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Impact and Mechanisms of Extracellular Electrical Stimulation on Peripheral Nerve Regeneration

Singh, Bhagat


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Impact and Mechanisms of Extracellular Electrical Stimulation on Peripheral Nerve Regeneration

by

Bhagat Singh

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
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Abstract

Peripheral nerve injuries are unfortunately very common and debilitating. Peripheral nerve regeneration is slow and regenerative outcomes following severe transection injuries remain limited. Intrinsic inhibitors of neurotrophin signalling diminish the regenerative ability of axotomized neurons and regulate regenerative responses. The overall theme of this thesis is to evaluate whether extracellular electrical stimulation (ES) enhances peripheral nerve regeneration after severe and challenging nerve injuries such as transection and superimposed diabetes, and to understand and exploit its molecular correlates. First, I describe the impact of ES in a severe transection injury model and demonstrate that ES enhances early axon outgrowth that later translates into earlier skin target reinnervation and recovery of sensory and motor function. Utilizing an in-house designed microelectrode array (MEA), I illustrate that ES enhances neurite outgrowth of adult sensory neurons in vitro (Chapter 3). Next, I describe the potential cellular and molecular mechanisms of ES-enhanced regeneration in cultured sensory neurons in vitro and in animal models. Specifically, I observed activation of the PI3-K pathway through downregulation of PTEN expression in response to ES. Other contributing mechanisms involve upregulation of regeneration-associated genes, and enhanced support from perineuronal satellite cells in DRGs (Chapter 4). Finally, in Chapter 5, I show that an ES paradigm has the potential to regenerate axons in a diabetic animal model known for its inherent neuroregenerative deficits (Chapter 5). The results suggest that ES modulates the intrinsic mechanisms of axon regeneration and has a remarkable impact on peripheral neuron plasticity. Overall, the findings support the concept that ES can be utilized as a therapeutic option for severe peripheral nerve injuries.
Acknowledgements

I sincerely want to thank my supervisor, Dr. Douglas Zochodne for his constant support, guidance, and supervision. I have learned a lot from him over the years and feel fortunate to have been under his mentorship.

Special thanks to my supervisory committee, Drs. Ray Turner and Rajiv Midha, your feedback and support were greatly appreciated. Thanks also to my internal external examiner, Dr. Alan Robertson Harrop and my external examiner, Dr. Ranjan Gupta.

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Finally, I would like to thank and express my love to my family for their everlasting love, support and understanding throughout this thesis.

Some of the work presented here has been published in the following manuscripts:


Dedication

To my parents, my wife Vandana and my daughter Ayanna.

My parents (Maa, Pitajee), and my brothers, your unconditional love and support made me who I am. Vandana: without you and your love and caring, this journey would not have been completed. I couldn’t have wished for more than this. Ayanna, you are the most beautiful part of my life. Your smile is always a stress-buster!
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<td>mTOR</td>
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Chapter One: Introduction
1.1 Peripheral Nervous System

The peripheral nervous system (PNS) is intricate and has specialized characteristic anatomy and functions. Peripheral nerves connect the central nervous system (CNS) inclusive of the brain and spinal cord to the rest of the body. Peripheral nerves are the essential connections for movement and sensation. Peripheral nerves are highly specialized structures, and axons travel from the cell bodies in dorsal root ganglia (DRG), spinal cord and autonomic ganglia to distal muscle and skin targets. This distance could be as long as one meter in humans (Zochodne, 2008b). Peripheral nerve injuries are unfortunately very common. In the United States alone, each year 360,000 patients undergo paralysis of the upper extremities as a result of a peripheral nerve injury (Schloshhauer et al., 2006). In the United States and Europe, approximately 100,000 patients undergo neurosurgery related to PNS injuries (Kelsey, 1997). In contrast, only 30,000 people suffer from spinal cord injuries annually (Marino, 1999). There are severe and fundamental limitations to axon regrowth in the PNS. Unlike the CNS, peripheral nerves have a potential to regenerate yet the progression is slow, painful and frequently incomplete, where full functional recovery is often never achieved (Fu and Gordon, 1997; Gordon et al., 2011). Despite advances in neurosurgery, a patient with a brachial plexus injury at the shoulder level is unlikely to ever regain hand function or sensation (Kelsey, 1997; Stoll and Muller, 1999). After many decades of intense research in the field, scientists and clinicians are still exploring mechanisms to find appropriate therapies to enhance the slow and frustrating process of peripheral nerve regeneration.

