Gene	Early (6 hours-2 days)	Middle (7 days)	Late (21 days)
ES			
bdnf	-	-	↓
gdnf	↑	-	-
TP			
bdnf	↑	-	-
ES + TP			
α_I-tubulin	↓ , ↑	-	↓
β_{II}-tubulin	-	-	↓
gap-43	-	-	↓
crmp2	↓	-	↓
bdnf	-	-	↓
gdnf	↑	-	-

Table 7: Summary of ipsilateral mRNA expression

Change in Expression	Facial Nerve Crush Injury	Sciatic Nerve Crush Injury
Differential	α_I-*tubulin*, β_{III}-*tubulin*, and *gap-43*	*bdnf*
Additive	*pacap*, *bdnf*, and *neuritin*	α_I-*tubulin*, β_{III}-*tubulin*, *gap-43,* g*dnf* and *crmp2*
No change	-	*pacap* and *neuritin*

Table 8: Comparison of the effects of ES and TP on gene expression after facial vs. sciatic nerve crush injuries.

**Facial nerve crush injury gene expression summarized from Sharma et al. (2010a).*

C. Effects of ES and TP on functional recovery

The following assessments were used to test functional recovery following sciatic nerve axotomy: rear foot falls (automatic foot misplacement apparatus), grip strength, gait analysis (CatWalk XT©), and EMG recordings. Testing of the automatic foot misplacement and CatWalk XT© apparatuses was completed on day 0 (3 days before injury), 2, 4, 7, 9, 11, 14, 16, 18, 21, 23, 25, 28, 30, 32, 35, 37, 39, 42, 44, 46, 49, 51, 53, and 56 days post-axotomy. Grip strength was tested before axotomy (0) and weekly for 8 weeks. EMG recordings were taken before axotomy and at 53 days post-axotomy.

A two-way repeated measures ANOVA revealed a significant difference in animal weight across time ($F_{27,509} = 355.54$; $P < 0.001$) and an interaction between treatment group x time ($F_{81,509} = 3.22$; $P < 0.001$) but no effect was observed between treatment groups ($F_{3,509} = 1.9$; $P = 0.162$; **Fig. 24**). Analysis revealed that animals treated with ES had a significantly higher weight than TP only treated animals from 32 days post-axotomy through 46 days post-axotomy ($P < 0.05$; **Fig. 24**). Since there was an effect of treatment on weight and since speed alters several gait parameters (Batka et al., 2014), we included weight and speed as dependent factors for all CatWalk XT© analysis.

1. Automatic foot misplacement apparatus

A two-way repeated measures ANOVA revealed a significant difference in the number of rear foot falls across time ($F_{24,429} = 9.36$; $P < 0.001$) and an

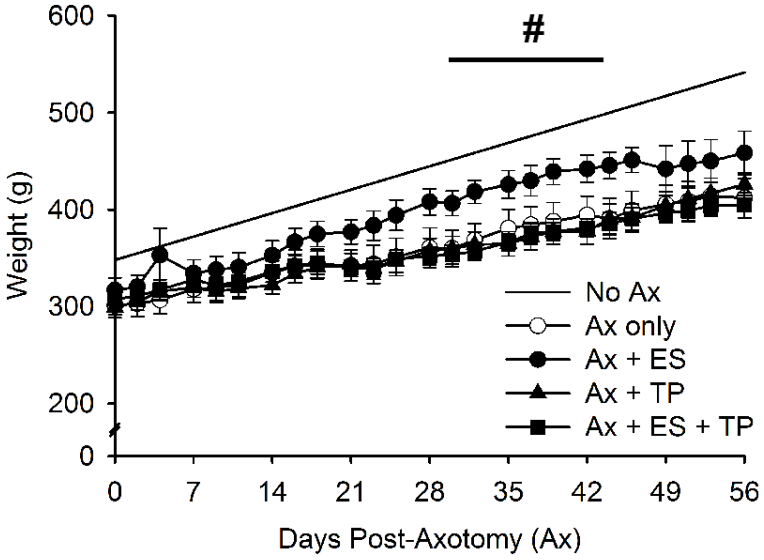

Fig. 24: Effect of ES and TP on animal body weight.

Shown is a time-course of the effect of ES and TP on animal body weight post-axotomy (Ax). Uninjured animals (solid line) represent a longitudinal baseline throughout the study. Control animals, which were axotomized but left untreated (Ax only), are compared to axotomized animals receiving only ES (A), only TP (B), or the combination of both ES and TP (C). (D) Represents the comparison between the combination of ES and TP relative to both ES and TP alone. Data is represented as weight (g) ± SEM. Statistical significance was evaluated using linear mixed effects models. Each model included random intercepts and slopes for speed and weight for each animal. Post hoc pairwise multiple comparisons were performed within time points using the Holm-Šidák method when group differences were detected. * represents a significant difference between ES, TP, or ES and TP compared to axotomy alone, at $P < 0.05$. # represents a significant difference between ES and TP relative to both ES and TP alone, at $P < 0.05$. For each experimental group, n = 5-6 animals/time-point.

interaction between treatment group x time ($F_{96,429}$ = 1.52; *P* = 0.003) but no effect was observed between treatment groups ($F_{4,429}$ = 2.27; *P* = 0.092; **Fig. 25**). At 2 days post-axotomy, all injured animals had a significantly higher number of rear foot fall errors relative to uninjured animals (*P* < 0.001; **Fig. 25**). However, axotomized, untreated animals by 4 days post-axotomy were similar to control, unaxotomized animals. Due to the fast recovery time of 2 days post-axotomy for injured, untreated animals with this particular motor test, treatment effects could not be determined.

2. Grip strength

a. Bar apparatus

Overall, we observed a main effect with time ($F_{8,170}$ = 66.25; *P* < 0.001) and between treatment groups ($F_{4,170}$ = 2.74; *P* = 0.05; **Fig. 26**); although, there was no interaction observed between treatment x time ($F_{32,170}$ = 1.26; *P* = 0.177; **Fig. 26**) when examining the mean force for the bar grip strength test. Additional analysis to determine the presence of a treatment effect was not completed since there was no statistical difference between treatment x time.

b. Grid apparatus

A two-way repeated measures ANOVA for mean force for the grid grip strength test revealed no difference between treatment groups ($F_{4,170}$ = 1.91; *P* = 0.143; **Fig. 27**). In contrast, statistical analysis revealed a significant difference

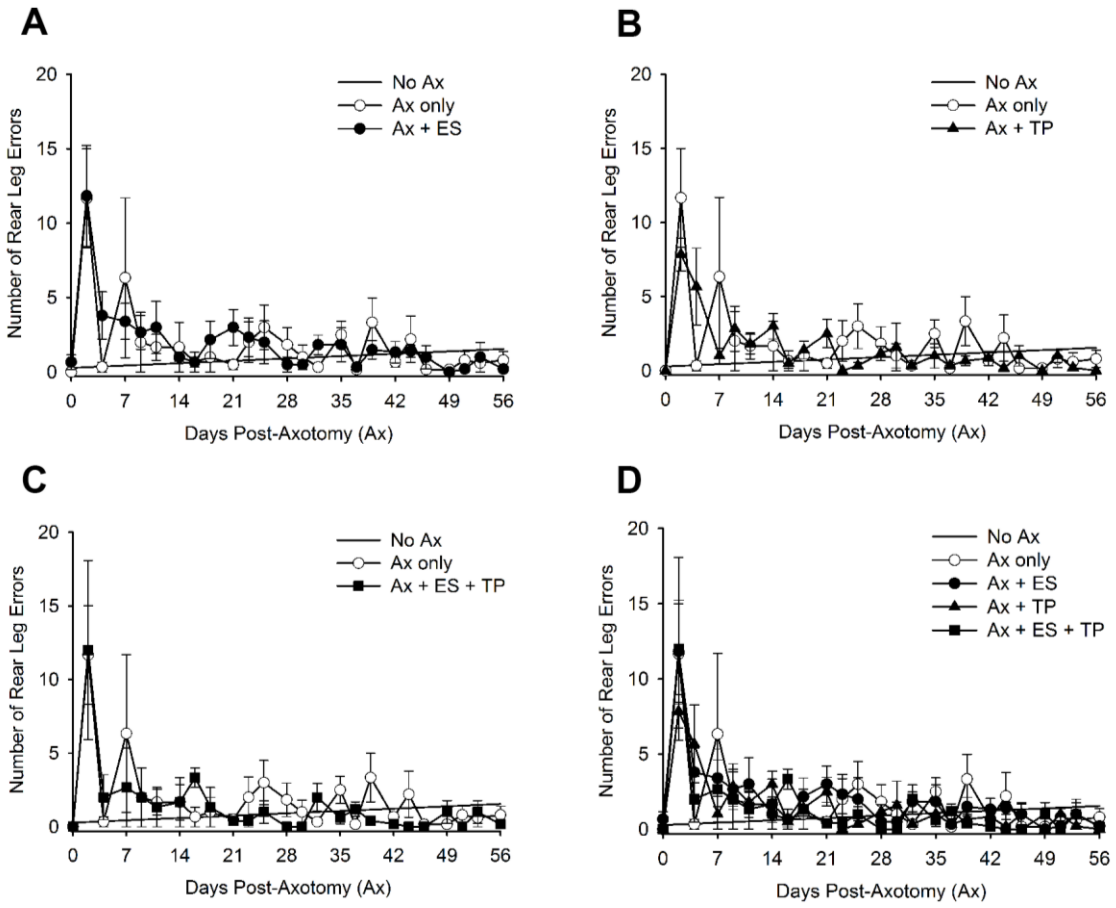

Fig. 25: Effect of ES and TP on the number of rear leg errors.

Shown is a time-course of the effect of ES and TP on the number of rear leg errors post-axotomy (Ax). Uninjured animals (solid line) represent a longitudinal baseline throughout the study. Control animals, which were axotomized but left untreated (Ax only), are compared to axotomized animals receiving only ES (A), only TP (B), or the combination of both ES and TP (C). (D) Represents the comparison between the combination of ES and TP relative to both ES and TP alone. Data is represented as the number of rear leg erros ± SEM. Statistical significance was evaluated using linear mixed effects models. Each model included random intercepts and slopes for speed and weight for each animal. Post hoc pairwise multiple comparisons were performed within time points using the Holm-Šidák method when group differences were detected. * represents a significant difference between ES, TP, or ES and TP compared to axotomy alone, at $P < 0.05$. # represents a significant difference between ES and TP relative to both ES and TP alone, at $P < 0.05$. For each experimental group, n = 5-6 animals/time-point.

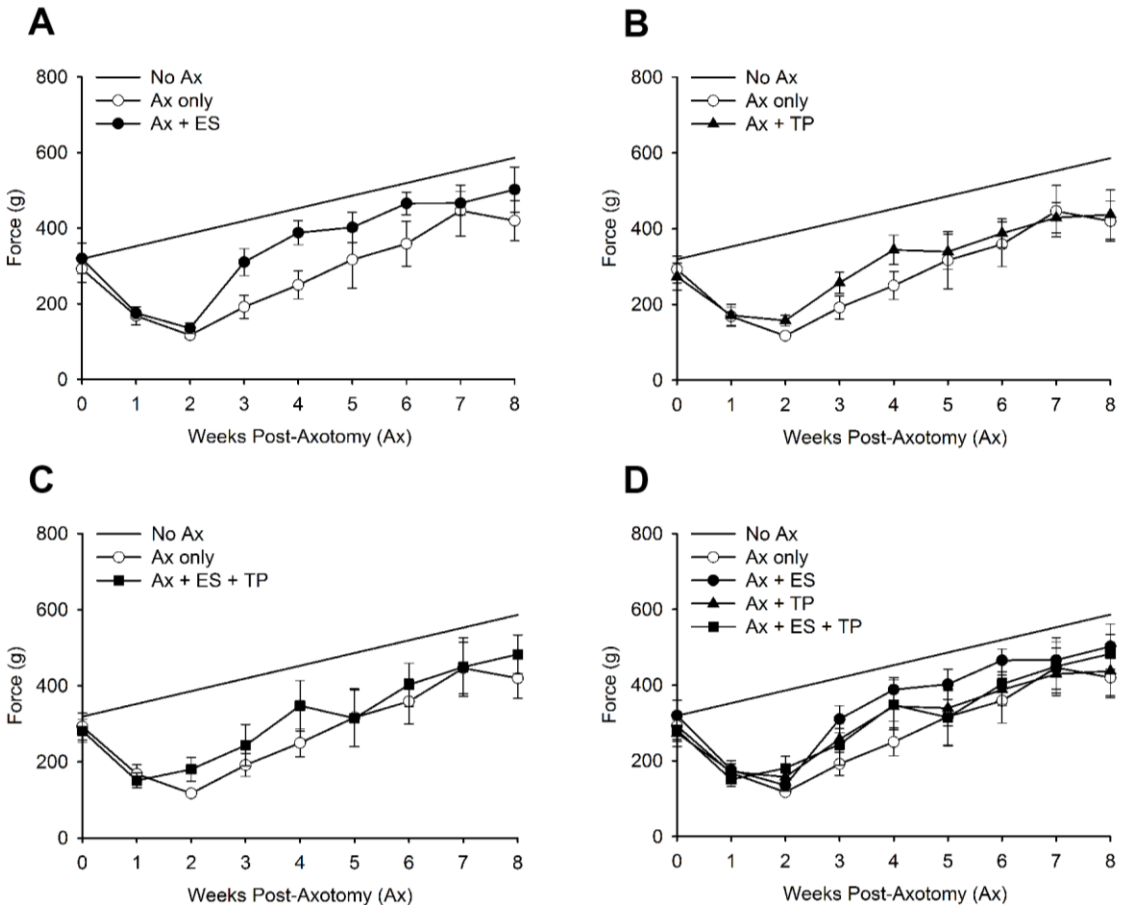

Fig. 26: Effect of ES and TP on hind paw grip strength utilizing the bar apparatus.

Shown is a time-course of the effect of ES and TP on hind paw grip strength utilizing the bar apparatus post-axotomy (Ax). Uninjured animals (solid line) represent a longitudinal baseline throughout the study. Control animals, which were axotomized but left untreated (Ax only), are compared to axotomized animals receiving only ES (A), only TP (B), or the combination of both ES and TP (C). (D) Represents the comparison between the combination of ES and TP relative to both ES and TP alone. Data is represented as force (g) ± SEM. Statistical significance was evaluated using linear mixed effects models. Each model included random intercepts and slopes for speed and weight for each animal. Post hoc pairwise multiple comparisons were performed within time points using the Holm-Šidák method when group differences were detected. * represents a significant difference between ES, TP, or ES and TP compared to axotomy alone, at $P < 0.05$. # represents a significant difference between ES and TP relative to both ES and TP alone, at $P < 0.05$. For each experimental group, n = 5-6 animals/time-point.

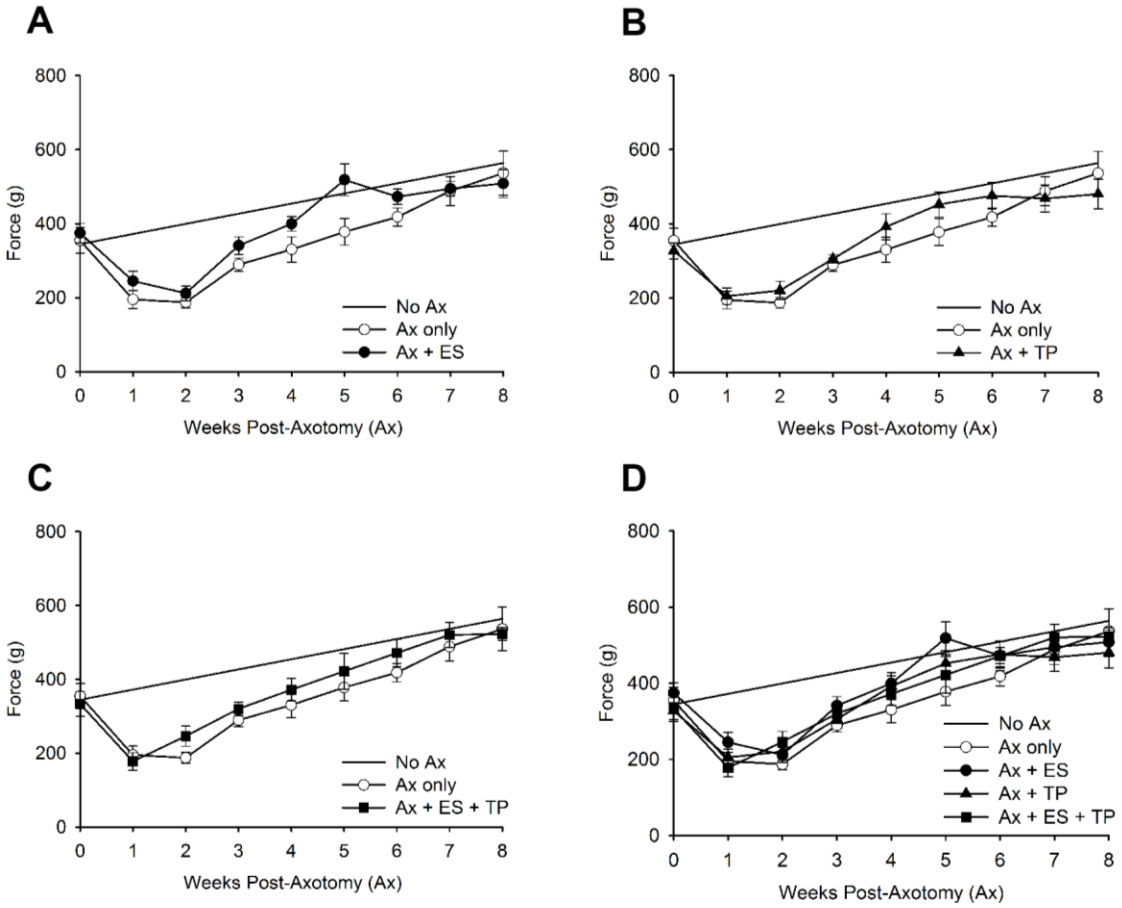

Fig. 27: Effect of ES and TP on hind paw grip strength utilizing the grid apparatus.

Shown is a time-course of the effect of ES and TP on hind paw grip strength utilizing the grid apparatus post-axotomy (Ax). Uninjured animals (solid line) represent a longitudinal baseline throughout the study. Control animals, which were axotomized but left untreated (Ax only), are compared to axotomized animals receiving only ES (A), only TP (B), or the combination of both ES and TP (C). (D) Represents the comparison between the combination of ES and TP relative to both ES and TP alone. Data is represented as force (g) ± SEM. Statistical significance was evaluated using linear mixed effects models. Each model included random intercepts and slopes for speed and weight for each animal. Post hoc pairwise multiple comparisons were performed within time points using the Holm-Šidák method when group differences were detected. * represents a significant difference between ES, TP, or ES and TP compared to axotomy alone, at $P < 0.05$. # represents a significant difference between ES and TP relative to both ES and TP alone, at $P < 0.05$. For each experimental group, n = 5-6 animals/time-point.

across time ($F_{8,170}$ = 119.90; P < 0.001) and an interaction between treatment x time ($F_{32,170}$ = 2.33; P < 0.001; **Fig. 27**). At 1 week post-axotomy, all axotomized groups had a significantly lower grip strength force relative to uninjured, untreated animals (No Ax: 393.8 ± 46.6 g; Ax only: 195.0 ± 24.1 g; ES: 245.0 ± 26.5 g; TP: 205.2 ± 22.3 g; ES + TP: 177.7 ± 23.9 g ; P < 0.05; **Fig. 27A-D**). By 2 weeks post-axotomy, the combined treatment group was similar to the No Ax and Ax only groups (246.1 ± 27.2 g, 367.8 ± 63.8 g, and 187.3 ± 14.9 g, respectively; **Fig. 27C**), but was similar to either treatment alone (ES: 211.8 ± 20.1 g; TP: 220.4 ± 25.0 g; **Fig. 27D**).

3. CatWalk XT© apparatus

A detailed list of analyzed CatWalk parameters with definitions can be found in **Table 4**.

a. Paw-related parameters

i. TP increases print length.

We found an overall main effect in the right hind (RH) print length between treatment groups ($F_{4,5767}$ = 13.80; P < 0.0001), time ($F_{26,5767}$ = 69.77; P < 0.0001), as well as an interaction between treatment x time ($F_{104,5767}$ = 10.54; P < 0.0001; **Fig. 28**). Print length was significantly decreased in injured, untreated animals at 2 days post-axotomy relative to uninjured control animals (0.25 ± 0.14 cm and 2.07 ± 0.17 cm, respectively; P < 0.001; **Fig. 28**). At 14 days post-axotomy, print

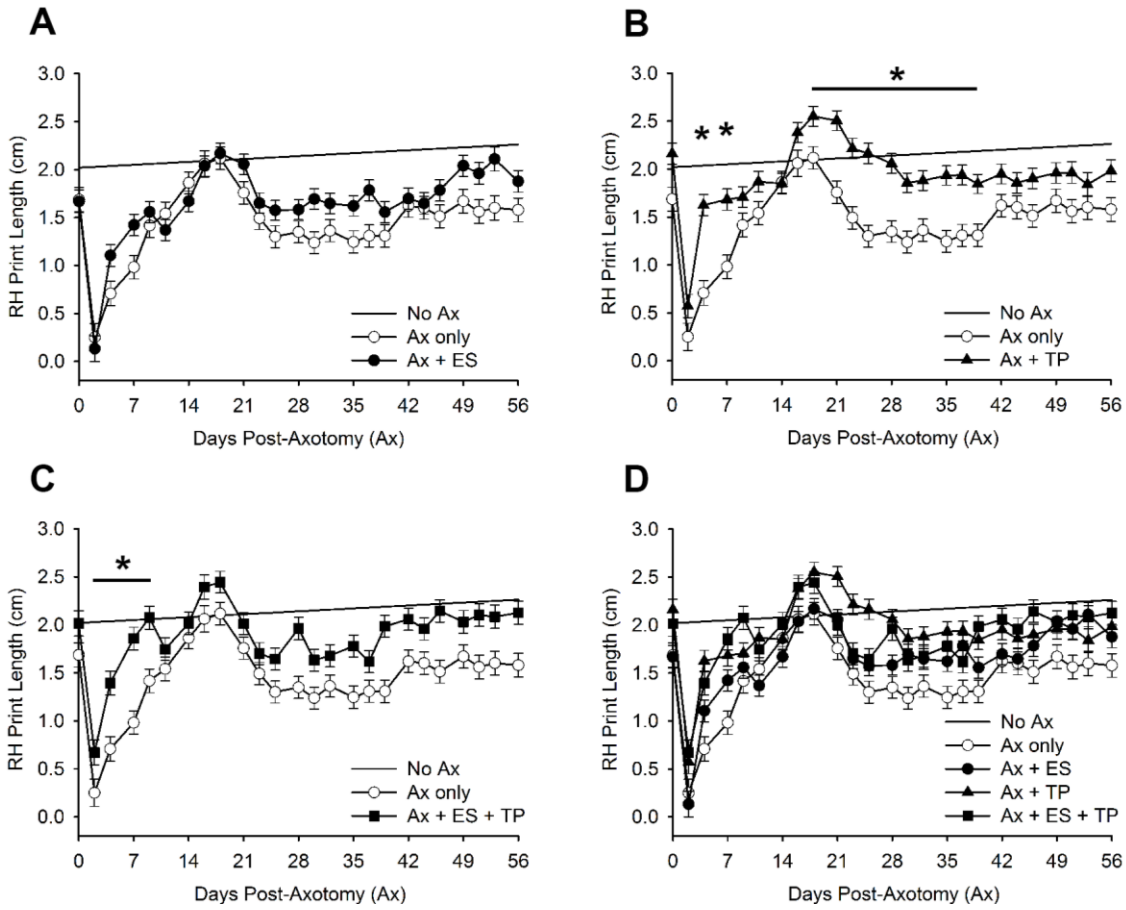

Fig. 28: Effect of ES and TP on RH print length.

