Motor cortex electrical stimulation in rats with a cervical spinal cord injury to promote axonal outgrowth

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Abstract

Objective

Many treatment regimens for improving recovery after spinal cord injury (SCI) in humans have been trialed with limited success. Electrical stimulation (ES) to promote spinal cord repair has been more recently examined as a SCI treatment modality in animals, though remains under investigated. Here, we examined the role of motor cortex ES on axonal re-growth, plasticity, and functional recovery in a SCI rat model.

Materials and Methods

A dorsal lateral quadrant transection at C4 in 48 rats was performed after Montoya grasping stairwell training. Rats were divided into 4 groups: 1) ES333 (n=14; 333Hz, biphasic pulse, 0.2ms duration every 500ms with 30 pulses per train); 2) ES20 (n=14; 20Hz, biphasic pulse, 0.2ms duration every 1s with 60 pulses per train); 3) SCI only (n=10); 4) sham (n=10; electrode insertion without ES). ES of the forelimb motor cortex corresponding to the injured corticospinal tract (CST) for 30 minutes at the time of SCI surgery was performed. Post-injury grasping scores were recorded weekly for 4 weeks. Axonal collateralization and dieback at multiple points were quantified using bright-field microscopy after CST labeling. Behavioural outcomes and histological outcomes were found to be no different between SCI only and sham rats, and as such the two groups were combined into one 'control' group. Significance level for between-group comparisons (ANOVA or Kruskal-Wallis for non-parametric data with subsequent post-hoc testing) was set at $p<0.05$.

Results

Post-SCI grasping success and furthest well reached scores were significantly lower than baseline values ($p<0.01$, Tukey test) for all groups. ES20 animals had significantly lower grasping scores and lower furthest well reached scores post-SCI than controls ($p=0.03$ for both, Tukey test).

Significantly more axonal collaterals (i.e., axonal sprouts rostral to the lesion) were found in the ES333 animals compared to control animals ($p<0.01$, Mann-Whitney test). No difference was found with respect to the number of collaterals between the two ES groups ($p=0.10$, Mann-Whitney test), nor between the ES20 and control groups (p-value 0.16, Mann-Whitney test).

Beginning at 100μm rostral to the injury, ES20 rats had significantly more axonal dieback (axon count rostral to SCI) than the ES333 rats ($p=0.03$, Tukey test). At both 50μm rostral to injury and at the injury site, ES20 rats had significantly more axonal dieback than controls and ES333 rats (50μm mark: $p=0.02$ and 0.02, respectively, Tukey test; lesion site: $p=0.01$ and 0.02, respectively, Tukey test).

Conclusion

We have demonstrated that motor cortex ES of the injured CST results in greater axonal collateralization. The extent of axonal retraction rostral to SCI and behavioural outcomes also seem to vary with different ES parameters. Collectively, our data suggests that ES represents a potentially promising SCI therapy to promote axonal outgrowth, but further investigation is required.

Preface

This thesis is an original work by Dr. Andrew Jack. No part of this thesis has been previously published. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta ACUC Health Sciences, Project Name “Repairing the injured spinal cord”, No. AUP00000254_REN4, January 4, 2015.

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Tables and Figures

Symbols and abbreviations

ABC-DAB: avidin-biotin complex-3,3'-diaminobenzidine

ANOVA: analysis of variance

ASIA: American Spinal Injury Association

BDA: biotin dextran amine

BDNF: brain derived neurotrophic factor

cAMP: cyclic adenosine monophosphate

CNS: central nervous system

CREB: cAMP response element binding protein

CREM: cAMP response element modulator

CSPG: chondroitin sulfate proteoglycan

CST: corticospinal tract

DC: direct current

DLQ: dorsal lateral quadrant

DRG: dorsal root ganglion

EF: electrical field

ES: electrical stimulation

ERK: extracellular receptor kinase

GABA: gamma-aminobutyric acid

GAG: glycosaminoglycan

GAP-43: growth-associated protein 43

GDNF: glial derived neurotrophic factor

GPI: glycolsylphosphatidyl-inositol

GTPase: guanosine-triphosphatase

ICCP: international campaign for cures of spinal cord injury paralysis

MAG: myelin-associated glycoprotein

MIASCI: minimum information about spinal cord injury

MVC: motor vehicle collision

NGF: nerve growth factor

NgR: Nogo receptor

NT: neurotrophin

OFS: oscillating field electrical stimulation

OMgP: oligodendrocyte myelin glycoprotein

PNS: peripheral nervous system

PVC: polyvinyl chloride

ROCK: Rho-kinase

SCI: spinal cord injury

SD: standard deviation

SSEP: somatosensory evoked potential

tSCI: traumatic spinal cord injury

TBS: triphosphate buffered solution

TrkB: tropomyosin-related kinase B

VAS: visual analogue scale

VGF: nerve growth factor inducible growth factor

Chapter 1:

Part 1 – An introduction to spinal cord injury

1.1.1 Epidemiology

Spinal cord injury (SCI) is a disastrous event when it occurs often leaving patients irreparably debilitated. The consequences of SCIs can be so pervasive because they may result in varying degrees of motor, sensory, and autonomic dysfunction that affect almost every aspect of one's life—psychological, social, economic, among others. Increased dependence on others, and decreased health-related quality of life are just a few of the resultant effects on individuals (Mahabaleshwarkar et al., 2014). SCI unfortunately is not an uncommon event. Its incidence rate within North America is reported to vary between 20.7 and 83 people per million (Furlan et al., 2013), and approximately 85,000 Canadians are currently living with a SCI (Wilson et al., 2013). The incidence and prevalence reported here are increased from previous years mainly due to improved field management and pre-hospital patient care of trauma patients with SCI (resulting in increased life expectancy post-SCI). Increased awareness of SCI related complications and treatment thereof, as well as an ageing population have also contributed to an increased incidence and prevalence of SCI (Noonan et al., 2012). This is especially true considering that falls are the leading cause of SCI among patients over the age of 60 (Devivo et al., 2012). SCI due to falls represented 16.2% of all SCIs in the 1970s, compared to 21.8% as of 2000 (Devivo et al., 2012). However, the overall leading cause of SCI remains motor vehicle collisions (48.3%), followed by acts of violence and sport-related SCI (12% and 10%, respectively) (Devivo et al., 2012). With motor vehicle collisions being the most common cause of SCI, it is not surprising that SCI incident rates are highest among people in the late teens and early twenties (Devivo et al., 2012). However, again, with an ageing population some studies have shown the emergence of a

second peak in the elderly (Acton et al., 1993; Hagen et al., 2010; Price et al., 1994). This coincides with an increasing mean and median age of people suffering SCI from 28.3 (1970) and 30 (1980) to 37.1 (2005-2008) and 36.9 (2010), respectively (DeVivo et al., 2011). If individuals suffering SCI are living longer and the cost of living with a SCI is higher, then undoubtedly the financial burden of suffering a SCI is going to increase both personally and societally. In addition, there will be more long-term complications and eventual hospitalizations required for SCI patients over the course of their lifetime.

Hospitalization of SCI patients is often more lengthy, complicated, and thus more expensive than those patients hospitalized for other reasons. For example, in a recent study, it was shown that hospitalization for SCI patients was approximately 2.5-times longer, had approximately 4-times higher charges associated with them, and had 2.5-times higher percentage of mortality than patients without SCI (Mahabaleshwarkar et al., 2014). Higher mortality in individuals with a SCI can result from a number of different problems. Long-term complications related to SCI depend somewhat on the severity of the injury, however may include: deep vein thrombosis and resultant embolic phenomena, pressure ulcers and skin breakdown, autonomic dysreflexia and hypertensive crises, urinary tract infections, gastrointestinal motility problems, upper and lower respiratory infections, spasticity and pain management issues, among others. Moreover, most deaths after SCI occur in an acute fashion in comparison to the general population. Leading causes of mortality within the SCI population include pneumonia and respiratory failure, infectious causes and sepsis, pulmonary emboli, and external causes (unintentional injuries, homicide/suicide) (DeVivo et al., 1993; DeVivo et al., 2011;

DeVivo et al., 1999). As mentioned, however, many of the long-term complications experienced by people living with a SCI depend on the extent of injury at the initial presentation due to both primary and secondary damage to the spinal cord.

1.1.2 Spinal cord injury pathophysiology

SCI is an extremely heterogeneous condition with many different classification schemes. The pathophysiological damage to the spinal cord at the time it occurs, for example, can be divided into primary and secondary damage. Primary damage occurs at the time the injury first takes place (in most circumstances this is a compressive force resulting in spinal cord damage). This compressive force may be instantaneous and transient or continuous and ongoing (Molliqaj et al., 2014; Wilson et al., 2013). The primary injury often incites a series of biomolecular and cellular changes that result in secondary damage (processes that occur as a result of the sentinel injury that exacerbate spinal cord tissue damage). These secondary insults causing damage begin within seconds of the primary damage and may last for a number of weeks thereafter (Wilson et al., 2013). For example, these secondary changes may include electrolytic and ionic signaling disturbances with calcium and sodium, neurotransmitter excitotoxicity and receptor mediated injury, cell membrane lipid peroxidation, apoptosis, ischemia, inflammation and immunological responses, among others (Hagg et al., 2006; Molliqaj et al., 2014; Wilson et al., 2013). Although not a lot can be done with respect to treating the primary damage, medical and surgical treatments are aimed at optimizing parameters to mitigate secondary damage (Fehlings et al., 2006; Molliqaj et al., 2014; Wilson et al., 2013). This includes, but is not limited to, oxygenation and avoiding hypercarbia, avoidance of

hypotension, maintaining euthermia and euglycemia, electrolytic homeostasis, and timely decompression of ongoing spinal cord compression.

As a result of the primary injury, secondary injury, and ensuing inflammation, axonal demyelination and degeneration occurs. This not only occurs at the site of injury, but also remotely (Emery et al., 1998). Several mechanisms for oligodendrocyte death and subsequent demyelination have been proposed including microglia-induced apoptosis (Emery et al., 1998; Li et al., 2005), and changes to local trophic factors (Barres et al., 1993; Beattie et al., 2002; Casaccia-Bonnefil et al., 1996; Krenz et al., 2000). Axonal damage and demyelination results in abnormal axonal function, impaired functional recovery, and Wallerian degeneration. After the acute SCI phase (first 48 hours) and as axonal degeneration is occurring in the sub-acute (2 days to 2 weeks) to intermediate/chronic phase (>2 weeks), astrocytes are among the last cells to infiltrate into the injury site (Rowland et al., 2008). These astrocytes proliferate and begin to wall-off the site of injury and inflammation by forming a glial scar. Although this may in one sense decrease further damage (Faulkner et al., 2004), it also impedes axonal regeneration. This glial scarring represents a significant deterrent to central nervous system (CNS) axonal regeneration (Rowland et al., 2008). Several factors are responsible for this. Notably, chondroitin sulphate proteoglycans (CSPGs) are expressed and secreted by astrocytes (Rowland et al., 2008). Structurally, CSPGs are made up of a core protein (after which it is named) with glycosaminoglycan (GAG) side-chains attached. Both the core protein and GAG chains have been proposed as being responsible for growth-inhibition through the Rho-ROCK pathway (Rowland et al., 2008). However, several

other inhibitory molecules are also present working to prevent axonal regeneration and recovery. Inhibitory factors such as myelin-associated inhibitors (myelin-associated glycoprotein or MAG, oligodendrocyte myelin glycoprotein or OMgP, Nogo-A) (Faulkner et al., 2004) have been found within the glial scar following SCI. Many of these molecules act by activating the Rho-ROCK inhibitory pathway leading to growth cone collapse through cytoskeletal rearrangement (Rowland et al., 2008). Further discussion of this molecular pathway and its different therapeutic targets to promote axonal regeneration can be found in chapter 1, part 2, of this thesis.

1.1.3 Spinal cord injury clinical-anatomical classification: syndromes, grades, and treatment schemes

Just as SCI pathophysiological events can be classified as being primary or secondary, SCI can also be categorized based on the anatomical structures involved and the resultant pattern of deficits. The classification of SCI into spinal cord syndromes reflects mainly the involvement of the lateral CST, the posterior somatosensory columns (gracile and

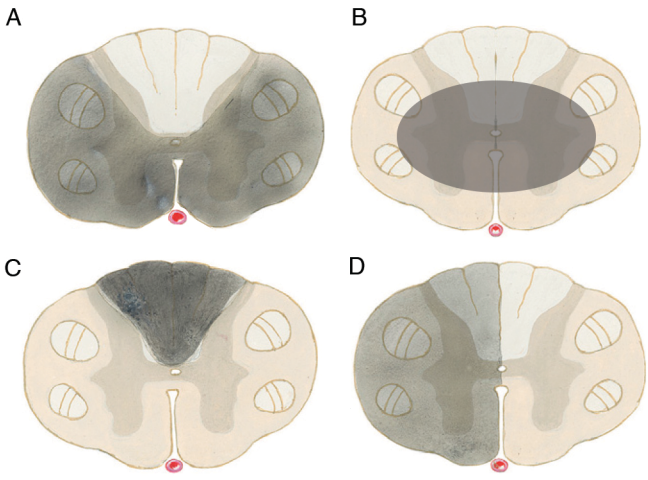

Figure 1: Anatomical schematic of tissue damage in spinal cord injury (SCI) syndromes (adapted from Netter's Concise Orthopaedic Anatomy (2nd Ed.). A) Anterior cord syndrome; B) central cord syndrome; C) posterior cord syndrome; D) Brown-Sequard syndrome

cuneate fasciculi), and the lateral spinothalamic tract (Figure 1). Spinal cord syndromes include Brown-Sequard syndrome, central cord syndrome, anterior cord syndrome, posterior cord syndrome, and conus medullaris syndrome. In addition to SCI classification schemes based on anatomically involved structures and presenting syndromes, SCI patients can also be categorized based on the severity of their injury. In the simplest of schemes, SCI can be separated into complete or incomplete injuries based on preservation of perianal function. Patients that have a complete SCI are classified as an 'A' according to the American Spinal Injury Association (ASIA) impairment classification. Incomplete SCI patients may be subcategorized based on the remaining motor strength below the neurological level. An ASIA B patient has sensory preservation and no motor strength, an ASIA C has less than a grade 3/5 power in over 50% of key muscle groups below the neurological level of injury, an ASIA D has at least a grade 3/5 power in over 50% of key muscle groups below the neurological level, and an ASIA E has normal sensory and motor testing. This method of classification according to the severity of neurological injury has been shown by many to correlate with SCI patient outcome (Flanders et al., 1999; Flanders et al., 1996; Freund et al., 2012).

ASIA classification scheme for SCI is based on presenting neurological deficits, which are in turn related to the specific spinal cord anatomy that is injured. The extent of neurological deficit is also dependent on the site of injury to the spinal cord (Figure 2), and importantly a more severe neurological deficit at baseline is likely reflective of extensive primary damage to the spinal cord and amount of functional tissue remaining.

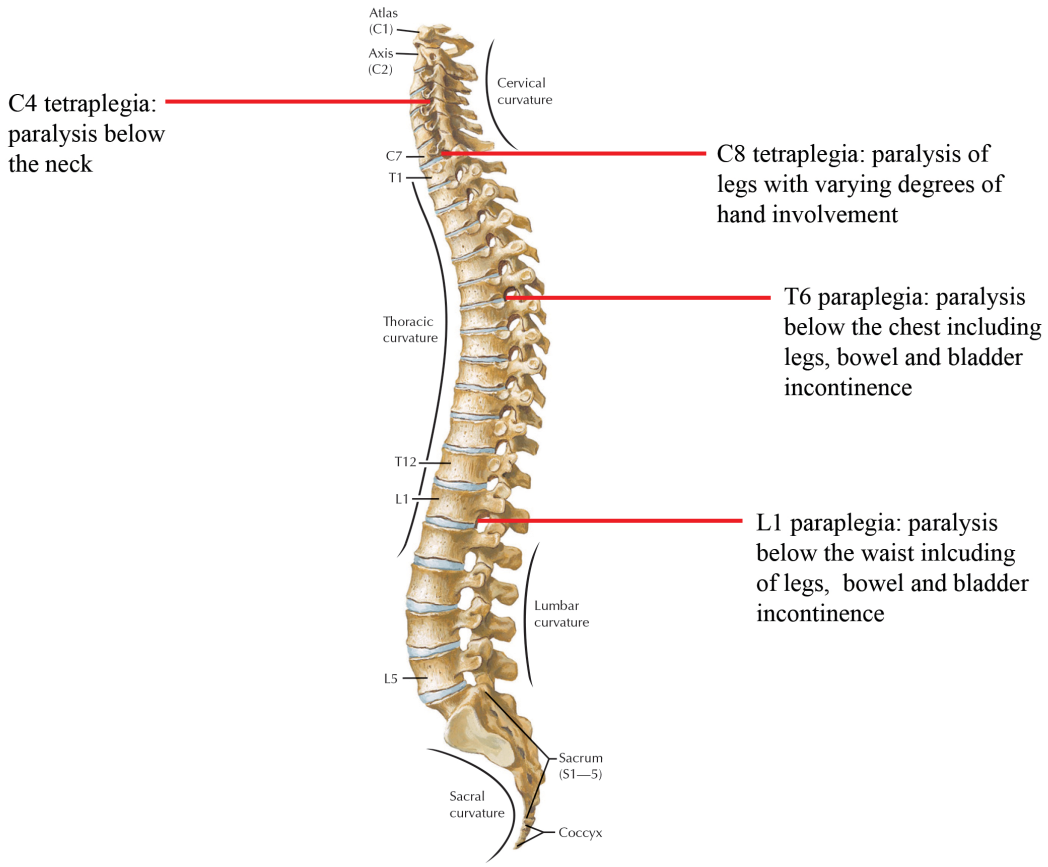

Figure 2: Schematic representation of spinal column and resultant neurological deficit in the event of complete SCI at each level (adapted from Netter's Concise Orthopaedic Anatomy (2nd Ed.).

As there is currently nothing that can be done to reverse the primary damage incurred, treatment of SCI in the acute phase is aimed at mitigating secondary damage to the spinal

cord. Treatments aimed at minimizing secondary damage are known as neuroprotective. This is in contrast to treatments aimed at repairing primary damage to the spinal cord, or neuroregenerative treatment strategies. Whether neuroprotective or neuroregenerative, optimizing the conditions for spinal cord cellular repair and regeneration of any remnant tissue is the goal of treatment. "After SCI, the difference between recoveries of substantial or minimal neurological function is determined by the preservation of only a fraction of the original tissue at the injury site" (Krishna et al., 2014). Rarely does a SCI result from complete transection of the cord with distinct rostral and caudal ends (resulting in an ASIA A, or complete SCI). More commonly, remnants of CNS tissue connect the superior and inferior ends of the spinal cord on either side of the lesion acting as a bridge (Schwab et al., 2002). This is called an anatomically incomplete SCI, in comparison to an anatomically complete SCI in which there is no CNS tissue bridge (transection of the spinal cord). It is these tissue bridges that remain a target for treatment following SCI with respect to preventing their destruction as well as promoting their repair and regeneration (Krishna et al., 2014).

1.1.4 Concluding remarks

Many treatment options have been investigated in animal models, and few trialed in the context of a clinical SCI. These can be subcategorized into neuroprotective and neuroregenerative strategies. Strategies for the treatment of SCI have traditionally been focused on avoidance of complications and historically consisted of patients being prescribed bed rest until mechanical stability was achieved in the case of bony injury (Wilson et al., 2013). However, therapies have more recently been undertaken looking at

the potential role for neuroprotective agents (methylprednisolone, naloxone, riluzole, minocycline, among others) and neuroregenerative strategies (cethrin, anti-nogo antibodies, cellular transplant, electrical stimulation, among others). Discussion of neuroprotective agents that have been investigated for SCI are beyond the scope of this thesis. In contrast, select neuroregenerative strategies are discussed in the following section, and although many demonstrate great promise in animal models, few have been shown to impart significant benefit with respect to improved outcomes in humans.