Through this thesis, I have made efforts to answer specific questions related to nerve regeneration, particularly the impact of extracellular electrical stimulation (ES) on early axonal and Schwann cell (SC) outgrowth, long-term reinnervation and functional recovery. The
mechanisms associated with the benefits of ES explored in this thesis include molecular properties that promote effective repair locally at the proximal stump and at the cell body level.

My research goals for this thesis are:

1. To analyze the impact of extracellular ES on early axonal outgrowth, maturation of axons, target reinnervation and functional recovery after sciatic nerve injury.
2. To explore the potential cellular and molecular mechanisms of ES enhanced peripheral nerve regeneration.
3. To analyze the role of ES on peripheral nerve regeneration in a challenging paradigm, a diabetic animal model, with limited regeneration capabilities.

1.2 Anatomy of the Peripheral Nervous System

Peripheral nerves are complex arrangements of thousands of axons in a single nerve. For example, the sciatic nerve is the longest nerve in the human body, in rats it contains around 30,000 axons (Schmalbruch, 1986). Axons are the principal component of peripheral nerves. Apart from axons, peripheral nerves are composed of many specialized cells including SCs, fibroblasts, immune cells such as resident macrophages and connective tissue. The peripheral nerve is composed of three layers of connective tissue. The nerve is wrapped with an outermost layer of connective tissue known as the external epineurium, which also is comprised of longitudinal blood vessels and other cell types including resident macrophages and fibroblasts. The internal epineurium differentiates fascicles inside the nerve. Axons are clustered in bundles called fascicles. The sciatic nerve in mouse contains 2-3 fascicles and each fascicle is wrapped by a perineurium, which also constitutes the blood-nerve barrier. Endoneurial basal lamina
surround SCs and closely associated axons (Figure 1.1). The cell bodies of peripheral axons reside in the spinal cord or dorsal root ganglia (DRG) depending on the modality of the axons. For example, the cell bodies of sensory neurons are located in DRGs adjacent to the spinal cord and these neurons extend axons to skin tissue and muscle spindles. The cell bodies of motor neurons which supply axons to skeletal muscles are in the ventral grey matter of the spinal cord, and these axons extends to the muscles of the limbs and trunk (Zochodne, 2008b; Birch, 2011).
Figure 1.1 Anatomy of the peripheral nerve trunk.

Myelin layers are formed by SCs that wrap axons. The nerve is composed of three layers: the epineurium, perineurium and endoneurium. Axons and SCs are closely associated and contained within individual fascicles, which also includes endoneurial basal lamina. Reproduced from (Berger, 2004).
1.2.1 Sensory neurons

In the peripheral nervous system, groups of neuronal cell bodies, or perikarya, are known as ganglia and they are divided into two types: sensory and autonomic ganglia. Autonomic ganglia are further subdivided into sympathetic and parasympathetic ganglia. Sensory ganglia, or DRGs, house the cell bodies of afferent sensory neurons. Distinctive populations of small, medium and large sensory neurons are intermixed in the DRG and each neuron is covered by several perineuronal satellite cells. These cells are described later in this Chapter. Sensory neurons are pseudounipolar that means that single axons from each cell body divide into afferent fibers to target organs and central branches that communicate the signal into the spinal cord through the dorsal root. Some of these axons make synaptic contact either with interneurons in the lateral horn or with motor neurons in the ventral horn of the spinal cord. Central afferent branches of large caliber sensory neurons enter the dorsal column and travel to brainstem relay nuclei (Figure 1.2) (Waxman et al., 1995; Zochodne, 2008b).