Shown is a time-course of the effect of ES and TP on right hind (RH) print length post-axotomy (Ax). Uninjured animals (solid line) represent a longitudinal baseline throughout the study. Control animals, which were axotomized but left untreated (Ax only), are compared to axotomized animals receiving only ES (A), only TP (B), or the combination of both ES and TP (C). (D) Represents the comparison between the combination of ES and TP relative to both ES and TP alone. Data is represented as RH print length (cm) ± SEM. Statistical significance was evaluated using linear mixed effects models. Each model included random intercepts and slopes for speed and weight for each animal. Post hoc pairwise multiple comparisons were performed within time points using the Holm-Šidák method when group differences were detected. * represents a significant difference between ES, TP, or ES and TP compared to axotomy alone, at $P < 0.05$. # represents a significant difference between ES and TP relative to both ES and TP alone, at $P < 0.05$. For each experimental group, n = 5-6 animals/time-point.

length for axotomized animals returned to control levels (1.86 ± 0.11 cm and 1.99 ± 0.16 cm, respectively; **Fig. 28**). At 23 days post-axotomy, print length of axotomized animals significantly decreased relative to uninured animals and remained steady for the remainder of the time-course ($P < 0.05$; **Fig. 28**). Animals treated with ES demonstrated no consistent significant pattern for print length across all time-points relative to that observed in uninjured animals (**Fig. 28A**). In contrast, animals treated with TP demonstrated a significantly higher print length as early as 4 days post-axotomy relative to injured, untreated animals ($P < 0.05$; **Fig. 28B**) until 9 days post-axotomy. From 25 to 39 days post-axotomy, TP-treated animals demonstrated a significantly elevated print length relative to axotomy alone ($P < 0.05$; **Fig. 28B**). The combinatorial treatment significantly elevated print length at the earlier time-points of 2, 4, 7, and 9 days post-axotomy relative to axotomy alone ($P < 0.05$; **Fig. 28C**), but demonstrated no consistent difference compared to axotomized animals throughout the rest of the time-course. Animals treated with ES and TP demonstrated no supplementary effect relative to either treatment alone throughout the time-course (**Fig. 28D**).

ii. TP increases print width.

Overall, we observed a main effect in the RH print width between treatment groups ($F_{4,5767} = 15.34$; $P < 0.0001$), time ($F_{26,5767} = 115.02$; $P < 0.0001$), as well as an interaction between treatment x time ($F_{104,5767} = 18.41$; $P < 0.0001$; **Fig. 29**). Print width was significantly decreased at 2 days post-axotomy

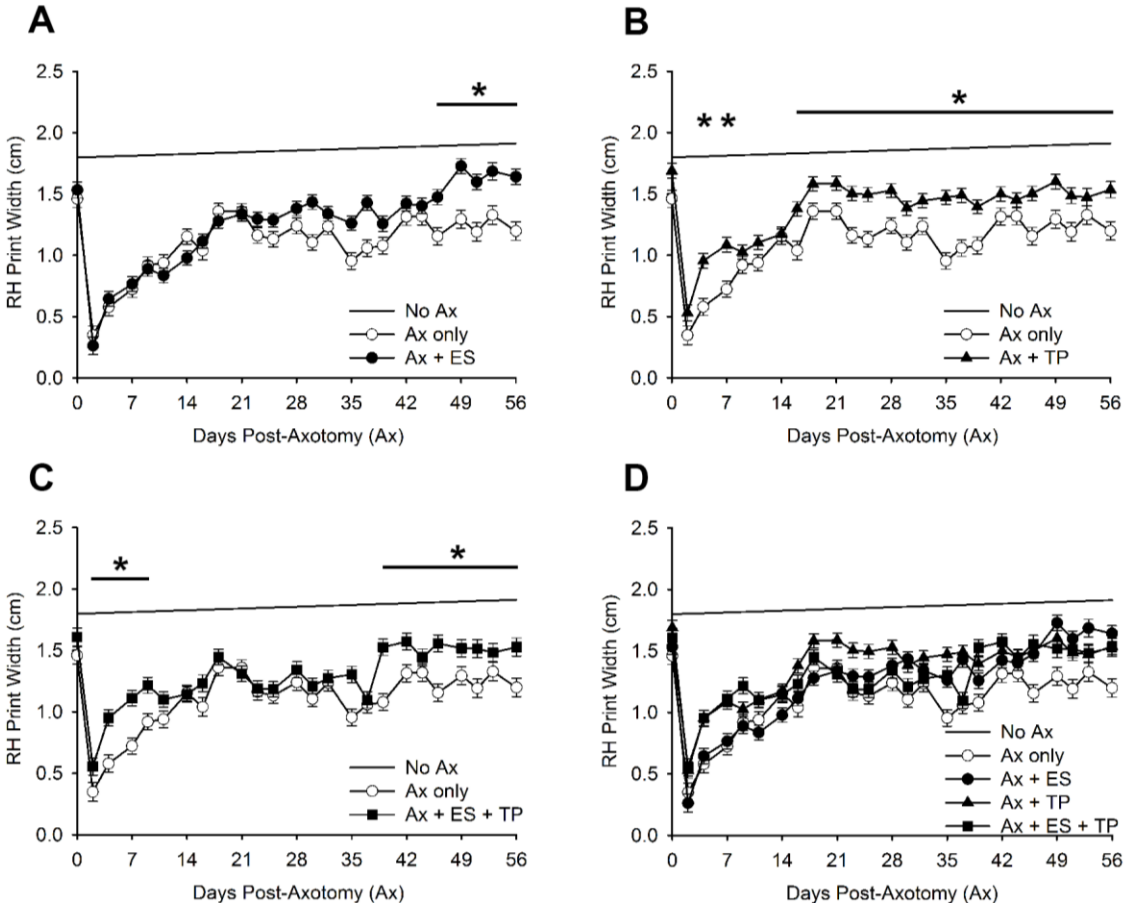

Fig. 29: Effect of ES and TP on RH print width.

Shown is a time-course of the effect of ES and TP on right hind (RH) print width post-axotomy (Ax). Uninjured animals (solid line) represent a longitudinal baseline throughout the study. Control animals, which were axotomized but left untreated (Ax only), are compared to axotomized animals receiving only ES (A), only TP (B), or the combination of both ES and TP (C). (D) Represents the comparison between the combination of ES and TP relative to both ES and TP alone. Data is represented as RH print width (cm) ± SEM. Statistical significance was evaluated using linear mixed effects models. Each model included random intercepts and slopes for speed and weight for each animal. Post hoc pairwise multiple comparisons were performed within time points using the Holm-Šidák method when group differences were detected. * represents a significant difference between ES, TP, or ES and TP compared to axotomy alone, at $P < 0.05$. # represents a significant difference between ES and TP relative to both ES and TP alone, at $P < 0.05$. For each experimental group, n = 5-6 animals/time-point.

in injured, untreated animals relative to uninjured control animals (0.35 ± 0.08 cm and 1.85 ± 0.09 cm, respectively; $P < 0.001$; **Fig. 29**) and never returned to that observed in uninjured animals. Animals treated with ES demonstrated no consistent significant pattern for print width until 46 days post-axotomy at which point levels were significantly elevated relative to that observed in uninjured animals ($P < 0.05$; **Fig. 29A**). In contrast, animals treated with TP demonstrated a significantly higher print width at 4 and 7 days post-axotomy relative to injured, untreated animals (TP: 0.95 ± 0.06 cm and 1.09 ± 0.06 cm, respectively; Ax only: 0.58 ± 0.07 cm and 0.72 ± 0.07 cm, respectively; $P < 0.001$; **Fig. 29B**). Similarly, TP-treated animals demonstrated a significantly elevated print width beginning at 16 days post-axotomy for the remainder of the time-course relative to axotomy alone ($P < 0.001$). The combinatorial treatment significantly elevated print width at the earlier time-points of 2, 4, 7, and 9 days post-axotomy relative to axotomy alone ($P < 0.05$). At 35 days post-axotomy, animals treated with ES and TP exhibited a consistent significant elevation relative to axotomy alone that was present through 56 days post-axotomy ($P < 0.05$; **Fig. 29C**). The combinatorial treatment had no supplementary benefit to either treatment alone (**Fig. 29D**).

iii. TP increases print area.

When investigating the effect of treatment on RH print area we observed a main effect between treatment groups ($F_{4,5767} = 12.64$; $P < 0.0001$), time ($F_{26,5767} = 76.29$; $P < 0.0001$), as well as an interaction between treatment x time ($F_{104,5767} = 18.52$; $P < 0.0001$; **Fig. 30**). Print area was significantly decreased in injured,

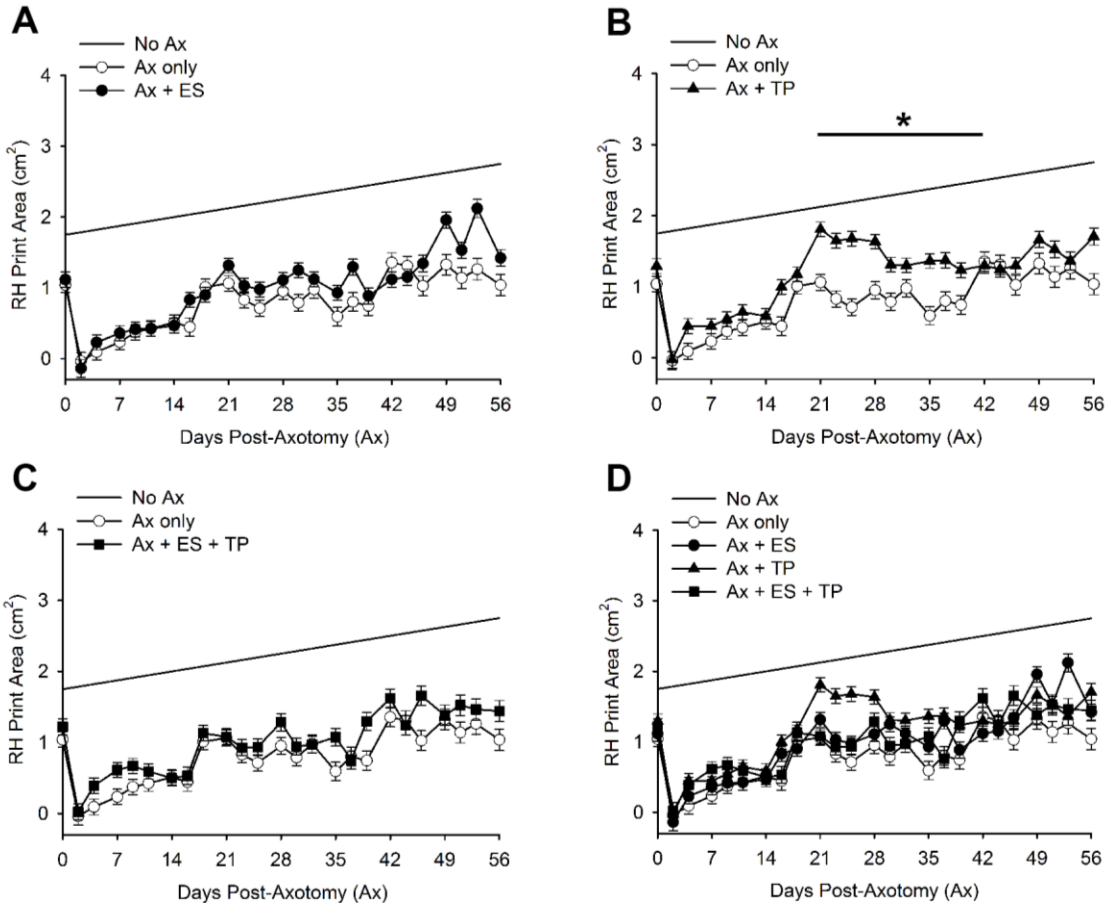

Fig. 30: Effect of ES and TP on RH print area.

Shown is a time-course of the effect of ES and TP on right hind (RH) print area post-axotomy (Ax). Uninjured animals (solid line) represent a longitudinal baseline throughout the study. Control animals, which were axotomized but left untreated (Ax only), are compared to axotomized animals receiving only ES (A), only TP (B), or the combination of both ES and TP (C). (D) Represents the comparison between the combination of ES and TP relative to both ES and TP alone. Data is represented as RH print area (cm^2) ± SEM. Statistical significance was evaluated using linear mixed effects models. Each model included random intercepts and slopes for speed and weight for each animal. Post hoc pairwise multiple comparisons were performed within time points using the Holm-Šidák method when group differences were detected. * represents a significant difference between ES, TP, or ES and TP compared to axotomy alone, at $P < 0.05$. # represents a significant difference between ES and TP relative to both ES and TP alone, at $P < 0.05$. For each experimental group, n = 5-6 animals/time-point.

untreated animals at 2 days post-axotomy and never returned to levels observed in that of uninjured control animals (-0.04 ± 0.13 cm^2 and 1.93 ± 0.15 cm^2, respectively; $P < 0.001$; **Fig. 30**). Animals treated with ES demonstrated no consistent significant pattern throughout the observed time-course (**Fig. 30A**). In contrast, animals treated with TP demonstrated a significantly higher print area at 21 days post-axotomy until 42 days post-axotomy relative to injured, untreated animals ($P < 0.05$; **Fig. 30B**). Similar to ES-treated animals, animals treated with ES plus TP demonstrated no consistent difference in print area relative to animals receiving axotomy alone (**Fig. 30C**). The combinatorial treatment had no supplementary benefit to either treatment alone (**Fig. 30D**).

iv. ES only and TP only increase swing

Statistical analysis revealed a main effect between treatment groups ($F_{4,5730} = 10.36$; $P < 0.0001$), time ($F_{26,5730} = 67.00$; $P < 0.0001$), as well as an interaction between treatment x time ($F_{104,5730} = 9.11$; $P < 0.0001$; **Fig. 31**) with respect to RH swing. Swing was significantly increased in injured, untreated animals at 2 days post-axotomy relative to uninjured control animals (0.31 ± 0.010 s and 0.10 ± 0.007 s, respectively; $P < 0.001$; **Fig. 31**). At 30 days post-axotomy, swing of axotomized animals returned to values similar to that of uninjured animals (0.14 ± .006 s and 0.11 ± 0.008 s, respectively) where they were maintained for the remainder of the time-course. ES of the sciatic nerve significantly decreased swing as early as 2 days post-axotomy relative to injury alone (ES: 0.18 ± 0.009 s; $P < 0.001$; **Fig. 31A**) but returned to values similar to

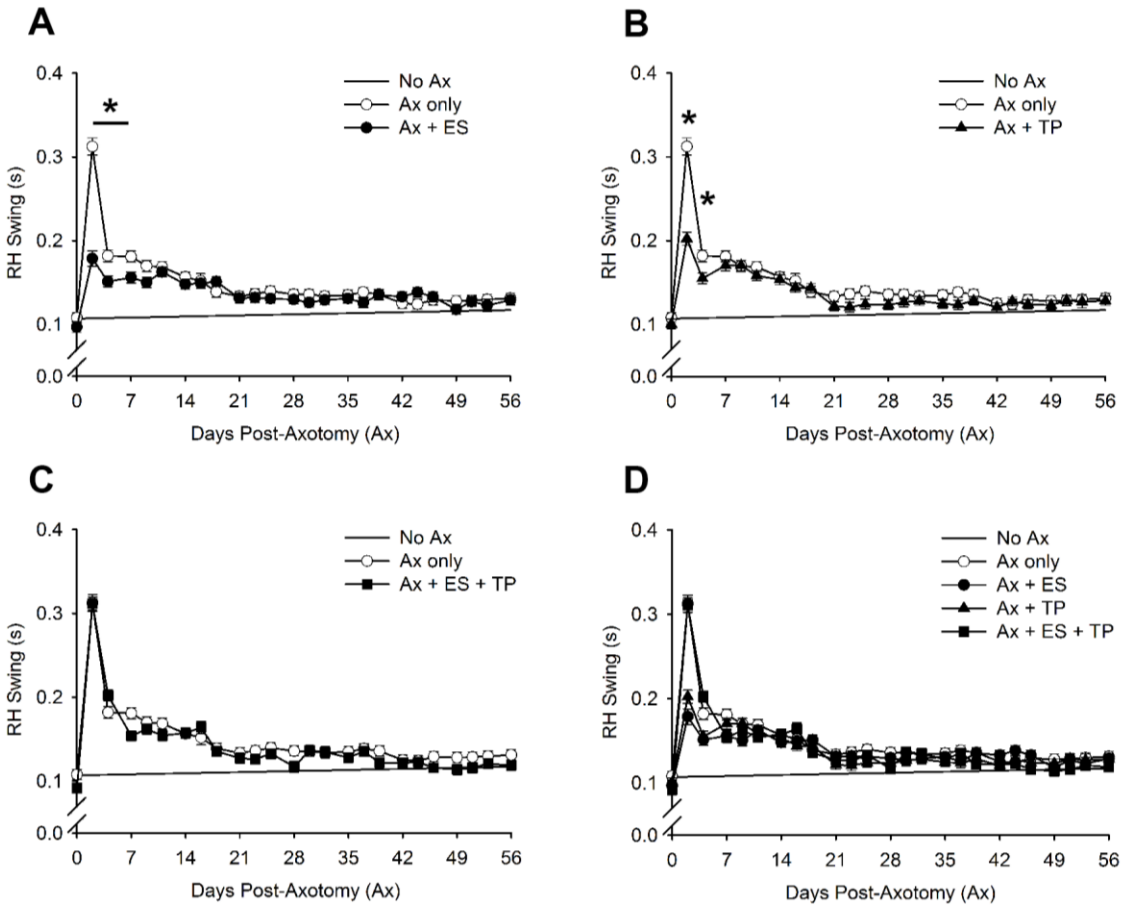

Fig. 31: Effect of ES and TP on RH swing.

Shown is a time-course of the effect of ES and TP on right hind (RH) swing post-axotomy (Ax). Uninjured animals (solid line) represent a longitudinal baseline throughout the study. Control animals, which were axotomized but left untreated (Ax only), are compared to axotomized animals receiving only ES (A), only TP (B), or the combination of both ES and TP (C). (D) Represents the comparison between the combination of ES and TP relative to both ES and TP alone. Data is represented as RH swing (s) ± SEM. Statistical significance was evaluated using linear mixed effects models. Each model included random intercepts and slopes for speed and weight for each animal. Post hoc pairwise multiple comparisons were performed within time points using the Holm-Šidák method when group differences were detected. * represents a significant difference between ES, TP, or ES and TP compared to axotomy alone, at $P < 0.05$. # represents a significant difference between ES and TP relative to both ES and TP alone, at $P < 0.05$. For each experimental group, n = 5-6 animals/time-point.

that of injury alone by 9 days post-axotomy. Animals receiving systemic TP demonstrated a significant decrease in swing at 2 and 4 days post-axotomy (0.20 ± .008 s, 0.15 ± .007 s, respectively) relative to injured, untreated animals ($P < 0.05$; **Fig. 31B**). There was no consistent difference in swing of animals that received reatment with the combination of ES plus TP compared to axotomy alone throughout the time-course (**Fig. 31C**). Therefore, ES and TP had no supplementary effect in swing relative to either treatment alone (**Fig. 31D**).

v. TP increases swing speed

Overall, we observed a main effect between treatment groups ($F_{4,5730} = 11.07$; $P < 0.0001$), time ($F_{26,5730} = 186.18$; $P < 0.0001$), as well as an interaction between treatment x time ($F_{104,5730} = 18.89$; $P < 0.0001$; **Fig. 32**) with respect to RH swing speed. Axotomy alone significantly decreased swing speed at 2 days post-axotomy relative to uninjured animals (94.8 ± 4.5 cm/s and 170.0 ± 4.0 cm/s, respectively; $P < 0.001$; **Fig. 32**) where it was maintained until 42 days post-axotomy. Animals treated with ES only had similar swing speed throughout the observed time-course compared to axotomized animals (**Fig. 32A**). In contrast, TP-treated animals demonstrated a significantly higher swing speed between 14 and 28 days post-axotomy relative to axotomized, untreated animals albeit this trend was not consistent ($P < 0.05$; **Fig. 32B**). Similar to treatment with ES, the combination of ES and TP did not consistently alter swing speed relative to axotomy alone (**Fig. 32C**). Thus, the combination of ES and TP demonstrated

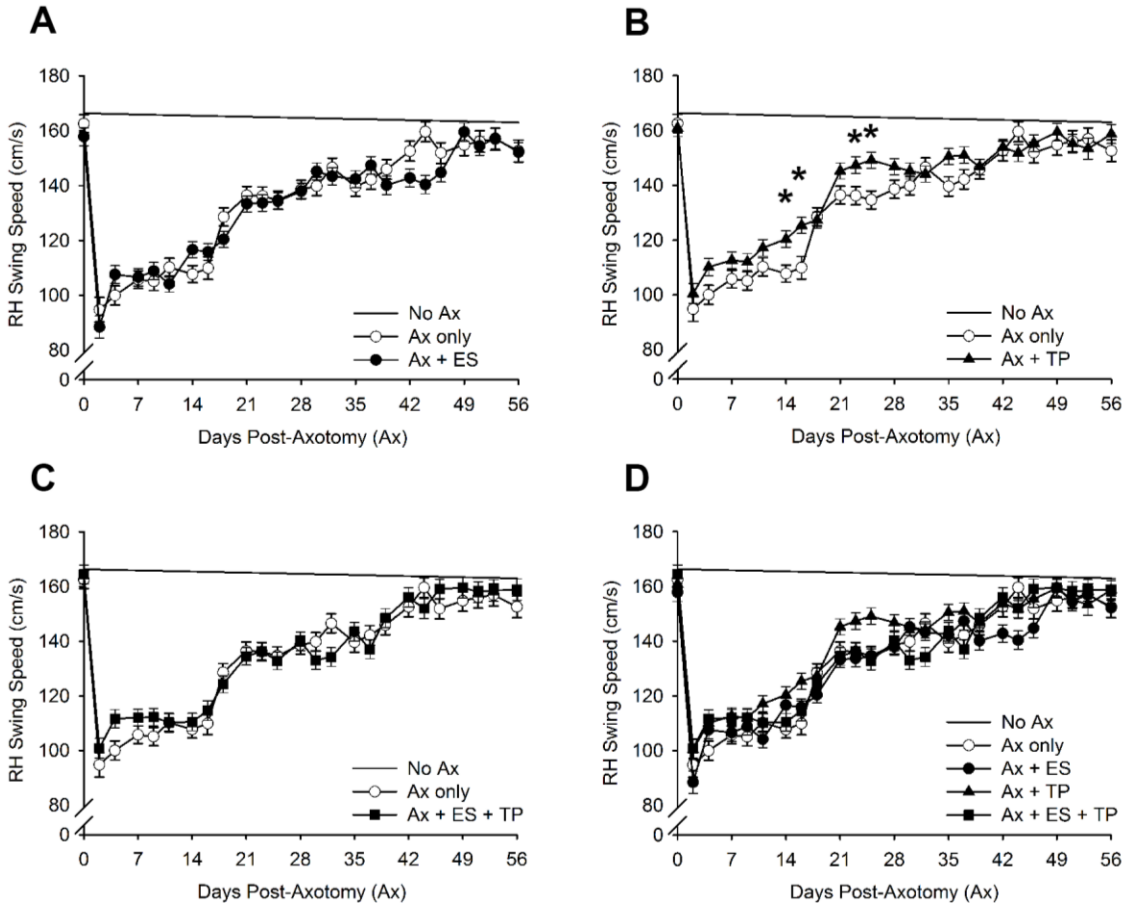

Fig. 32: Effect of ES and TP on RH swing speed.