Chapter 1:

Part 2 – Spinal cord injury neuroregenerative treatment strategies

1.2.1 Rationale for neuroregenerative treatments

Despite the gravity of SCI, there have been no significant regenerative therapeutic advancements resulting in substantially improved functional outcomes in humans. Neuroregenerative treatments are those that are aimed at repairing the incurred primary damage and resulted in axontomesis (complete interruption of an axon and its myelin sheath with preservation of its supporting structures), neurontomesis (complete nerve interruption without preservation of its supporting structures), and other neural cell injuries. More specifically, neuroregenerative therapy is an umbrella term for a host of different treatments with effects that may include increased axonal outgrowth. Axonal outgrowth includes both regeneration (re-growth of a severed axon from its terminal, lesioned, end) and collateralization (new sprouting from a severed axon rostral to its injury). In order to increase axonal outgrowth, neuroregenerative therapy can act on mechanisms that either inhibit or promote axonal outgrowth. The following discussion focuses on neuroregenerative strategies that have been tried for SCI in animal models (and subsequently humans in select cases). In order to discuss neuroregenerative strategies however, prerequisite knowledge of neuroregenerative mechanisms and how to promote them must first be considered.

1.2.2 Neuroregenerative treatment mechanisms and their targets

Understanding neuroregenerative treatment mechanisms and their targets starts with first comprehending the hostile growth environment within the injured spinal cord. After SCI, axons were initially described to retract into ‘dystrophic end-balls’ that were thought to be incapable of growth and regeneration (Cajal, 1928). However, the dystrophic end-balls

have more recently been demonstrated to not necessarily be incapable of growth, but rather be inhibited from growth by the surrounding CNS environment (Tom et al., 2004). This inhibition stems from a variety of sources, including for example CNS myelin, myelin-associated inhibitors, and CSPGs which have been previously demonstrated to cause growth cone collapse (Filbin et al., 2003; Kottis et al., 2002; Mukhopadhyay et al., 1994). In addition, neutralization of these myelin-associated inhibitors has been shown to enhance axonal growth (Schnell et al., 1990; Zorner and Schwab, 2010; McKerracher et al., 2015). Decreasing the extrinsic inhibition to axonal growth in the surrounding environment by inhibiting myelin-associated growth inhibitors is one strategy employed to promote neural repair and regeneration. Another consideration with respect to promoting axonal outgrowth is attempting to change the intrinsic state of the neuron to one that is pro-growth, akin to that seen in embryonic CNS neurons (Spencer et al., 2004).

Adult CNS axons have a limited capacity for outgrowth relative to the immature, embryonic CNS neurons (Spencer et al., 2004; Lu et al., 2012). A number of factors account for this difference. For example, endogenous cAMP levels (an important attractive growth cone guidance cue) (Ming et al., 1997; Song et al., 1998; Song et al., 1997) are decreased in mature neurons compared to younger ones. Moreover, this drop in cAMP has also been shown to coincide with the inability of older neurons to regenerate (Filbin et al., 2003), and blocking cAMP action in young animals post-axotomy can block the normal regenerative process (Cai et al., 2001). In this sense, cAMP can be thought of

as a “common pathway” leading to axonal outgrowth for different upstream growth promoters (Figure 3).

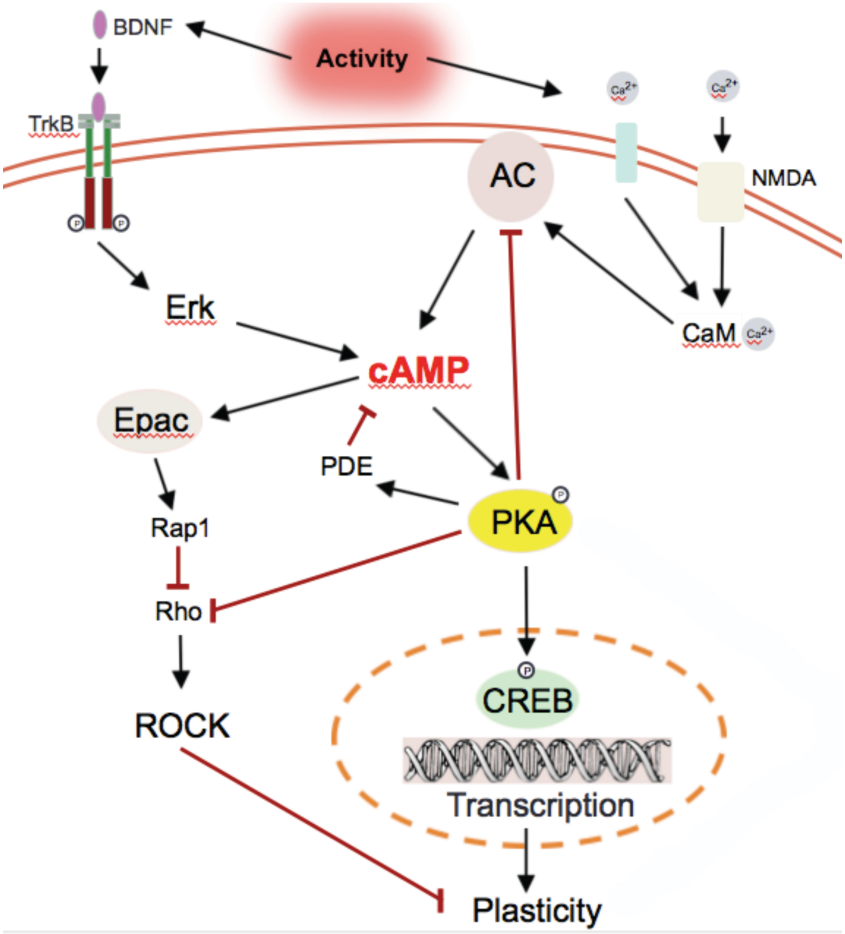

Figure 3: Schematic representation of the mechanism of action of neurotrophic signaling (shown here, BDNF) through which the effect of activity, or electrical stimulation, results in increased cAMP, PKA activity, and upregulation of growth-associated genes (figure courtesy Dr. Karim Fouad).

For example, the outgrowth effects from BDNF administration and subsequent behavioural improvement are thought to be mediated through, among others, downstream cAMP. BDNF is a neurotophic factor that stimulates axonal outgrowth, and exogenous BDNF administration after SCI has been shown to increase outgrowth (Vavrek et al., 2006) and promote behavioural recovery. Other intracellular signaling pathways related to BDNF and cAMP that are integral to growth and development are also differentially regulated between mature and immature neurons. These pathways include extracellular signal-regulated kinase (ERK) and protein kinase B (Akt) which are also thought to be important for and partially explain the reduced potential for axonal regeneration following SCI. It goes without saying then that differential regulation of ERK and cAMP pathways also leads to differential expression of products downstream in those pathways. Regeneration associated genes, and gene expression protein products such as growth-associated protein 43 (GAP-43), microtubule-associated protein and myelin oligodendrocyte protein are only a few examples of products from these pathways (Hannila et al., 2008; Gil-Perotin et al., 2009). Simply put, different treatments and strategies that result in greater cAMP levels could potentially increase axonal outgrowth by increased expression of downstream growth-associated genes and their respective protein products. Neuroregenerative treatments attempting to increase cAMP levels include, for example, growth factor supplementation such as BDNF, direct cyclic nucleotide supplementation, or as discussed in chapter 2 of this thesis, electrical stimulation (ES). From the basic growth (cAMP, growth factors, etc.) and inhibitory growth (myelin-associated inhibitors, CSPGs, etc.) mechanisms above, it becomes

obvious that promoting neuroregeneration can come in the way of either inhibiting growth inhibitors or promoting growth promoters.

1.2.3 Inhibiting the inhibitors: CSPGs, Myelin-associated inhibitors, and Rho-ROCK

Chondroitin sulfate proteoglycans

CSPGs have been shown to be a significant inhibitor to axonal regeneration, and make up a major component of the extracellular matrix within the CNS (Rowland et al., 2008). CSPGs also make up the perineuronal net (PNN), a dense part of the extracellular matrix ensheathing neuronal somata and proximal dendrites that plays a role in synaptic stabilization and neuronal protection (Zhao et al., 2013). Upon injury to the spinal cord, astrocytic proliferation takes place which results in upregulation and production of CSPGs forming a glial scar (Rowland et al., 2008). This scar acts as a significant physical and chemical barrier to axonal regeneration. Use of the bacterial enzyme chABC has been shown to degrade CSPGs, and as a result has been investigated as a potential method of promoting axonal outgrowth post-SCI, as reviewed by Kwon and colleagues (Kwon et al., 2011). The majority of these have involved a rat thoracic SCI (either contusion or transection), although cervical SCI models have also been investigated to a lesser extent (Kwon et al., 2011). The behavioural and histological outcomes, in general, for the use of chABC have been positive. Most studies investigating the use of chABC and its influence on outgrowth have shown increased axonal outgrowth compared to controls (whether thoracic or cervical SCI models were used) (Kwon et al., 2011; Vavrek et al., 2007). Behavioural data are more varied, however most show improved functional

outcomes with the administration of chABC (Garcia-Alias et al., 2008). ChABC has also been shown to be beneficial when used as an adjunct to, for example, stem cell transplantation with respect to behavioural outcomes (Fouad et al., 2005; Garcia-Alias et al., 2008; Kwon et al., 2011).

Nogo

Inhibiting the factors responsible for inhibiting neuronal outgrowth primarily involves agents that act extrinsic to the neuron itself. Insight into what was responsible for preventing neuronal outgrowth post-SCI (extrinsic inhibitory factors or innate neuronal properties), and the stage for future spinal cord regeneration experiments was set by seminal work like that of Sam David and Albert Aguayo (David and Aguayo, 1981). The two demonstrated that CNS neuronal growth could occur in a peripheral nerve graft after SCI (David and Aguayo, 1981). Results from their experiment led them to postulate that it was a lack of CNS growth factors that was the culprit for a lack of axonal outgrowth after SCI. Subsequent experiments built on the work of David and Aguayo, but also changed the focus of the cause for poor re-growth post-SCI to there being a presence of inhibitory factors in the CNS (Schwab et al., 1985). Eventually, the Schwab group created a monoclonal antibody (IN-1) against an inhibitory portion of myelin that was believed to be responsible for the decreased CNS neuronal growth. Indeed, the group demonstrated that this antibody was able to curb the effects of the inhibitory myelin-associated molecule (now known as Nogo) *in-vitro* (Schwab et al., 1988), and also *in-vivo*. In a rat SCI model, the injection of IN-1 resulted in CST regeneration and axonal growth through a SCI and into the distal spinal cord stump (Schnell et al., 1990). The

formal Nogo protein was soon after sequenced (GrandPre et al., 2000). Moreover, it was discovered that Nogo-A (one of three Nogo isoforms) was expressed in CNS oligodendrocytes, but not peripheral nervous system (PNS) schwann cells (GrandPre et al., 2000). Many studies soon after followed investigating this molecule and its therapeutic potential. Inhibition of Nogo-A to promote axonal outgrowth has been studied *in-vivo* alone, and as a combinatorial treatment. For example, in a thoracic SCI rat model, Schwab et al. demonstrated that anti-nogo antibody administration resulted in increased axonal regeneration as well as an improved functional outcome (Liebscher et al., 2005). In light of the positive effects documented from extensive animal testing of anti-nogo antibodies (Zorner and Schwab, 2010), human trials were planned and are underway. Currently, a phase 1 clinical trial investigating the use of intrathecal anti-nogo antibodies has been completed in thoracic and cervical incomplete SCI patients showing excellent tolerance and no adverse effects (AE)—although these results have not yet been published (Zorner and Schwab, 2010). Currently, a phase 2 trial is underway investigating its efficacy in tSCI (Zorner and Schwab, 2010).

Myelin associated glycoprotein

The discovery of MAG dates back to the mid-1990s. Two separate groups identified the inhibitory protein independently using different biochemical and cell culture techniques (McKerracher et al., 1994; Mukhopadhyay et al., 1994). This was the first myelin-associated growth inhibitory protein identified, and led to almost a decade of work being dedicated to the clarification of MAG's influence on axonal growth inhibition. This discovery initially created several problems. Firstly, myelin was found in both the CNS

and PNS, however, injured peripheral nerves retained their capacity to regenerate. A study published by David et al. demonstrated that the basement membrane protein laminin was capable of neutralizing the inhibitory effect of MAG (David et al., 1995). Furthermore, MAG is predominantly found in compact, mature myelin close to the axolemma (Trapp, 1990). As such, in order to exert its inhibitory influence, growth cones would require coming in contact with MAG after injury resulting in their collapse. Because laminin is present in abundance in the PNS and relatively lacking in the CNS, upon nerve injury the amount of laminin in the PNS is able to overcome the inhibitory MAG and allow axonal regeneration. Its paucity in the CNS, however, prevents this neutralization where the inhibitory action of MAG then predominates (McKerracher and Rosen, 2015). Furthermore, it has also been shown that MAG also exists in a much more potent soluble form, which upon release from damaged myelin, can result in inhibition of axons that are not in direct contact with injurious environment. A number of animal studies have been done to date looking at the inhibition of MAG and attempting to overcome it (Barbay et al., 2015; Bartsch et al., 1995; Filbin, et al., 1996). Many of the studies have involved utilizing knock-out mice in an attempt to better characterize the role of MAG and its influence on axonal growth. Through these studies and seminal work done by the Filbin group, a dual role for MAG and its influence on axonal inhibition has become clearer. MAG in fact exerts a pro-growth effect on pre-natal neurons and an inhibitory effect on post-natal neurons (Cai et al., 2001; McKerracher and Rosen, 2015; Mukhopadhyay et al., 1994). As such, it acts as a developmental switch. This work eventually led to the discovery that MAG and other myelin-associated inhibitors work through a signal transduction pathway that results in a decrease in cAMP levels and

downstream PKA (protein kinase A) activity in mature neurons (Cai et al., 2001). To date, there have been very few translational studies in which MAG inhibition had been investigated in a pre-clinical setting. A monoclonal antibody to MAG (GSK249320) was trialed in squirrel monkeys 1-3 days after a stroke. Treated monkeys showed a faster recovery of dexterity in a pellet-grasping task as early as 3 days post-treatment. Although too early to be explained by axonal regeneration, it is postulated that the improvement seen is due to a neuroprotective effect (Barbay et al., 2015).

Rho and ROCK

Downstream in the signal transduction pathway of both the above mentioned inhibitors, is Rho and Rho-kinase. Increased production of Rho, and activation of the Rho-kinase enzyme leads to axonal growth inhibition. This represented another step in the signal transduction pathway that can be potentially inhibited in order to promote axonal regeneration. The McKerracher group was the first to show that the application of Rho-inhibitors such as Y27632 and C3 transferase into a SCI in the setting of a dorsal over-hemisection rat model resulted in improved functional recovery (Dergham et al., 2002). Soon after, it was shown that the administration of C3 transferase resulted in decreased RhoA activation after a thoracic contusion incomplete SCI in an animal model (Dubreuil et al., 2003). However, other studies investigating the use of such antagonists were not as optimistic. Some reported severe animal emaciation requiring subsequent euthanization, worsening or lack of effect with respect to behavioural outcomes, and a lack of observed effects on axonal outgrowth (Fournier et al., 2003; Sung et al., 2003). Improvement in the delivery of the Rho-antagonist through coupling it to a transport protein known as BA-

210 allowed for more direct SCI penetration. This agent has been commercialized as Cethrin (BioAxone Therapeutique, Montreal, QC, CAN), and has been demonstrated to result in improved behavioural outcomes in a mouse hemisection model (Lord-Fontaine et al., 2008). Similarly, Y27632, a direct Rho-kinase inhibitor, has been demonstrated to result in improved behavioural outcomes in a dorsal hemisection rat SCI model (Sung et al., 2003; Dergham et al., 2002; Fournier et al., 2003). Of the two therapeutics, BA-210 has been trialed in a phase 1/2a study which demonstrated safety and no AEs attributable to the drug. Improved neurological function was also reported, although the results with respect to functional outcomes are difficult to interpret in preliminary safety and dosing studies (Fehlings et al., 2011). A phase 2b study is currently underway.

1.2.4 Promoting the promotors: cAMP and growth factors

Promoting axonal growth can either be accomplished through neutralizing inhibitory factors such as Nogo, MAG, and Rho/ROCK, or alternatively through the alteration of CNS microenvironment to one that is actively conducive to axonal growth. In order to do so, strategies may include the supplementation of growth promoting factors or by altering the intrinsic state of the neuron to one that is pro-growth. As mentioned, mature neurons have been shown to have a decreased level of cAMP and PKA activity compared to younger ones (Cai et al., 2001; Filbin et al., 2003). Attempted strategies to "switch" the neuronal state to one that is pro-growth act mainly by attempting to increase intracellular cAMP levels that then facilitate growth cone elongation and growth cone stabilization (Ming et al., 1997; Song et al., 1998; Song et al., 1997). These have included the use of

cAMP supplementation and drugs such as rolipram, growth factors like BDNF, and electrical stimulation (ES).

Cyclic nucleotides

As a very simple analogy: the fulcrum of the balance between axonal growth inhibition and promotion after SCI appears to be cAMP. Prior to glial scar formation, there appears to be a time sensitive window during which axonal outgrowth may be most conducive. Prior to scar formation, or after digestion of the scar component CSPGs with chABC as discussed earlier, the main impediment to axonal regeneration is the presence of myelin-associated inhibitors. Although these myelin-associated inhibitors may have very different molecular structures, they also all have a relatively conserved binding sequence that can bind to the glycosylphosphatidyl-inositol (GPI)-linked receptor protein, Nogo-66 receptor (NgR) (McKerracher and Rosen, 2015). In order for binding to the receptor to result in signal transduction from the extracellular environment intracellularly, simultaneous binding with the neurotrophic receptor p75 (p75NTR) is required. Much of what follows in the pathway remains to be elucidated, however, what is known is that the GTPase enzyme RhoA becomes active and then ROCK with subsequent growth cone collapse (Hannila et al., 2008). In order to overcome this pathway, neurotrophic cues are required. Investigations into the mechanistic signaling of neurotrophins (an example of which is illustrated in Figure 3) led to the discovery that cAMP analogues (db-cAMP) resulted in a similar effect (Spencer et al., 2004). Furthermore, intracellular levels of cAMP and PKA activity (a cAMP dependent enzyme involved in the activation of numerous transcription factors) increase upon exposure to neurotrophins. This implies

that cAMP is the downstream signal for these growth promoters. The result of this pathway is the upregulation and transcription of, potentially, a plethora of growth promoting genes that enable axonal outgrowth. In order to do so, cAMP must first interact with cAMP response element binding protein (CREB) resulting in its phosphorylation. CREB is then responsible for causing the increased transcription of genes resulting in the ability of the axon to overcome myelin-associated inhibition. The genes that are then upregulated and transcribed that lead to increased axonal outgrowth have also more recently been the subject of study (for example, CREM [cAMP response element modulator], neuropeptide Y, VGF [nerve growth factor inducible growth factor], and GAP-43). From the wealth of knowledge gained from studies investigating cAMP and the signal transduction pathway that it is implicated in, several therapeutic targets for promoting axonal regeneration have become evident. In order to increase cAMP levels, strategies may include: cAMP analogues or forskolin (an adenylyl cyclase agonist) that have been shown to increase axonal growth both *in-vitro* and *in-vivo* animal models (Hannila et al., 2008; Lu et al., 2004), phosphodiesterase inhibitors such as rolipram (Nikulina et al., 2004; Pearse et al., 2004) that have also been shown to overcome myelin inhibition of axonal outgrowth, and neurotrophin administration such as BDNF and NT-3. A myriad of studies exist documenting use of neurotrophic factors to increase axonal outgrowth. In order to understand their use and success in promoting axonal outgrowth in animal models of SCI, it is first important to understand their mechanism of action in this pathway.