Sensory neurons range in size from 10-100 microns in diameter and are divided into three major types based on their morphology, trophic requirement and their expression of a distinctive repertoire of receptors and ion channels, although there is an overlap amid these neuronal populations (McMahon et al., 1994; Averill et al., 1995; Gavazzi et al., 1999; Petruska and Mendell, 2004). The first group makes up approximately 40% of the sensory neurons in the lumbar DRG and includes large and medium diameter neurons. These neurons are myelinated and act as mechanoreceptors and proprioceptors and typically express the heavy neurofilament chain NF-200 and tropomyosin related kinase (Trk) family of receptors A and C (Priestley et al., 2002; Ishikawa et al., 2005). The second group is the peptidergic sensory neurons that involve
approximately 30% of the lumbar DRG neuronal population. Peptidergic neurons are primarily small unmyelinated C fibers and typically express calcitonin-gene related peptide (CGRP) and are heat sensitive, also called nociceptors. This neuronal population also includes medium-sized myelinated (A\(\delta\)) fibers. Similar to larger neurons, they also express Trk receptors (Averill et al., 1995; Priestley et al., 2002; Ishikawa et al., 2005). The third group represents approximately 30% of the lumbar DRG sensory neurons, are non-peptidergic neurons and are IB4 positive (binds to the isolectin *Griffonia Simplex* IB4) (Molliver et al., 1997; Bennett et al., 1998; Baloh et al., 2000). These neurons are smaller in size and extend small unmyelinated axons that innervate the skin epidermis and function as nociceptors. These neurons are glial cell-derived neurotrophic factor (GDNF) responsive and express the tyrosine kinase receptor RET and one of the co-receptors GDNF receptor-\(\alpha\) (GFR-\(\alpha\)) and also label for ionotropic P2X3 purinergic receptors (ATP sensitive) (Bennett et al., 1998; Bradbury et al., 1998). Sensory neurons could be divided based on their neurotrophic receptor expression pattern. Approximately 41% are TrkA positive, 33% TrkB, and 43% TrkC (Karchewski et al., 1999). Neurons which are negative for Trk receptors are peptidergic neurons and correspond to the third category as described above (Karchewski et al., 1999; Zochodne, 2008b). Conversely, sensory neurons can express multiple Trk receptors and their expression can change following injury (Karchewski et al., 2002; Gratto and Verge, 2003).

### 1.2.2 Motor neurons

The motor neuron cell body resides in the anterior horn of the ventral spinal cord. Spinal nerves, or mixed nerves, originating from spinal column through intervertebral foramina carry
motor, sensory and autonomic axons and connect the ventral (efferent motor) and dorsal (afferent sensory) spinal cord to the target organs, muscle and skin. In humans there are 31 pairs of spinal nerves. Motor neurons are divided into lower and upper motor neurons. Lower motor neurons have their cell bodies located in the anterior horn of the spinal cord and are further divided into alpha motor neurons and gamma motor neurons (Figure 1.2). Alpha motor neurons are myelinated, fast conducting, and innervate motor endplates of extrafusal striated muscle fibers. These fibers serve as the connection between the CNS and voluntary muscle (Hulliger, 1984; Waxman et al., 1995; Riemann and Lephart, 2002). Gamma motor neurons are slow conducting, are thinly myelinated and innervate intrafusal striated muscles of the muscle spindles. Upper motor neurons originate in the cerebral cortex of the brain and make monosynaptic connections to both alpha and gamma motor neurons in the ventral horn (Hulliger, 1984; Riemann and Lephart, 2002). Peripheral nerve injury leads to degeneration of lower motor neurons, abolished voluntary and reflex responses, atrophy, and flaccid paralysis of the muscles innervated. Reinnervation of muscle after successful regeneration requires arrival and connection of the alpha motor neurons to the motor end plate and gamma motor neurons to muscle spindles (Figure 1.3) (Waxman et al., 1995; Stoll and Muller, 1999; Zochodne, 2008b).
Figure 1.2 Schematic of DRG and spinal cord.