Shown is a time-course of the effect of ES and TP on right hind (RH) swing speed post-axotomy (Ax). Uninjured animals (solid line) represent a longitudinal baseline throughout the study. Control animals, which were axotomized but left untreated (Ax only), are compared to axotomized animals receiving only ES (A), only TP (B), or the combination of both ES and TP (C). (D) Represents the comparison between the combination of ES and TP relative to both ES and TP alone. Data is represented as RH swing speed (cm/s) ± SEM. Statistical significance was evaluated using linear mixed effects models. Each model included random intercepts and slopes for speed and weight for each animal. Post hoc pairwise multiple comparisons were performed within time points using the Holm-Šidák method when group differences were detected. * represents a significant difference between ES, TP, or ES and TP compared to axotomy alone, at $P < 0.05$. # represents a significant difference between ES and TP relative to both ES and TP alone, at $P < 0.05$. For each experimental group, n = 5-6 animals/time-point.

no supplementary effect in swing speed compared to either treatment alone (**Fig. 32D**).

vi. ES only and TP only decrease stride length

Evaluation of RH stride length did not reveal a main effect of treatment group ($F_{4,5730}$ = 1.62; P = 0.1651) but did reveal an effect of post-operative time ($F_{26,5730}$ = 8.14; P < 0.0001) and an interaction between treatment x post-operative time ($F_{104,5730}$ = 2.89; P < 0.0001; **Fig. 33)**. Injury alone significantly increased stride length at 2 days post-axotomy relative to uninjured animals (20.8 ± 0.6 cm and 17.3 ± 0.5 cm, respectively; P < 0.001; **Fig. 33**) but was similar for the remainder of the observed time-course. In contrast, ES-treated animals demonstrated a significant decrease in stride length at 2 days post-axotomy animals (13.7 ± 0.5 cm) compared to axotomy alone (P < 0.001; **Fig. 33A**) but was similar to axotomy alone at all other time-points. Similar to ES-treated animals, systemic treatment of TP significantly decreased stride length only at 2 days post-axotomy (15.9 ± 0.5 cm) relative to injury alone (P < 0.001; **Fig. 33B**). Animals treated with both ES and systemic TP demonstrated a significant increase in stride length only at 4 days post-axotomy relative to axotomy alone (18.2 ± 0.4 cm and 16.2 ± 0.5 cm, respectively; P < 0.05; **Fig. 33C**). The combination of ES plus TP had no additional benefit to either treatment alone throughout the time-course (**Fig. 33D**).

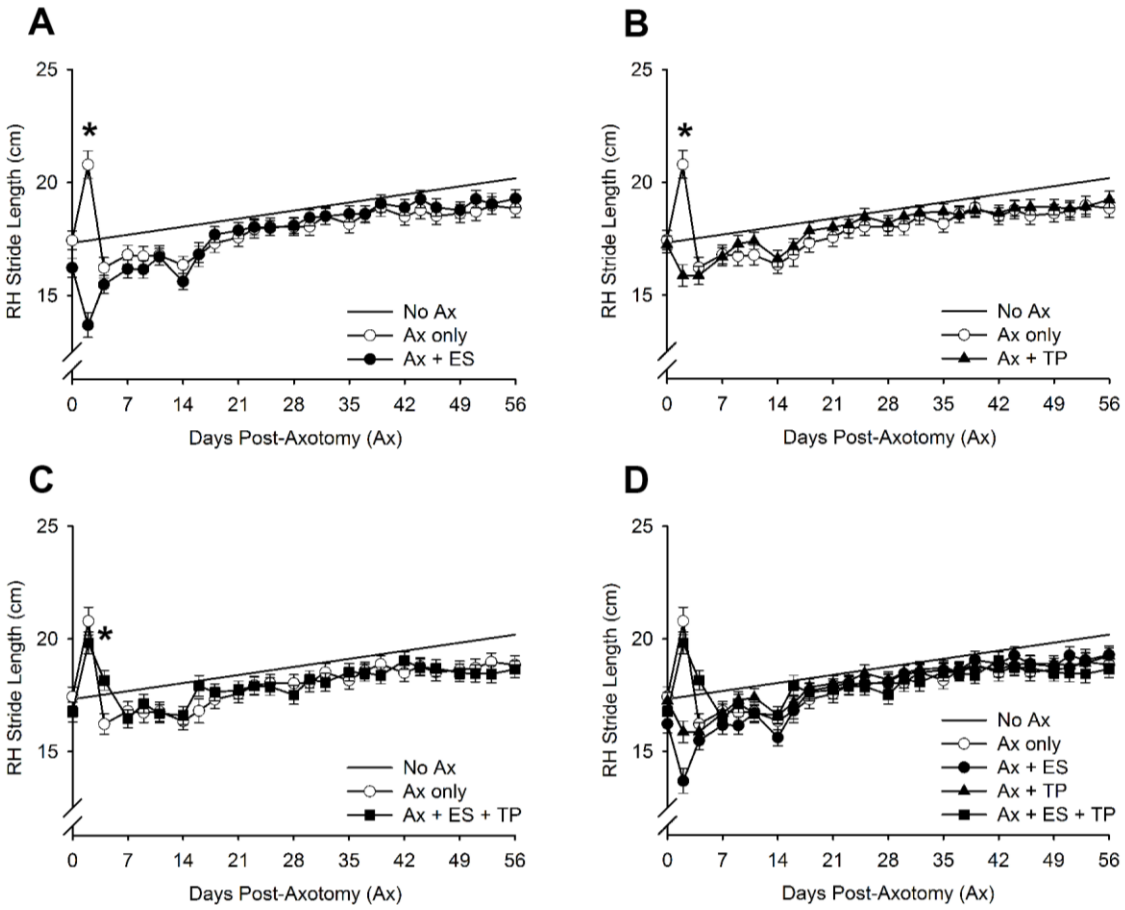

Fig. 33: Effect of ES and TP on RH stride length.

Shown is a time-course of the effect of ES and TP on right hind (RH) stride length post-axotomy (Ax). Uninjured animals (solid line) represent a longitudinal baseline throughout the study. Control animals, which were axotomized but left untreated (Ax only), are compared to axotomized animals receiving only ES (A), only TP (B), or the combination of both ES and TP (C). (D) Represents the comparison between the combination of ES and TP relative to both ES and TP alone. Data is represented as RH stride length (cm) ± SEM. Statistical significance was evaluated using linear mixed effects models. Each model included random intercepts and slopes for speed and weight for each animal. Post hoc pairwise multiple comparisons were performed within time points using the Holm-Šidák method when group differences were detected. * represents a significant difference between ES, TP, or ES and TP compared to axotomy alone, at $P < 0.05$. # represents a significant difference between ES and TP relative to both ES and TP alone, at $P < 0.05$. For each experimental group, n = 5-6 animals/time-point.

vii. ES only and TP only decrease step cycle

In the current study, the extended time course of RH step cycle revealed a main effect between treatment groups ($F_{4,5730} = 4.09$; $P = 0.0026$), time ($F_{26,5730} = 6.83$; $P < 0.0001$), as well as an interaction between treatment x time ($F_{104,5730} = 4.15$; $P < 0.0001$; **Fig. 34**). Injury alone significantly increased step cycle at 2 days post-axotomy relative to uninjured animals (0.32 ± 0.01 s and 0.23 ± 0.008 s, respectively; $P < 0.001$; **Fig. 34**) but was similar for the remainder of the observed time-course. In contrast, ES-treated animals demonstrated a significant decrease in step cycle at 2 days post-axotomy (0.17 ± 0.01 s) compared to axotomy alone ($P < 0.001$; **Fig. 34A**) but was similar to axotomy alone at all other time-points. Similar to ES-treated animals, systemic treatment of TP significantly decreased step cycle only at 2 days post-axotomy (0.20 ± 0.008 s) relative to injury alone ($P < 0.001$; **Fig. 34B**). Animals treated with both ES and systemic TP demonstrated a significant increase in step cycle only at 4 days post-axotomy relative to axotomy alone (0.25 ± 0.007 s and 0.21 ± 0.007 s, respectively; $P < 0.001$; **Fig. 34C**). The combination of ES plus TP had no additional benefit to either treatment alone throughout the time-course (**Fig. 34D**).

viii. TP increases duty cycle

Statistical analysis revealed a main effect between treatment groups ($F_{4,5730} = 14.39$; $P < 0.0001$), time ($F_{26,5730} = 345.63$; $P < 0.0001$), as well as an interaction between treatment x time ($F_{104,5730} = 30.51$; $P < 0.0001$; **Fig. 35**) with

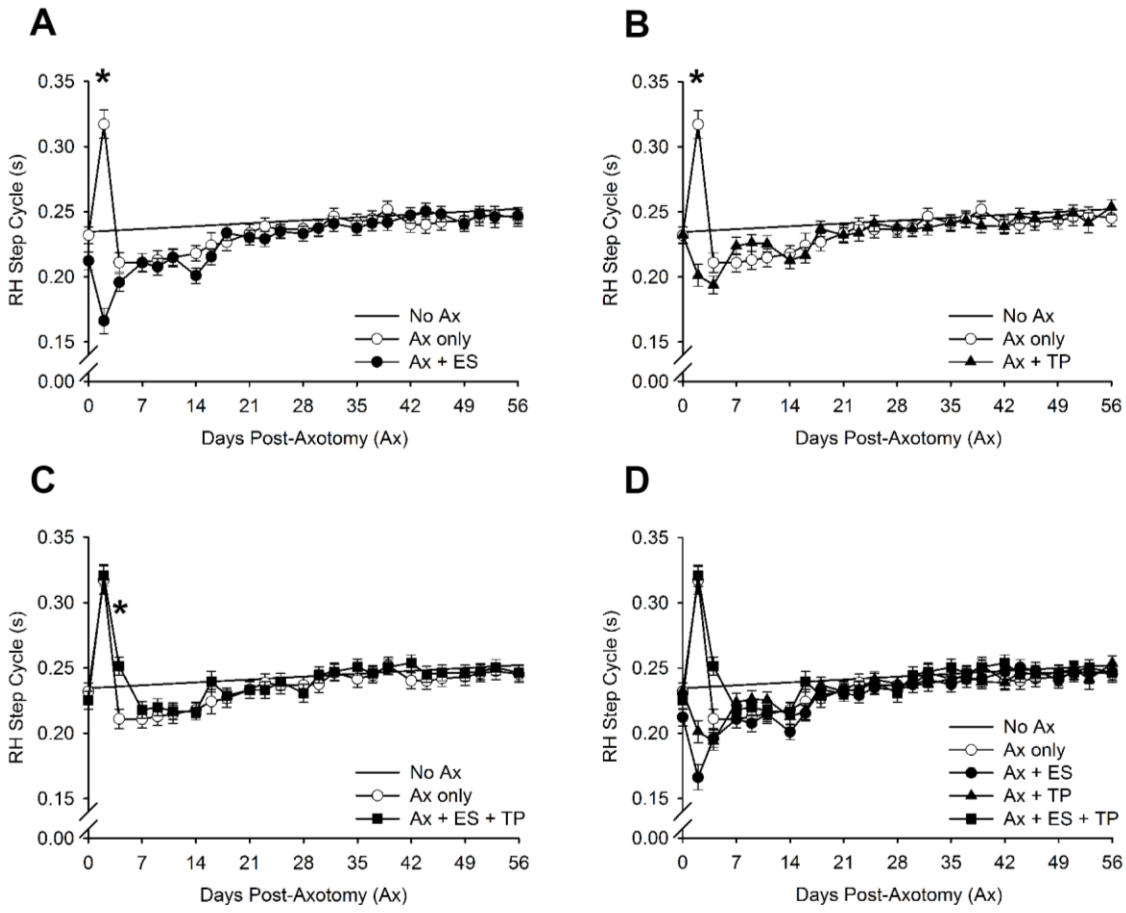

Fig. 34: Effect of ES and TP on RH step cycle.

Shown is a time-course of the effect of ES and TP on right hind (RH) step cycle post-axotomy (Ax). Uninjured animals (solid line) represent a longitudinal baseline throughout the study. Control animals, which were axotomized but left untreated (Ax only), are compared to axotomized animals receiving only ES (A), only TP (B), or the combination of both ES and TP (C). (D) Represents the comparison between the combination of ES and TP relative to both ES and TP alone. Data is represented as RH step cycle (s) ± SEM. Statistical significance was evaluated using linear mixed effects models. Each model included random intercepts and slopes for speed and weight for each animal. Post hoc pairwise multiple comparisons were performed within time points using the Holm-Šidák method when group differences were detected. * represents a significant difference between ES, TP, or ES and TP compared to axotomy alone, at $P < 0.05$. # represents a significant difference between ES and TP relative to both ES and TP alone, at $P < 0.05$. For each experimental group, n = 5-6 animals/time-point.

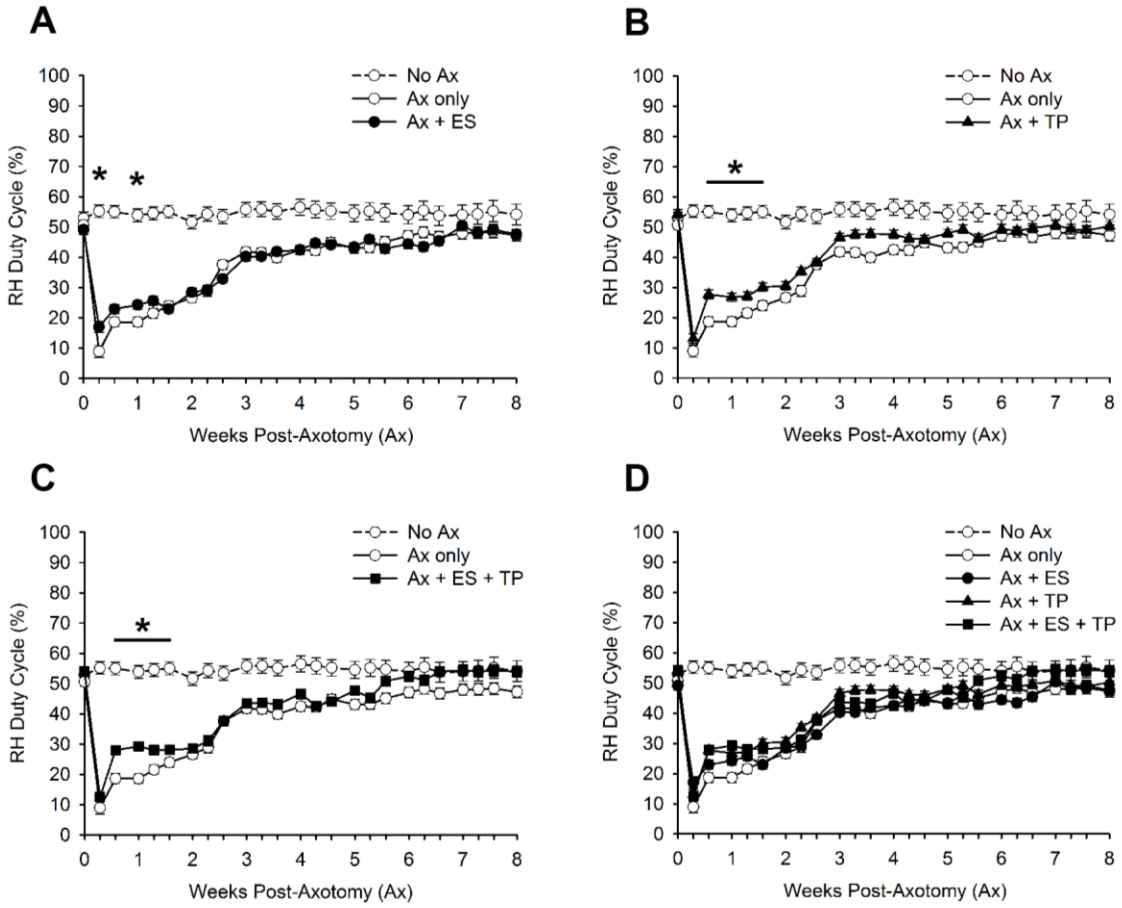

Fig. 35: Effect of ES and TP on RH duty cycle.

Shown is a time-course of the effect of ES and TP on right hind (RH) duty cycle post-axotomy (Ax). Uninjured animals (solid line) represent a longitudinal baseline throughout the study. Control animals, which were axotomized but left untreated (Ax only), are compared to axotomized animals receiving only ES (A), only TP (B), or the combination of both ES and TP (C). (D) Represents the comparison between the combination of ES and TP relative to both ES and TP alone. Data is represented as RH duty cycle (%) ± SEM. Statistical significance was evaluated using linear mixed effects models. Each model included random intercepts and slopes for speed and weight for each animal. Post hoc pairwise multiple comparisons were performed within time points using the Holm-Šidák method when group differences were detected. * represents a significant difference between ES, TP, or ES and TP compared to axotomy alone, at $P < 0.05$. # represents a significant difference between ES and TP relative to both ES and TP alone, at $P < 0.05$. For each experimental group, n = 5-6 animals/time-point.

respect to RH duty cycle. Duty cycle significantly decreased in injured, untreated animals at 2 days post-axotomy relative to uninjured control animals (8.9 ± 2.0% and 55.2 ± 2.0%, respectively; $P < 0.001$; **Fig. 35**). At 42 days post-axotomy, duty cycle of axotomized animals returned to values similar to that of uninjured animals and was maintained for the remaining time-points. ES of the sciatic nerve significantly increased duty cycle at 2 and 7 days post-axotomy relative to injury alone ($P < 0.001$; **Fig. 35A**) but was similar at all other time-points. Animals receiving systemic TP demonstrated a significant increase in duty cycle at 4, 7, 9, and 11 days post-axotomy relative to injured, untreated animals ($P < 0.05$; **Fig. 35B**). Similar to TP treatment, treatment with the combination of ES plus TP significantly increased duty cycle at 4, 7, 9, and 11 days post-axotomy compared to axotomy alone throughout the time-course ($P < 0.05$; **Fig. 35C**). However, the combination of ES and TP had no supplementary effect in duty cycle relative to either treatment alone (**Fig. 35D**).

ix. ES and TP increase max contact max intensity mean

When investigating the effect of treatment on RH max contact max intensity mean we observed a main effect between treatment groups ($F_{4,5767} = 7.48$; $P < 0.0001$), time ($F_{26,5767} = 109.30$; $P < 0.0001$), as well as an interaction between treatment x time ($F_{104,5767} = 16.13$; $P < 0.0001$; **Fig. 36**). Max contact max intensity mean was significantly decreased in injured, untreated animals at 2 days post-axotomy relative to uninjured control animals (53.7 ± 10.1 and 206.4 ± 11.6, respectively; $P < 0.001$; **Fig. 36**) and did not return to that observed in

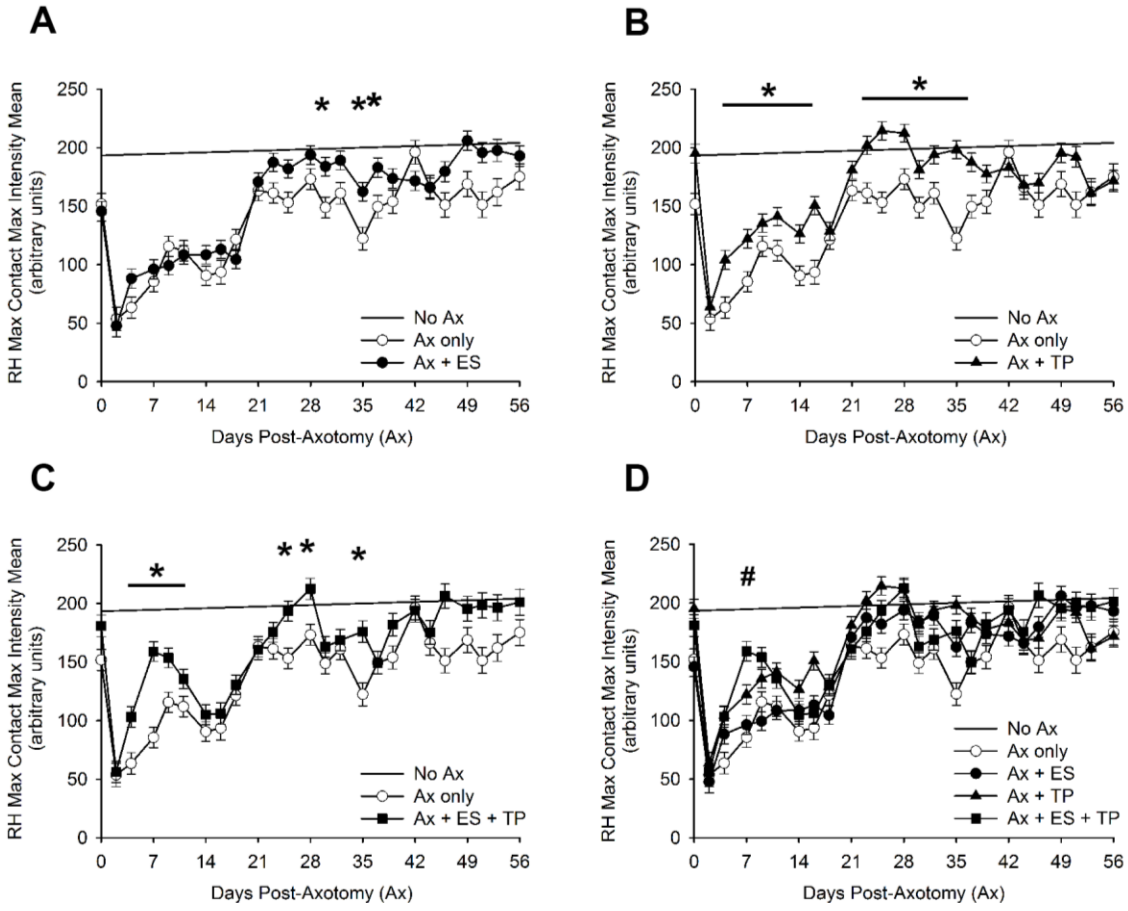

Fig. 36: Effect of ES and TP on RH max contact max intensity mean.