Growth Factors

Neurotrophins are potent neuronal growth factors, and the neurotrophin family members include BDNF, NT-3, NGF, GDNF, and NT-4/5. However, due to the inhibition imposed by myelin-associated inhibitory factors post-SCI, they are by themselves relatively ineffective as a treatment for axonal transection due to SCI. The mechanism through which many of these growth factors promote axonal outgrowth hinges on their ability to increase cAMP levels intracellularly. Here, we will focus primarily on BDNF and its signaling pathway. BDNF's ability to promote axonal regeneration starts by it first binding to its high affinity receptor tropomyosin-related kinase B (TrkB). This causes the start of a signal transduction cascade that includes autophosphorylation of intracellular tyrosine residues and can activate several pathways. Activation of one of these pathways leads to axonal growth being stimulated through extracellular signal regulated kinase (ERK) and cAMP (upon the ERK pathway becoming activated, an increase in intracellular cAMP production follows) (Hannila et al., 2008). Increased cAMP levels are the result of ERK inhibiting phosphodiesterase-4 (PDE4) and preventing the hydrolysis of cAMP (Gao et al., 2003; Hannila et al., 2008). The use of BDNF has been shown in many studies, both *in-vitro* and *in-vivo*, to promote axonal outgrowth and functional improvement. For example, after SCI BDNF has been shown to increase CST outgrowth (Vavrek et al., 2006). The effect of BDNF has been relatively modest, however, with some studies showing no effect (Blesch et al., 2004; Lu et al., 2001). The benefit in the functional read-out of these studies has also often been small to moderate (Tobias et al., 2003). Theories as to why the results from such studies have been somewhat lackluster have included, for example, the differential distribution of TrkB receptors (more highly expressed close to the cell body, resulting in spinally delivered BDNF having less

efficacy) (Weishaupt et al., 2012). Translation of neurotrophic use into a human model of SCI to promote axonal outgrowth and functional recovery has, to date, been unsuccessful. Many hurdles exist preventing their use. For example, logistical obstacles such as the cost of using growth factors and their limited half-life, or the widespread effects of agents such as BDNF and its implication in issues such as post-SCI hyperreflexia, spasticity, and pain (Weishaupt et al., 2012) have all hampered their clinical applicability. Although translation of neurotrophins into clinical practice has not been seen to date, alternatives to increase cAMP level post-SCI such as rolipram would seem more promising. Because rolipram has shown promise in *in-vitro* and *in-vivo* animal models, has already been approved by the Food and Drug Adminstration for human use, and is able to cross the blood-brain barrier, use in human trials would overcome many of the obstacles posed by other regenerative strategies.

2.5 Concluding remarks

Axonal regeneration represents one neuroregenerative strategy for promoting repair and recovery after SCI. As discussed, this can be achieved through inhibition of the molecules and factors that stunt axonal growth and plasticity, or by increasing the innate growth ability of the neuron itself. One therapeutic modality that "switches" the neuron to a pro-growth state is electrical stimulation (ES). The use and effects of ES, in addition to its potential therapeutic value after SCI has been the subject of more recent research and the subject of the next chapter of this thesis.

Chapter 2

A review of electrical stimulation to promote axonal outgrowth after spinal cord injury

2.1 Introduction: electrical stimulation and the corticospinal tract

The CST is the predominant motor pathway in humans connecting the upper motoneurons in the primary motor cortex to their end-target lower motoneuron circuits in the spinal cord. Through this connection, voluntary movement is generated. Loss of voluntary movement in the way of paralysis or weakness is often the result when the CST is damaged. The combined extent of primary and secondary damage to the CST in human SCI is not only correlative of neurological deficit and functional outcome, but also dictates the capacity for spinal cord tissue repair and healing (Raineteau et al., 2001; Stinear et al., 2007). Because of its importance with respect to movement, the CST has been one of the primary targets for treatment of SCI in animals and humans. However, after SCI a host of impediments within the central nervous system (CNS) micro-environment, including but not limited to the generation of inhibitory growth factors, decreased levels of growth promoting factors, and astroglial scarring hinder the effects of treatments for SCI. Different strategies have been employed to promote spinal cord repair including trying to overcome the inhibitory CNS growth environment (Fouad et al., 2005; Liebscher et al., 2005; Schnell et al., 1990) and altering the neuronal state to one more conducive to repair and regeneration. In keeping with the latter strategy, ES has been examined in both animals and humans to promote axonal outgrowth and behavioural recovery following SCI. Here, we review the use of epidural and intraparenchymal, spinal and cortical ES and its effect on the CST post-SCI. Furthermore, ES putative mechanisms for repair and recovery of the CST that serve as the basis for ES SCI animal and human experiments will be discussed. The ES SCI models previously investigated will be discussed as rationale for our model of intraparenchymal motor cortex ES to

promote CST outgrowth and post-SCI recovery in rodents. Finally, we then review the use of ES in a clinical context and its applicability to human SCI.

2.2 Mechanisms: neuronal outgrowth responds to electrical fields

The study of ES effects on neuronal growth dates back nearly a century (Ingvar, 1920; Williams, 1936), however its application to SCI in animals (Borgens et al., 1986b; Wallace et al., 1987a; Borgens et al., 1999; Politis and Zanakis, 1988) and humans (Shapiro et al., 2005) has been much more recent. With a few exceptions (Wallace et al., 1987a; Wallace et al., 1987b; Politis and Zanakis, 1988), the majority of studies examining the use of ES post-SCI have emanated from two main laboratories—that of Borgens et al. and Martin et al. Each body of work is based on a similar, but different ES mechanistic premise. Because of this, direct comparison between the different studies is difficult. The models used in studies from each lab (anatomical location of ES, lesion type, and animals used) have varied substantially (a list of which, with ES parameters and major results highlighted, can be seen in Table 2). Furthermore, the type of stimulation and stimulation parameters (for example frequency, duration, and amplitude) utilized has also varied. Studies published by the Borgens group (Borgens et al., 1987; Borgens et al., 1990; Borgens et al., 1986a; Borgens et al., 1986b; Borgens et al., 1997; Borgens et al., 1993; Borgens et al., 1999) served as the foundation for much of the human ES studies that have been subsequently carried out. Much of the work from the Borgens lab has been based on the idea that neurons respond to electrical fields (EFs) *in-vitro*. The idea that *in-vitro* neurons demonstrate outgrowth toward a cathode and regress away from an anode has been around for quite some time (Jaffe and Poo, 1979; McCaig, 1990). In an *in-vitro*

experiment with EF polarity being reversed, Mccaig found that there was an asymmetrical response in neurite outgrowth (cathode-facing outgrowth was faster than anode-facing regression) (McCaig, 1987). This suggested that by alternating the EF polarity, neuronal outgrowth could be achieved in opposite directions. These *in-vitro* experiments demonstrating neuronal outgrowth in response to EFs is what forms the basis for some of the ES SCI research. Indeed, Borgens et al. applied this idea to their own model of SCI experiments. Here, we will discuss their use use of epidural ES in the way of an EF at the site of SCI in order to promote axonal outgrowth into the lesion. However, this is not to be mistaken for epidural ES or intraspinal microstimulation below the SCI used to excite local motoneurons circuits and central pattern generators to produce patterned movements (beyond the scope of this thesis).

2.3 Electrical stimulation to create an electrical field in an experimental model of spinal cord injury

One of the first studies examining the effects of an imposed EF on SCI was completed in 1981 in a compete spinal cord transection lamprey animal model by the Borgens group (Borgens et al., 1981). This experiment consisted of 11 larval lamprey and 13 control animals undergoing ES by having a 10μA direct current (DC) applied across the spinal cord lesion for 5 or 6 days. A significantly higher number of the stimulation group was found to conduct action potentials across the lesion and have axonal processes growing into or across the lesion. Subsequent studies from the same lab investigating ES and SCI involved using a complete and partial transection guinea pig model. These studies showed increased axonal growth in the experimental groups into, but not across the lesion

(Borgens et al., 1986a; Borgens et al., 1986b). Although these former studies did not include behavioural data, a follow-up study conducted by the lab did demonstrate functional recovery in a group of electrically stimulated guinea pigs when the EF was applied rostral to the site of lesioning (Borgens et al., 1990). Moreover, further experimentation with DC ES involved the implantation of an ES pack in dogs (Borgens et al., 1999). This served as one of the first models for implantable electrical stimulators for potential use in humans. The results of these experiments were again favorable showing increased axonal growth into and through the spinal cord lesion. In the dog model, in keeping with the idea that alternating the EF polarity can result in neuronal outgrowth in opposite directions (and thus promote outgrowth of ascending sensory fibers in one-direction and descending motor fibers in the other), an oscillating electric field stimulator (OFS) was used and several neurological outcomes were followed up to 6-months post-implantation. These included superficial and deep pain sensation, proprioception, urological and urodynamic testing, ambulation and evoked potential testing. At the 6-month follow-up, the OFS treated group was found to have a significantly better combined neurological outcome score (Borgens et al., 1999). It was this latter work using the dog model and an implantable OFS that led to preliminary clinical studies of OFS use in humans with SCI.

2.4 Electrical stimulation and electrical fields: clinical applicability

Shapiro et al. published the first clinical trial of electrical stimulation in humans in 2005 (Shapiro et al., 2005). This was a phase 1a study, and consisted of an OFS device implanted in 10 complete SCI patients (level C5-T10). The device was placed within 18

days of injury with the electrodes spanning one segment on either side of the level of injury in an extra-spinal location (no contact with any neural elements). This was left implanted in patients for 15 weeks after which time it was removed and its function tested. Outcome measures from this study included ongoing neurological examination by an unblinded study neurologist, surgeon, and research nurse using the ASIA Impairment Scale, somatosensory evoked potentials (SSEPs) and the Visual Analog Scale (VAS) for pain at baseline, 6-weeks, 6-months, and 1-year post-implantation. The trial concluded that the surgical procedure and OFS implant was well tolerated, safe, and resulted in improvement in all parameters assessed. This resulted in approval of further patient recruitment, in which an additional 4 patients were included and compared to 14 historical control patients. Results from this expanded study were subsequently published in a report to the Securities and Exchange Commission by the owner of the technology (Walters, 2010; Cyberkinetics Neurotechnology Systems Inc., 2007). With the exception of motor recovery, patients treated with OFS showed marked improved in all domains assessed.

The lack of motor improvement between the two groups represents a significant limitation to this trial. That being said however, overemphasizing improvements solely in ASIA motor scores ignores other aspects of SCI individuals' quality of life deemed to be important (Fawcett et al., 2007; Walters, 2010). For example, the decreased pain scores reported in the OFS group represents a large determinant of quality of life that could be overlooked. This is an aspect of previously completed animal studies that should not be under-appreciated, and shows promise with respect to their human applicability.

The studies completed investigating the use of epidural electrical stimulation (Borgens et al., 1990; Borgens et al., 1986b; Borgens et al., 1993; Borgens et al., 1999; Carmel et al., 2010; Carmel et al., 2013; Carmel et al., 2014) have reported no harmful effects on the animals studied—no motor deterioration of the intact limbs, nor pain observed in the stimulated cohort. These are important aspects to consider in light of the fact that the full effects of increased CST outgrowth are as of yet unknown. In addition to the Borgens et al. group, the other studies reporting results pertaining to use of epidural ES have emanated from the Martin lab. Although their results have as of yet, for better or for worse, not translated into a human model of ES for SCI, results from their studies completed to date are equally as impressive. The ES in SCI model that they employ, however, is also drastically different. The group focuses primarily on ES of the intact, or uninjured, CST whether at the level of the medullary pyramids or motor cortex. As could be expected from using such a different model, the proposed mechanism by which ES promotes axonal outgrowth is also substantially different from that seen above with EFs.

2.5 Mechanisms: electrical stimulation to increase growth promoters and messengers

Although not yet completely elucidated, the use of ES as a method of increasing neuronal activity remote from the site of SCI (whether of motor neurons upstream of the injury or downstream sensory neurons) is thought to promote axonal outgrowth by acting primarily through the signaling messengers cAMP, calcium and growth factor BDNF. Much of the work supporting this has come from a Fouad et al. collaboration and conditioned lesion studies. Conditioned lesions consist of lesioning the peripheral branch of a sensory axon

from the dorsal root ganglion (DRG), and subsequently the CNS branch in a delayed fashion. In the absence of the conditioned lesion, the CNS axons fail to regenerate. However, by lesioning the peripheral branch, increased axonal outgrowth into the central lesion is seen (Filbin et al., 2003; Neumann et al., 1999; Richardson et al., 1984). Interestingly, in a combined conditioned lesion and ES experiment, peripheral nerve ES was shown to increase axonal outgrowth of the injured nerve's central projection into a central spinal cord lesion and increase cAMP levels to the same extent as a conditioned lesion (Udina et al., 2008). The mechanism by which increased axonal outgrowth (whether after a conditioned lesion or ES) occurs is thought to be mediated through cAMP. In fact, injecting cAMP into the DRG can mimic the effect seen by the conditioned lesion. In addition to acting through cAMP, ES has also been shown to increase the expression of BDNF and its receptor tyrosine receptor kinase B (TrkB) (Al-Majed et al., 2000). BDNF is a multifaceted neurotrophin whose breadth of effects is beyond the scope of this thesis, however a full review can be found elsewhere (Weishaupt et al., 2012). Among its many actions, BDNF has been shown to stimulate axon growth via the extracellular signal regulated kinase (ERK) pathway (Weishaupt et al., 2012). Furthermore, the administration of BDNF in the vicinity of axotomized neuronal cell bodies in rats with a SCI has been shown to increase axonal growth and contacts with propriospinal interneurons (PrIs). This correlated with an improved functional behavioural outcome compared to control animals (Vavrek et al., 2006). It is on the basis of the aforementioned studies and their findings that ES is believed to play a role in promoting axonal outgrowth and functional recovery following SCI. These proposed mechanisms serve as the foundation and rationale for the use of ES in the SCI

studies completed by the Martin group who use cortical ES to stimulate the uninjured CST post-SCI.

2.6 Electrical stimulation of the uninjured corticospinal tract to drive outgrowth in spinal cord injury

Much of the more recent work investigating the use of ES has originated from the Martin group. Their work has focused on the use of epidural and cortical (primary motor cortex, M_1, corresponding to the forelimb motor cortex) ES from which the uninjured CST originates (Carmel et al., 2010; Carmel et al., 2013; Carmel et al., 2014), although some have focused on ES of the CST at the level of the medullary pyramids (Brus-Ramer et al., 2007). The rationale for stimulation of the uninjured CST is founded on the concept of competition for end-target synaptic terminals between the two CSTs. Following injury to the CST, efforts to improve functional outcome and recovery have traditionally centered on trying to repair damaged connections or preserve spared connections from the injured CST. Alternatively, treatment can also be aimed at strengthening and expanding connections from the uninjured CST so that it assumes control of both sides of the body.

It is this uninjured CST that has been the target for axonal growth and improving functional recovery in rodent SCI experiments from the Martin group. The uninjured CST has been shown to be much larger in primates than rodents (Brosamle et al., 2000; Rosenzweig et al., 2009) suggesting it to be an optimal target for promoting recovery in primates. Furthermore, neurophysiological microstimulation experiments investigating the use of the uninjured CST have shown that at higher current thresholds, ipsilateral

cortical stimulation (of the uninjured CST) can generate similar movement to those seen contralaterally (Brus-Ramer et al., 2009). This in combination with CST developmental studies indicating that CST development and end-target synaptic density can be enhanced through activity, has led to the theory that competition exists for end-target synaptic terminals between the two CST tracts (Martin et al., 2007; Martin et al., 1999; Salimi et al., 2004). This can then be influenced through ES to increase axonal outgrowth (either regeneration or sprouting).

2.7 Activity drives competition and thus axonal outgrowth resulting in improved behavioural outcomes in animal spinal cord injury models

Axonal regeneration is defined as outgrowth of an axotomized neuron from its terminal, lesioned, end at the site of injury, whereas sprouting is defined as outgrowth from spared axons or axotomized neurons proximal to the site of injury (Dietz et al., 2014). This outgrowth has also been shown to correlate with functional recovery (Girgis et al., 2007; Vavrek et al., 2006). It has been well established by the Tuszynski lab and others that a SCI induces CST sprouting in animal models (Weidner et al., 2001). Interestingly, the Martin group has shown that while both ES and SCI both promote axonal sprouting in rats, the effects are in fact additive (Brus-Ramer et al., 2007). Furthermore, in keeping with the idea of axonal growth being largely determined by activity-dependent competition, this stimulation results in increased axonal growth both ipsilaterally and contralaterally. However, the largest increase in growth was seen on the denervated side of the animal (where there would be less competition for new axonal growth and synaptic connections being formed) (Carmel et al., 2013; Carmel et al., 2014). Finally, again in the

same vein as competition determining laterality of axonal growth in the spinal cord, local axonal outgrowth also appears to be governed by competition. The pattern of axonal outgrowth into the spinal cord grey matter was greatest in the regions denervated by pyramidotomy (Carmel et al., 2013). Contralateral to stimulation, axonal outgrowth of the stimulated CST fibers was greatest in regions outside the normal CST termination pattern suggesting that stimulation was not enough to overcome competition presented by the intact CST terminations in that region. Unlike the experiments from the Borgens lab, the majority of the ES investigations from the Martin lab included behavioural data. Experiments used the horizontal ladder test, and consisted of measuring the performance of the rats once every 5 days for 30 days. The Martin group found that the rats undergoing stimulation and injury (pyramidotomy) improved significantly with a decreased stepping-error rate compared to the rats with injury only. Furthermore, by the end of the post-operative testing period there was no significant difference between the stimulation group and uninjured rats (Carmel et al., 2010; Carmel et al., 2014). The errors that did occur by the stimulation group were also comparable to their baseline errors arguing against behavioural compensation or adaptation (Carmel et al., 2014). These experiments demonstrate that the ipsilateral motor cortex (and thus uninjured CST) is a viable target for ES in order to promote recovery following CST injury. Questions that remain, however, include: is there a time-dependent window following CST injury in which electrical stimulation needs to be implemented to promote axonal growth and behavioural recovery? And is the stimulated cortex truly responsible for the effects seen?

2.8 Electrical stimulation timing after spinal cord injury

The Martin group has gone on to clarify these questions through inclusion of another series of ES experiments. In one experiment, they applied cortical, epidural, ES at 8-weeks time post-pyramidotomy. Similar to the application of ES immediately post-injury, the stimulated group's behavioural error rate returned to their baseline level (Carmel et al., 2014). To investigate whether or not the stimulated motor cortex was responsible for the observed histological and behavioural effects, muscimol ($GABA_A$ agonist) was injected into the stimulated motor cortex. This resulted in the reversal of the behavioural improvements seen in the stimulated rats compared to controls. Once the muscimol effects had worn off the rats' motor deficits once again improved to baseline (Carmel et al., 2014; Carmel and Martin, 2014). Issues that remain unresolved from these studies include the relative contributions of the CST and RST to behavioural recovery. Although increased axonal growth of each tract has been observed, the relative amount that each contributes to the improvement of motor impairments remains unknown. In addition, although increased axon varicosity density correlates with increased axon density, the exact role of these new axon growth terminals has not yet been established. Whether they are synapsing onto motoneurons or interneurons, play an excitatory role or inhibitory role for example, has yet to be determined. Presumably, the increased axon density correlating with increased axon varicosities (synaptic boutons) (Brus-Ramer et al., 2007; Li et al., 2001) would mean that these stimulated CST axons have formed new synaptic connections resulting in behavioural amelioration. However, the exact cellular and biomolecular mechanisms and pathways to show causation for this have not been worked out. As such, although many promising findings from the Borgens, Martin, and Fouad group have been discovered, the application of such strategies to humans should still be

far from reality. The need for more investigation, including elucidation of the optimal electrical stimulation parameters (including frequency, electrode placement location, uninjured or injured CST ES) has yet to be determined.