Sensory neuronal cell bodies reside in dorsal root ganglia and make synaptic connections in the spinal cord through the dorsal root. Motor neurons are in the ventral horn of the spinal cord and innervate muscles in the limb through myelinated motor axons (obtained from http://quizlet.com/2660674/neuroscience-ch2-flash-cards).
Figure 1.3 Schematic of sensory and motor neurons.

Motor neurons extend their peripheral axons from the ventral horn of the spinal cord and innervate muscles in the periphery. Motor neurons receive several synaptic inputs. The motor neuron cell body is large and hexagonal in shape compared to the circular sensory neuronal cell body. Sensory neurons are pseudounipolar: a central branch makes synaptic connections in the spinal cord and the peripheral branch innervates skin terminals and muscle spindles. Motor axons are myelinated whereas sensory axons consist of both myelinated and unmyelinated fibers (obtained from: http://3pmganatomy.files.wordpress.com/2012/08/f0429-01.jpg).
1.2.3 The axon

The axon is the fundamental unit of the PNS. Axons conduct electrical signals known as action potentials. Action potentials jump from one node of Ranvier to another and this process is called saltatory conduction. The conduction velocity of axons is directly proportional to the axon thickness and myelination, and ranges from 1 m/sec to 120 m/sec. Impulses can move in both directions from (anterograde) and to (retrograde) the cell body but physiological activation is centrifugal for motor axons and centripetal for sensory axons (Thomas, 1982; Waxman et al., 1995). A rat sciatic nerve contains approximately 7000 motor, 5000 sensory, and the rest are autonomic axons (Schmalbruch, 1986).

Axons consist of neuronal cytoplasm, axoplasm, and the neuronal membrane, the axolemma. Axoplasm includes neurofilaments, microfilaments and microtubules. Neurofilaments are the neuronal-specific intermediate filament proteins of the axon cytoskeleton and provide structural support to the axon and maintain neurofilament spacing: subunits of the neurofilament protein contain sidearm extensions that determine the caliber of axons. Neurofilaments consist of three subunits: light, medium and heavy (Lariviere and Julien, 2004; Zochodne, 2008b). Axonal neurofilaments are mainly phosphorylated whereas neurofilaments in the neuronal cell body are not (Waxman et al., 1995). The majority of the cellular machinery resides in the neuronal cell body that is often several hundred centimeters away from the most peripheral parts of the axon. To transport intracellular material to and from the cell body, peripheral nerves have developed a refined strategy (Lundborg, 1988; Brashart, 2011). Anterograde transport occurs from cell body to the target organs and can be subdivided into slow axonal transport and fast axonal transport. Slow axonal transport is further divided into two
types: the slowest, type “a” (0.2-1 mm/day), which is responsible for the movement of cytoskeletal elements such as microtubules and neurofilaments; and slow component type “b” (2-8 mm/day), which transports enzymes and molecules such as actin (Hoffman and Lasek, 1975; Brown, 2003). Fast axonal transport occurs along axonal microtubules and carries cell organelles such as mitochondria, membrane protein, and neurotransmitter vesicles. Fast anterograde axoplasmic transport is approximately 400 mm/day (Lubinska, 1964; Zochodne, 2008b; Brushart, 2011). Neurotrophins such as nerve growth factor (NGF) from muscle and recycled components from skin and muscle are taken up by the terminal axons and carried along microtubules, with the assistance of a dynein motor, to the cell body through retrograde transport (Lundborg, 1988). Retrograde transport is approximately 200-300 mm/day. Injury signals including transcription factors also travel through retrograde cargos. The rate of axonal elongation is thought to be dependent on the rate of transport of cytoskeleton proteins (Lundborg, 1988; Hanz and Fainzilber, 2006).