Shown is a time-course of the effect of ES and TP on RH max contact max intensity mean post-axotomy (Ax). Uninjured animals (solid line) represent a longitudinal baseline throughout the study. Control animals, which were axotomized but left untreated (Ax only), are compared to axotomized animals receiving only ES (A), only TP (B), or the combination of both ES and TP (C). (D) Represents the comparison between the combination of ES and TP relative to both ES and TP alone. Data is represented as intensity (arbitrary units) ± SEM. Statistical significance was evaluated using linear mixed effects models. Each model included random intercepts and slopes for speed and weight for each animal. Post hoc pairwise multiple comparisons were performed within time points using the Holm-Šidák method when group differences were detected. * represents a significant difference between ES, TP, or ES and TP compared to axotomy alone, at $P < 0.05$. # represents a significant difference between ES and TP relative to both ES and TP alone, at $P < 0.05$. For each experimental group, n = 5-6 animals/time-point.

uninjured animals, with the exception of 42 days post-axotomy. Animals treated with ES demonstrated a significantly elevated max contact max intensity mean at some of the later time-points between 28 and 42 days post-axotomy, but this effect was not consistently maintained ($P < 0.05$; **Fig. 36A**). In contrast, animals treated with TP demonstrated a significantly higher max contact max intensity mean as early as 4 days post-axotomy relative to axotmy alone that lasted for several weeks ($P < 0.05$; **Fig. 36B**). Animals treated with ES plus TP demonstrated a significant increase in max contact max intensity mean 4 days post-axotomy relative to animals receiving axotomy alone ($P < 0.05$; **Fig. 36C**) that lasted until 11 days post-axotomy. This effect returned at the later time-point of 25 days post-axotomy, but was not consistently maintained. The combinatorial treatment significantly increased max contact max intensity mean at 7 days post-axotomy relative to either treatment alone ($P < 0.05$; **Fig. 36D**) but was similar at all other time-points.

b. Non paw-related parameters

i. ES only and TP only significantly increase SFI

Evaluation of sciatic functional index (SFI) score did not reveal a main effect of treatment group ($F_{3,1429} = 0.91$; $P = 0.4336$) but did reveal an effect of post-operative time ($F_{7,1429} = 106.44$; $P < 0.0001$) and an interaction between treatment x post-operative time ($F_{21,1429} = 3.91$; $P < 0.0001$; **Fig. 37**). Axotomized animals demonstrated a recovery in SFI over the extended 8 week time-course

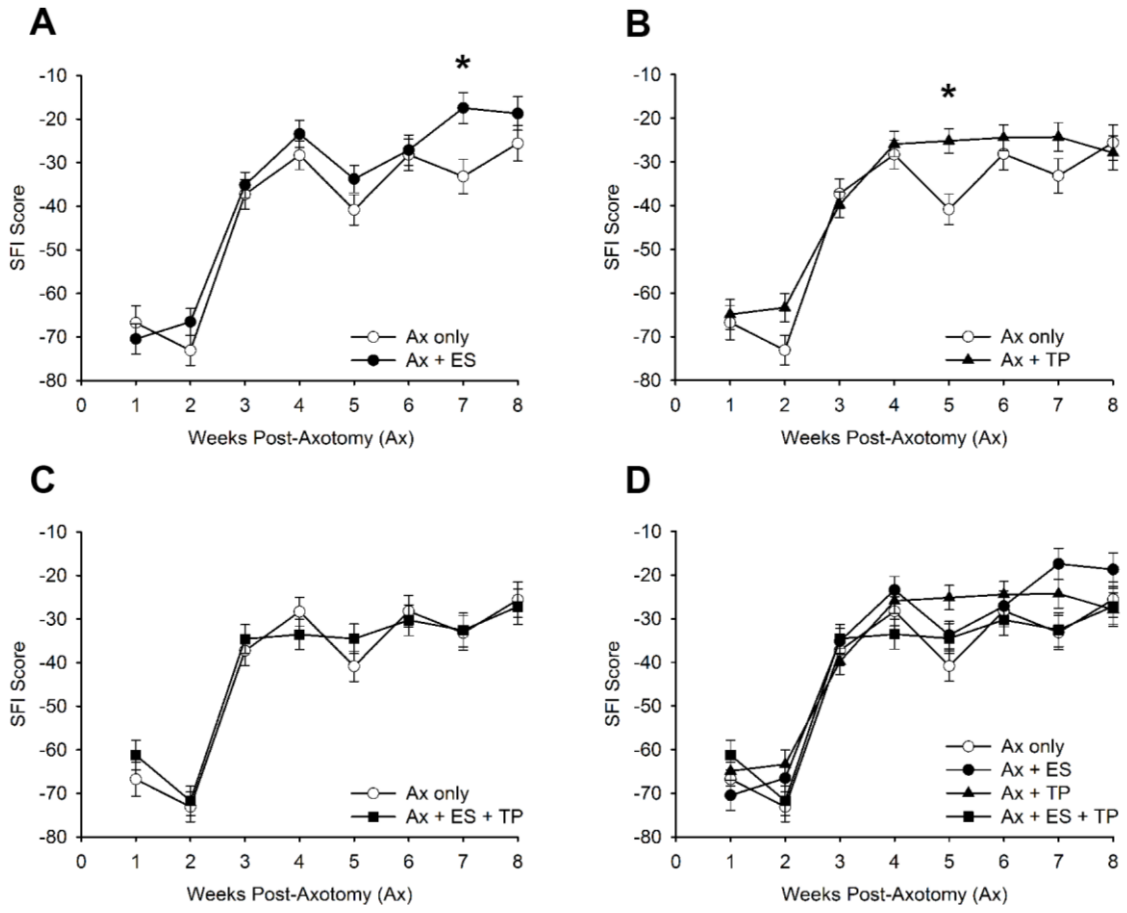

Fig. 37: Effect of ES and TP on SFI.

Shown is a time-course of the effect of ES and TP on SFI score post-axotomy (Ax). Control animals, which were axotomized but left untreated (Ax only), are compared to axotomized animals receiving only ES (A), only TP (B), or the combination of both ES and TP (C). (D) Represents the comparison between the combination of ES and TP relative to both ES and TP alone. Data is represented as SFI score ± SEM. Statistical significance was evaluated using linear mixed effects models. Each model included random intercepts and slopes for speed and weight for each animal. Post hoc pairwise multiple comparisons were performed within time points using the Holm-Šidák method when group differences were detected. * represents a significant difference between ES, TP, or ES and TP compared to axotomy alone, at $P < 0.05$. # represents a significant difference between ES and TP relative to both ES and TP alone, at $P < 0.05$. For each experimental group, n = 5-6 animals/time-point.

(**Fig. 37**). ES treatment significantly elevated SFI levels only at 7 weeks post-axotomy relative to axotomy alone (-17.4 ± 3.6 and -33.2 ± 3.9; $P < 0.05$; **Fig. 37A**). TP-treated animals demonstrated a significant increase in SFI only at 5 weeks post-axotomy relative to axotomy alone (-25.2 ± 2.8 and -40.8 ± 3.5; $P < 0.05$; **Fig. 37B**). The combinatorial treatment had no effect on PFI relative to axotomy alone (**Fig. 37C**). Analysis revealed the combination treatment of ES plus TP did not have any supplementary benefit to either treatment alone (**Fig. 37D**).

ii. ES only and TP only significantly increase PFI

Overall, we observed a main effect of treatment group ($F_{3,1429} = 2.68$; $P = 0.0459$), time ($F_{7,1429} = 47.32$; $P < 0.0001$) as well as an interaction between treatment x post-operative time ($F_{21,1429} = 8.17$; $P < 0.0001$; **Fig. 38**) with respect to peroneal functional index (PFI) score. Axotomy alone demonstrated a recovery in PFI over the extended 8 week time-course (**Fig. 38**). ES treatment significantly elevated SFI levels at the later time-points of 5, 6, and 7 weeks post-axotomy ($P < 0.05$) relative to axotomy alone (Ax + ES: -43.6 ± 10.5, -21.2 ± 11.3, and -8.7 ± 11.5; Ax only: -97.8 ± 12.8, -73.4 ± 12.7, and -65.0 ± 13.1; $P < 0.05$; **Fig. 38A**). TP-treated animals demonstrated a significant increase in SFI at 1, 5, and 6 weeks post-axotomy (-99.2 ± 14.5, -40.1 ± 10.6, and -32.3 ± 10.6) relative to axotomy alone (Ax only at 1 week post-axotomy: -184.3 ± 19.2; $P < 0.05$; **Fig. 38B**). The combinatorial treatment had no effect on PFI relative to axotomy alone (**Fig. 38**). Analysis revealed the PFI score for animals treated with both ES plus

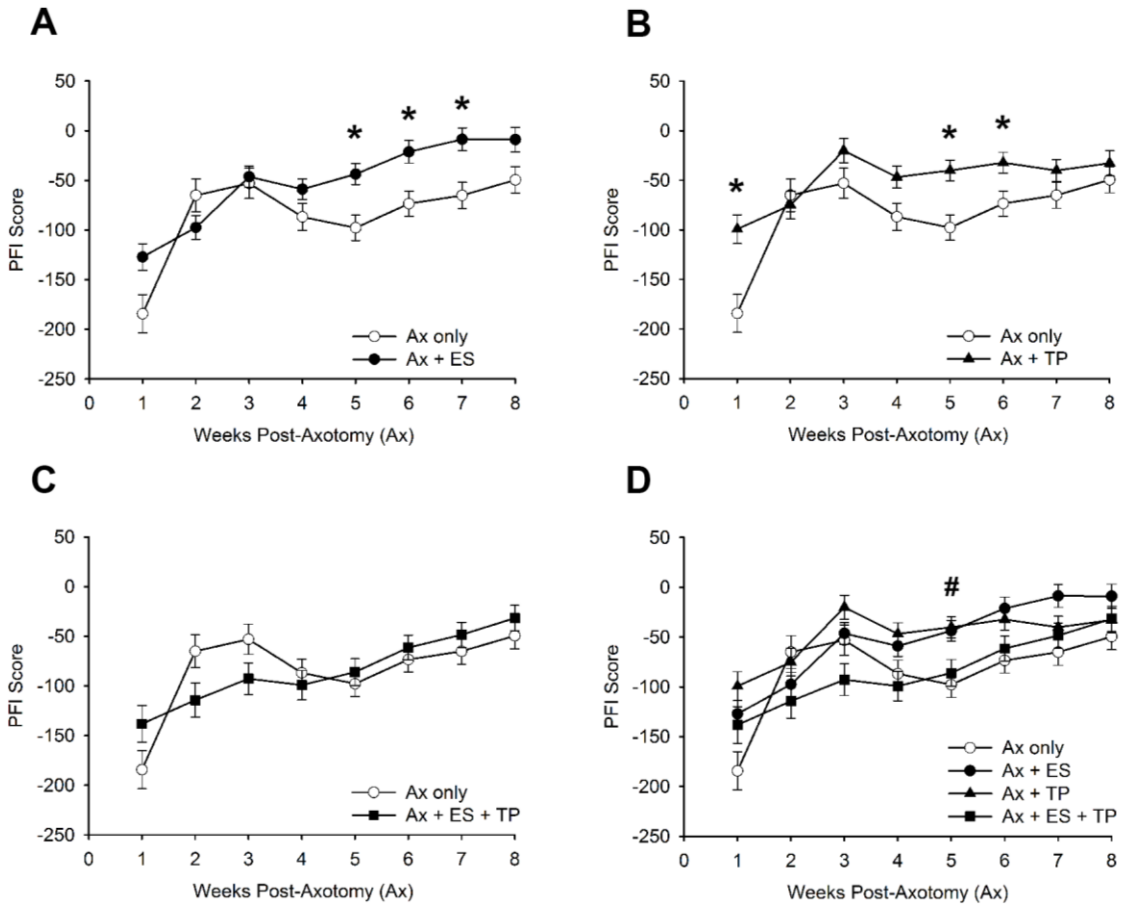

Fig. 38: Effect of ES and TP on PFI.

Shown is a time-course of the effect of ES and TP on PFI score post-axotomy (Ax). Control animals, which were axotomized but left untreated (Ax only), are compared to axotomized animals receiving only ES (A), only TP (B), or the combination of both ES and TP (C). (D) Represents the comparison between the combination of ES and TP relative to both ES and TP alone. Data is represented as PFI score ± SEM. Statistical significance was evaluated using linear mixed effects models. Each model included random intercepts and slopes for speed and weight for each animal. Post hoc pairwise multiple comparisons were performed within time points using the Holm-Šidák method when group differences were detected. * represents a significant difference between ES, TP, or ES and TP compared to axotomy alone, at $P < 0.05$. # represents a significant difference between ES and TP relative to both ES and TP alone, at $P < 0.05$. For each experimental group, n = 5-6 animals/time-point.

TP was significantly lower at 5 weeks post-axotomy (-85.9 ± 13.4) relative to either treatment alone ($P < 0.05$; **Fig. 38D**).

iii. ES only and TP only significantly increase TFI

Overall, we did not observe a main effect between treatment groups ($F_{3,1429} = 1.73$; $P = 0.1591$; **Fig. 39**); however, analysis revealed an effect with time ($F_{7,1429} = 143.74$; $P < 0.0001$) and an interaction between treatment x post-operative time ($F_{21,1429} = 86.74$; $P < 0.0001$; **Fig. 39**) with respect to posterior tibial functional index (TFI) score. Axotomy alone demonstrated a recovery in TFI over the extended time-course of 1, 2, 3, 4, 5, 6, 7, and 8 weeks post-axotomy (**Fig. 39**). At 5 and 7 weeks post axotomy, treatment with either ES (-33.4 ± 3.3 and -18.3 ± 3.8, respectively; **Fig. 39A**) or TP (-25.9 ± 3.0 and -22.2 ± 3.4, respectively; **Fig. 39B**) significantly elevated TFI score relative to injury alone (-45.3 ± 3.6 and -35.5 ± 4.0, respectively, $P < 0.05$). In contrast, ES + TP animals were similar to axotomy alone at all observed time-points (**Fig. 39C**). At 4 weeks post-axotomy, the combination treatment of ES and TP (-39.7 ± 3.7) demonstrated a significantly lower TFI score than either treatment alone ($P < 0.05$; **Fig. 39D**).

iv. TP decreases BOS of front paws

We found an overall main effect between treatment groups ($F_{4,5863} = 3.00$; $P = 0.0173$), time ($F_{26,5863} = 8.70$; $P < 0.0001$), as well as an interaction between

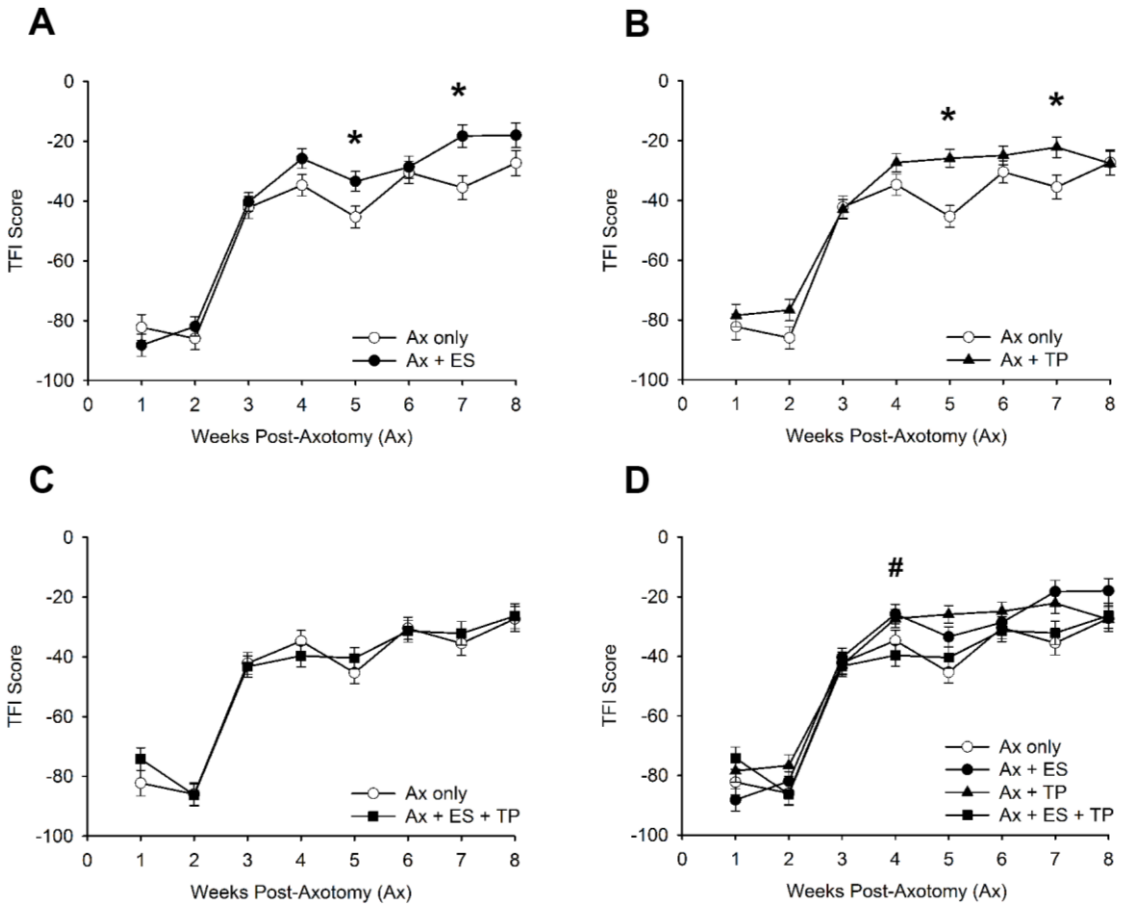

Fig. 39: Effect of ES and TP on TFI.

Shown is a time-course of the effect of ES and TP on TFI score post-axotomy (Ax). Control animals, which were axotomized but left untreated (Ax only), are compared to axotomized animals receiving only ES (A), only TP (B), or the combination of both ES and TP (C). (D) Represents the comparison between the combination of ES and TP relative to both ES and TP alone. Data is represented as TFI score ± SEM. Statistical significance was evaluated using linear mixed effects models. Each model included random intercepts and slopes for speed and weight for each animal. Post hoc pairwise multiple comparisons were performed within time points using the Holm-Šidák method when group differences were detected. * represents a significant difference between ES, TP, or ES and TP compared to axotomy alone, at $P < 0.05$. # represents a significant difference between ES and TP relative to both ES and TP alone, at $P < 0.05$. For each experimental group, n = 5-6 animals/time-point.

treatment x time ($F_{104,5863}$ = 3.12; P < 0.0001; **Fig. 40**) with respect to the base of support (BOS) of the front paws. Although the BOS of the front paws was significantly elevated at 2 days post-axotomy in axotomized animals relative to uninjured animals (2.18 ± 0.10 cm and 1.70 ± 0.15 cm, respectively) this effect was not consistent across all time-points (P < 0.05; **Fig. 40**). The BOS of the front paws of ES-treated animals was significantly lower at 21 and 23 days post-axotomy (1.67 ± 0.09 cm and 1.65 ± 0.10 cm, respectively) relative to axotomy alone (2.09 ± 0.10 cm and 2.06 ± 0.10 cm) but was similar at all other time-points (P < 0.05; **Fig. 40A**). In TP-treated animals, the BOS of the front paws was significantly decreased at multiple time-points between 7 and 14 days post-axotomy and 21 and 28 days post-axotomy relative to axotomy alone (P < 0.05; **Fig. 40B**). The combination of ES plus TP significantly decreased BOS of the front paws at 2 days post-axotomy, but this effect was not consistent across time (P < 0.05; **Fig. 40C**). In addition, the combination of both ES and TP demonstrated no additional benefit to either treatment alone throughout the time-course (**Fig. 40D**).

v. Treatment does not affect BOS of hind paws

We observed an overall main effect between treatment groups ($F_{4,5767}$ = 4.55; P = 0.0011), time ($F_{26,5767}$ = 137.50; P < 0.0001), as well as an interaction between treatment x time ($F_{104,5767}$ = 12.71; P < 0.0001; **Fig. 41**) with respect to the BOS of the hind paws. The BOS of the hind paws was significantly increased in injured, untreated animals at 28 days post-axotomy relative to uninjured

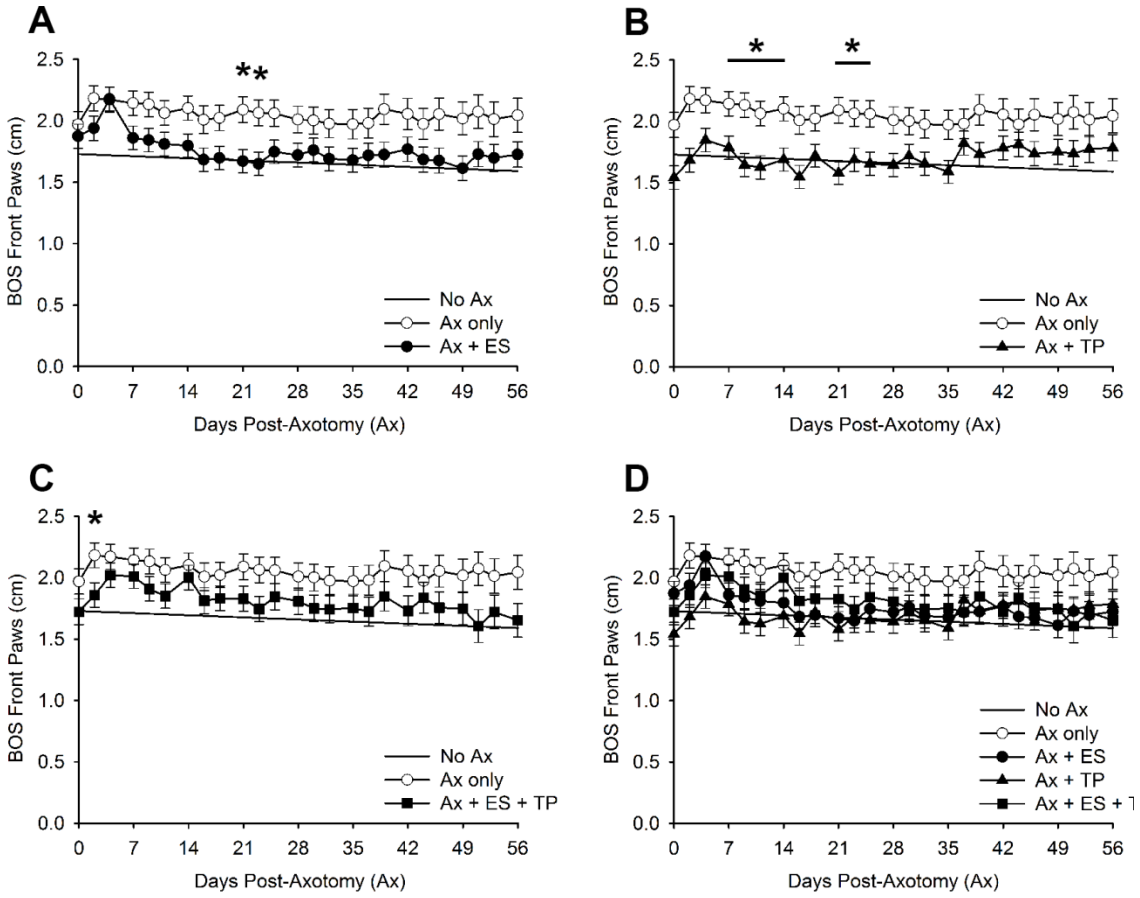

Fig. 40: Effect of ES and TP on BOS of the front paws.