2.9 Concluding remarks: rationale for short-term electrical stimulation in spinal cord injury, our experience

Due to the uncertain effects of several factors pertaining to the use of ES to promote axonal outgrowth and SCI recovery, we sought to systematically investigate some of these variables and further elucidate their influence on outgrowth of the injured CST post-SCI. We did so by adopting different methodological aspects from a few of the previous studies published by the Fouad and Martin groups on the presumed mechanistic basis of ES increasing cAMP, BDNF, and thus outgrowth as discussed previously. More specifically, the Martin group has shown that cortical epidural high-frequency ES (333Hz) of the uninjured primary motor cortex after SCI (transection pyramidotomy) has resulted in increased functional recovery and axonal outgrowth. Similar results have been found with low-frequency (20Hz) PNS stimulation with respect to increasing dorsal root ganglion CNS projection outgrowth after SCI (dorsal spinal cord transection injury) (Udina et al., 2008). In the next chapter of this thesis we describe our investigation of both high- and low-frequency (333Hz and 20Hz) ES applied to the primary motor cortex corresponding to the injured CST in a dorsal lateral quadrant sectioning SCI model.

Study	**Animal and injury model**	**Electrical Stimulation model**	**Parameters**	**Experimental Groups**	**Outcome (histological and behavioural)**
Borgens, Blight et al. (1986)	-Model: Hartley female guinea pigs -Injury: Low-thoracic dorsal transection	-Implanted direct current (DC) ES (epidural) across the SCI for mean 51 days	-Power supply: DC, 9V (3x3V in series) LiMn battery -Electrodes: Ag/AgCl on either side of the SCI	-ES: 1uA (n=4) -ES: 5uA (n=6) -ES: 10uA (n=11) -Sham ES (n=11)	-Histological: ES showed more regeneration into scar; 10uA group extended into spinal cord below the lesion
Wallace, Tator et al. (1987)	-Model: Wistar female rats -Injury: T6-7 extradural clip compression (125g for 1min)	-Implanted continuous DC ES (epidural) across the SCI for 15-20 weeks	-Voltage applied: <340mV (minimum for muscle twitch) -Electromagnetic field: 460kHz -Power supply: DC 9V ES group -Frequency: 10Hz -Electrodes: Pt on each side of the SCI (cathode proximal and anode distal)	-Sham ES with SCI (n=10) -ES with SCI: <340mV (n=10)	-Behavioural: 1. Inclined plane: no difference between groups -Neurophysiological: no difference between groups -Histological: no difference in regeneration, scarring, or lesions between groups -Adverse Events: 9/20 with single electrode dislodgement; 6/20 both electrodes dislodged; no changes in EM field based on position/location of rat
Maiman, Myklebust et al. (1987)	-Model: cats -Injury: T8 contusion with 20g weight drop from 25cm	-Intermittent DC ES monopolar below and bipolar (epidural) across the SCI, 3-5 months post-SCI examining effects	-Intensity: <1.0mA -Frequency: 100Hz for 25ms -Electrodes: Pt	-Multiple trials of ES in same experiment (n=14): -Monopolar ES 100Hz for 25ms with negative	-Behavioural: 100Hz group had less intense spasms than 25Hx group; Bipolar ES across SCI less effective than monopolar ES -Histological: gliosis and cyst formation at SCI site in all

		on spasticity		electrode below SCI -Monopolar ES 25Hz for 25ms with negative electrode below SCI -Bipolar ES 100Hz for 25ms with negative electrode below SCI	groups, with no changes with electrode use
Politis and Zanakis (1988)	-Model: Wistar female rats -Injury: T8 contusion with 350g weight drop from 1cm against ventral plate	-Implanted continuous DC ES (epidural) across the SCI for 3 weeks	-Intensity: 3uA -Power supply: 1.5V zinc/alkaline/silver oxide battery -Electrodes: 90/10 Pt:Ir attached to epidural pad	-SCI only (n=6) -No ES or SCI (normal) (n=4) -ES with SCI anode rostral (n=6) -ES with SCI cathode rostral (n=6)	-<u>Behavioural:</u> 1. Hindlimb use: no difference between ES groups, both better than control 2. Inclined plane: Cathode rostral group better than anode rostral ($p<0.05$), which were better than SCI only ($p<0.05$) -<u>Histological:</u> Cathode rostral group had more neurofilament stained axons than other groups

Borgens, Toombs et al (1999)	-Model: dogs (various species) -Injury: complete thoracic SCI dogs after intervertebral disk herniation or trauma	-Implanted OFS (epidural) across the SCI for 6 months	-Intensity: 600μA from constant current stimulator alternating polarity every 15min -Power supply: DC, 3.6V Li battery -Electrodes: Pt:Ir on either side of the SCI	-ES: 600uA (n=20) -Sham OFS (n=14)	-Behavioural (6-month follow-up): 1. Superficial pain: more recovery in OFS group (p=0.0002) 2. Deep pain: no difference between groups (p=0.11) 3. Proprioception and ambulation: no difference between groups (p=0.43 and 0.22, respectively) 4. Combined score: more improvement in OFS group (p=0.047) -Neurophysiological: No difference between groups (p=0.13) -Adverse Effects: no
Moriarty, Borgens et al. (2001)	-Model: Sprague-Dawley female rats -Injury: T10 dorsal penetrating SCI	-Implanted oscillating field stimulation (OFS) (epidural) across the SCI for 4 weeks	-Intensity: 40μA alternating polarity every 15min -Power supply: DC, 3V Li Cell -Electrodes: 90:10 Pt:Ir on either side of the SCI	-ES: OFS (n=7; 4 replaced due to stimulator malfunction) -Sham OFS	-Histological: OFS decreased astrocytes in SCI region
Fujiki, Kobayashi et al. (2004)	-Model: Sprague-Dawley female rats -Injury: T7 hemisection	Intermittent bipolar ES (epidural) across the SCI, 24h prior to lesioning and repeated every	-Voltage applied: double voltage threshold for motor evoked potential -Frequency: 500Hz, 10 pulses/train every 10	-ES with no SCI (n=3) -ES with SCI surviving 6h (n=3) -ES with SCI	-Histological: ES groups had increased GFAP staining (diffusely and focally at electrode site) 1. 24h post-SCI: ES with SCI group had less necrosis,

		24h for 7 days	seconds for 2h (720 trains)	surviving 24h (n=3) -ES with SCI surviving 1 week (n=6) -ES with SCI surviving 3 weeks (n=3) -ES with SCI surviving 8 weeks (n=6)	hemorrhage and neutrophilic infiltration with smaller lesion /cavity 2. 1st and 3rd week post-SCI: ES with SCI group had smaller lesion/cavity, increased GFAP, vimentin, and GAP-43 staining 3. 8th week: no difference in lesion size/cavity between groups
Brus-Ramer, Carmel et al. (2007)	-Model: Sprague-Dawley female rats -Injury: unilateral pyramidotomy (PTx) transection contralateral to ES	-Intermittent constant current bipolar ES (epidural) on intact pyramid (contralateral to the lesion) for 6h daily for 10 days beginning post-operative day 1	-Intensity: minimum required for forelimb contraction (35-120μA) -Power supply: constant current stimulator -Frequency: 333Hz for 45ms every 2seconds -Electrodes: stainless steel implanted at the time of PTx	-Sham ES with no PTx (n=9) -PTx only (n=8) -ES only (n=7) -ES and PTx (n=8)	-Neurophysiological: lower activation threshold for ES+PTx group (strong ipsilateral motor activation; the sum of effects of PTx alone and ES alone were equal to the ES+PTx group -Histological: ES with PTx group had greatest increase in CST axon terminations (equal to sum effects of PTx alone and ES alone), axon length, and axon terminal varicosity density in ipsilateral grey matter; ES and PTx group had greatest topographical outgrowth in the ventral grey matter, contralateral CST axons re-crossing midline, and

					contralateral grey matter CST axon density
Hentall, Burns (2009)	-Model: Sprague-Dawley (ES experiment) and Fisher (neurophysiology experiment) female rats -Injury: thoracic contusion (neurophysiology experiment) via impactor (4mm tip causing 3kdyn force for 20ms displacing cord 0.95mm) and T8 contusion (ES experiment) via 10g weight (2mm diameter) drop from 12.5mm	-Implanted intermittent DC ES (to NRM) every 5 minutes for 12 h every day beginning 30min-1 h after SCI (stimulator implanted within 60min of SCI or 5-7 days prior)	-Intensity: 30uA -Power supply: 3V (2 x1.5V in series) silver oxide battery -Frequency: 8Hz every 5 minutes for 12h every day for approximately 3d (mean battery life 3.2d) -Electrodes: Tungsten cathode and stainless steel anode	-SCI only (n=6) -ES and no SCI (n=4) -No ES or SCI (normal) (n=6) -Sham ES with SCI (n=13) -ES with SCI (n=14)	-Behavioural: 1. BBB score: no difference between groups from having implants 2. Tail-flick test: Prolonged latencies 7-15 days after implant in ES group 3. Von Frey allodynia test: ES with SCI group had less forepaw (hindpaw, no difference) and allodynia than sham ES with SCI, which had more allodynia than normal group in forepaw and hindpaw -Neurophysiological: Normal group had more neutral cells than SCI only group and less spontaneous firing from on-cells and off-cells than ES with SCI group; sham ES with SCI rats had weaker on- and off-cell responses to noxious stimuli above lesion than normal rats -Histological: More myelination in ES with SCI, but no difference in lesion size/cavity or NeuN staining,

					though less GFAP
Li, Brus-Ramer et al. (2010)	-Model: Sprague-Dawley female rats -Injury: unilateral PTx transection contralateral to ES	-Intermittent constant-current bipolar ES (epidural) on intact pyramid for 6h every day for 10 days beginning post-operative day 1	-Intensity: minimum required for forelimb contraction (35-120uA) -Power supply: constant current stimulator -Frequency: 333Hz for 45ms every 2seconds -Electrodes: stainless steel implanted at time of PTx	- ES and PTx (n=4) -Sham ES and PTx (n=4)	-Histological: ES with PTx group had more BrdU+ cells (proliferating) in dCST, BrdU+ cells apposing axons, OPCs, mature proliferating OLs, and OPCs differentiating into OLs; No difference in proliferating astrocytes or endothelial cells between groups
Carmel, Berrol et al. (2010)	-Model: Sprague-Dawley female rats -Injury: unilateral PTx contralateral to cortical ES	-Intermittent constant-current bipolar ES (epidural) on intact CST motor cortex (contralateral to the lesion) for 6h every day for 10 days beginning post-operative day 1	-Intensity: minimum required for forelimb contraction (1.1-1.8mA) -Power supply: constant current stimulator -Frequency: 333Hz for 45ms every 2seconds -Electrodes: stainless steel implanted >1 week prior to PTx	-ES and PTx (n=5) -Sham ES with PTx (n=5)	-Behavioural: 1. Horizontal ladder test: ES and PTx group had improvement of affected forelimb over time and returned to baseline scores; Sham ES had no improvement of forelimb with worsening from baseline; difference between groups at days 20 and 30; ES with PTx group had reduction in all error types beginning at day. -Histological: ES with PTx group had increased axon density in ipsilateral grey matter, overall length of axons with a similar topographic distribution, had

					dorsal horn outgrowth (muscle and cutaneous receptor terminations), motor laminae outgrowth but not superficial dorsal laminae (nociceptive afferent terminations)
Carmel, Kimura et al. (2013)	-Model: Sprague-Dawley female rats -Injury: unilateral PTx contralateral to ES	-Intermittent constant-current bipolar ES (epidural) on intact CST motor cortex (contralateral to the lesion) for 6h every day for 10 days beginning post-operative day 1	-Intensity: minimum required for forelimb contraction (1.1-1.8mA) -Power supply: constant current stimulator -Frequency: 333Hz, 45ms, for 0.2ms duration every 2 seconds -Electrodes: stainless steel implanted >1 week prior to PTx	-ES with PTx -PTx alone -Total: n=17	-Histological: no difference in cortical cellular architecture or GFAP staining between groups due to implants 1. Spinal Cord: ES with PTx group had greater total axon length in ipsilateral grey matter; ES drives outgrowth in areas of lower axonal density and areas having lost innervation due to lesioning 2. Cuneate nuclei: ES with PTx group had greater total axon length in ipsilateral and contralateral grey matter; ES drives axon outgrowth in cuneate nucleus in areas of lower density ipsilaterally and contralaterally 3. Parvocellular nuclei: ES with PTx group had greater total axon length in ipsilateral and contralateral

					grey matter; ES drives greatest outgrowth in densely innervated areas of nucleus 4. Magnocellular nuclei: ES with PTx group had greater total axon length in ipsilateral and contralateral grey matter; ES increases growth bilaterally with similar topographical distribution (thus greatest outgrowth in red nucleus that restore function) -Number of axons re-crossing midline in SC greater in ES with PTx.
Carballosa-Gonzalez, Vitores et al. (2013)	-Model: Sprague-Dawley female rats -Injury: T8 contusion via 10g weight (2mm diameter) drop from 12.5mm	-Single session ES (to nucleus raphe magnus(NRM)) every 5minutes for 2h starting 72h after SCI	-Intensity: 30uA -Frequency: 8Hz every 5 minutes for 2h with a pulse width of 1ms -Electrodes: Tungsten	-Sham ES and SCI (n=4) -No ES or SCI (normal) (n=4) -ES with SCI (n=4) <u>CREB/PKA and pCREB/pPKA experiment</u> -No ES or SCI (normal) (n=7) -No ES with SCI	-Histological: ES with SCI group had higher cAMP levels in cervical, thoracic and lumbar tissue with return to near-normal levels; pimozide use reduced cAMP levels those in SCI (ES failed to increase it afterward) -PKA and CREB higher in SCI animals with ES reversing this increase -pPKA/PKA and pCREB/CREB reduced after

				(n=8) -ES with SCI (n=7)	SCI and increased with ES
Carmel, Kimura et al. (2014)	-Model: Sprague-Dawley female rats -Injury: unilateral PTx contralateral to ES	Intermittent constant-current bipolar ES (epidural) on intact CST motor cortex (contralateral to the lesion) for 6h every day for 10 days beginning 8 weeks after PTx	-Intensity: minimum required for forelimb contraction (0.9-1.7mA) -Frequency: 333Hz, 0.2ms biphasic pulse, 45ms duration, every 2 seconds -Electrodes: stainless steel implanted 5 weeks after PTx -Power supply: constant current stimulator	-ES with PTx (n=5) -Sham ES+PTx (n=5)	-Behavioural: 1. Horizontal ladder test: ES with PTx group had more improvement with errors back to baseline at week 11; muscimol use in ES with PTx resulted in transient re-emergence of errors with no change in subtypes of errors (ES after chronic PTx improves recovery through intact M1 to impaired forelimb connections)

Table 1: Electrical stimulation studies. Table outlining previous electrical stimulation for spinal cord injury animal studies and highlighting histological evidence of axonal outgrowth and behavioural outcomes.

Chapter 3

Motor cortex electrical stimulation in rats with a cervical spinal cord injury to promote axonal outgrowth

Key Words: Electrical stimulation, Spinal cord injury, CNS plasticity, Axonal Regeneration, Axonal collaterals

Category of paper: Original basic science research

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3.1 Introduction

Traumatic spinal cord injury affects approximately 40 people per million annually in North America (Furlan et al., 2013), and often leaves patients irrevocably injured due to axonal damage. Treatments aimed at inducing axonal outgrowth (whether by axonal regeneration or/and collateralization), have, to date, failed to translate into significant functional improvement in individuals with SCI.

As discussed in chapter 2, axonal outgrowth post-SCI is hampered by an inhibitory environment in the adult mammalian spinal cord (Cajal, 1928). Myelin-associated inhibitors (e.g., Nogo-A, myelin-associated glycoprotein (MAG), oligodendrocyte myelin glycoprotein (OMgP)), and extracellular matrix and post-injury scar component chondroitin sulfate proteoglycans (CSPGs) are mainly responsible for this inhibitory environment (Filbin et al., 2003; Jones et al., 2002). Neutralization of some of these factors has previously been attempted, with evidence of increased axonal outgrowth and associated functional recovery in animal models (Fouad et al., 2005). However, in addition to these extrinsic inhibitory factors, mature neurons also have a reduced regenerative proclivity. Many factors contribute to this decreased ability to regenerate including, decreased expression of growth-associated proteins (GAP-43), microtubule associated proteins, and cAMP (Filbin et al., 2003; Han et al., 2004; Hannila et al., 2008; Ming et al., 1997; Song et al., 1997). In order to promote robust neuronal outgrowth that will translate into significant behavioural improvements, overcoming the post-SCI inhibitory environment and increasing neuronal outgrowth ability is likely necessary as seen with the increasing number of combinatorial treatment studies (McCall et al., 2012).

Due to the complexity of combinatorial treatment studies however, here, we focus on a single approach to SCI repair and recovery by increasing the neuronal capacity for outgrowth. We do so by using ES of the injured CST in the motor cortex to promote axonal outgrowth in the injured spinal cord. The proposed mechanism by which ES may achieve this effect is through increased levels of growth factors such as BDNF and second messengers such as cAMP and calcium (Bregman et al., 1997; Vavrek et al., 2006; Kater et al., 1991). So far, ES in the setting of SCI has largely been confined to stimulating the uninjured CST that descends contralateral to the side of injury in a hemisection SCI model (Carmel et al., 2013; Carmel et al., 2014; Carmel and Martin, 2014). It is not yet known whether cortical ES targeting the injured CST may be able to promote axonal outgrowth comparable to what has been shown for sensory fibers (Udina et al., 2008). Here, we investigate the effect of ES in promoting repair and recovery in a rodent cervical SCI model by applying ES with different parameters to the primary motor cortex. Furthermore, we examine the use of an implantable ES unit in a pilot project, designed and fabricated in-house, to investigate the feasibility of longer-term ES in our rodent model of SCI (appendix).

3.2 Materials and methods

All rats were cared for and experiments conducted according to the following methods section. Additional steps specific to long-term ES experiments are outlined in the appendix following this thesis.

Animals and experimental cohorts:

Experiments were conducted on adult female Lewis (Charles River Laboratories, Canada) rats weighing 180-220g and group housed (5 rats per cage) subject to a 12h:12h light-dark cycle. Surgeries (cortical stimulation and spinal cord lesioning) were performed under general anaesthesia (70mg/kg ketamine; 0.3mg/kg buprenorphine; 7mg/kg xylazine, i.p.) and sterile conditions. Local animal care and use committee (ACUC-HS) approval was obtained for all procedures. Animals were fed ad libitum with the exception of the day prior to behavioural training and testing (food restriction of 10g/rat).