In peripheral nerves, axons are further classified based on their fiber diameter. In sensory nerves such as the sural nerve, they are classified into three types: small, intermediate and large diameter axons. Aα are the large myelinated sensory axons with a diameter that ranges from 5-12 microns, and their conduction velocity is 40-80 m/sec. Aβ are the intermediate-sized sensory fibers, which innervate the skin and are responsible for nociception. Small myelinated axons (1-4 micron diameter) are known as Aδ fibers and their conduction velocity ranges from 12-30 m/sec. Unmyelinated fibers ‘C’ are smaller in diameter (0.4-1.2 microns) and conduct at velocities that range from 0.5-2.0 m/sec (Table 1.1) (Waxman et al., 1995; Zochodne, 2008b; Brushart, 2011)
<table>
<thead>
<tr>
<th>Fiber Type</th>
<th>Erlanger-Gasser Classification</th>
<th>Fiber Diameter Range (mm)</th>
<th>Conduction Velocity (m/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Myelinated</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Large</td>
<td>Aα</td>
<td>12-20</td>
<td>72-120</td>
</tr>
<tr>
<td>Medium</td>
<td>Aβ</td>
<td>6-12</td>
<td>36-72</td>
</tr>
<tr>
<td>Small</td>
<td>Aδ</td>
<td>1-6</td>
<td>4-36</td>
</tr>
<tr>
<td><strong>Unmyelinated</strong></td>
<td></td>
<td>0.2-1.5</td>
<td>0.4-2.0</td>
</tr>
</tbody>
</table>

Table 1-1 Classification of nerve fiber types.

Modified from (Terzis and Smith, 1990; Gardner et al., 2000).
1.2.4 Schwann cells

Schwann cells (SCs) are the principal supporting cells or glia of the peripheral nervous system (Jessen and Mirsky, 2005). The counterparts of SCs in the CNS are oligodendrocytes. The normal SC to myelinated axon ratio is 1:1 in the PNS. SCs are broadly divided into two types: myelinating and non-myelinating. Myelinating SCs wrap myelin around axons from one node of Ranvier to the next, placed around 100 µm apart (Armati, 2007). Nodes of Ranvier have clusters of voltage gated sodium channels, specifically Na\textsubscript{v} 1.2, Na\textsubscript{v} 1.6, Na\textsubscript{v} 1.8 and Na\textsubscript{v} 1.9. These regions are also enriched with the cytoskeletal proteins spectrin and ankyrin. The juxtaparanode region is lateral to the paranode and contains: potassium channels, particularly Kv 1.1, Kv 1.2 and Kv 1.5; connexin 32; and E-cadherin (Scherer, 1996). Remak bundles, groups of non-myelinated axons, are each associated with a single non-myelinating SC and basal lamina (Griffin and Thompson, 2008). Non-myelinating SCs are essential for the survival and normal physiological maintenance of these axons (Scherer, 1997; Jessen and Mirsky, 2005). These cells are also involved in the maintenance of C-fiber sensory neurons and thereby maintain pain sensation (Chen et al., 2003). Recently, Frieboes et al. (2010) reported that Nav 1.8 is also expressed in endoneurial SCs following peripheral nerve injury and mediates noninflammatory neuropathic pain. The molecular repertoire is different between SCs associated with unmyelinated or myelinated axons. Myelinating SCs express antigenic markers such as S-100, myelin basic protein (MBP) and myelin protein P0, whereas non-myelinating SCs express glial fibrillary acidic protein (GFAP), p75 neurotrophic receptor, and CNPase (Jessen and Mirsky, 2002).
Two more types of SCs are described: satellite cells in the DRG and perisynaptic SCs at the neuromuscular junction. Satellite cells tightly wrap around sensory neurons in the DRG and serve essential roles for physiological maintenance of sensory neurons, provide trophic support during the regeneration process and partially protect the DRG which otherwise lacks a blood brain barrier (Tusscher et al., 1989; Armati, 2007). Perisynaptic SCs are important for the normal functioning and development of the tripartite synapse and express neurotransmitter receptors (Reynolds and Woolf, 1992; Armati, 2007). The pathophysiology of SC disorders includes, but is not limited to, neuropathies such as: Charcot-Marie-Tooth (CMT), Guillain-Barré syndrome (GBS), chronic inflammatory demyelinating polyneuropathy (CIDP) and others (Armati, 2007).