Shown is a time-course of the effect of ES and TP on BOS of the front paws post-axotomy (Ax). Uninjured animals (solid line) represent a longitudinal baseline throughout the study. Control animals, which were axotomized but left untreated (Ax only), are compared to axotomized animals receiving only ES (A), only TP (B), or the combination of both ES and TP (C). (D) Represents the comparison between the combination of ES and TP relative to both ES and TP alone. Data is represented as BOS of the front paws (cm) ± SEM. Statistical significance was evaluated using linear mixed effects models. Each model included random intercepts and slopes for speed and weight for each animal. Post hoc pairwise multiple comparisons were performed within time points using the Holm-Šidák method when group differences were detected. * represents a significant difference between ES, TP, or ES and TP compared to axotomy alone, at $P < 0.05$. # represents a significant difference between ES and TP relative to both ES and TP alone, at $P < 0.05$. For each experimental group, n = 5-6 animals/time-point.

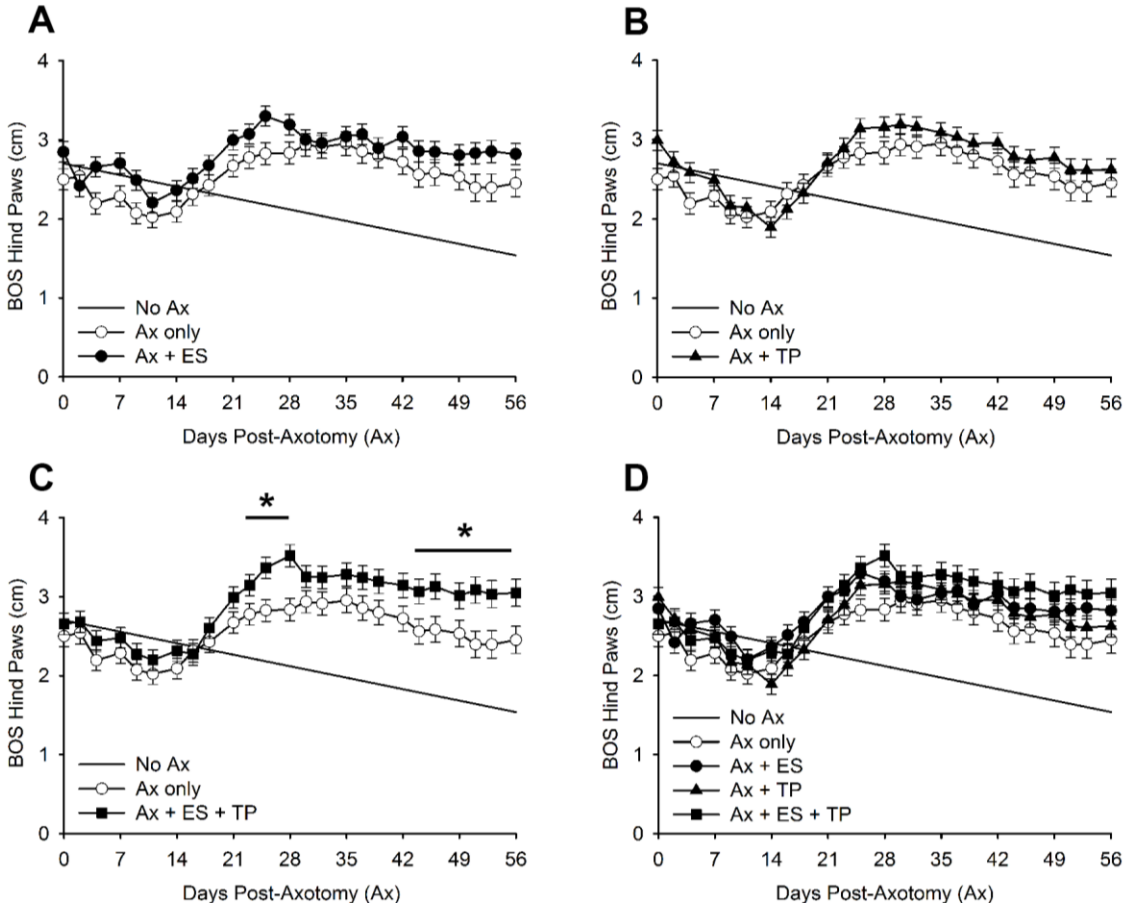

Fig. 41: Effect of ES and TP on BOS of the hind paws.

Shown is a time-course of the effect of ES and TP on BOS of the hind paws post-axotomy (Ax). Uninjured animals (solid line) represent a longitudinal baseline throughout the study. Control animals, which were axotomized but left untreated (Ax only), are compared to axotomized animals receiving only ES (A), only TP (B), or the combination of both ES and TP (C). (D) Represents the comparison between the combination of ES and TP relative to both ES and TP alone. Data is represented as BOS of the hind paws (cm) ± SEM. Statistical significance was evaluated using linear mixed effects models. Each model included random intercepts and slopes for speed and weight for each animal. Post hoc pairwise multiple comparisons were performed within time points using the Holm-Šidák method when group differences were detected. * represents a significant difference between ES, TP, or ES and TP compared to axotomy alone, at $P < 0.05$. # represents a significant difference between ES and TP relative to both ES and TP alone, at $P < 0.05$. For each experimental group, n = 5-6 animals/time-point.

control animals (2.84 ± 0.14 cm and 2.09 ± 0.24 cm, respectively; $P < 0.05$; **Fig. 41**) and was maintained throughout the remaining time-course. The BOS of the hind paws of ES-treated animals demonstrated no consistent difference relative to injury alone (**Fig. 41A**). In TP-treated animals, the BOS of the hind paws was similar to axotomy alone throughtout the time-course (**Fig. 41B**). The combination of ES plus TP significantly increased BOS of the hind paws between 21 and 28 days post-axotomy ($P < 0.05$; **Fig. 41C**). Similarly, the BOS of the hind paws in ES and TP treated animals was significantly elevated shortly after 42 days post-axotomy throughout the remaining time-points relative to injury alone ($P < 0.05$) with the exception of 49 days post-axotomy. In contrast, the combination of both ES and TP demonstrated no additional benefit to either treatment alone throughout the time-course (**Fig. 41D**).

vi. TP increases regularity index

Evaluation of the regularity index revealed an overall main effect between treatment groups ($F_{4,5863} = 2.98$; $P = 0.0181$), time ($F_{26,5863} = 149.18$; $P < 0.0001$), as well as an interaction between treatment x time ($F_{104,5863} = 12.20$; $P < 0.0001$; **Fig. 42**). Axotomy alone significantly decreased the regularity index at 2 days post-axotomy relative to uninjured animals (31.3 ± 2.1% and 99.6 ± 2.1%, respectively, $P < 0.001$) and returned to levels similar to that observed in uninjured animals at 11 days post-axotomy (**Fig. 42**). At the earlier time-points of 2 and 4 days post-axotomy, ES of the sciatic nerve (40.2 ± 2.0% and 90.8 ± 1.8%, respectively) significantly increased the regularity index relative to axotomy

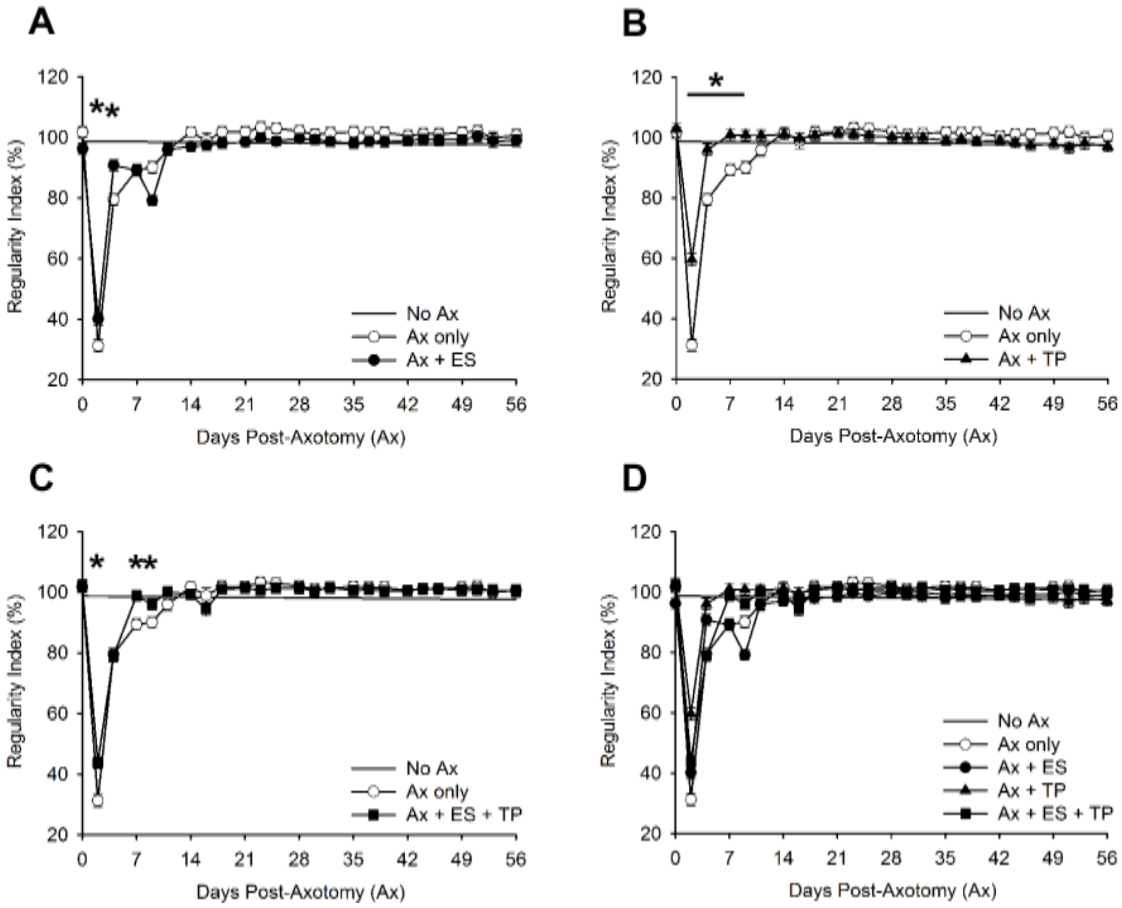

Fig. 42: Effect of ES and TP on regularity index.

Shown is a time-course of the effect of ES and TP on regularity index post-axotomy (Ax). Uninjured animals (solid line) represent a longitudinal baseline throughout the study. Control animals, which were axotomized but left untreated (Ax only), are compared to axotomized animals receiving only ES (A), only TP (B), or the combination of both ES and TP (C). (D) Represents the comparison between the combination of ES and TP relative to both ES and TP alone. Data is represented as regularity index (%) ± SEM. Statistical significance was evaluated using linear mixed effects models. Each model included random intercepts and slopes for speed and weight for each animal. Post hoc pairwise multiple comparisons were performed within time points using the Holm-Šidák method when group differences were detected. * represents a significant difference between ES, TP, or ES and TP compared to axotomy alone, at $P < 0.05$. # represents a significant difference between ES and TP relative to both ES and TP alone, at $P < 0.05$. For each experimental group, n = 5-6 animals/time-point.

alone ($P < 0.05$; **Fig. 42A**). Similarly, TP treatment demonstrated a significant elevation at 2, 4, 7, and 9 days post-axtomy (59.6 ± 2.0%, 96.1 ± 1.9%, 101.0 ± 1.8%, and 100.8 ± 1.7%, respectively) relative to axotomy alone ($P < 0.05$; **Fig. 42B**). At 2, 7, and 9 days post-axotomy, the combination of ES and TP (43.7 ± 1.9%, 98.9 ± 1.7%, and 96.1 ± 1.8%, respectively) significantly elevated the regularity index relative to axotomy alone ($P < 0.05$; **Fig. 42C**). The combinatorial treatment of ES and TP did not demonstrate a supplementary effect in regularity index relative to either treatment alone (**Fig. 42D**).

vii. Treatment does not affect cadence

The extended time course did not reveal any differences between treatment groups ($F_{4,5863} = 1.23$; $P = 0.2958$) but did reveal a significant effect between time ($F_{26,5863} = 21.69$; $P < 0.0001$) as well as an interaction between treatment x time ($F_{104,5863} = 3.94$; $P < 0.0001$; **Fig. 43**). Axotomy alone demonstrated a brief and delayed significant increase in the cadence from 11 through 21 days post-axotomy relative to uninjured animals ($P \leq 0.05$; **Fig. 43**). Treatment with ES (**Fig. 43A**), TP (**Fig. 43B**), or the combination of ES and TP (**Fig. 43C**) demonstrated no consistent difference across time relative to axotomy alone. Thus, the combination of ES and TP showed no supplementary benefit relative to either treatment alone **Fig. 43D**).

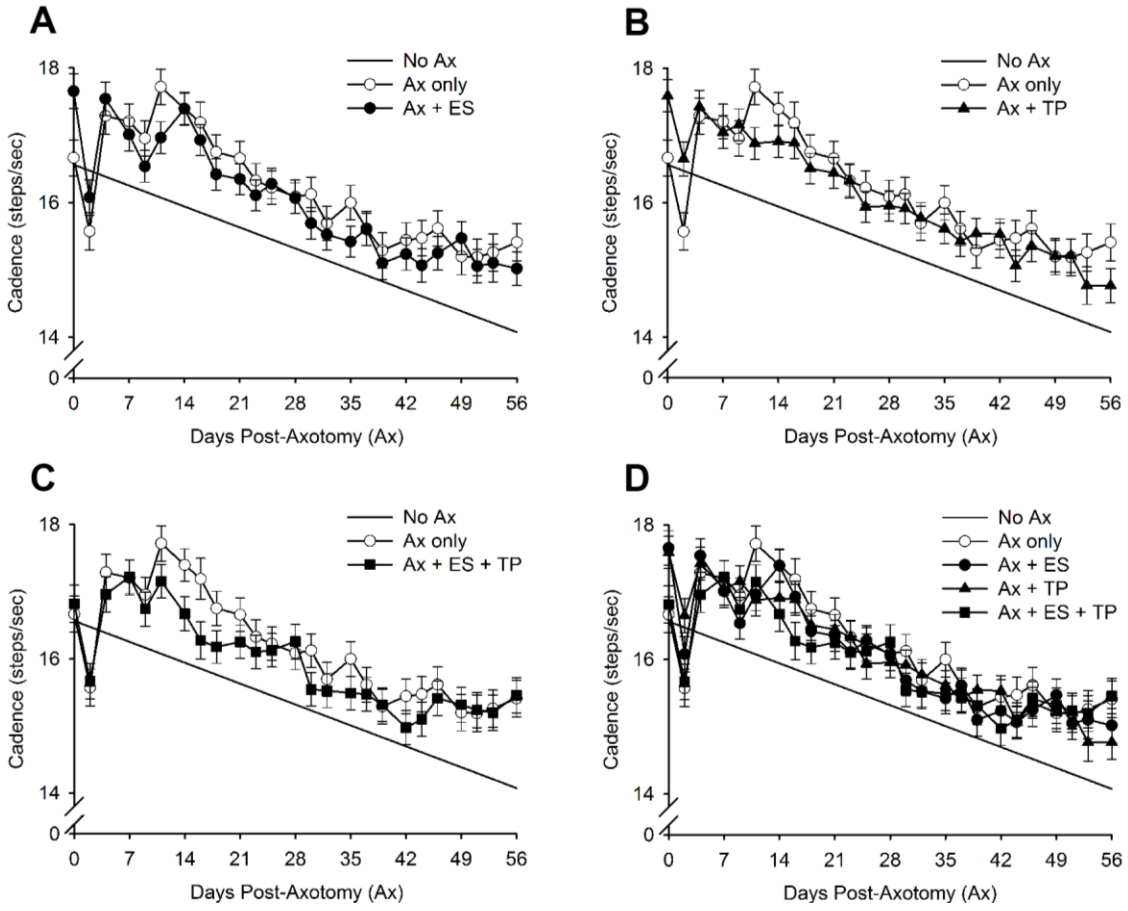

Fig. 43: Effect of ES and TP on cadence.

Shown is a time-course of the effect of ES and TP on cadence post-axotomy (Ax). Uninjured animals (solid line) represent a longitudinal baseline throughout the study. Control animals, which were axotomized but left untreated (Ax only), are compared to axotomized animals receiving only ES (A), only TP (B), or the combination of both ES and TP (C). (D) Represents the comparison between the combination of ES and TP relative to both ES and TP alone. Data is represented as cadence (steps/sec) ± SEM. Statistical significance was evaluated using linear mixed effects models. Each model included random intercepts and slopes for speed and weight for each animal. Post hoc pairwise multiple comparisons were performed within time points using the Holm-Šidák method when group differences were detected. * represents a significant difference between ES, TP, or ES and TP compared to axotomy alone, at $P < 0.05$. # represents a significant difference between ES and TP relative to both ES and TP alone, at $P < 0.05$. For each experimental group, n = 5-6 animals/time-point.

4. EMG recordings

Overall, we observed a main effect with time ($F_{1,16}$ = 4.72; *P* = 0.05; **Fig. 44**); although there was no difference between treatment groups ($F_{3,16}$ = 1.08; *P* = 0.385) or an interaction observed between treatment x time ($F_{3,16}$ = 0.20; *P* = 0.895; **Fig. 44**) when examining the EMG amplitude of the tibialis anterior muscle. Additional analysis to determine the presence of a treatment effect was not completed since there was no statistical difference between treatment x time.

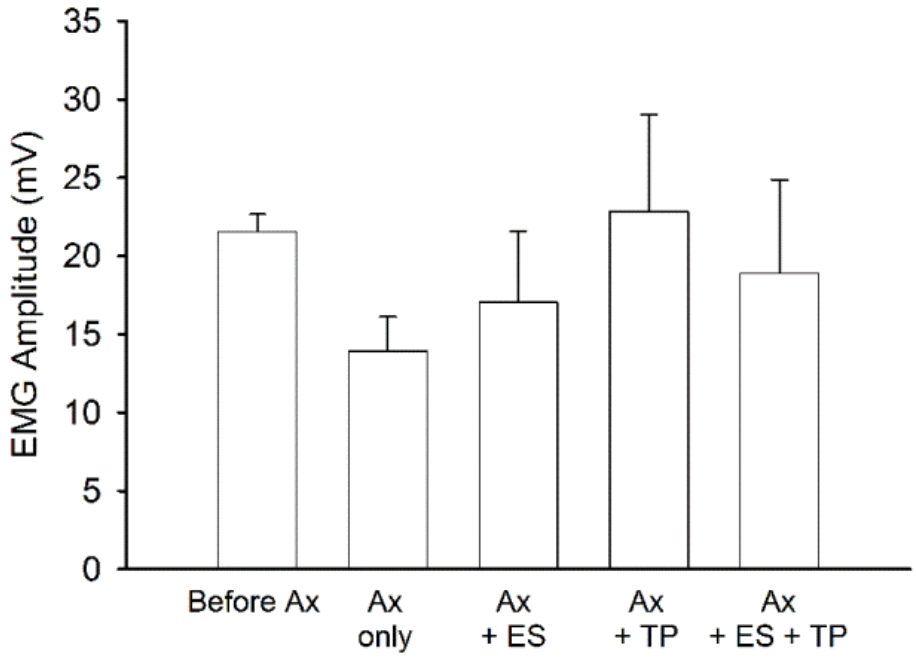

Fig. 44: Effect of ES and TP on tibialis anterior EMG amplitude.

Shown is the effect of ES and TP on electromyography (EMG) amplitude post-axotomy (Ax). EMG amplitudes of animals that were axotomized but left untreated (Ax only) and axotomized animals receiving only ES, only TP, or the combination of both ES and TP are compared to their respective EMG amplitude before Ax. Bars represent means ± SEM. For graphical purposes, the EMG amplitudes before axotomy for all treatment groups were averaged together. For each experimental group, *n* = 4-6 animals/time-point.

5. Summary

In this study, we characterized the effects of ES and TP on functional recovery following sciatic nerve crush axotomy. We found that animals treated with systemic TP exhibited the greatest recovery then either treatment with ES only or the combination of both ES and TP. Moreover, our results indicate that treatment with only ES improved many gait parameters but not to the same extent as treatment with only TP. Our results demonstrate that ES and TP together significantly improved one parameter, max contact max intensity, relative to either treatment individually.

D. Effects of ES and TP on dendritic morphology

1. Axotomy and treatment do not alter motoneuron counts

In normal animals, the number of motoneurons within the identified tibialis range averaged 125.83 (± 13.01, Mean ± SEM) on the left side and 134.91 (± 17.62) on the right side (right/left ratio X 100 = 116.65 ± 21.06). Nerve crush with or without subsequent treatment had no effect on the number ($F_{(4,25)}$ = 0.74, ns) or ratio ($F_{(4,25)}$ = 0.56, ns) of tibialis motoneurons (Ax only left, 153.14 ± 16.03, right, 150.29 ± 23.07, right/left ratio = 98.33± 11.76; Ax + ES left, 179.91 ± 19.55, right, 155.46 ± 26.41, right/left ratio = 86.44 ± 11.52; Ax + TP left, 154.20 ± 15.80, right, 137.54 ± 17.81, right/left ratio = 97.38 ± 18.45; Ax + ES + TP left, 144.24 ± 22.81, right, 156.48 ± 27.04, right/left ratio = 112.72 ± 15.43; **Fig. 45**).