Behavioural training

Upon arrival, rats were placed in a cage containing a polyvinyl chloride (PVC) tube, wood chip bedding, a wood gnawing block, and food and water sources. Rats were acclimated and handled for 1 week prior to commencement of behavioural training. Rats were then trained in a Montoya staircase test (Montoya et al., 1991) daily to grasp and eat sucrose pellets (45mg; Research Diets, New Brunswick, NJ, USA). Rats were trained for 2 weeks, after which they were tested in the trained task and their average overall score of total pellets grasped (3 pellets per well), as well as lowest well reached used as their baseline behavioural ability. Rats then underwent spinal cord lesioning (see spinal cord lesion section), and were allowed to recover for 1 week. The rats then underwent post-lesion Montoya testing weekly for 4 weeks (experimental timeline shown in Figure 4). The average overall score of total pellets grasped (expressed as a percentage of total pellets available and standardized according to their baseline grasping ability) and lowest

well reached were used as their reaching outcome measures. Rats unable to learn the behavioural task (defined as a successful grasping attempt on at least two separate occasions prior to injury) were not included in the study.

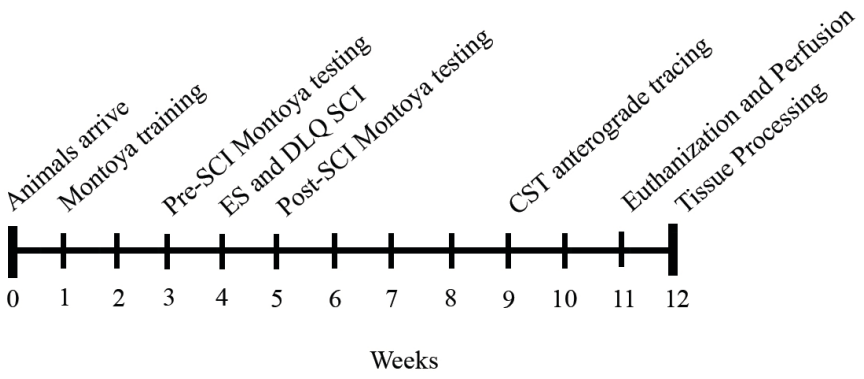

Figure 4: Timeline of electrical stimulation experiment.

Short-term electrical stimulation

Upon being anaesthetized with ketamine and xylazine, the surgical sites for incision were shaved and prepared with 10% chlorhexidine digluconate (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada). The rats were placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA) and their eyes protected with Lacri-lube (Allergan, Inc., Irvine, CA, USA). Physiological monitoring of the rats was performed throughout the surgery using pulse oximetry, and body temperature was maintained at approximately 37°C using a water heating blanket. For the stimulation portion of the operation (preceding spinal cord lesioning), an incision was made in the scalp and dissection carried down through subcutaneous tissue and periosteum to reveal the skull. Retractors were then used to maintain the exposure and calvarial sutures identified (coronal, sagittal, and bregma). A small craniotomy was performed using a dental drill on the side contralateral to the preferred paw (identified via Montoya testing) and side of the spinal cord to be lesioned.

Two insulated tungsten electrodes (5μm diameter, World Precision Instruments, Inc., Sarasota, FL, USA), spaced approximately 1.5mm apart were inserted into the forelimb area of the motor cortex to a depth of 1mm. The electrodes were situated 1mm rostral to bregma, and the lateral positions were 1mm and 2.5mm, respectively, relative to bregma. This corresponded to the forelimb area of the motor cortex (Brus-Ramer et al., 2007; Carmel et al., 2014; Girgis et al., 2007). To confirm proper placement of the electrodes, forelimb movement was visualized upon the brain being stimulated using an isolation unit (A.M.P.I., Jerusalem, Israel) prior to spinal cord lesioning. Stimulation occurred almost exclusively between 0.5-0.8mA (minimum current required to evoke contralateral paw movement). Experimental cohorts were as follows (Figure 5): ES333 cohort receiving trains of biphasic stimuli at 333Hz for 0.2ms duration every 500ms and 30 pulses per train (n=14 rats), ES20 cohort receiving trains of biphasic stimuli at 20Hz for 0.2ms duration every 1s with 60 pulses per train (n=14 rats), ES sham cohort with cortical electrode insertion contralateral to the SCI, but no stimulation (n=10 rats), and SCI control cohort undergoing SCI, but no cortical electrode insertion (n=10 rats). The rationale for including the different cohorts consisted of ensuring any observed treatment effect of ES was not due to simple lesioning of the spinal cord (SCI only group), and was not due to insertion of the parenchymal electrodes (ES sham group). Differing stimulation parameters were included according to previous studies completed (ES333 and ES20 groups). Stimulation occurred for 30 minutes immediately preceding spinal cord lesioning. Upon completion of the stimulation, the scalp was closed in a single layer with interrupted, 5-0, prolene sutures (Ethicon, Somerville, NJ, USA).

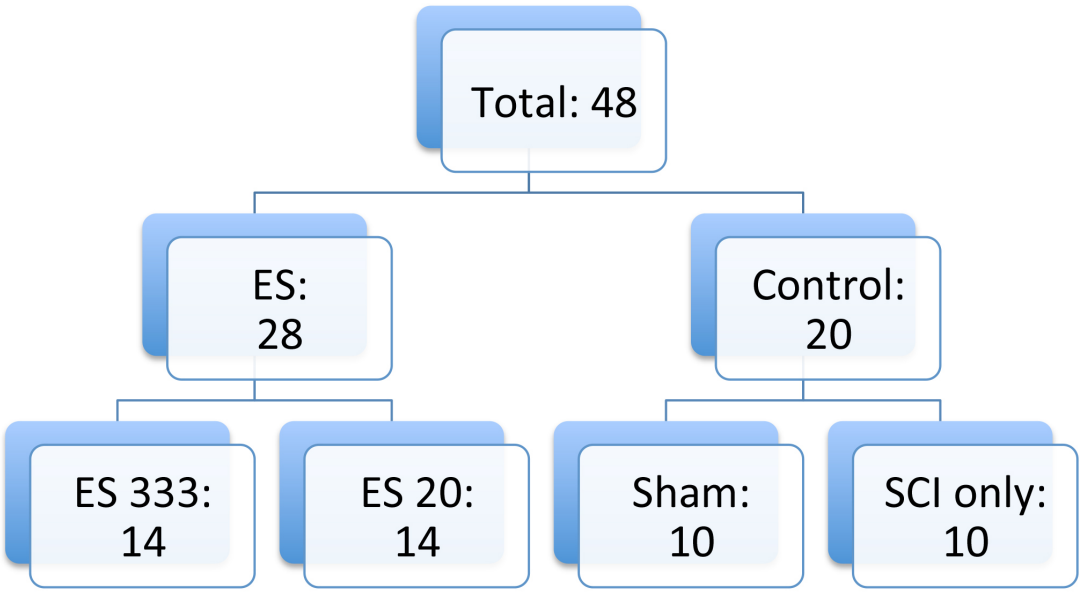

Figure 5: Flowchart of animals included, and their respective experiment cohorts.

Spinal cord lesioning

SCI consisted of a unilateral dorsal-lateral quadrant (DLQ) lesion at the fourth cervical level (C4) on the side ipsilateral to the rat's preferred paw. This lesion was completed during the same general anaesthetic administration as the cortical stimulation. An incision was made in the skin and dissection carried down through the subcutaneous layer and paraspinal musculature revealing the underlying C4 vertebrae. A retractor was placed to maintain exposure of the surgical site. A laminotomy was performed to expose the dura underneath. A durotomy was then performed, and a customized microblade was inserted into the midline of the spinal cord. At a depth of 1mm, the microblade was moved laterally completing the DLQ lesion. This lesion results in ablation of the dorsal CST, the majority of the rubrospinal tract (RST), as well as dorsal column ascending sensory

fibers. Upon completion of the lesion, local hemostasis of the surgical site was ensured and the incision closed in layers using interrupted, 4-0, vicryl sutures (Ethicon, Somerville, NJ, USA) for the fascial layer and surgical clips (Ethicon, Somerville, NJ, USA) for the cutaneous layer.

Anterograde labeling of the CST (injection and staining)

Upon completion of Montoya testing after SCI (4 weeks following SCI), biotinylated dextran amine (BDA; 10%, Life Technologies, Grand Island, New York, USA) was injected into the forelimb area of the primary motor cortex. Rats were anaesthetized using isoflurane (4% for induction, 2.5% for maintenance of anaesthesia), a 50:50 mixture of air and oxygen, as well as buprenorphine (0.3mg/kg). Rats were placed in a stereotaxic frame. The previous incision was opened sharply, and the dissection carried down to the skull. Self-retaining retractors were placed to maintain the exposure and reveal the underlying dura. Durotomy was then performed and the injection needle inserted to a depth of 1.5mm into the primary mortor cortex remote from the previous electrode insertion sites. This was repeated for a total of 3 injections of 1µl over 6 minutes. Once completed, the incision was closed as a single layer with interrupted, 5-0, prolene. Rats were perfused 2 weeks later as described below.

Perfusion and histological sectioning

Rats were anaesthetized with isoflurane (4%) and were then euthanized using pentobarbital (3.2ml/kg; Euthanyl, Biomeda-MTC, Cambridge, ON, Canada). This was followed by a transcardial perfusion performed using warm saline, and subsequently a

4% cold formalin solution with 5% sucrose. The spinal cord and brain from each rat were then dissected and post-fixed in a 4% formalin solution for 24 hours. The tissue was then placed in a 30% sucrose solution for 3 days for cryoprotection. The spinal cord tissue was cut into the following sections: lumbar, thoracic, cervical, C1 spinal cord, and brain stem. The tissue sections were mounted on filter paper using Tissue Tek (Sakura Finetek, CA, USA) after which they were frozen using methylbutane and dry ice at -45°C, and then stored at -80°C. For histological sectioning, the tissue was cut to a thickness of 25μm on a cryostat at -20°C. The mounted tissue was cut in axial cross sections at the C1 level to allow traced CST fiber quantification, and the cervical spinal cord section was cut horizontally to allow lesion size analysis and axonal collateralization quantification.

Histological staining

The histological sections of the spinal cord were placed on coated slides (Fisher Scientific, Ottawa, ON, Canada) and stored at -20°C until stained.

For cresyl violet (CV) staining, the slides were dehydrated for 1 hour at 37°C, followed by two-cycles rehydration in tris-buffered saline (TBS) for 10 minutes. The slides were then placed in 0.5% CV for 3 minutes, and dipped in double-distilled water (ddH_20) to remove any excess stain. The slides were subsequently serially dehydrated in 50%, 75%, 99%, 99% ethanol, for 2 minutes each, respectively. The tissue slides were next cleared in xylene twice for 2 minutes each time, and cover-slipped with permount (Fisher Scientific, Ottawa, ON, Canada).

For CST axonal staining and BDA visualization, the slides were dehydrated for 1 hour at 37°C, followed by re-hydration in TBS for 10 minutes (repeated once). The slides were then washed twice in TBS-triton (TBS-TX, 0.5%) for 45 minutes each wash. Avidin-biotin complex (ABC) reagent (ABC kit; Vector Laboratories, Burlingame, CA, USA) was then added to the slides, and left to incubate for 2 hours at room temperature. After incubation, the slides were rinsed three times, 10 minutes per rinse, in TBS to remove any excess ABC. The slides were next incubated with the chromogen diaminobenzidine (DAB; Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) for 2-4 minutes. Excess DAB reagent was then removed, and the reaction halted by immersing in ddH_20. The slides were washed again in TBS, 3 times for 10 minutes per wash, followed by serial dehydration in 50%, 75%, 99% ethanol twice for 2 minutes each concentration. The slides were then cleared twice for 2 minutes in xylene, and cover-slipped with permount.

Lesion size analysis

Spinal cord sections stained with CV were examined using light microscopy at 10x magnification to assess the lesion size within each rat. Bright field and phase contrast settings were used to examine every eighth horizontal section. Landmarks such as the central canal, grey and white matter tracts, as well as anterior and posterior median sulci were used to analyze the site and size of each lesion which was transferred onto a schematic cross-section representation of the spinal cord (Figure 6). Each lesion was then calculated as a percentage of the total spinal cord cross sectional area upon reconstruction with ImageJ computer software (National Institutes of Health software, Bethesda, MD, USA). The size of the CST lesion was also analyzed, calculated and expressed as a

percentage of the total CST area upon reconstruction with ImageJ. Previous studies have shown that lesions not injuring approximately 50% of the CST can have quite variable effects with respect to behavioural deficits and recovery observed (Fouad et al., 2013). As such, one of the inclusionary criteria utilized for this study included a minimum of 50% of the CST having been lesioned.

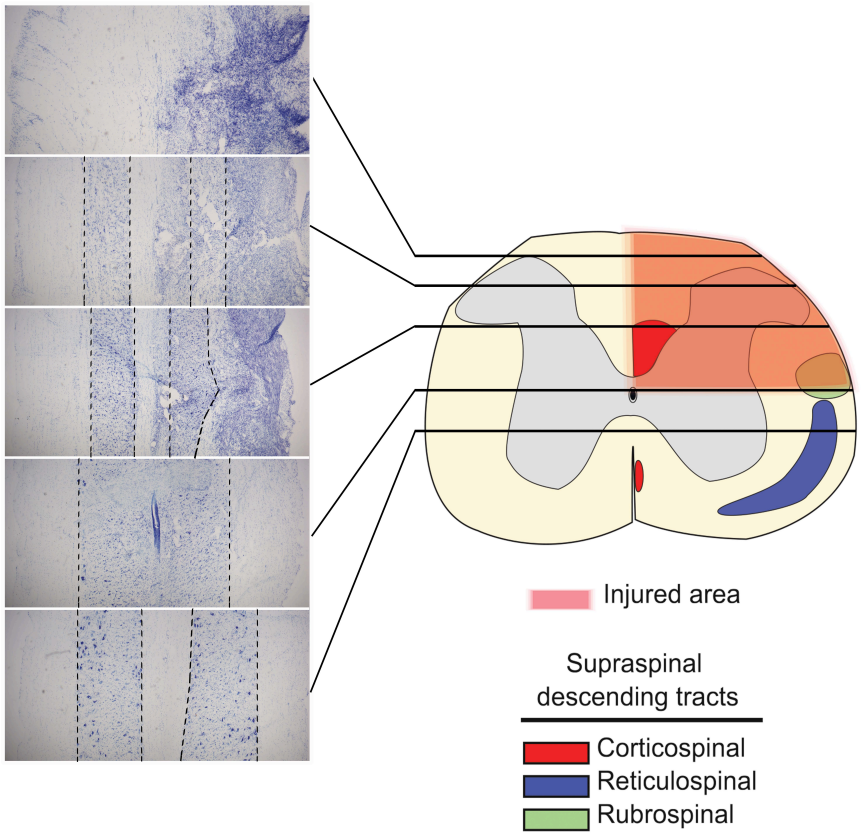

Figure 6: Cross-section schematic of cervical spinal cord depicting the dorsal lateral quadrant lesion region (right) and corresponding horizontal histological sections stained with cresyl violet.

Neurite outgrowth (collateralization and regeneration)

Spinal cord sections stained with DAB were examined using light microscopy at 10x and 40x magnification to assess the extent of axonal collateralization. Bright field microscopy

was used to examine every eighth horizontal section as well as one axial cross-section located at C1. Axonal sprouting (collaterals) above the lesion was calculated by counting the number of axonal collaterals from C1 to a point 5mm rostral to the SCI (point at which all groups were found to have approximately the same number of axons as discussed in the dieback analysis), and crossing the boundary between the dorsal CST and into the grey matter at least 10μm (as delineated by a line drawn between the grey matter and dorsal CST) on horizontal sections. The total number of collaterals was then expressed as a proportion of the total length of the horizontal section analyzed (in mm). The number of total collaterals/mm was then divided by the total number of axons traced as determined by counting the number of dorsal CST axons stained at C1 (as shown in Figure 7) on an axial cross section. This last calculation was completed to normalize and control for the possibility of a discrepancy in the number of dorsal CST axons stained between rats. The farthest regenerated axon into the lesion was also measured for each rat according to criteria previously published (Tuszynski et al., 2012). The average farthest regenerated axon of all rats from each group was then used for statistical analysis.

Axonal dieback

Spinal cord sections stained with DAB were examined using light and phase contrast microscopy at 10x and 40x magnification to assess axonal dieback. The number of axons for each rat was also counted at: 25μm into the lesion, the rostral border of the lesion, 50μm rostral to the lesion, 100μm rostral to the lesion, 1mm rostral to the lesion, and 5mm rostral to the lesion. The above measurements were then expressed as a proportion of the total number of axons labeled as described in the axonal collateralization section.

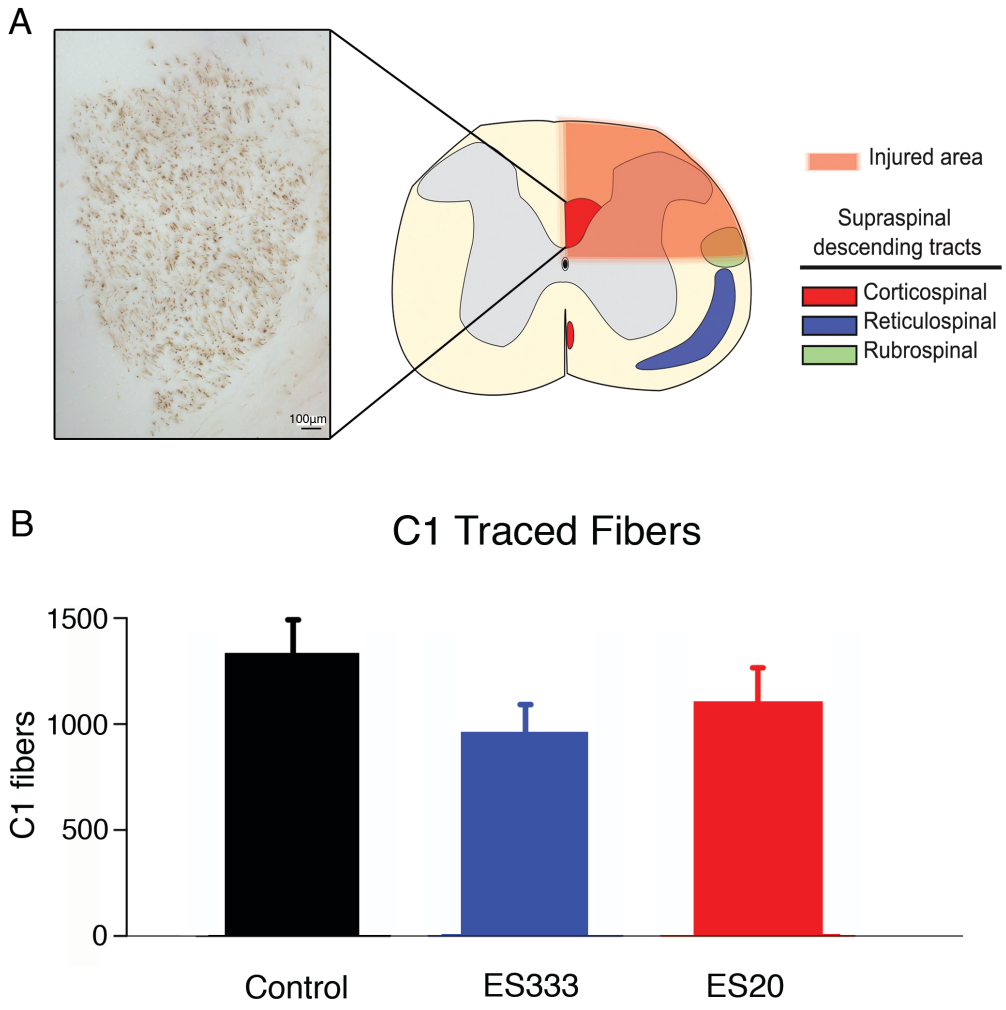

Figure 7: A) Cross-section schematic of cervical spinal cord with corresponding histological cross-section with stained corticospinal tract fibers. B) Bar graph representation of the average number of traced fibers from each group (no significant difference was found between groups).