The relationship between axons and SCs is intimate and bidirectional (Chen et al., 2005). Axons provide signals for the initiation of myelination, control the number of myelin lamellae and help in the regular maintenance of SCs. SCs control axonal diameter and neurofilament spacing (Taveggia et al., 2005). Neuregulins (NRG) are critical signals between axons and SCs during development and myelination (Jessen et al., 2008). NRG is also known as glial growth factor (GGF) and is expressed by sensory and motor axons. It acts as a mitogen for SCs by binding to its receptor tyrosine-protein kinases, ErbB2 and ErbB3 (epidermal growth factor receptor family) expressed on SCs (Marchionni et al., 1993; Meyer and Birchmeier, 1995). The overall functions of SCs are diverse and play an important role in axon regeneration. After injury, SCs in the distal stump dedifferentiate to immature SCs (proliferating non-myelinating phenotype), express GFAP and upregulate the production of neurotrophins and cell adhesion molecules (Fu and Gordon, 1997; Boyd and Gordon, 2003). This SC proliferation is key to aiding the process of regeneration and repair (Chen et al., 2007). SCs provide a large number of molecules that influence regeneration by supporting both themselves and partnering axons. This
includes essential growth factors (NGF, BDNF, NT-3, GDNF, NT-4/5), neurotrophic cytokines, other neurotrophic factors (insulin, IGF-1), fibroblast growth factors (FGFs), cytokines (CNTF, IL-6, LIF), cell adhesion molecules, such as NCAM and N-cadherin, and extracellular matrix proteins (Murphy et al., 1995; Hoke et al., 2000; Boyd and Gordon, 2003).

1.2.5 Target organs

Skeletal muscle or voluntarily controlled muscles are innervated by both motor and sensory neurons. Intermediate to large myelinated sensory axons innervate intrafusal muscles (muscle spindles). Large afferent fibers are sensitive to dynamic movements, (Group Ia) and intermediate-sized to muscle length (Group II). These muscles are innervated by gamma motor neurons. Intrafusal muscles are further divided into nuclear bag and nuclear chain fibers. Alpha motor neurons innervate extrafusal muscle and generate tension in the muscle. There is also widespread innervation by unmyelinated nociceptive axons that mediate intramuscular pain. Axons communicate with muscle through the neuromuscular junction (NMJ) that includes motor neuron terminals, muscle fiber endplates and perisynaptic SCs. These synapses are cholinergic in nature, and acetylcholine (Ach) acts as a main neurotransmitter (Waxman et al., 1995; Zochodne, 2008b; Birch, 2011). Another major peripheral target organ is skin. The outermost layer of skin is composed of proliferating basal and differentiated suprabasal keratinocytes that comprise the epidermis. The skin has a rich supply of nerve fibers. Unmyelinated C fiber free nerve endings terminate in the epidermis and transmit thermal, mechanical and chemical pain sensations. Aδ fibers that convey mechanical pain innervate dermis. Reinnervation of the skin is challenging and it is likely that numerous barriers obstruct regrowth of newly regenerating fibers. Skin
reinnervation is never achieved completely after transection injuries and patients may experience ongoing loss of sensation as well as abnormal sensory symptoms as a result of failed reinnervation. Terminal SCs in skin direct regrowing axons to their destined target (Reynolds and Woolf, 1992). Measurement of intraepidermal nerve fiber density (IENFD) is considered the gold standard to assess reinnervation of the skin and is also necessary for the diagnosis of small-fiber neuropathy (Figure 1.4) (Polydefkis et al., 2001; Rajan et al., 2003).
Unmyelinated sensory nerve endings innervating the dermis and epidermis. These fibers subserve mechanical and thermal sensation. Other cell types are also visible (obtained from http://hospitals.unm.edu/burn/skin_anatomy.shtml).
1.3 Injuries to the Peripheral Nervous System

Nerve injury is a complex problem with only limited treatments available to facilitate reinnervation of target tissues. The timing and success of the peripheral nerve repair process depends on the extent of injury. Although there is potential for regeneration in the peripheral nervous system, the recovery from injury to peripheral nerves is incomplete and frequently renders severe and irreversible disability (Fu and Gordon, 1997; Chen et al., 2007; Zochodne, 2012).