2. Motoneuron morphometry

Injection of BHRP into the right anterior tibialis successfully labeled ipsilateral motoneurons in all groups (**Fig. 46**). Labeled motoneurons were located in the lateral motor column in the L3 spinal segment (Nicolopoulos-Stournaras & Iles, 1983). Dendritic arbors were strictly unilateral, with extensive ramification along the ventrolateral edges of the gray matter and in the lateral funiculus, as well as throughout the ventral horn. An average of 35.56 ± 3.34 motoneurons per animal were labeled with BHRP, and did not differ by group ($F_{(4,22)}$ = 0.07, ns).

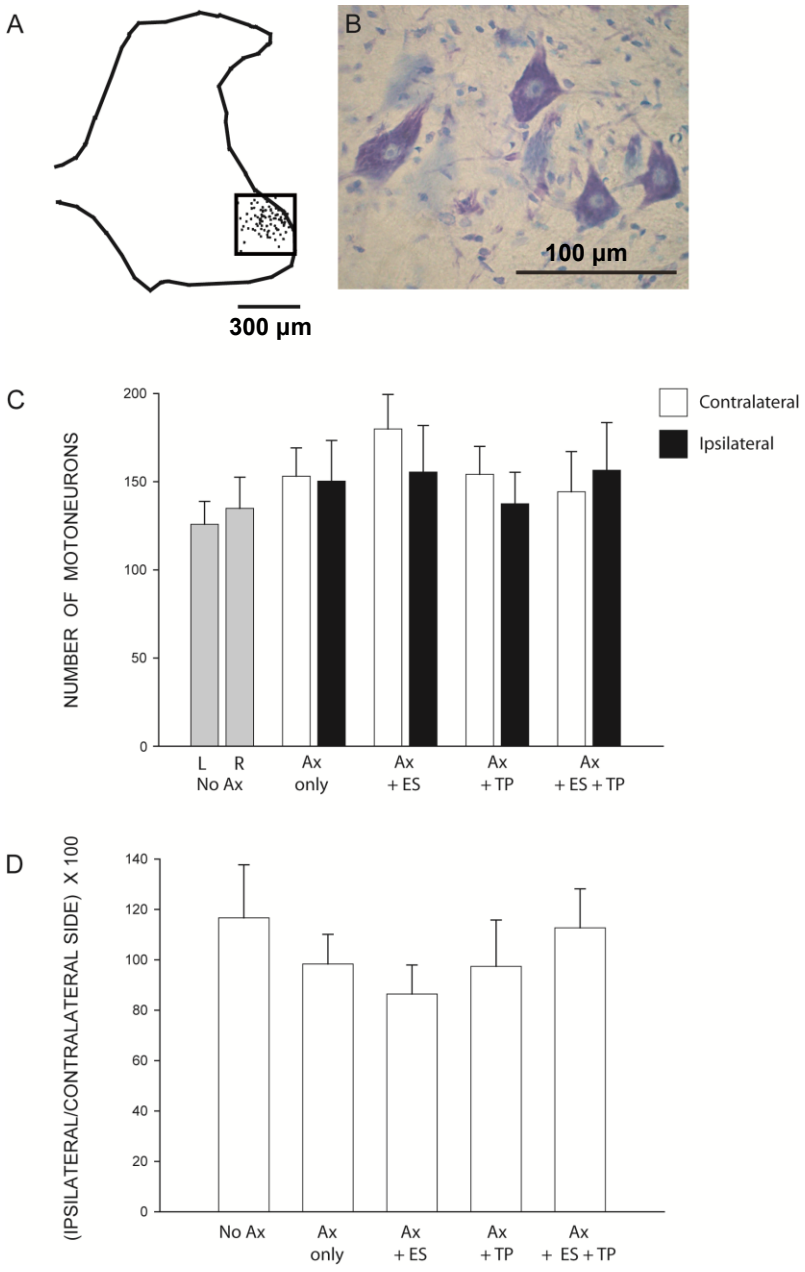

Fig. 45: Effect of ES and TP on number of tibialis anterior motoneurons.

Shown are the number of tibialis anterior motoneurons of normal controls (No Ax) and axotomized animals (Ax only) that were either treated with ES, TP, or ES + TP. (A) Location of tibialis anterior motoneurons in column 4 of the lateral motor column in the L3 spinal segment. (B) Image of tibialis anterior motoneurons. (C) Number of contralateral and ipsilateral tibialis anterior motoneurons at 58 days post-axotomy. (D) Number of tibialis anterior motoneurons as a percent ratio of ipsilateral/contralateral side. Bar heights represent means ± SEM.
* indicates significantly different from normal controls.

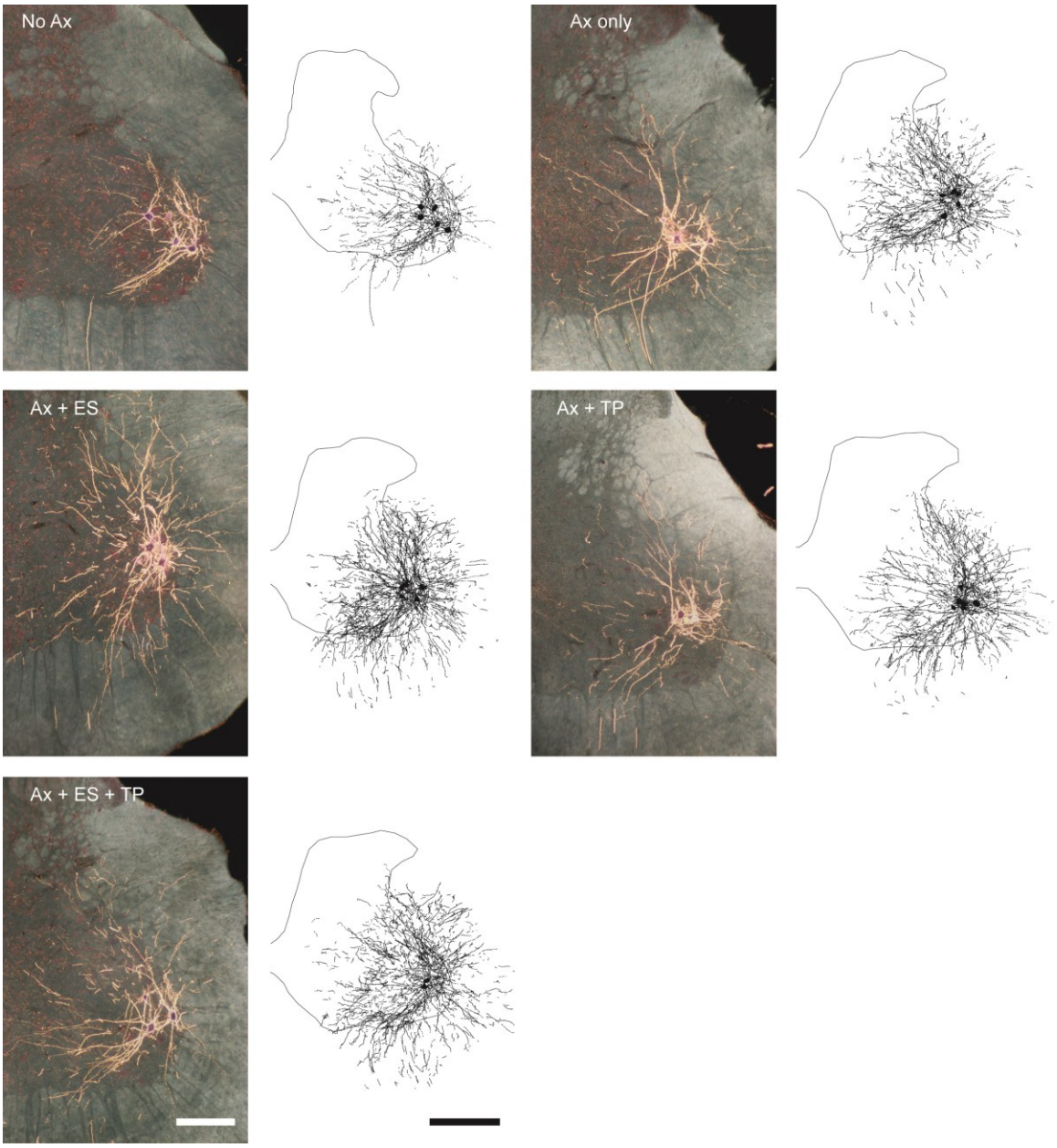

Fig. 46: Darkfield digital micrographs and somata composites demonstrating the effect of ES and TP on the dendritic arbor of tibialis anterior motoneurons.

Shown are darkfield digital micrographs of transverse hemisections through the lumbar spinal cord and corresponding computer-generated composites of labeled somata and processes after BHRP injection into the right tibialis anterior muscle. Composites of BHRP-labeling were selected as representative of the respective group average dendritic lengths. Scale bars = 250 µm (micrographs), 500 µm (composites).

a. Axotomy and treatment do not alter soma volume

In normal animals, tibialis motoneuron somata were typical in size (24985.63 ± 1946.05 μm^3), and did not differ from those of Ax only (22143.52 ± 2507.01 μm^3), Ax+ES (24107.88 ± 1176.06 μm^3), Ax + TP (25849.02 ± 2268.86 μm^3), or Ax + ES + TP (29511.34 ± 2470.25 μm^3) animals ($F_{(4,25)}$ = 1.49, ns; **Fig. 47**).

b. TP attenuates axotomy-induced dendritic hypertrophy

Following nerve crush, tibialis motoneurons underwent marked dendritic hypertrophy. Dendritic length increased by 174.57% (11741.62 ± 1931.11 μm in Ax only animals compared to 4276.42 ± 928.54 μm for normal animals, LSD, $P < 0.05$; overall test for the effect of group on arbor per cell $F_{(4,22)} = 5.15$, $P < 0.05$; **Fig. 48**). Treatment with ES or ES + TP had no effect on dendritic hypertrophy: dendritic lengths in Ax + ES animals (11233.80 ± 2449.38 μm) and Ax + ES + TP (13345.94 ± 2449.38 μm) were 162.69% and 212.08% (respectively) longer than those of normal animals (LSDs $P < 0.05$), and did not differ from each other (LSD, ns). In contrast, treatment with TP attenuated crush-induced hypertrophy: dendritic lengths in Ax+TP animals (8385.91 ± 1686.28 μm) were 96.10% longer than those of normal animals but this difference did not reach statistical significance (LSD, ns). Dendritic length per bin was nonuniform across radial bins, and a repeated-measures ANOVA revealed a significant effect of radial

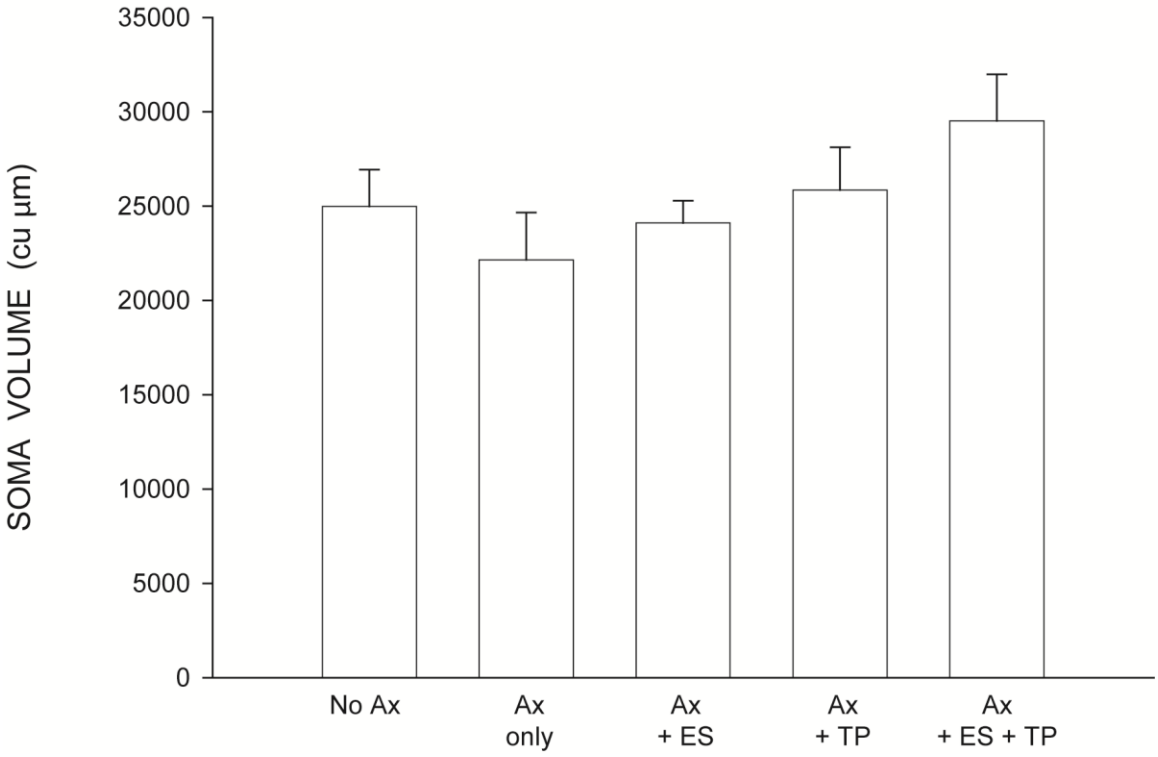

Fig. 47: Effect of ES and TP on soma volume.

Shown is the cross-sectional soma areas of tibialis anterior motoneurons of normal controls (No Ax) and axotomized animals (Ax only) that were either treated with ES, TP, or ES + TP. Bar heights represent means ± SEM
* indicates significantly different from normal controls.

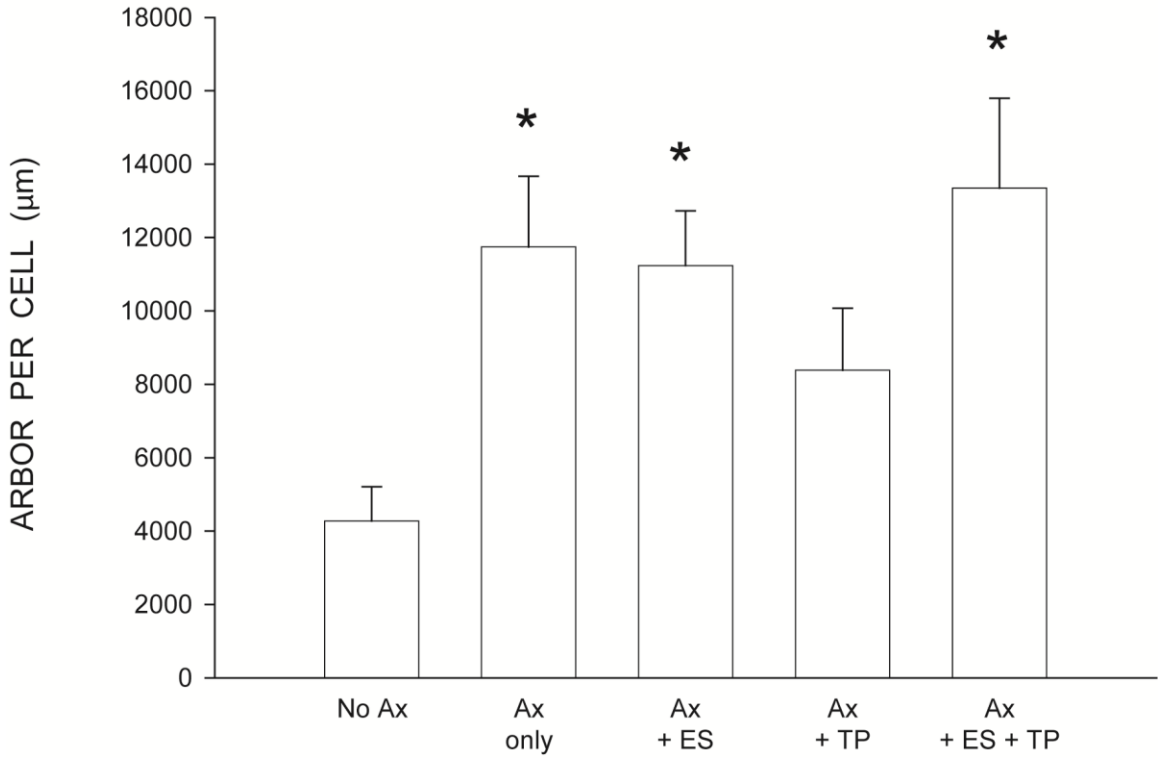

Fig. 48: Effect of ES and TP on dendritic arbor.

Shown is the dendritic lengths of tibialis anterior motoneurons of normal controls (No Ax) and axotomized animals (Ax only) that were either treated with ES, TP, or ES + TP. Bar heights represent means ± SEM. * indicates significantly different from normal controls.

location ($F_{(11,242)}$ = 41.51, P < 0.05; **Fig. 49**). Consistent with the results of the arbor per cell analysis, there was also a significant effect of group ($F_{(4,242)}$ = 5.17, P < 0.05). Increases in dendritic length occurred throughout the radial distribution in Ax only animals compared to that of normal animals (an average of 275.91%; $F_{(1,110)}$ = 14.46, P < 0.05). Similar increases in length throughout the radial distribution of the dendritic arbor were present in animals treated with ES (averaging 271.18%) or ES + TP (326.87%) ($Fs_{(1,110)}$ > 15.30, Ps < 0.05). Consistent with the attenuation in crush-induced dendritic hypertrophy seen after TP treatment, smaller but statistically significant ($F_{(1,110)}$ = 5.27, P < 0.05) increases in length throughout the radial distribution of the dendritic arbor were present in Ax + TP animals (averaging 198.12%).

c. TP attenuates axotomy-induced dendritic extent

Consistent with the nonuniform dendritic distribution of tibialis motoneurons apparent in **Fig. 49**, radial dendritic extent differed across bins (**Fig. 50**), and repeated-measures ANOVA revealed a significant effect of location ($F_{(11,242)}$ = 69.83, P < 0.05). Radial dendritic extent also differed across groups ($F_{(4,242)}$ = 8.73, P < 0.05). Increases in dendritic extent occurred throughout the radial distribution in Ax only animals compared to that of normal animals (an average of 159.59%; $F_{(1,110)}$ = 20.12, P < 0.05). Similar increases in extent throughout the radial distribution of the dendritic arbor were present in animals treated with ES (averaging 154.09%), TP (137.22%), or ES + TP (160.62%)

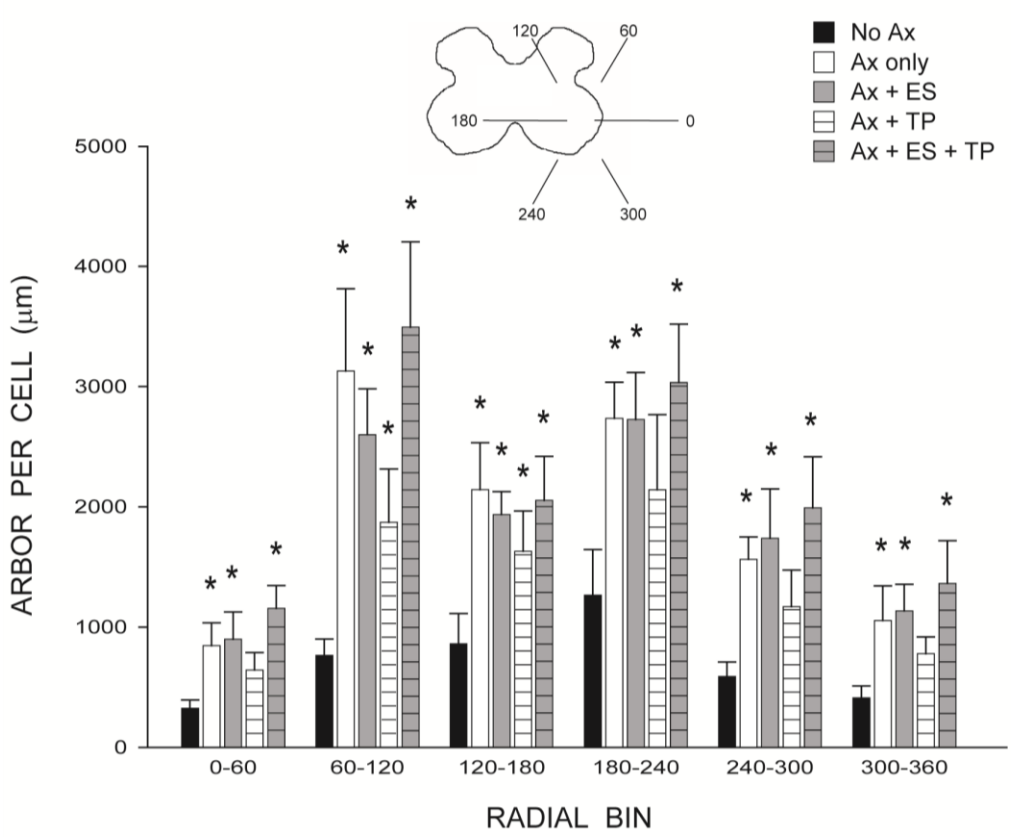

Fig. 49: Effect of ES and TP on dendritic arbor across radial bins.

Top: Drawing of gray matter divided into radial sectors for measure of tibialis anterior motoneuron dendritic distribution.
Bottom: Shown is the length per radial bin of tibialis anterior motoneurons of normal controls (No Ax) and axotomized animals (Ax only) that were either treated with ES, TP, or ES + TP. For graphical purposes, dendritic length measures have been collapsed into 6 bins of 60° each. Bar heights represent means ± SEM. * indicates significantly different from normal controls.

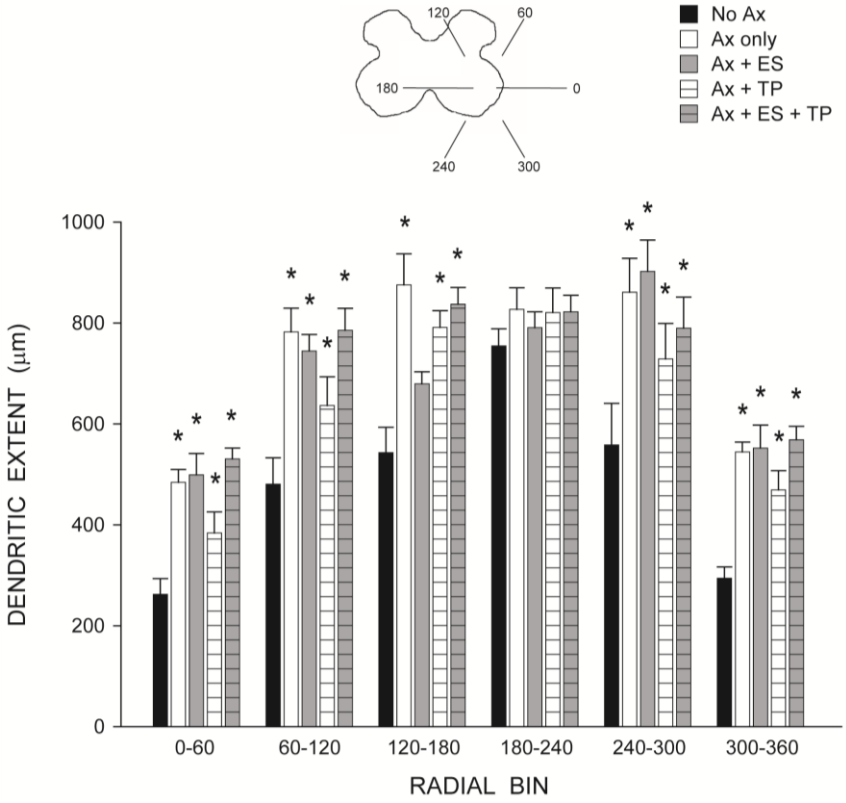

Fig. 50: Effect of ES and TP on dendritic extent.