Statistical analysis

Behavioural outcomes were compared using repeated measures two-way ANOVA with appropriate post-hoc testing. All other between group comparisons were done with a one-way ANOVA, or Kruskal-Wallis test in the case of non-parametric data, with appropriate Tukey or Mann-Whitney post-hoc testing. All histological data collection and analyses

were completed in a blinded fashion without knowing which experimental group each animal belonged to. Behavioural outcomes and histological outcomes were found not to be different between SCI control rats and ES sham control rats, and as such the two groups were combined in order to increase statistical power. Statistical power is the probability that a null hypothesis will be rejected in the event that it is truly false. The power analysis was completed using G*Power software (Faul et al., 2007). Given a desired power of 0.8 and a significance level for between-group comparisons set at $p<0.05$, the required sample size was calculated to be 44 animals. Results are reported as mean (± standard deviation, SD).

3.3 Results

A total of 48 rats were included in the analysis of this experiment. Figure 5 shows the total number of animals in each of the experimental groups with 14 rats in the 333Hz electrical stimulation cohort (ES333), 14 in the 20Hz electrical stimulation cohort (ES20), 10 in the sham stimulation cohort (control), and another 10 in the SCI alone cohort (control). The spinal cords from each rat were analyzed for the extent of SCI, axonal collateralization rostral to the site of injury, extent of axonal regeneration into and dieback from the site of injury. Behavioural data (including grasping success and furthest well reached) were also analyzed and compared between groups.

3.3.1 Lesion analysis revealed no difference between groups

SCI lesion and CST lesion size analyses were completed with no significant difference being found between groups in spinal cord lesion size, expressed as a percentage of the

total cross-sectional area of the spinal cord at the level of injury (p=0.21). No significant difference was found between groups in CST lesion size either, expressed as a percentage of the total cross-sectional area of the CST at the level of injury (p=0.11) (Figure 8). The homogeneity of the lesion sizes is likely related to the fact that animals were selected for inclusion in the study based on a pre-requisite lesion size threshold of having at least 50% of the CST ablated. The average lesion size for the control group was 25.0% (SD 6.8%), for ES333 group was 26.7% (SD 7.9%), and the ES20 group was 30.0% (SD 11.4%). The average CST lesion sizes for the control, ES333, and ES 20 groups were 90.3% (SD 15.9%), 91.9% (SD 13.1%), and 99.8% (SD 0.9%), respectively.

3.3.2 Axonal outgrowth analysis revealed increased collateralization in electrical stimulation groups

Axonal outgrowth can consist of collateralization (in our experiment, quantified as the number of sprouts rostral to SCI emanating from either injured fibers or spared fibers) or regeneration (re-growth of a transected axon from its terminal, injured end). Both of these outgrowth parameters were quantified and analyzed in this study. All groups were found to have approximately the same number of traced axons 5mm rostral to the SCI (as discussed in the following axonal dieback analysis), and as such the number of collaterals were counted between C1 and 5mm rostral to the SCI, expressed as a function of both the total number of C1 fibers traced, as well as distance over which they were measured

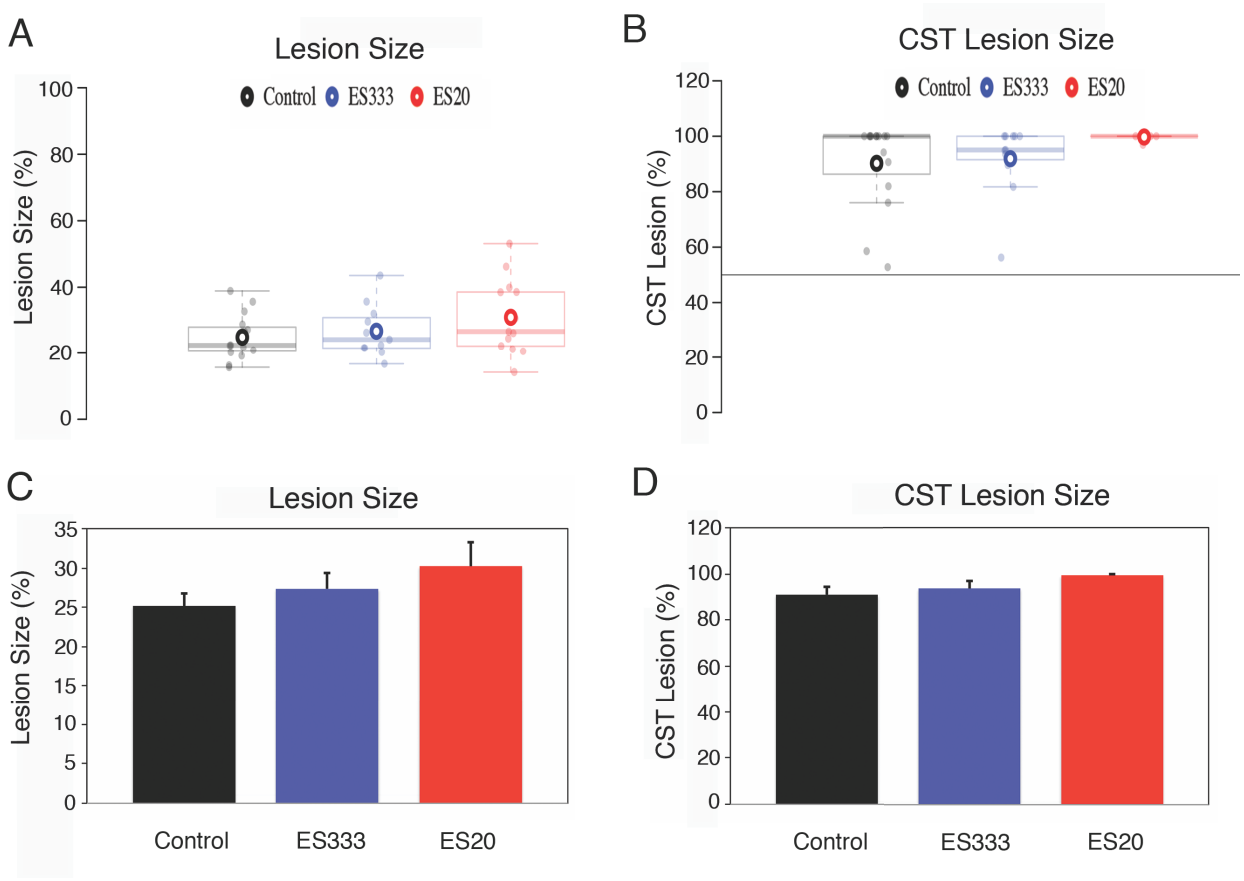

Figure 8: A) Scatter plot of dorsal lateral quadrant lesion size for each group. B) Scatter plot depicting the corticospinal tract lesion size for each group. C) Bar graph of lesion size for each experimental group with no statistical difference found between groups. D) Bar graph depicting the corticospinal tract lesion size for each experimental group with no difference being found between groups. Open Circle: average; bar: median; closed circles: individual animals; box: 95% confidence interval; hashed line: range.

rostral to the SCI. The average number of normalized collaterals for the control, ES333, and ES20 group were as follows: 0.0063/fiber/mm (SD 0.0048/fiber/mm), 0.0161/fiber/mm (SD 0.0137/fiber/mm), 0.0170/fiber/mm (SD 0.0234/fiber/mm), respectively. Significantly more axonal collaterals (i.e., axonal sprouts rostral to the lesion) were found in the ES333 animals compared to the control animals ($p<0.01$, M-W post-hoc test) (Figure 9).

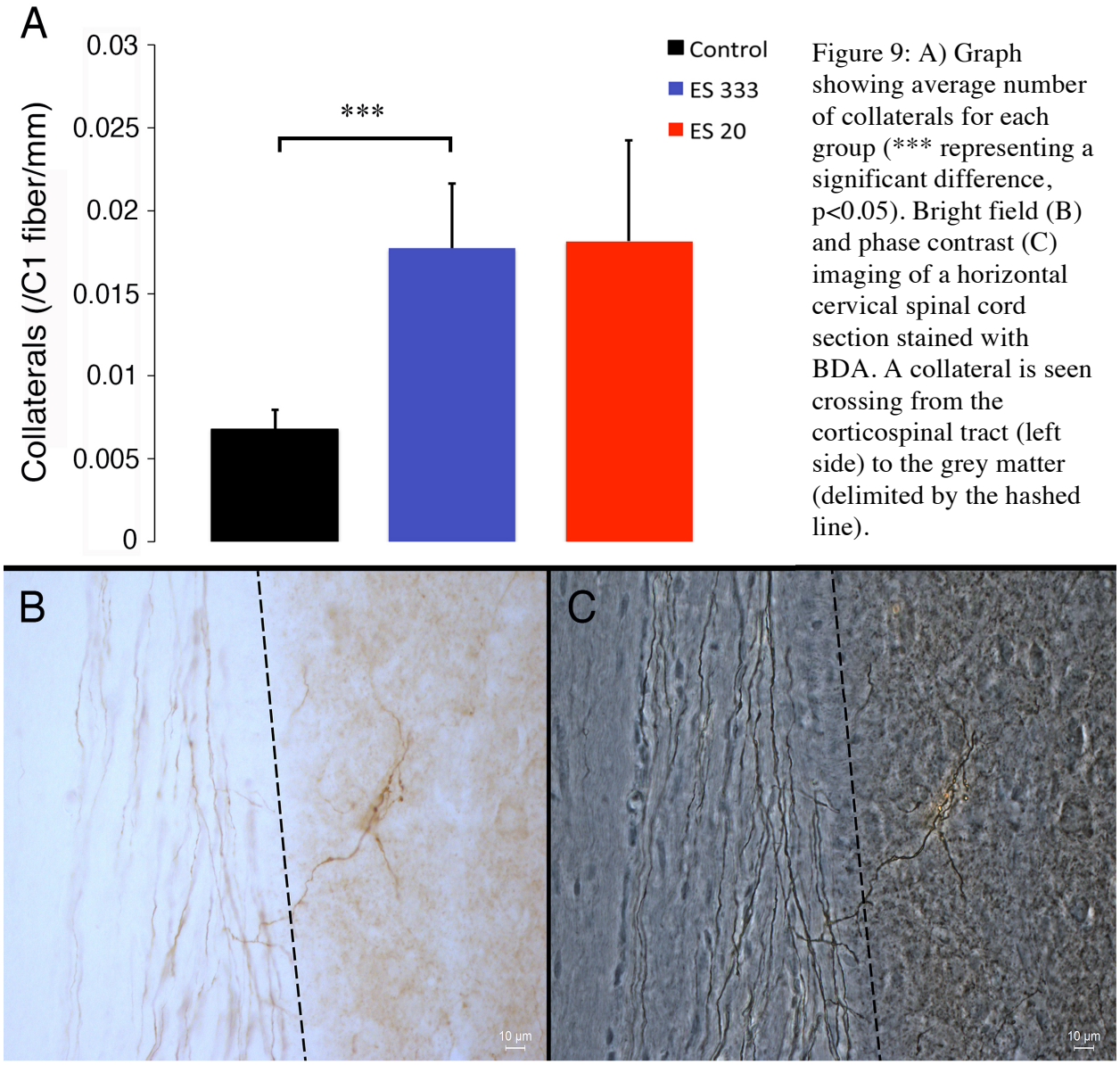

Figure 9: A) Graph showing average number of collaterals for each group (*** representing a significant difference, $p<0.05$). Bright field (B) and phase contrast (C) imaging of a horizontal cervical spinal cord section stained with BDA. A collateral is seen crossing from the corticospinal tract (left side) to the grey matter (delimited by the hashed line).

No difference was found with respect to collateralization between the two ES groups ($p=0.10$, M-W post-hoc test), nor between the ES20 and control groups ($p=0.16$, M-W post-hoc test). It is suggested that no differences were found to be significant due to the high variability observed within groups. Axonal regeneration was measured and calculated according to the average farthest axon into the lesion measured from each

group (in keeping with previously published guidelines, Tuszynski et al., 2012). The average farthest axon into the lesion from each group was: 43.27µm (SD 41.23µm) for the control animals, 73.36µm (SD 76.76µm) for ES333 animals, and 26.69µm (SD 46.84µm) for ES20 animals. No significant difference was found between groups with respect to the degree of axonal regeneration into the lesion (i.e., axonal re-growth from its terminal, lesioned end) (p=0.13, Figure 10).

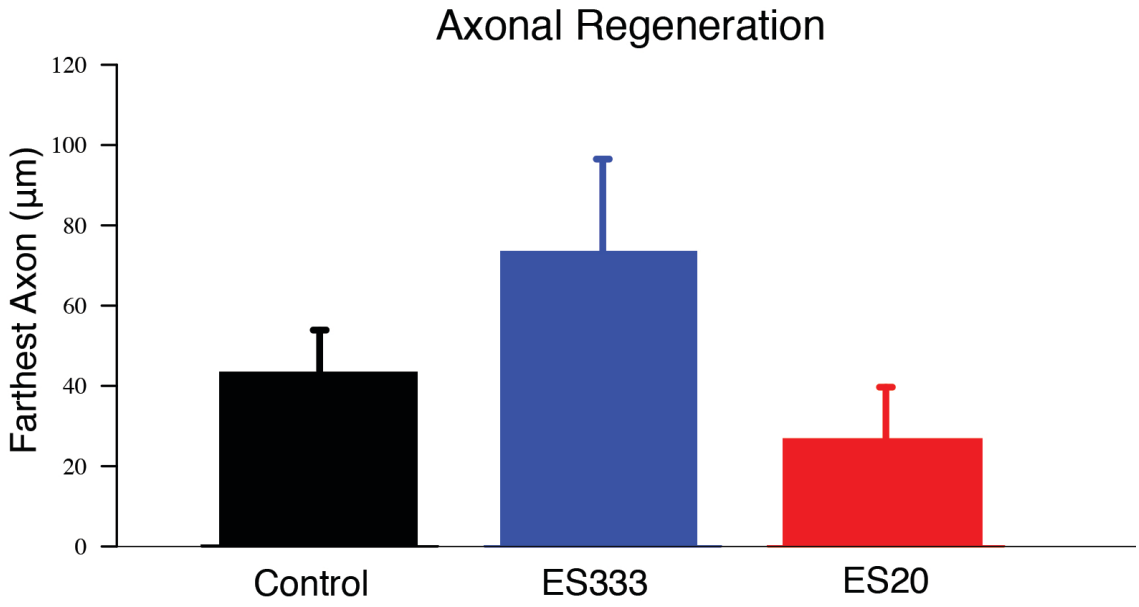

Figure 10: Bar graph of average length of axon fiber regeneration into lesion site measured for each group (no statistical difference being found between groups).

3.3.3 Axonal dieback analysis revealed more dieback in low-frequency electrical stimulation animals

Axonal dieback is a phenomenon in which axons, upon being injured (usually through axonotmesis or neurontmesis), begin to retract and "dieback" more proximally from the

injury site. We observed varying degrees of axonal dieback amongst animals upon completion of our outgrowth analysis, and as such axonal dieback was assessed and measured in each experimental group. The average number of axons in each group was calculated at various points rostral to the SCI and between group comparisons were done at each point for any significant differences (Figure 11 and Table 2). Here, there was a significant difference found with respect to the degree of axonal dieback (axon count rostral to the SCI representative of degeneration) at multiple points between groups. All animal groups were found to have approximately equal numbers of axons 5mm rostral to the lesion with no difference between them ($p=0.36$), and a trend toward a significant difference in axon numbers beginning at the 1mm mark rostral to the SCI was noticed ($p=0.06$). Beginning at 100μm rostral to the injury, the ES20 rats had significantly more axonal dieback (i.e., worse histological outcome) than the ES333 cohort ($p=0.03$, Tukey post-hoc test). At both 50μm rostral to injury and at the injury site, ES20 animals had significantly more axonal dieback than both controls and ES333 animals (50μm mark: $p=0.02$ in both cases, Tukey post-hoc test; lesion site: $p=0.01$ and 0.02, respectively, Tukey post-hoc test). Finally, 25μm into the spinal lesion ES20 rats also had significantly fewer axons than the ES333 rats ($p<0.01$, Tukey post-hoc test).

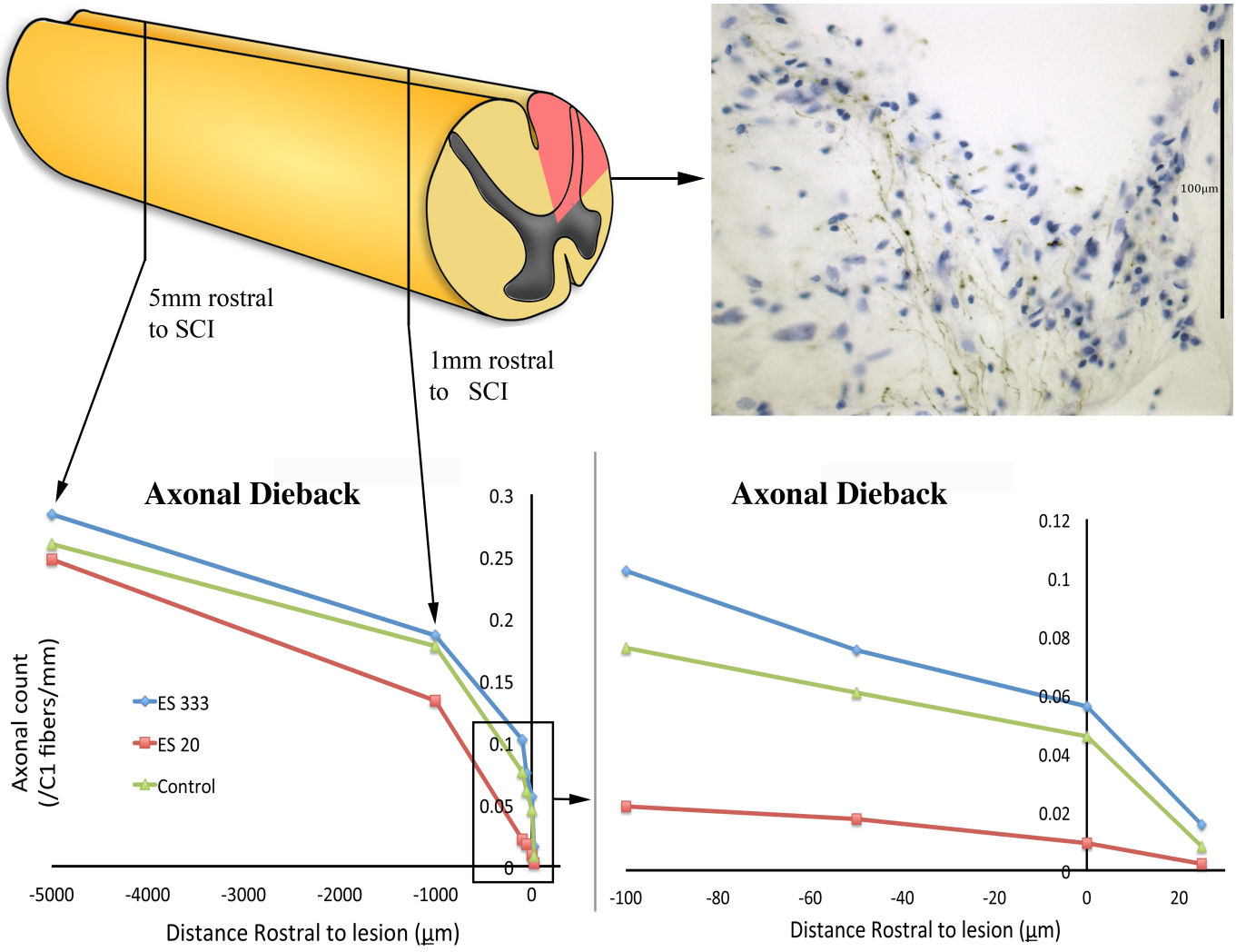

Figure 11: Schematic representation of spinal cord showing points rostral to the injury where axonal dieback measurements were made (top left), and graphical representation of results between groups (significant difference found between the ES20 and other groups starting 100µm rostral to injury, $p<0.05$) (bottom row). Histological section of a spinal cord at the site of injury from an ES333 animal with BDA stained axons and cresyl violet lesional staining (top right).