1.3.1 Types of injuries

Primary mechanisms involved in peripheral nerve injury include transection, crush, ischemia, chemotherapy, laceration and stretch. Example of stretch injuries include obstetrical brachial plexus palsy, of transection include penetrating trauma and of compression is Saturday night palsy, which occurs by prolonged compression of a limb segment and either mechanical distortion or ischemia in the compressed region (Noble, 1998; Zochodne, 2008b). Common sequellae of nerve injury are pain (ranging from a tingling pain to intense burning pain), numbness or inappropriate sensation, muscle weakness in the involved body part, and loss of function or active movement from motor dysfunction (e.g. wrist drop and foot drop) (Stoll and Muller, 1999).

The regenerative outcome of nerve injuries depends on the type and extent of injury and the degree of neural tissue involved. The two most widely accepted classification schemes for nerve injury are the Seddon system (Seddon, 1943) and the Sunderland (detailed and contrasted in Figure 1.5 and 1.6) (Sunderland, 1978). According to Seddon, peripheral nerve injuries are
classified into three types. 1) Neurapraxia: This is the mildest form of nerve injury. It involves local demyelination, usually segmental with conduction block. Recovery develops without surgical intervention and is more rapid than other forms of injury without Wallerian or Wallerian-like degeneration. Stretch injuries or mild compression injuries are common examples. Conduction is usually restored in a few weeks. 2) Axonotmesis: This involves the loss of axon continuity with preservation of the connective tissue framework of the nerve and involves Wallerian-like degeneration. Common examples of these injuries include mechanical trauma, ischemia, and others. The distal axons degenerate, lose their connection to the target organ and have a loss of axonal excitability. These injuries recover slowly, and functional recovery may or may not be complete. The endoneurial Bands of Bungner remain intact to support some degree of successful regeneration. 3) Neurotmesis: This is the most severe lesion and involves complete transection of axons and their connective tissue framework. The distal axons undergo Wallerian degeneration and often require surgical repair to remove associated scar tissue. The outcomes associated with injuries are often poor and recovery of sensation is impeded (Waller, 1850; Griffin et al., 1996). Another major classification of nerve injuries was described by Sunderland who divided injuries into five classifications. A first-degree injury is similar to Seddon’s neurapraxia, with local conduction block but no structural damage to the axons. Second-degree injury is equivalent to Seddon’s axonotmesis, defined by injury to axons with preserved connective tissue. Sunderland’s third and fourth degrees are intermediate stages between Seddon’s axonotmesis and neurotmesis. Third degree injuries are reflective of a partial loss of connective tissue (endoneurium) with axonal disconnection. Sunderland’s fourth degree includes disruption of axons, endoneurium and perineurium but the nerve trunk is still connected because of sparing of the epineurium. The regeneration outcomes are meager and recovery takes longer.
Sunderland’s fifth degree classification of injury is equivalent to the neurotmesis of Seddon, and includes retraction of proximal and distal stumps; the regenerative process in this setting is very limited and the functional recovery is inadequate (Figures 1.5 and 1.6).

Thus, peripheral nerve injury may result in demyelination or axonal degeneration (Wallerian or Wallerian-like). Clinically, both demyelination and axonal degeneration result in disruption of the sensory and/or motor function of the injured nerve. For neurotmesis, treatment is usually surgical intervention, which includes nerve repair, nerve grafts and nerve transfer, although all these treatments have limited success (Seddon, 1963; Kline and Hudson, 1995; Mackinnon and Novak, 1999).
Figure 1.5 Peripheral nerve injury types according to the Seddon and Sunderland classifications.