Top: Drawing of gray matter divided into radial sectors for measure of tibialis anterior motoneuron radial dendritic extent.
Bottom: Shown is the radial extents of tibialis anterior motoneurons of normal controls (No Ax) and axotomized animals (Ax only) that were either treated with ES, TP, or ES + TP. For graphical purposes, dendritic length measures have been collapsed into 6 bins of 60° each. Bar heights represent means ± SEM. * indicates significantly different from normal controls.

($Fs_{(1,110)}$ > 6.65, *Ps* < 0.05). Rostrocaudal dendritic extent also differed across groups ($F_{(4,22)}$ = 3.72, *P* < 0.05; **Fig. 51**), spanning 2605.70 ± 147.00 μm in normal animals, and increasing to 4000.00 ± 572.40 μm in Ax + ES animals, and 4128.00 ± 478.4 μm in Ax+ES+TP animals (LSDs, *Ps* < 0.05). Rostrocaudal extent in Ax only (3424.00 ± 164.7 μm) and Ax + TP (3328.00 ± 163.2 μm) animals did not differ from that of normal animals (LSDs, ns).

6. Summary

In this study, we characterized the effects of ES and TP on motoneuron morphology following sciatic nerve crush axotomy. We found that crush axotomy and treatment did not alter the number of tibialis motoneurons. Interestingly, our results indicate crush axotomy leads to hypertrophy of the dendritic arbor of tibialis motoneurons which is attenuated with treatment of TP.

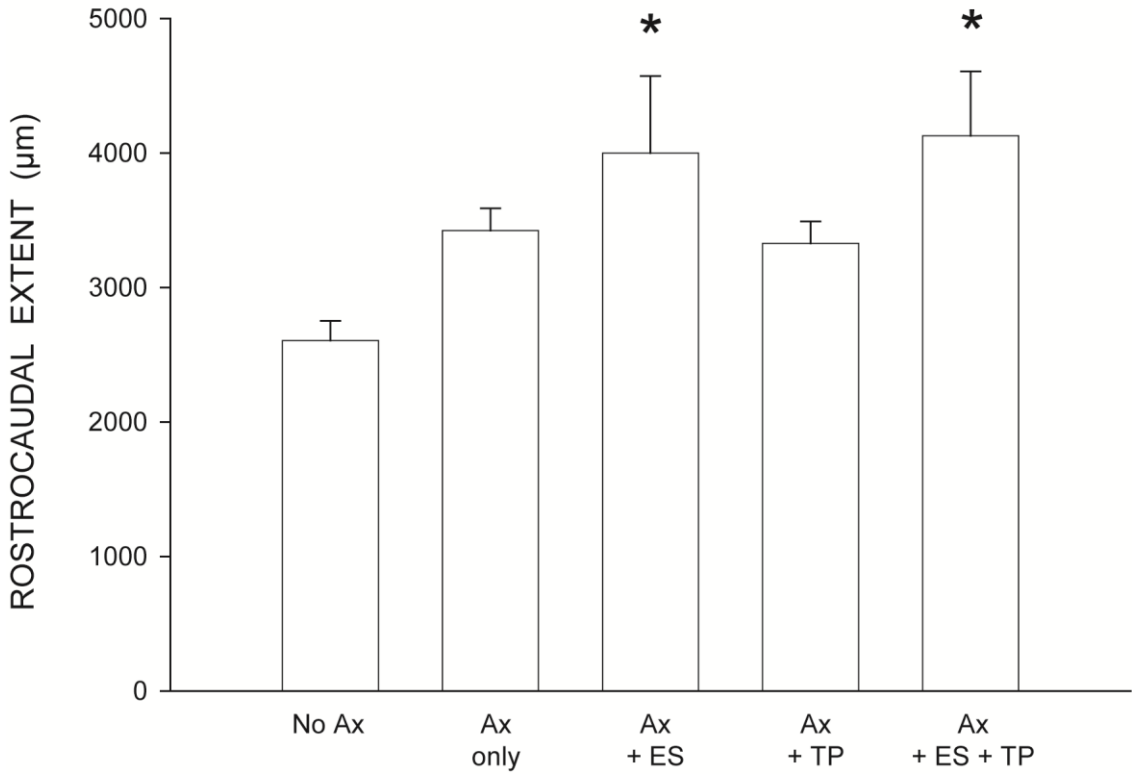

Fig. 51: Effect of ES and TP on rostrocaudal extent of the dendritic arbor.

Shown is the rostrocaudal extent of tibialis anterior motoneurons of normal controls (No Ax) and axotomized animals (Ax only) that were either treated with ES, TP, or ES + TP. Bar heights represent means ± SEM
* indicates significantly different from normal controls.

CHAPTER 5: DISCUSSION

It is well-established that functional recovery is suboptimal following peripheral nerve injury (PNI), especially with transection injuries, with full functional recovery seen in approximately 50% of individuals (Kallio & Vastamäki, 1993). The use of electrical stimulation (ES), gonadal steroids, growth factors, and exercise as therapies following axotomy have had great success in rodents, but few have shown promise in a clinical setting (Gordon et al., 2010). It is likely that the greatest success in achieving full functional recovery will come from the use of a combinatorial approach that has the potential to stimulate multiple targets and pathways. Several objectives must be accomplished to have successful regeneration and complete functional recovery: 1) maintaining the internal neuronal growth state, 2) increasing regeneration-associated genes to promote axon elongation across the injury site, 3) removing inhibitory debris to promote a permissive environment, 4) increasing axonal regeneration by decreasing the time before sprout formation and/or accelerating regeneration rate, 5) increasing regeneration specificity, and 6) maintaining viable and intact target end-organs. Many of the aforementioned treatments target one or more of these goals, although two treatments show more promise than the others. Both ES and testosterone propionate (TP) address several of the goals listed above. ES decreases the time before sprout formation and increases regeneration specificity (Brushart, 1988; Sharma et al., 2009). On the other hand, TP maintains the internal growth state and target end-organs while also enhancing

axonal regeneration rate (Kujawa et al., 1993; Tetzlaff et al., 2007; Verhovshek et al., 2010; Verhovshek & Sengelaub, 2013). Together, ES and TP increase regeneration-associated gene expression (Jones et al., 1999; Al-Majed et al., 2000a; Fargo et al., 2008; Sharma et al., 2010a). From a translational perspective, ES has demonstrated therapeutic potential in the clinic following axotomy (Gordon et al., 2010) and TP is currently utilized in the clinic to treat other disorders, thus making it an easily translational therapy for treatment of PNI. The results presented in the preceding chapter have provided insight into the mechanisms by which ES and TP promote functional recovery following sciatic nerve crush injury. Following is a discussion of some of those key results and their impact on the current literature.

A. Validation Experiments

As previously discussed, functional recovery following nerve injury is dependent upon several factors: type and location of injury, age of individual, and regeneration distance to target (Birch & Raji, 1991; Perry et al., 1992; Fu & Gordon, 1995a, 1995b). In the current study, we demonstrated that there is a significant increase in β_{II}-*tubulin* mRNA expression with proximal sciatic nerve crush injury relative to a distal sciatic nerve crush axotomy; findings which have been previously documented (Fernandes et al., 1999). Therefore, we can conclude that the strength of the motoneuron response following injury is in direct proportion to the proximity of injury to the cell body.

The peripheral nervous system exhibits a high degree of symmetry with side-to-side integration particularly extensive in the spinal cord. Some animal PNI models, such as the facial nerve injury model, have historically utilized the contralateral side as an internal control for analysis, such as normalizing gene expression data (Haulcomb et al., 2014). However, numerous examples of unilateral injury have been reported to produce bilateral effects, including alterations in gene expression and nociceptive response (McGregor et al., 1984; Sugimoto et al., 1990; Booth & Brown, 1993; Koltzenburg et al., 1999; Pettersson et al., 2004). In contrast to motor nuclei in the brainstem, the spinal cord is anatomically predisposed to exhibit contralateral effects to unilateral nerve injury (Kiernan & Rajakumar, 2013).

In the literature, there are contradictory data on whether nerve injury can lead to a contralateral effect in gene expression in the spinal cord (Booth & Brown, 1993; Fernandes et al., 1999; Pettersson et al., 2004). The current study revealed alterations in the expression of α_I-*tubulin*, β_{II}-*tubulin*, and *collapsin response mediator protein 2* (*crmp2*) with axotomy alone. Some studies have reported altered mRNA levels of *pituitary adenylate cyclase-activating peptide* (*pacap*) and *43-kiloDalton growth-associated protein* (*gap-43*) in intact motoneurons contralateral to a sciatic nerve lesion (Booth & Brown, 1993; Pettersson et al., 2004). Similar to our findings, Pettersson and colleagues did not detect a change in *pacap* expression on the contralateral side with a distal sciatic nerve injury (level of mid-thigh), but did observe a slight increase in *pacap* expression after a proximal sciatic nerve injury (Pettersson et al., 2004). Given

the discrepancy in the literature and our current results, we did not use the contralateral side as an internal control.

B. ES and TP differentially alter regeneration-associated gene expression

It is well-established that motoneurons undergo a robust response to axotomy and shift from a state of maintenance to growth/repair (Lieberman, 1971). This morphological shift is critical for motoneurons to regenerate and characterized by several changes in the somata, including chromatolysis, swelling, and an upregulation of genes critical for neuronal recovery and repair (regeneration-associated genes; Lieberman, 1971). In the current study, we analyzed the effects of ES and TP on axotomy-induced expression of the following regeneration-associated genes: *α_I-tubulin*, *β_{II}-tubulin*, *gap-43*, *crmp2*, *brain derived neurotrophic factor* (*bdnf*), *glial cell derived neurotrophic factor* (*gdnf*), pacap, and *neuritin*.

Over the extended post-axotomy time-course, we observed an increase in *gap-43* mRNA expression at 7 days post-axotomy. Previous studies from our laboratory and others support the upregulation of *gap-43* mRNA following axotomy (Tetzlaff et al., 1991; Jones et al., 1997a; Sharma et al., 2010a). In addition, we observed a downregulation in the expression of *α_I-tubulin* and *crmp2* after axotomy that contradicts what has been previously reported (Pasterkamp et al., 1998; Sharma et al., 2010a), and which could be attributed to differences in the molecular response of cranial versus spinal neurons. Initially, it was

surprising that we did not observe significant changes in the expression of β_{III}-*tubulin*, *bdnf*, and *pacap* with axotomy alone, as all of these genes have been reported to increase after facial or sciatic nerve axotomy (Brown et al., 1999; Zhou et al., 1999; Pettersson et al., 2004; Sharma et al., 2010a; Haulcomb et al., 2014). However, these contradictory findings could be due to differences in tissue source and extraction methodologies (i.e. motoneuron cell body vs. entire ventral horn) and also the lesion site. Similar to previous studies, we found no axotomy-induced increase in neuritin mRNA (Höke et al., 2000; Fargo et al., 2008; Sharma et al., 2010a).

Several groups have reported an increase in *gdnf* expression after axotomy in the DRG, dorsal horn, and sciatic nerve (Höke et al., 2000; Dong et al., 2005; Cobianchi et al., 2013). In contrast, resultant low *gdnf* expression within the whole spinal cord has been documented following axotomy (Cobianchi et al., 2013), which is in agreement with our current results. ES has been demonstrated to increase *gdnf* expression in the DRG, but not the ventral horn, at 1 and 3 days following sciatic nerve injury (Cobianchi et al., 2013). Similarly, we did not detect a change in *gdnf* expression at 1 day post-axotomy. Although we discovered ES-treated animals displayed a significant increase in ventral horn *gdnf* expression at 2 days post-axotomy, this discrepancy is most likely due to temporal differences.

We also reported ES treatment has no effect on early *bdnf* expression. In contrast, Cobianchi et al. published an increase in *bdnf* ventral horn expression

following axotomy and ES treatment (Cobianchi et al., 2013); however, their analysis was relative to sham animals and not to injured animals.

An earlier study from our laboratory concluded that one 30 minute session of low frequency ES (20 Hz) was just as effective as daily, 30 minute ES at the same low frequency (Foecking et al., 2012). Therefore, for the current study we used one 30 minute session of low frequency ES (20 Hz). It is possible that a longer stimulation session or multiple sessions would lead to more robust increases in regeneration-associated gene expression following sciatic nerve crush injury (Sharma et al., 2010a).

Previously, our laboratory reported that systemic TP treatment enhanced *bdnf* mRNA after facial nerve injury at 7 days post-axotomy (Sharma et al., 2010a). Similarly, we observed an upregulation in *bdnf* expression with systemic TP treatment but at an earlier time-point of 2 days post-axotomy. Most likely, the temporal delay observed by Sharma et al. is due to the fact they utilized a proximal nerve injury model and thus more time was necessary for nerve degeneration. In addition, they examined a mostly pure motoneuron population and could detect smaller changes in mRNA expression.

To our knowledge, we are the first to investigate the combined effects of ES and TP on the expression of both *gdnf* and *crmp2* after sciatic nerve injury. Our results indicate that together ES and TP together act in an additive manner to elevate *gdnf* levels 1 day post-axotomy. Cobianchi et al. found that ES in combination with treadmill exercise led to a synergistic increase in *gdnf* ventral

horn expression following sciatic nerve axotomy (Cobianchi et al., 2013). It is possible that multiple pathways need to be activated to achieve substantial elevation in *gdnf* expression, which could explain the greater effect on *gdnf* expression with the combinatorial treatment of ES and TP rather than with ES alone. Moreover, we observed an early and late decrease in mRNA expression of *crmp2* in response to ES and TP treatment. Similar to *gap-43*, *crmp2* is important in axonal growth and shows a decreased expression level at a late time-point similar to that of *gap-43*. Decreased expression of both *gap-43* and *crmp2* could indicate a decrease in axonal outgrowth, resulting in a slower rate of axon regeneration.

The work by Sharma et al. effectively described the differential and combinatorial effects of ES and TP on regeneration-associated gene expression after facial nerve axotomy (Sharma et al., 2010a). For ease of discussion, a comparison of the previous study by Sharma et al. and the current study is summarized in **Table 8**. Sharma and colleagues demonstrated that ES and TP differentially altered the expression of *α_I-tubulin*, *β_{II}-tubulin*, and *gap-43*, whereas the combination of ES and TP increased the expression of *bdnf*, *neuritin*, and *pacap*. Interestingly, our results with a different injury model, crush injury to the sciatic nerve, contrasted significantly with those reported by Sharma and coworkers. ES has been reported to enhance *α_I-tubulin*, *gap-43*, and *pacap* mRNA expression (Al-Majed et al., 2004; Sharma et al., 2010a). In addition, findings from our laboratory have established that TP enhances *β_{II}-tubulin* expression following facial and sciatic nerve axotomy (Jones & Oblinger, 1994;

Jones et al., 1999; Brown et al., 2001; Storer et al., 2002; Sharma et al., 2010a). Yet, in the current study, we did not observe any changes in *α_I-tubulin* or *β_{II}-tubulin* expression with ES or systemic TP treatment alone, but a combinatorial treatment of ES and TP resulted in early increases in *α_I-tubulin* expression and late downregulation in both *α_I-tubulin* and *β_{II}-tubulin*. Furthermore, in the current study we did not find an altered expression of *gap-43* with either ES or TP treatment alone (Jones et al., 1997a; Al-Majed et al., 2004; Sharma et al., 2010a) but did observe a decrease in *gap-43* levels after concerted administration of ES and TP. Previously, Sharma et al. found the expression levels of *bdnf* were unchanged by the individual treatments of ES or TP but were elevated after treatment with both ES and TP following facial nerve axotomy (Sharma et al., 2010a). In contrast to their findings, results from our current study indicate that administering TP led to an early increase, while ES treatment led to a late downregulation in *bdnf* expression. Sharma and colleagues report that when ES and TP were administered together, ES led to an early increase in *neuritin* mRNA whereas TP elevated *neuritin* levels in a delayed fashion. Most notably, the current study revealed no change in the expression of *neuritin* after sciatic nerve crush axotomy. In the facial nerve axotomy model, treatment with the combination of ES and TP elevated the expression of *pacap* in a synergistic manner and demonstrated the most robust change out of all of the regeneration-associated genes that were examined in the study (Sharma et al., 2010a). Interestingly, treatment did not alter *pacap* expression in the sciatic nerve injury model used in the current study.

The numerous findings our study which contradict what has been reported in other PNI models raise several interesting questions that should be considered when guiding future investigations for therapeutic strategies and whether ES and/or TP are beneficial treatments following PNI. First, the remarkable dichotomy in differential and combinatorial effects observed between the facial and sciatic nerve injuries supports the theory that cranial and spinal motoneurons, respectively, molecularly respond differently to treatment following PNI. Second, our results support the idea that gene expression levels in direct proportion to injury distance from the motoneuron cell body. In our model of distal sciatic nerve crush injury, we observed smaller changes in expression compared to what has been reported by others using a proximal nerve crush injury model (Fernandes et al., 1999; Pettersson et al., 2004; Sharma et al., 2010a). Furthermore, our results demonstrate ES and TP alone lead to an early increase in expression of regeneration-associated genes; however, when ES and TP are administered in combination they elicit a late suppressive effect in regeneration-associated gene expression. These results agree with a rapid regenerating response at the level of the motoneuron cell body accompanied by rapid functional recovery, which will be discussed in more detail below. Lastly, the type of injury should be considered when choosing the best therapeutic strategy. Although our experiments were restricted to crush axotomy, a more severe transection injury may respond differently to ES and/or TP treatment. The latter will be discussed in more detail in the section outlining Future Directions.

C. TP improves functional recovery following sciatic nerve crush axotomy

Rodent gait analysis has been long utilized as a method to assess therapeutic efficacy and extrapolate to humans (Medinaceli & Wyatt, 1988). Moreover, rodent locomotion has been examined in the context of treatment efficacy on several disease and injury models, including Parkinson's disease, amyotrophic lateral sclerosis, and PNI (Deumens et al., 2007; Hampton & Amende, 2009; Westin et al., 2012).The CatWalk gait analysis system is an automated unforced gait analysis method and has been utilized to measure functional recovery following sciatic nerve crush and transection injuries (Deumens et al., 2007; Bozkurt et al., 2008). From these studies, it was discovered that sciatic nerve injury altered several gait parameters, including but not limited to print length, print width, print area, swing, swing speed, step cycle, duty cycle, max contact max intensity mean, sciatic functional index (SFI), peroneal functional index (PFI), posterior tibial functional index (TFI), and base of support (BOS) of the hind paws. Although CatWalk examines 162 gait parameters, we limited our analysis to clinically relevant parameters, such as those described above with the addition of stride length, BOS of the front paws, and cadence. It should be noted, for all paw related parameters only the right hind (RH) paw was analyzed.

In addition to gait analysis, other behavioral assessments have been utilized to examine functional recovery after nerve injury, such as grip strength and electromyography (EMG) recordings (Galtrey & Fawcett, 2007; Hamilton et al., 2011). Both grip strength and EMG measurements correlate with nerve

reinnervation to intrinsic muscles of the paws. We adapted these methods to measure functional recovery of the hind limbs following sciatic nerve axotomy.

For assessment of the effects of ES and TP on functional recovery, we employed a variety of behavioral tests, including automatic foot misplacement, grip strength, CaWalk©, and EMG recordings. Below, I will discuss our findings in context with the current literature and our original hypothesis.

The foot misplacement apparatus tests the ability of the rat to use all four paws in a coordinated manner. An increase in the number of rear leg errors indicates a decrease in coordinated movement. Although all axotomized groups demonstrated a significant increase in the number of rear leg errors 2 days post-axotomy, few rear legs errors were observed for the remaining time-course. These data suggest axotomized animals quickly adapted to the behavioral test and altered their gait. Likewise, axotomized animals exhibited a significant decrease in grip strength relative to uninjured animals. Although no significant treatment effect was observed, ES had the strongest effect in returning grip strength levels to those of uninjured animals.

The time-line for functional recovery following a sciatic nerve crush injury has been well documented (Forman & Berenberg, 1978; Alberghina et al., 1985; Brown et al., 1999; Lago & Navarro, 2006). Based on our results, axotomoized animals returned to levels of uninjured animals in all of the behavioral parameters analyzed, including EMG activity, with the exception of BOS hind paws, print length, print width, and print area. These findings suggest that, in the absence of

treatment, animals still exhibit deficits at 8 weeks post-axotomy, which substantiates the need for therapeutic intervention to achieve full functional recovery.

Print length and print width are used to calculate the total area of the paw that is in contact with the glass floor (print area). A larger print area indicates that a larger portion of the foot is in contact with the glass floor. A decrease in print length, print width, and print area has been reported following sciatic nerve transection and crush (Deumens et al., 2007; Bozkurt et al., 2008). Systemic TP treatment beginning immediately after injury resulted in the most pronounced improvement throughout the time-course for print length, print width, and thus print area, compared to either treatment with only ES or the combination of ES and TP following axotomy. An increase in all three of these parameters indicates reinnervation of the calf and foot muscles (de Medinaceli et al., 1982; Brown et al., 1999). The abililty of TP treatment to improve print length, print width, and print area better than ES treatment or the combination of ES and TP, suggests TP treatment alone can accelerate regeneration of the sciatic nerve (Brown et al., 1999).

Stride length is the distance between two successive placements of the same paw, in this case the RH paw. Axotomy has been shown to have no effect on stride length when examined as early as 3 days post-axotomy (Deumens et al., 2007). We observed a significant increase in stride length with injury at 2 days post-axotomy. In line with previous findings, this effect diminished by 4 days post-axotomy (Deumens et al., 2007). Interestingly, treatment with either ES or

TP alone significantly decreased stride length relative to the combination of ES and TP. This suggests that ES and TP individually can decrease the distance between two consecutive placements of the RH paw. Despite the paw being placed more frequently, there was no significant difference in cadence among any of the groups.