	Distance from spinal cord injury site					
Group	**5mm rostral**	**1mm rostral**	**100µm rostral**	**50µm rostral**	**Lesion border**	**25µm caudal**
Control	0.2564	0.1770	0.0764	0.0638	0.0489	0.0088
ES333	0.2812	0.1771	0.1027	0.0705	0.0502	0.0142
ES20	0.2437	0.1292	0.0221	0.0178	0.0094	0.0021
p-value (post-hoc testing)	0.36	0.06	0.03 (ES20 vs ES333	0.02 (ES20 vs ES333 and Control)	0.02 (ES20 vs ES333) 0.01 (ES20 vs Control)	0.01 (ES20 vs ES333)

Table 2: Axonal dieback analysis results. Table showing axonal dieback for experimental groups with the average number of axons at various distances rostral to the spinal cord injury. Significant differences were found between the ES20 group and the other two groups starting at 100µm rostral to the lesion as calculated on post-hoc statistical tests.

3.3.4 Behavioural analysis revealed worse outcomes with low-frequency electrical stimulation

Behavioural pre- and post-SCI Montoya stairwell grasping success scores and furthest well reached scores were recorded. In order to account for potential differences between animals with respect to pre-operative grasping success and furthest well reached, each animal's post-SCI grasping score and lowest well reached score was normalized to their respective pre-lesioning score. Post-SCI average grasping success for control, ES333 and ES20 groups were as follows: -0.38 (SD 1.03), -0.71 (SD 0.43), -0.74 (SD 0.41), respectively (expressed as a proportion of baseline ability). Post-SCI average furthest well reached scores for control, ES333, and ES30 animal were as follows: 2.07 (SD 1.33), 1.73 (SD 1.27), 2.15 (SD 1.41), respectively. Post-SCI grasping scores and furthest well reached scores were found to be significantly lower than baseline values ($p<0.01$ for both grasping scores and furthest well reached scores) for all groups (Figure 12). The ES20 animals were also found to have significantly lower average grasping scores and lower average furthest well reached scores (i.e., worse behavioural outcome scores) post-SCI than controls ($p=0.03$ for both, Tukey post-hoc test) (Figure 12) with no difference between the two ES groups ($p=0.99$ for both Tukey post-hoc test).

3.4 Discussion

Previous ES animal experiments have shown promising results with respect to increased axonal outgrowth (Brus-Ramer et al., 2007; Carmel et al., 2013). Functional improvement has also been reported (Carmel et al., 2010; Carmel et al., 2014; Carmel and Martin, 2014). In an attempt to demonstrate that the observed behavioural

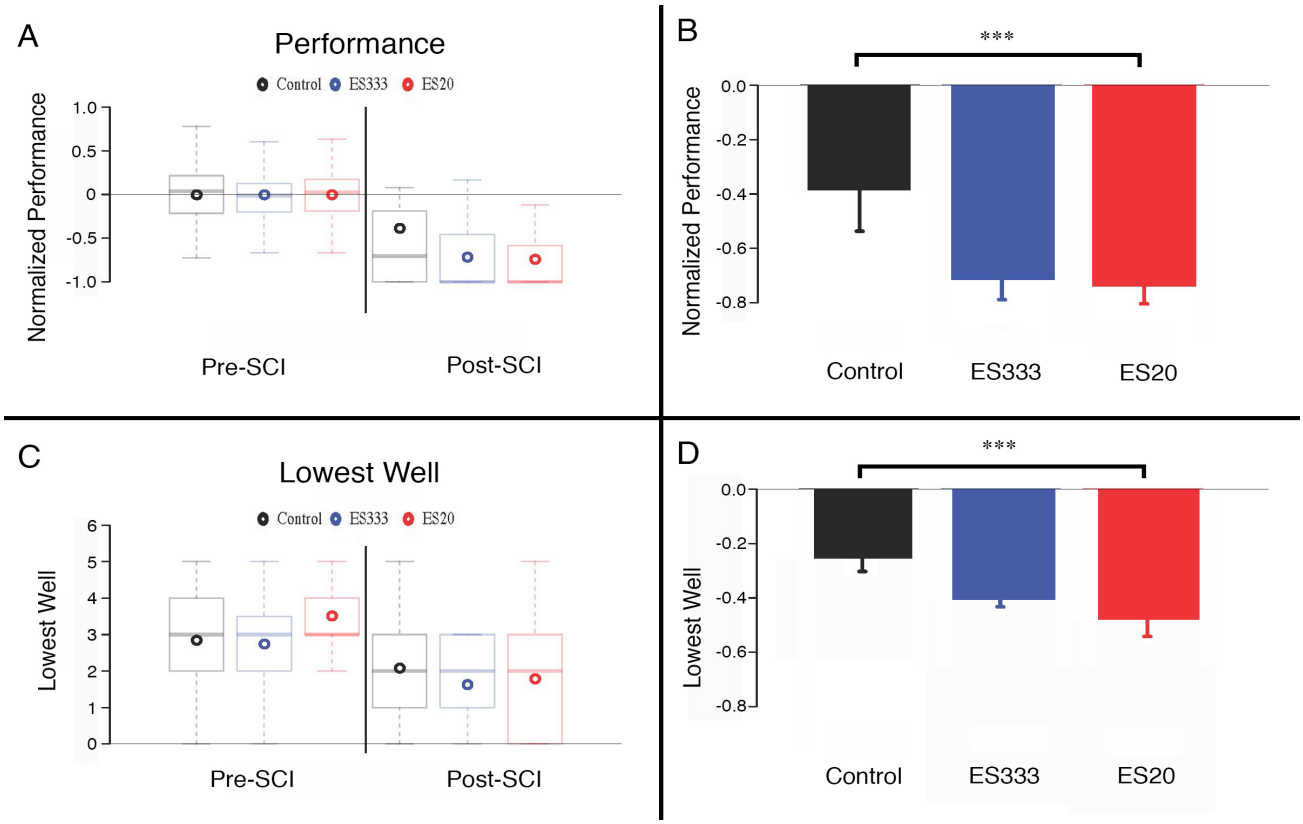

Figure 12: A) Bar plot of Montoya stairwell grasping results (normalized to each animal's pre-lesioning score) from each group pre- and post-spinal cord injury (SCI). B) Bar graph of each group's post-SCI results from panel A. C)) Bar plot of Montoya stairwell lowest well reached results from each group pre- and post-SCI. D) Bar graph of each group's post-SCI results (normalized to each animal's pre-lesioning score) from panel C. Open circle: average; bar: median; box: 95% confidence interval; hashed line: range. Significant difference denoted by *** ($p<0.05$).

improvements were due to ES, subsequent experiments showed that inhibition of the electrically stimulated axons resulted in loss of any behavioural improvements over control animals (Carmel et al., 2014).

These studies, however, have focused on ES of the uninjured CST (either through ES at the level of the medullary pyramid or primary motor cortex), and as such there is a paucity of research investigating the use of ES post-SCI and its proposed benefits as it

applies to the injured CST. Furthermore, experiments examining axonal regeneration in the spinal cord with peripheral nerve stimulation have shown increased central axon projection outgrowth after ES (Udina et al., 2008). Both of these experimental ES paradigms (CNS ES and PNS ES) consisted of quite divergent parameters. CNS ES made use of higher frequency ES parameters (333Hz) on descending motor fibers of the CST, whereas PNS ES consisted of lower frequency ES parameters (20Hz) on ascending sensory fibers. Here, we sought to systematically investigate several of these parameters and examine the influence that they exert on axonal outgrowth of the injured CST. ES was used to stimulate the primary motor cortex contralateral to the SCI at both high- (333Hz) and low- (20Hz) frequencies to investigate its effect on axonal outgrowth (both collateralization and regeneration) and behavioural outcome in a Montoya stairwell reaching task. Data from our experiment have supported previous histological results in which ES promotes axonal outgrowth (Al-Majed et al., 2000; Carmel et al., 2010; Carmel et al., 2013; Carmel et al., 2014; Udina et al., 2008; Harvey et al., 2005): (1) at both high- and low-frequency (increases axonal collateralization), and (2) at both high- and low-frequency does not result in significant regeneration differences. However, low-frequency ES also results in increased axonal dieback from the lesion, and ES at both high- and low-frequency does not translate into a better behavioural outcome (in fact, low-frequency ES resulted in significantly worse outcomes).

3.4.1 Stimulation drives increased axonal collateralization

Previous studies have found that low-frequency ES of a peripheral nerve promotes axonal outgrowth of its central branch within the spinal cord after SCI in a conditioned lesion

experiment (Udina et al., 2008). In addition, CNS motor cortex stimulation of the uninjured CST post-SCI has also promoted axonal outgrowth (Brus-Ramer et al., 2007; Carmel et al., 2010; Carmel et al., 2013; Carmel et al., 2014). In general, our results stimulating the injured CST have been similar to those previously shown in which ES of the uninjured CST also increases outgrowth (collateralization). We found that ES of the forelimb motor cortex at both high- and low-frequencies (333Hz and 20Hz) resulted in increased collateralization compared to control animals. Furthermore, our study also found that there was no significant difference between animal groups with respect to axonal regeneration. As such, although ES increased axonal collateralization, no significant effect on axonal regeneration into the lesion was found. One possible explanation for the discrepancy in our results between collateralization and regeneration could be related to the mechanism through which ES exerts its effect. ES has been shown to result in increased levels of cAMP, upregulation of BDNF, its receptor TrkB, actin and tubulin (Al-Majed et al., 2000; Neumann et al., 2002; Udina et al., 2008; Han et al., 2004). It is possible that cAMP may be, in part or wholly, responsible for axonal collateralization and the commencement of axonal outgrowth via small axonal collaterals, however not for more long-distance axonal growth like regeneration across a SCI lesion from the primary, severed, axon (Al-Majed et al., 2000; Brushart et al., 2002; Han et al., 2004; Udina et al., 2008). The idea that distinct mechanisms are responsible for short-distance sprouting and collaterals versus long-distance regeneration has also been demonstrated in peripheral nerve studies and conditioned lesion experiments (Han et al., 2004; Wujek and Lasek, 1983). Long-distance regeneration depends on elevated expression of at least two growth-associated proteins (i.e., GAP-43 and CAP-23, but not

GAP-43 alone) (Bomze et al., 2001). Cyclic AMP analogues have also failed to elevate expression of both resulting in its inability to increase axonal growth capacity (Bomze et al., 2001; Han et al., 2004). In the same vein, for more long-distance axonal growth (i.e., more than simple neurite sprouting, or collateralization), re-organization of cytoskeletal components such as slow axonal transport proteins (SCa and SCb) is necessary to facilitate transport toward the elongating end of the axon (Han et al., 2004; Stenoien et al., 1999). In fact, the rate of axonal regeneration is identical to the rate of transport of tubulin in SCb (Han et al., 2004). Previous *in-vitro* experiments examining the rate of regeneration of DRG axons after the administration of a cAMP analogue have shown that the analogue failed to increase the rate of tubulin transport (and thus regeneration) (Wujek and Lasek, 1983). Based on findings from these latter experiments, it is not surprising then that increases in cAMP do not translate into increased axonal regeneration (Han et al., 2004). With this in mind, results from our experiment also become clearer. If ES has been shown to increase cAMP which is involved in initiating axonal collaterals (but not longer distance axonal regeneration), then it makes sense that ES resulted in increased collateralization rostral to SCI, but not regeneration into the SCI.

3.4.2 Stimulation affects axonal dieback differently than collateralization

As discussed in 3.4.1, axonal collateralization and regeneration are likely two different phenomena with distinct cellular mechanisms. Although there was no significant difference found between groups in our study with respect to regeneration, it did appear as though there was a differential effect based on the ES frequency used. The 333Hz group trended toward more regeneration into the lesion site compared to the control

group and low-frequency (ES20) group. Similarly, in a previous conditioned lesion experiment, high- and low-frequency stimulation also resulted in varying degrees of axonal regeneration into a SCI lesion (Udina et al., 2008). As such, it is possible that stimulation with different ES frequencies results in intra-neuronal changes that affect axonal regeneration differently. The idea that different ES parameters (high- vs. low-frequency) may affect axons and their capacity for elongation from their terminal end (regeneration, for example) differently also seems to be supported by our axonal dieback results. Axonal dieback is the retraction of an axon from a site of injury. If it is thought of as the opposite process of axonal elongation or regeneration, then the net length of an axon is simply the balance of the two processes. Taken in this context, regeneration and dieback results from our experiment become clearer. Just as they did in the regeneration analysis, the two stimulation frequencies had divergent effects with respect to axonal dieback from the SCI lesion. While the ES333 animals were found to have the least amount of axonal dieback from the lesion site, followed by the control group, the ES20 group had significantly more dieback from the lesion site. Several possible explanations could account for this. ES and neuronal depolarization induce calcium entry into the cell body. This calcium influx is known to be associated with upregulation and expression of immediate-early genes involved in regeneration (Kocsis et al., 1994). Calcium influx also plays a regulatory role in growth cone motility (Kater and Mills, 1991; Ming et al., 1997) through its action on actin polymerization and depolymerization (Lankford and Letourneau, 1989). However, whether or not calcium acts as a growth promoting or retraction/dieback cue is also influenced by both the rate of calcium influx and total amount of calcium influx into the cell (Forbes et al., 2012; Kater and Mills, 1991; Song et

al., 1997). With this in mind, it is possible that the different ES frequencies used in our experiment affect intra-axonal calcium homeostasis differently, and thus growth-associated gene expression and cytoskeletal behaviour differently as well. Here, high-frequency ES may result in calcium influx in a manner that is conducive to axonal elongation and regeneration, whereas low-frequency ES may result in calcium influx acting as an axonal retraction/dieback signal.

3.4.3 Stimulation adversely affects behavioural outcomes

Rehabilitation is a crucial factor in determining functional outcome after SCI. Here, we sought to determine the effect of ES on axonal outgrowth and behavioural outcomes in the absence of rehabilitation. In our experiment, animals that underwent ES were found to have worse behavioural outcome scores than control animals (although only the ES20 group was significantly worse). The two ES groups were also found to have increased collateralization than the control group. ES increased axonal collateralization, however in the absence of rehabilitation to solidify, consolidate, and strengthen functional synaptic connections (in addition to pruning unwanted ones), these potentially useful collaterals may become dysfunctional and hinder recovery post-SCI. Support for this is found in examining results from developmental studies. As the CST develops, it uses neural activity to refine its initially crude pattern of synaptic connections (Alisky et al., 1992; Li et al., 2000; Martin et al., 2005). Furthermore, if activity in neurons emanating from the motor cortex is inhibited, the CST fails to grow into most areas of the spinal gray matter (Martin et al., 2005). Similarly, in ES experiments investigating stimulation of the uninjured CST after SCI, increased outgrowth of the spared CST can be seen into regions

that have become denervated in a competition-driven process (Carmel et al., 2013). Activity in the way of ES of these fibers makes them more competitive for synaptic terminals. In our experiments, ES resulted in increased collateralization, however the non-directed refinement of these collaterals possibly resulted in their becoming detrimental and "out-competing" more functional connections from being formed (preventing behavioural improvement). In addition to unrefined growth hampering functional connections from forming, other ES effects may have contributed to this impaired recovery. ES has been shown to increase BDNF and upregulate its receptor, TrkB (Al-Majed et al., 2000). However, prior to BDNF becoming active and exerting pro-growth effects through TrkB binding, post-translational cleavage by plasmin is necessary. The immature form of BDNF (pro-BDNF) has also been shown to bind to the pro-apoptotic p75 receptor (Lu et al., 2005). If ES results in excessive pro-BDNF release and activation of the p75 pathway, increased neuronal apoptosis could occur resulting in decreased behavioural recovery. Similarly, exercise in SCI has been shown to restore spinal cord BDNF levels (Endo et al., 2009). However, exercise post-SCI has also been demonstrated to result in aberrant sprouting of C-fibers that stain positive for TrkB. Subsequent blocking of TrkB then resulted in decreased neuropathic pain (Endo et al., 2009). Moreover, BDNF has also been demonstrated to result in increased excitability of spinal motor neurons and hyperreflexia (Weishaupt et al., 2012). This increased tone could also hinder functional behavioural recovery post-SCI. "There seems to be a delicate balance between the BDNF induced beneficial effects and the excessive excitability of spinal motor neurons, which may lead to hyperreflexia and spasticity" (Weishaupt et al., 2012). Although we did not witness any specific cases of decreased pain threshold or

spasticity in our animals, we also did not test for it. As such, it is entirely possible that these factors, coupled with non-selective axonal outgrowth due to inadequate rehabilitation, resulted in decreased functional behavioural outcomes. Finally, the significantly worse behavioural outcomes seen with respect to the ES20 group could also be related to the degree of axonal dieback this group incurred. The low-frequency group displayed significantly more axonal dieback approaching the lesion site, and as such had fewer collaterals in the vicinity of the SCI. The ES20 group's inability to perform the task as well as the control group may be simply related to having fewer axonal collaterals at the site of injury. The importance of collateralization in influencing functional outcomes post-SCI has been previously demonstrated (Vavrek et al., 2006; Krajacic et al., 2010).

3.4 Conclusion

ES represents a promising approach to increasing axonal outgrowth after SCI. Here, we found that both high- and low-frequency ES resulted in increased axonal collateralization. However, a differential effect was found upon examining the effect of ES frequency on axonal regeneration and dieback. This difference of effects is postulated to be due to more cAMP-dependent mechanisms for collateralization and more cAMP-independent mechanisms for regeneration and dieback. Furthermore, although both ES groups demonstrate more collateralization, behavioural outcomes seem to be worse than controls. This is possibly due to a lack of refinement of collaterals into functionally useful connections with rehabilitation. ES represents a potentially promising SCI therapy, but further elucidation of its cellular mechanisms and effects of varying ES parameters is required before translation to humans should occur.

Chapter 4

Concluding remarks

4.1 Summary of Results

The previous chapters describe both the state of the literature pertaining to ES use to promote repair and recovery after SCI, as well as our experiment investigating the use of motor cortex ES to improve axonal outgrowth after cervical SCI in a rat model. Results from our study were promising in the sense that ES successfully increased axonal collateralization, however regeneration, dieback, and behaviour were differentially affected. Prior to any sort of clinical translation, several significant limitations must be addressed. However, before discussing limitations of our study, consideration of the difficulties in translating SCI study results in general, and subsequently ES use in animal SCI studies specifically will be addressed.