Swing has been reported to increase with axotomy and is defined as the duration in seconds of no contact of the RH paw with the glass plate (Vrinten & Hamers, 2003; Deumens et al., 2007; Bozkurt et al., 2008). Similarly, we observed an increase in swing following crush injury to the sciatic nerve. Individually, ES and TP decreased swing by 2 days post-axotomy relative to no treatment. However, the combination of both ES and TP had no supplementary benefit. These data suggest ES and TP individually decrease the amount of time the RH paw is in the air between consecutive placements of that paw.

Stand is defined as the duration of time the RH paw is in contact with the glass plate. Step cycle is defined as the time in seconds between two consecutive initial contacts of the same paw and can be calculated as the sum of both stand and swing. Given that minor differences were observed across treatment groups in swing, it is not surprising we did not detect substantial treatment differences with step cycle.

Duty cycle expresses stand as a percentage of step cycle. In the current study, duty cycle significantly increased within the first 2 weeks post-axotomy across all treatment groups. Since small treatment effects were detected with

step cycle, the treatment effect observed with duty cycle is mostly attributed to an increase in stand or the time the RH paw was placed on the glass. In contrast to our results, Bozkurt and colleagues report a decrease in duty cycle following sciatic nerve crush injury (Bozkurt et al., 2008). It should be noted that their data is reported as a percent ratio of ipsilateral to contralateral hind paws and the current study did not examine the duty cycle of the contralateral hind paw. Taken together, these data suggest that either ES or TP treatment alone is beneficial in improving gait parameters following injury.

Swing speed takes into account both stride length and swing and is the speed of the RH paw during swing. Since there were no substantial differences among treatment groups in stride length, swing, or cadence, it was surprising to us that TP-treated animals exhibited significant increases in swing speed between the second and fourth weeks post-axotomy. One explanation for these differences is that TP-treated animals had a shorter swing, albeit not significant, during the second and fourth weeks post-axotomy. Furthermore, a decrease in swing would lead to an increase in swing speed. Our results contradict what has been reported previously by Bozkurt et al. (2008); however, it should be noted their data is expressed a ratio of the ipsilateral to contralateral hind paw and the current study did not examine swing speed of the contralateral hind paw.

In 1988, Bain et al. modified formulas to calculate functional recovery of the sciatic nerve and its branches (Bain et al., 1989). The formulas proposed by Bain et al. are the basis for calculation of SFI, PFI, and TFI with the CatWalk system (**Fig. 4**). A decrease in SFI, PFI, or TFI indicates a deficit in the injured

hind paw and the inability of the rat to correctly spread their toes. Regardless of treatment, animals showed early improvement in SFI, PFI, and TFI. ES of the sciatic nerve significantly improved SFI, PFI, and TFI during the later phases of recovery, an observation reported by others (Gigo-Benato et al., 2010; Zhang et al., 2013). Although we observed improvements with ES at slightly later time-points than what has been previously published, these differences are most likely due to differences in location of injury and duration of ES treatment. TP-treated animals exhibited PFI recovery relatively early and all three parameters were significantly improved in later recovery phases (Brown et al., 1999). In contrast, the combination of ES plus TP did not enhance the benefits of individual treatments only. Together, these data suggest with a distal sciatic nerve injury, either treatment alone is sufficient to improve functional recovery; however, the combination of ES and TP gives no additional therapeutic advantage.

BOS indicates stability of the trunk of the animal and is defined as the average width between either the front or hind paws. In agreement with the literature describing gait alterations after spinal cord injury, we observed no consistent difference in BOS of the front paws of axotomized animals compared to uninjured animals (Hamers et al., 2001). Treatment with either ES or TP resulted in a decreased front paw BOS that was similar to uninjured animals. In contrast to results within the literature, hind paw BOS increased at 3 weeks post-axotomy relative to uninjured animals (Deumens et al., 2007), which is most likely due to differences in type of injury. For example, Deumens et al. measured the BOS of hind paws using a transection injury, rather than a crush injury as

presented in the current study. It is likely that a more severe nerve injury results in a shorter hind paw BOS. Interestingly, we observed no treatment effect on hind paw BOS. Moreover, the combination of ES plus TP resulted in an even wider hind paw BOS, relative to axotomy alone. Taken together, these data suggest either treatment alone is effective in treating distal sciatic nerve injury; whereas, the combinatorial treatment of both ES and TP provided no additional benefit.

Regularity index measures the degree of interlimb coordination and is expressed as the number of normal step sequence patterns and paw placements. In healthy uninjured rats, the regularity index is valued at 100%. After injury, there is a loss in interlimb coordination and decrease in regularity index. Relative to uninjured animals, we observed a significant decrease in the regularity index all axotomized groups regardless of treatment; accordingly, these data suggest injured animals exhibited extra paw placements and irregular walking patterns (Hendriks et al., 2006). Minor, yet significant, improvements were detected in all treated animals within the first two weeks following axotomy. These results are similar to our observations with swing, step cycle, and stride length. Together, these results indicate an alteration in gait immediately after injury. Deumens et al. (2007) report no change in regularity index following sciatic nerve resection in comparison to our findings. It should be noted that our results indicate recovery of regularity index by 7 days post-axotomy which is in accordance with their data.

Max contact max intensity is the average maximum intensity when the paw is in maximum contact with the glass floor. Intensity ranges from 0 to 255 and depends on the degree of contact between the paw and glass floor. Vrinten and Hamers examined paw intensity utilizing a sciatic nerve constriction injury model, which causes neuropathic pain (Vrinten & Hamers, 2003). Following nerve injury, they observed a decrease in paw intensity of the injured hind limb that gradually returned to pre-injury levels over time. In addition, Vrinten and Hamers demonstrated a high correlation between intensity and mechanical withdrawal thresholds.

In the current study, all axotomized groups, regardless of treatment, exhibited a significant decrease in max contact max intensity mean of the injured paw. These results are similar to what have been observed by others (Vrinten & Hamers, 2003; Deumens et al., 2007; Bozkurt et al., 2008). ES of the sciatic nerve led to a delayed increase in the max contact max intensity mean, relative to systemic treatment with TP. In addition, the combination of ES and TP significantly increased the max contact max intensity mean similar to that observed with treatment of TP. Surprisingly, the combinatorial treatment led to a synergistic effect in intensity at 1 week post-axotomy relative to either individual treatment, an effect that was not observed with any other behavioral measure.

From these tests, our results indicate that either ES or TP alone demonstrate therapeutic potential following sciatic nerve crush injury. In addition, the combination of ES and TP did not demonstrate any improvements in functional recovery, with the exception of max contact max intensity mean.

Despite this difference, the combinatorial difference appeared to worsen recovery in some measurements. In accordance with our molecular data, the inability of the combinatorial treatment to enhance functional recovery may be due to suppressed expression of regeneration-associated genes.

D. TP attenuates axotomy-induced dendritic hypertrophy

There is a dichotomy in motoneuron death in rodents following injury that is contingent upon age. It is well-established that newborn rat motoneurons are susceptible to cell death following spinal nerve axotomy (Schmalbruch, 1984); however, there is very limited motoneuron death in adult rats (Gu et al., 1997). One study published by Gu et al. (1997) investigated the effect of spinal nerve axotomy on motoneuron loss in adult rats. They discovered there was no motoneuron loss in the spinal cord if the lesion site was at least 4 mm distal to the soma. In the current study, we observed a comparable number of HRP-labeled tibialis anterior motoneurons to that observed in the literature (Nicolopoulos-Stournaras & Iles, 1983). Moreover, crush injury did not affect motoneuron number (Gu et al., 1997), and motoneurons were healthy with respect to similar soma volumes across all groups.

Dendritic morphology is directly correlated with the electrophysiological response of a neuron (Rumberger et al., 1998; Lu et al., 2001; Grudt & Perl, 2002). Moreover, the dendritic morphology of motoneurons is influenced by injury (Standler & Bernstein, 1982; Wellman & Sengelaub, 1995). For example, the

dendritic fields of spinal motoneurons undergo a cyclic pattern of degeneration and regeneration following ventral root crush (Standler & Bernstein, 1982). In the current study, we found that crush axotomy led to hypertrophy of the dendritic arbor that extended in a radial and rostrocaudal direction. In addition, we discovered that TP treatment attenuated the axotomy-induced dendritic hypertrophy. At first, we were surprised that our results contradict what others have reported (Bowe et al., 1992; O'Hanlon & Lowrie, 1995); however, these morphological differences could expain why animals do not regain normal functional recovery by 2 months post-axotomy. Consequently, attenuation of the dendritic tree by TP treatment may reflect a restoration of synaptic inputs similar to that of an uninjured state and is one possible explanation for why TP treatment demonstrated the greatest therapeutic effect in regards to our behavioral data.

After closer examination, several differences exist between the current and preceding studies. The study led by Bowe et al. (1992) investigated the effects of a distal sciatic nerve crush injury in adult female rats on motoneuron morphology, including the dendritic arbor, at 5 and 10 months post-axotomy. It should be noted that their distal sciatic nerve injury was at mid-thigh level and proximal to the lesion performed in the current study. At 5 months post-axotomy, Bowe et al. observed no differences in the morphology of ipsilateral sciatic motoneurons relative to contralateral motoneurons. However, at 10 months post-axotomy ipsilateral sciatic motoneurons displayed an increase in perikaryal area relative to contralateral sciatic motoneurons. The results from the current study demonstrated sciatic nerve axotomy resulted in dendritic hypertrophy of tibialis

anterior motoneurons which are a subset of sciatic motoneurons. It is highly possible that sciatic nerve axotomy, regardless of injury location, leads to a hypertrophy in dendritic arborization by 2 months post-axotomy that regresses by 5 months post-axotomy. This question could be answered with further experimentation.

In opposition to our findings, O'Hanlon and colleagues discovered that sciatic nerve crush injury led to a smaller dendritic tree of hallucis longus motoneurons at 1 and 2 months post-axotomy (O'Hanlon & Lowrie, 1995). Several similarities exist between the study executed by O'Hanlon et al. and the current study, such as rat age and sex and location of injury. However, it should be noted the authors examined dendritic properties of a different pool of motoneurons. It is highly possible that different pools of spinal motoneurons respond differently to axotomy and could explain the contradictory results. If we assume that all spinal motoneurons respond the same to axotomy, several other differences between the two studies are worthy of discussion. First, O'Hanlon et al. limited their analysis to isolated motoneurons and did not take into account the dendritic tree from motoneurons that had overlapping dendritic arbors. Second, their analysis was restricted to within one tissue section. As a result, their analysis was biased toward smaller motoneurons that were isolated. Moreover, the entire dendritic length of each cell (in both the transverse and longitudinal planes) was not considered in their final analysis. Together, these differences could have led to a decrease in total dendritic length and thus a smaller dendritic tree.

E. Conclusion

Decades of regeneration studies have impacted our detailed understanding of the mechanisms by which ES and/or TP treatment mediate axonal regeneration and improve functional recovery following axotomy. This dissertation analyzed whether ES and TP enhance axon regeneration and functional recovery following a distal sciatic nerve crush injury, and evaluated the effects of ES and TP on axotomy-induced molecular expression of genes involved in motoneuron regeneration. Our results indicate that treatment with TP accelerates functional recovery and attenuates axotomy-induced dendritic hypertrophy following a distal sciatic nerve crush injury. In addition, we demonstrated that either treatment alone or in combination led to an early increase in regeneration-associated gene expression, but the combination treatment exhibited a late suppression in regeneration-associated gene expression. In support of our molecular results, we observed no added therapeutic benefit with administration of both ES and TP in combination. Based on these results, we propose two separate mechanisms by which ES and TP ultimately enhance functional recovery. First, systemic treatment of TP may activate the internal growth state by upregulating the expression of *bdnf* to maintain synaptic homeostasis. As a result, axonal elongation is enhanced and successful functional recovery is achieved. Second, we propose ES upregulates the expression of gdnf to increase regeneration specificity and promote functional recovery. Together, the combination of ES and TP would activate both of these mechanisms to enhance functional recovery.

With respect to earlier findings from our laboratory, either treatment alone may be beneficial for treatment of distal nerve injuries (Monaco et al., 2013); however, the combination of both ES and TP may be an advantageous treatment for proximal nerve injuries (Hetzler et al., 2008; Sharma et al., 2010b). Together, our results support the concept that not all injuries respond equally to treatment. Furthermore, our data support the importance of treatment strategy development in an injury-dependent manner and based upon distinct functional characteristics of spinal vs. cranial nerves.

F. Future Directions

In the preceding chapters, the effects of ES and TP on regeneration-associated gene expression and functional recovery following sciatic nerve crush injury in rats were delineated. The results from this dissertation present several avenues to complete our understanding of the therapeutic benefits of ES and TP following axotomy.

It is likely that the change in mRNA levels of regeneration-associated genes reflect concomitant alterations in with protein expression, however future studies will need to be done to validate the mRNA expression data with protein analysis. The current study provided a time-line of functional recovery where recovery was observed as early as 2 weeks post-axotomy. Future studies will need to be done to examine intact NMJ and dendritic morphology on a weekly

basis after axotomy for 8 weeks, and correlate these morphological changes with improvements in functional recovery.

This dissertation primarily focused on motor recovery. Since the sciatic nerve is a mixed nerve, the effects of ES and TP on sensory recovery should be examined. This could be accomplished by repeating the study and measuring mechanical and thermal nociceptive responses of the injured paw. We are aware that nerve cuffs have been reported to elicit pain-like behavior in rodents (Pitcher et al., 1999; Benbouzid et al., 2008). To rule out this effect, the study should be repeated without the use of a nerve cuff.

Future experiments could also include multiple and/or longer sessions of ES. Importantly, it should be determined whether ES and TP elicit a combinatorial effect after a proximal nerve injury. These findings could be correlated with regeneration-associated gene expression changes to elucidate potential mechanisms by which ES and TP may be mediating their effects. Based on the molecular results, future studies could include genetic mouse models with the ability to overexpress/knockout candidate genes and assess functional recovery as an outcome measure.

Although the combination of ES and TP did not demonstrate a therapeutic effect after distal sciatic nerve crush injury, ES and TP may prove to be beneficial after transection injury. One substantial challenge that remains with transection injuries is guiding regenerating nerves back to functionally appropriate Schwann cell tubes. The work by Bittner et al. demonstrates great promise at maintaining a

conduit for the axons to regenerate into after sciatic nerve transection with the use of microsuture, hypotonic Ca^{2+}, methylene blue, and PEG (Bittner et al., 2012). Combining surgical and non-surgical approaches, such as surgical repair with membrane fusion followed by ES and TP administration could have significant potential in maintaining an appropriate channel for axons to regenerate back to their appropriate target. Moreover, ES and TP would decrease the time before sprout formation and accelerate axon regeneration rate, respectively.

In summary, there are multiple aspects of the post-injury response that are amenable as treatment targets, including the severity, location, and type of nerve injury, rapidity of recovery, and regeneration distance to target. The data presented in this dissertation do not support the concept of a “blanket” treatment approach that could be applied in a generalized manner to PNI, regardless of the nature of that injury. Instead, we propose different treatment strategies, depending on injury severity. With a severe nerve injury, in close proximity to the cell body and far from target, there is a prolonged regenerative response needed to accommodate protracted recovery times accompanying proximal lesions. Moreover, prolonged target disconnection times and longer regeneration distances compromise target end-organ viability and suitability for re-innervation. As such, administration of a combinatorial treatment, targeting multiple post-injury stages or phases following proximal nerve injury, is essential for maximal enhancement of axon regeneration and, by extrapolation, functional recovery. In contrast, distal nerve injuries have relatively rapid recovery time, as axons have

shorter distances to reach and reinnervate target tissues. Due to fast recovery times, less damaging effects occur at the target end-organ. Administration of selected treatments targeting a single phase or stage can, therefore, reduce the time to functional recovery in less severe PNI effectively, thus eliminating the need to build multi-tiered treatment approaches necessary in more severe injury situations. An important future direction will be to translate the conclusions obtained in this study using a crush axotomy injury paradigm to a complete transection and surgical repair paradigm that reflects the clinical PNI situation.

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CURRICULUM VITAE

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Education

2003-2006	Undergraduate studies, Youngstown State University, Youngstown, OH, B.S. in Combined Sciences
2006-2008	Graduate studies, Youngstown State University, Youngstown, OH, M.S. in Biology **Advisor: Mark Womble, Ph.D**
2008	Certificate in Bioethics, Youngstown State University, Youngstown, OH
2008-2014	Graduate Studies, Indiana University, Indianapolis, IN, Ph.D in Medical Neuroscience **Advisor: Kathryn J. Jones, Ph.D.**

Honors

2005	Undergraduate Research Grant
2005	Black Family Scholarship Fund
2006-2008	Graduate Student Teaching Award & Stipend
2007, 2008	Summer Research Assistantship Award
2011	IUSM travel Award
2010-2013	Educational Enhancement Grant Travel Award
2013	American Association of Anatomists Award

Peer-Reviewed Research Publications

1. **Meadows RM**, Batka RJ, McMillan KP, Brown RJ, Sengelaub DR, and Jones KJ. Electrical stimulation and testosterone propionate enhance functional recovery after sciatic nerve crush injury. *Manuscript in preparation*

2. **Meadows RM**, McMillan KP, Batka RJ, Brown RJ, Sengelaub DR, and Jones KJ. Electrical stimulation and testosterone propionate enhance regeneration-associated genes after sciatic nerve injury. *Manuscript in preparation*

3. *Haulcomb MM, ***Meadows RM**, Beahrs TR, Sanders, VM, Jones KJ. Existence of a resilient population of motoneurons following neuronal injury and disease. Journal of Neuroscience. *Manuscript in preparation*

4. Haulcomb MM, Mesnard NA, Batka RJ, Brown TJ, McMillan KP, **Meadows RM**, Sanders VM, Jones KJ. Identification of two rates of

disease progression in the mSOD1 transgenic mouse. Experimental Neurology. *Manuscript in preparation*

5. Haulcomb MM, Batka RJ, Brown TJ, McMillan KP, **Meadows RM**, Sanders VM, Jones KJ. Disease progression patterns in a murine model of ALS. Amyotrophic Lateral Sclerosis. *Manuscript in preparation*

6. Batka RJ, Brown TJ, McMillan KP, **Meadows RM**, Jones KJ, and Haulcomb MM. Speed-dependency of locomotion parameters in unforced mouse gait analyses. Anatomical Record. *Accepted*

7. Schmutzler BS, Roy S, Pittman SK, **Meadows RM**, Hingtgen CM. Ret-dependent and Ret-independent mechanisms of Gfl-induced sensitization. Mol. Pain. 30:7-22. (2011)

Book Chapters

1. **Meadows RM**, Sengelaub DR, and Jones KJ. Sunderland's Nerves and Nerve Injuries: Cellular Aspects of Nerve Injury and Regeneration. *Submitted*

Presentations

Invited Talks

1. **Meadows RM**. The effects of electrical stimulation and testosterone treatment on axon regeneration and functional recovery after sciatic nerve crush injury. Motoneuron Seminar. Indiana University School of Medicine, Indianapolis, IN, USA. (2013)

2. **Meadows RM**. An in vitro and in vivo model of schwannomatosis-related pain. Public Seminar. Indiana University School of Medicine, Indianapolis, IN, USA. (2012)

3. **Meadows RM**. Joints of the Upper Limb and Vertebral Column. Guest Lecturer. Youngstown State University, Department of Physical Therapy, Youngstown, OH, USA. (2008)

4. **Meadows RM**. SP receptor activation and desensitization as monitored by M current inhibition. Public Seminar. Youngstown State University, Department of Biological Sciences, Youngstown, OH, USA. (2006)

Abstracts / Poster Presentations

1. **Meadows RM**, McMillan KP, Batka RJ, Brown RJ, Sengelaub DR, and Jones KJ. Electrical stimulation and testosterone propionate enhance regeneration-associated genes after sciatic nerve injury. Abstract for poster presentation, Society for Neuroscience Meeting. San Diego, CA, USA. (2013)

2. **Meadows RM**, McMillan KP, Batka RJ, Brown RJ, and Jones KJ. The effect of electrical stimulation and testosterone on the expression of regeneration-associated genes. Abstract for poster presentation, Experimental Biology, American Association of Anatomists Meeting. Boston, MA, USA. (2013)

3. Batka RJ, Haulcomb MM, **Meadows RM**, Jones KJ, and Brown TJ. Exploration of the relationship between speed and other locomotion parameters using the CatWalk gait analysis system in wild type mice. Abstract for poster presentation, Society for Neuroscience. New Orleans, LA, USA. (2012)

4. Haulcomb MM, Batka RJ, Brown TJ, McMillan KP, **Meadows RM**, Sanders VM, and Jones KJ. Evolution of motor deficits in SOD1 mice using a combination of behavioral tests. Abstract for poster presentation, Society for Neuroscience Meeting. New Orleans, LA, USA. (2012)

5. **Meadows RM**, Monaco GN, Batka RJ, Jones KJ, and Brown TJ. Assessment of gait parameters after combined treatment of electrical stimulation and testosterone following sciatic nerve injury. Abstract for poster presentation, Society for Neuroscience Meeting. New Orleans, LA, USA. (2012)

6. Monaco GN, **Meadows RM**, Batka RJ, Brown TJ, and Jones KJ. The effects of combinatorial treatment of electrical stimulation and androgen on pain-associated gait parameters after sciatic nerve injury. Abstract for poster presentation, Indianapolis Veterans Affairs Medical Center Research Day. Indiana University School of Medicine, Indianapolis, IN, USA. (2012)

7. **Meadows RM**, Nguyen M, Kalpana GV, and Hingtgen CM. An in vitro model of schwannomatosis-related pain. Abstract for poster presentation, Neurofibromatosis Conference sponsored by the Children's Tumor Foundation. New Orleans, LA, USA. (2012)

8. **Meadows RM** and Hingtgen CM. Schwann cells modulate the release of calcitonin-gene related peptide from sensory neurons. Abstract for poster presentation, Society for Neuroscience Meeting. Washington, DC, USA. (2011)

9. **Meadows RM** and Hingtgen CM. The effects of conditioned-Schwann cell media on capsaicin-stimulated release of calcitonin-gene related peptide from sensory neurons. Abstract for poster presentation, Society for Neuroscience Meeting. San Diego, CA, USA. (2010)

Membership in Professional Societies:

2010 – 2013	American Association for the Advancement of Science (AAAS)
2012 – present	American Association of Anatomists (AAA)
2010 – present	Society for Neuroscience (SfN)

Teaching Experience

Fall 2012 & 2013	Teaching Assistant, Department of Anatomy and Cell Biology, Indiana University School of Medicine, Indianapolis, IN, *Courses: Neuroscience & Clinical Neurology (D505/852), Indianapolis Campus Gross Anatomy (630), Ball State Campus*
August 2013	Guest Lecturer, Department of Biology, Indiana University-Purdue University, Indianapolis, Indianapolis, IN, *Presented 4 lectures covering the following topics: digestive physiology, signal transduction, endocrinology, reproduction, metabolism, and energy balance*
Fall 2013	Teaching Assistant, Marian University College of Osteopathic Medicine, Indianapolis, IN, *Assisted students through laboratory assignments and prepared cadaver prosections*