4.2 Translating spinal cord injury research: trials and tribulations

The use of ES seems like a promising therapeutic avenue because it helps to promote axonal outgrowth, and as such may help with repair and recovery post-SCI. Like many modalities before it, however, many difficulties exist in trying to clinically translate any SCI basic science research. Prior to translation of basic science SCI research into clinical practice, replication of results is crucial. One of the single largest difficulties is the inability to reproduce results. Despite an overwhelming majority within the SCI research community agreeing that independent replication of results is important (Kwon et al., 2010), traditionally little effort has been put into this pursuit. One explanation for this lack of reproducibility is the variability of animal models which makes it difficult to replicate findings prior to their being trialed in more expensive and arduous human studies. More recently, increased awareness and funding has been dedicated to ensuring

SCI research is being done in a more consistent and reproducible fashion. For example, the National Institute of Health (NIH) in the United States (US) has started to address this concern by allocating grants to the replication of promising positive results. These have included pharmacological therapies such as minocycline, Nogo receptor antagonists, and erythropoietin (Pinzon et al., 2008a; Pinzon et al., 2008b; Steward et al., 2008). Although these latter studies had relatively lackluster results in comparison to their original publications, the incongruent findings only serve to highlight the importance of investing in the thorough investigation of these therapies prior to more expensive non-human primate and human clinical trials. Unfortunately, it also underscores the notion that very few scientific SCI research findings are reproducible (Begley and Ioannidis, 2015). With the goal of increasing SCI research reproducibility and translation of truly promising therapies, reporting guidelines such as the minimum information about spinal cord injury (MIASCI) and international campaign for cures of spinal cord injury paralysis (ICCP) were created. Endeavors to standardize the SCI literature is a positive step, however do not overcome other ubiquitous hurdles such as lesion model variability.

Animal models of SCI have allowed much progress to be made with respect to understanding SCI pathophysiology and treatment options. However, different models of SCI exist with potentially different resulting lesions (for example, transection versus contusion lesions). It should be noted that, as discussed in chapter 1, most human SCI occurs due trauma meaning that the contusion lesion model is likely more clinically relevant. However, contusion lesions can be more heterogeneous due to a higher likelihood of multi-level injury secondary to spreading grey matter ischemia and greater

inflammatory response resulting in more hemorrhagic, cavitation, and demyelination sequelae (Siegenthaler et al., 2007; Tator, 2006). This heterogeneity and increasing lesion severity makes it more difficult to study in rodent SCI models (especially of the cervical spine) because of the extensive damage it imparts on the spinal lower motoneuron pool. With damage to the local spinal grey matter, plasticity of the descending CST tract and its contribution to recovery becomes difficult to study. Behavioural outcomes such as reaching are also harder to study if the final common pathway (lower motoneurons) for motor control is damaged. Finally, different lesion models are used in SCI experiments, often times, based on the experimental focus of the study. To illustrate, if the objective of the experiment is to study axonal regeneration and plasticity then a transection model is likely more ideal to enable more accurate study of axons. In our study, we were focused on investigating the effect of ES on CST plasticity and regeneration. Because of this, we chose to use a transection model of SCI in keeping with the more recent ES literature.

4.3 Translation of spinal cord injury electrical stimulation findings

The publication of studies examining the role of ES post-SCI is certainly not immune to the aforementioned challenges. As seen in chapter 3 of this thesis, there has been a resurgence of interest and studies examining the use of ES post-SCI. It is often difficult to compare results amongst these studies as many use various ES frequencies, materials, animals, injury models, etc. The field of ES specific to SCI research, for better or for worse however, has still managed to translate into a clinical trial. A phase 1a trial was completed examining oscillating field ES (OFS) in humans and demonstrated improved sensory scores, without motor improvement (Shapiro et al., 2005). This study opened

more than 10 years ago, was completed more than 5 years ago, and no indication of similar on-going clinical trials exists. With the more recent publication of animal studies investigating the use of CNS ES post-SCI from groups such as the Martin lab, and findings from our own experiments, hopefully further investigation and translation of this potential therapy is not too far away. However, substantial work is needed before this can be considered a reality. Here, both ES groups faired worse with respect to their behavioural outcome. Not examined in our study, however, is the effect of rehabilitation in conjunction with ES. If ES is able to increase axonal collateralization post-SCI and rehabilitation is able to sculpt and refine these anatomical connections into functionally beneficial motor circuits, then the combination of the two could prove to be a powerful therapeutic modality. Support for the notion that rehabilitation plays a key role in determining functional outcome post-treatment also exists with respect to the administration of BDNF post-SCI (Weishaupt et al., 2013). In this study, BDNF treatment alone was unable to impart a functional benefit in rats with a cervical SCI, however the combination of BDNF and rehabilitation resulted in a significantly improved behavioural outcome (Weishaupt et al., 2013). With that in mind, several limitations specific to this project exist necessitating further investigation.

4.4 Limitations of electrical stimulation

One of the obvious limitations of our study with respect to its clinical applicability is the timing of stimulation. In our experiment, ES occurred just prior to SCI. For a given tSCI patient, activity to promote recovery of their SCI can by definition not take place prior to the injury actually occurring. The experiment was designed in this fashion to ensure

proper placement of the electrodes (visualizing contralateral paw movement in real-time). However, with the experience we have gained placing electrodes from this experiment, its replication in a more clinically relevant fashion (ES occurring post-SCI) would be possible. Other limitations of the short-term ES experiment include the relevance of quantifying collateralization, and what constitutes a significant effect size with respect to post-operative SCI. No absolute threshold exists as to what should be considered a significant cut-off regarding the number of collaterals, although their correlation with functional outcome in a rat model has been reported (Carmel et al., 2010; Carmel et al., 2014; Vavrek et al., 2006; Girgis et al., 2007; Krajacic et al., 2010). The quantity of collaterals may be beneficial if it translates into improved motor recovery, however it may also be detrimental if it results in aberrant synapses and increased pain sensation (Endo et al., 2009). The latter was not observed in our study, however we were also not testing for it. Finally, there is a paucity of previous reports documenting a model for intraparenchymal cortical ES. As such, further investigation with respect to issues such as charge density, current spread, optimal electrode materials, etc. are needed. As seen in our long-term ES pilot experiment of this thesis (appendix), electrode insertion caused significant histological changes to occur. However, the resultant functional neurological deficit remains to be seen. With that in mind, several disadvantages to intraparenchymal insertion of electrodes exist. For example, with long-term hardware implantation there is always a concern for an increased risk of infection and material failure. Although the infection rate between an ES set-up using epidural and intraparenchymal insertion of electrodes is likely going to be similar, it will without a doubt be higher than non-invasive techniques such as transcranial magnetic stimulation (TMS). Hardware

malfunction is also a concern as highlighted in a study by Wallace et al. (Wallace et al., 1987a). However, several advantages to our ES set-up also exist. The main advantage of intraparenchymal electrode placement is more direct and specific stimulation of the CST emanating from the primary motor cortex (versus cortical epidural stimulation, spinal stimulation, and TMS). Furthermore, continuous and long-term stimulation at higher frequencies is also possible with an implantable device, whereas with TMS it is not. The eventual stereotactic insertion of electrodes, as is well established within the functional neurosurgical literature, should be the long-term goal.

4.5 Electrical stimulation: unanswered questions and future directions

In addition to studying optimal timing, frequency, and ES duration of our model, the above limitations also serve as a template for future studies. Although a clinical trial has already been completed with respect to the use of OFS in the spinal cord, as discussed in chapter 2, the rationale for ES use across the SCI is different then the rationale for its use to specifically stimulate the CST. As such, consideration should be given as to whether or not these two modalities of ES are even comparable. Before the use of ES to stimulate CST axonal outgrowth can occur in the clinical setting, several questions require investigation and further elucidation is necessary with respect to the effects of varying ES parameters on axonal outgrowth, dieback and behavioural read-outs. We observed a diverging effect on axonal dieback upon varying the ES frequency. Investigation regarding the mechanism for this, whether related to collateralization or not, would be an essential follow-up experiment. To determine the link between cAMP, BDNF, ES, and axonal dieback, loss-of-function experiments in which cAMP and/or BDNF are blocked

would be sensible. Follow-up ES experiments measuring and examining potential therapeutic biomarkers such as cAMP, BDNF, or GAP-43 would also be an important next step. By clarifying the relationship between ES and the above factors, as well as understanding the full scope ES effects, avoidance of AEs may be possible prior to its premature clinical implementation. Furthermore, many of the cortical ES experiments (including stimulation of the uninjured CST) (Carmel et al., 2010; Carmel et al., 2013; Carmel et al., 2014) completed to date have utilized a transection model of SCI. With the observed effects of ES on axonal outgrowth from this experiment, a future step in evaluating ES for SCI repair and recovery should be its use in a contusion injury. Contusion injuries also introduce more lesion variability into our experimental paradigm. That being said, substantial variation due to a multitude of uncontrolled factors exists within the clinical SCI literature. Because of this variation, an argument for a more pragmatic experimental approach with limited exclusion criteria would more closely resemble SCI in humans. The obvious risk with this paradigm is a greater likelihood of non-significant findings. Finally, the most glaring limitation and thus future direction for our study is to replicate the experiment with the caveat of including rehabilitation post-SCI. Here, ES resulted in a worse behavioural outcome. We believe this may be in part due to a lack of rehabilitation. Further investigation as to why this occurred and the incorporation of rehabilitation post-SCI will serve to help determine the relationship between collateralization and whether rehabilitation is able to refine this axonal outgrowth in a functionally meaningful manner. Moreover, rehabilitation is a clinical standard of care after SCI, and as such should be included in future ES experiments. ES represents a potentially promising therapy for SCI. With further systematic investigation

optimizing the parameters of its use for SCI, its translation to humans will be more conducive to a positive outcome and less likely lead to its premature abandonment due to methodological flaws and AEs.

Although further investigation is required prior to translation, the application of our ES set-up to a clinical SCI scenario would not be a significant reach from many of the different neurosurgical procedures and technologies currently in use. For example, intraparenchymal depth electrodes for epilepsy monitoring and implantation of intraparenchymal electrodes for deep brain stimulation (DBS) are currently common practice. Furthermore, animal model DBS experiments to improve recovery and decrease pain post-SCI have been performed (Hentall et al., 2009). Finally, other models of ES post-SCI involve motor cortex stimulation of the uninjured CST. The advantage of our approach in its application to human SCI over other ES and SCI models (Brus-Ramer et al., 2007; Carmel et al., 2010; Carmel et al., 2013; Carmel et al., 2014; Carmel and Martin, 2014) includes: (1) the fact that most human tSCI results in bilateral damage with a mix of both injured and uninjured fibers post-SCI, and we are stimulating injured axons (and thus the benefits of both cortical ES paradigms are seen), and (2) the fact that our injury model is at the cervical level where the majority of human tSCI occurs (Pickett et al., 2006), and is then more applicable than a medullary injury model. Although our ES set-up may be more amenable to clinical practice, many challenges still exist.

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Appendix

Long-term electrical stimulation

A preliminary project consisting of rats without spinal lesioning was conducted to ensure the development of a methodologically reliable long-term ES model. This included assessing the feasibility of stimulation pack installation with the ability to stimulate the rats for up to 10 days consecutively, the tolerance of the rats to its implantation, durability and structural integrity of the long-term ES pack, and parenchymal changes associated with long-term electrode implantation. The stimulation packs were tested with respect to ensuring no short-circuits exist within them under normal conditions, immediately after being submerged in a saline bath to rule-out any immediate short-circuiting, as well as after remaining in a saline bath for 8 hours to ensure no delayed malfunctioning occurred.

Specific long-term electrical stimulation methods

Long-term electrical stimulation pack

Multiple designs for the stimulation pack were created and trialed. The final design (Figure 13) consisted of plastic housing measuring 1.6cm by 1.0cm created via 3D-printer (Makerbot Replicator 2, Makerbot Industries, LLC, Brooklyn, NY, USA) that was sutured on to the upper-thoracic region of a rat's back. Within the stimulation pack housing, there was a female-type connector (Connector Receptacle 50 Position 0.079" (2.00mm) Gold Surface Mount, Millmax, Oyster Bay, NY, USA) with two stainless steel, insulated and braided, lead wires soldered to it that were tunneled subcutaneously to the

craniotomy site. Two insulated electrodes (California Fine Wire Company, Grover Beach, CA, USA), 70μm in diameter, comprised of 90:10 Pl:Ir were soldered to the end of the stainless steel lead wires (A-M Systems, Inc., Carlsborg, WA, USA). Approximately 1mm of their tips were de-insulated. The solder joints were insulated using electronic grade silicon adhesive at the connector-lead wire joint and acrylic enamel at the electrode-lead wire joint. These electrodes were then inserted into the forelimb motor cortex as previously mentioned in the methods section in chapter 3 of this thesis. The electrodes were then affixed to the skull using cyanoacrylate and the scalp incision closed in layers. The spinal cord of the rat was next lesioned as previously outlined in the methods section of chapter 3, the incision closed in layers, and the stimulation pack sutured to dorsum of the rat's back.

Histological Analysis

Upon completing the 10-day stimulation experiment, the electrodes were removed, the brain preserved, sectioned at 25μm, and mounted on slides. The brain sections were stained using GFAP and Iba-1 to examine for parenchymal gliosis and inflammation that may occur with long term electrode implantation.

Results

Long-term electrical stimulation analysis

Histological study of brain tissue surrounding the site of the implantable electrodes was carried out, including GFAP and Iba-1 immunofluorescent staining, in rats undergoing long-term ES. Increased staining of GFAP (indicative of astrocytosis and gliosis) was

evident surrounding the site of implantation. Furthermore, increased staining with Iba-1 (indicative of macrophage/microglia presence) was also observed in comparison to normal brain parenchyma (Figure 14) indicative of potentially deleterious effects with long-term implantation of ES electrodes.

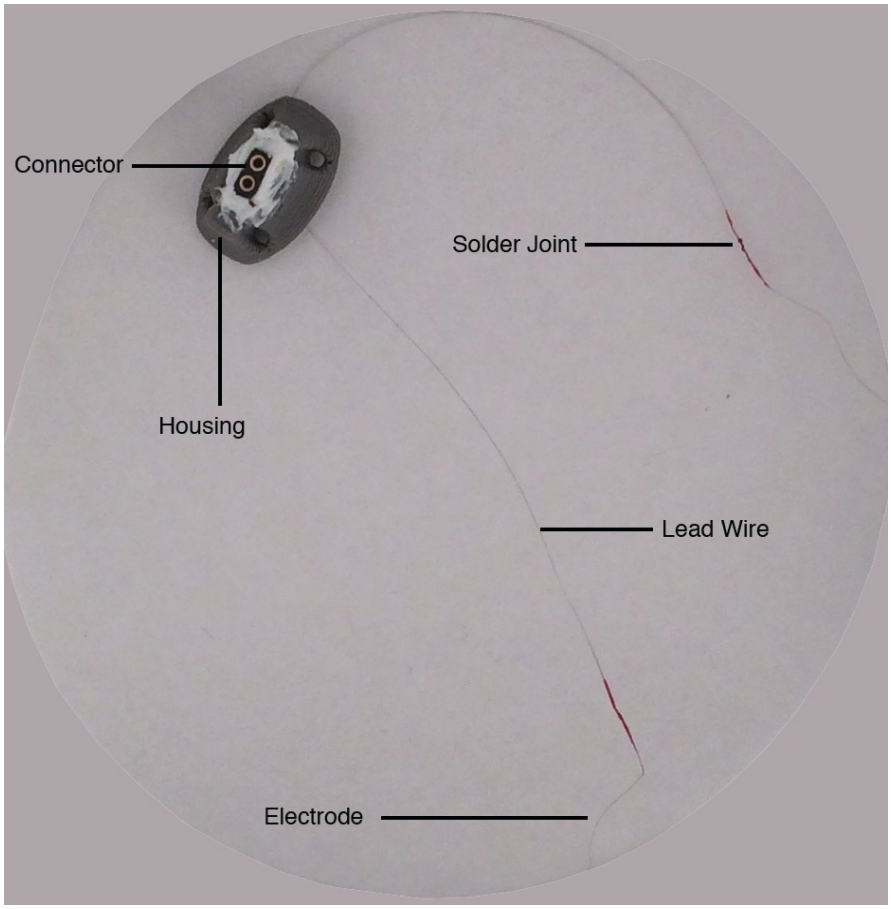

Figure 13: Prototype of long-term electrical stimulation unit.

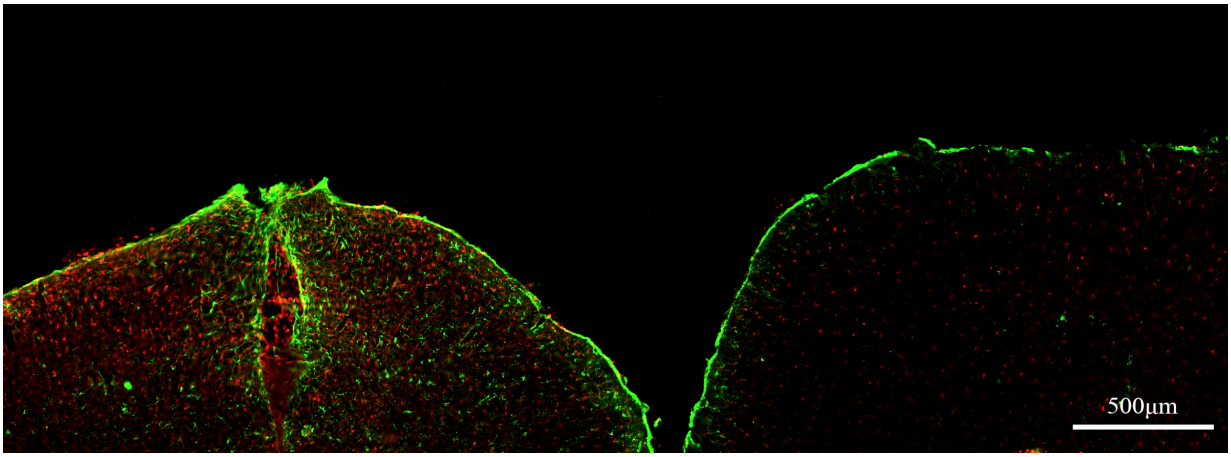

Figure 14: Coronal section of a brain with electrodes implanted into the primary motor cortex from one hemisphere (left) for long-term electrical stimulation. Immunofluroescent staining was completed for glial fibrillary acid protein (GFAP) (green) and Iba-1 (red). Increased signal for both GFAP (indicative of astrocytosis and gliosis) and Iba-1 (indicative of macrophages/microglia and inflammation) can be seen surrounding the site of electrode implantation (left) compared to the contralateral hemisphere (right).

Discussion

Long-term electrical stimulation with electrode implantation causes parenchymal gliosis and inflammation

Most studies examining the use of implantable electrical stimulation units do so using epidural ES. The majority of these studies mention no untoward effects from the long-term use of electrodes upon histological analysis (Borgens et al., 1986; Borgens et al., 1999; Carmel et al., 2013; Carmel et al., 2014; Wallace et al., 1987). It is undoubtedly unfair to compare electrodes situated in the epidural space to those implanted intraparenchymally (as in this study). Although there is a paucity of literature examining the use of intraparenchymal primary motor cortex electrodes in the setting of SCI, there

are some studies investigating the implantation of intrabulbar electrodes to stimulate the nucleus raphe magnus (NRM) (Hentall et al., 2009), as well as intraspinal microstimulation electrode implantation (Bamford et al., 2010). It is difficult to compare such studies to this one as well due to different electrode materials, stimulation parameters, and tissue in which the electrodes reside. Unfortunately, no mention of histological changes occurring around the electrode site in the medulla is mentioned regardless of the aforementioned factors. However, similar to the histological findings in the intraspinal microstimulation study, in our study a clear difference existed between cerebral tissue surrounding the electrodes compared to contralateral (control) cerebral tissue. Increased astrocytosis and gliosis (increased GFAP staining) and increased inflammation (increased presence of macrophages and microglia identified through Iba-1) were evident in the primary motor cortex surrounding the site of electrode implantation (Figure 14). Further study is required in order to determine the effects of the astrocytosis and increased microglia on stimulator reliability, and other factors such as stimulation thresholds, electrode materials, ES frequencies, and potentially resultant behavioural motor deficits.