All types of nerve injuries are depicted in the right side of the graph (obtained from http://www.neurosurgery.tv/wallerian.html).
Figure 1.6 The graph represents the association between nerve injury and subsequent tissue damage and recovery in the Seddon and Sunderland classifications.

Reproduced from (Burnett and Zager, 2004).
1.4 The Neural Response to Injury: Early Regenerative Events

Injuries to the peripheral nerves bring changes at all levels - cell body, proximal stump, distal segment and target organs. These changes are described below in detail (Figure 1.7).

1.4.1 Wallerian and Wallerian-like degeneration distal to an axon injury

August Waller in 1851 described the pathological changes in the distal stump to the injury site after transection of the glossopharyngeal nerve in frog. This process, termed Wallerian degeneration, refers to the series of events that begins in the distal nerve terminals of the transected nerve. Wallerian-like degeneration refers to a similar degenerative process that occurs distal to other forms of axon injury, for example following crush, stretch or neuropathy (Waller, 1850; Griffin et al., 1996; Stoll et al., 2002). Initially Wallerian degeneration was considered a passive process where simple disconnection of axons from their cell body would cause disruption in the supply of nutrients essential for the survival, and the axons would finally disintegrate. The discovery of the spontaneous mutant wld mouse with slow Wallerian degeneration associated with intact and excitable axons altered this dogma. In this mouse model, because of a mutation, ubiquitin regulatory enzyme related protein known as Wlds was overexpressed, which is composed of chimeric proteins, ubiquitin assembly protein Ufd2a and the nicotinamide adenine dinucleotide (NAD) biosynthetic enzyme Nmnat1 (Mack et al., 2001; Araki et al., 2004). Increased Nmnat activity and its downstream target, SIRT1, a mammalian ortholog of Sir2, prevents axonal degeneration (Araki et al., 2004).

During Wallerian or Wallerian-like degeneration SCs and macrophages interact to remove debris, specifically myelin and the damaged axons, to make the environment permissive
for nerve regrowth. Immediately following transection injury, an influx of calcium into the axon activates cysteine proteases and leads to proteolytic digestion of the cytoskeleton axonal framework (Stoll et al., 2002; Buki and Povlishock, 2006). Within a few hours, SCs express MAC-2, a galactose-specific lectin, which targets the myelin and is responsible for initial phagocytosis (Stoll et al., 1989; Reichert et al., 1994; Stoll and Muller, 1999; Stoll et al., 2002). Within 2-3 days of the nerve injury, SCs recruit macrophages in the distal stump by secreting MCP-1 (monocyte chemoattractant protein-1) and LIF (leukemia inhibitory factor) (Stoll et al., 1989; Tofaris et al., 2002). Macrophage influx enhances the efficacy of regeneration not only by clearing inhibitory myelin but also by inducing the proliferation of SCs in the distal stump by elaboration of local mitogens (chemical substances that encourage a cell to commence cell division) such as transforming growth factor-β1 (TGF-β1) (Perry et al., 1987; Perry and Brown, 1992). Macrophages also secrete growth factors including NGF and IGF-1 (insulin-like growth factor-1) that promote nerve regeneration (Sjoberg and Kanje, 1989; Griffin et al., 1993).

By day 5-7, SC proliferation begins in the distal stump with proliferation of mast cells, endothelial cells and fibroblasts (Stoll et al., 1989; Zochodne and Nguyen, 1997; Zochodne and Cheng, 2000). In contrast, in the CNS, this response is significantly attenuated, contributing to the limited regeneration of axons in white matter (George and Griffin, 1994). The extracellular inhibitory molecules that contribute to the limited regrowth in the CNS are myelin associated molecules NOGO-66, myelin-associated glycoprotein (Chen et al.), and oligodendrocyte myelin glycoprotein (OMgp) or chondroitin sulfate proteoglycans (CSPGs) (Mukhopadhyay et al., 1994; GrandPre et al., 2002; Niederost et al., 2002). RhoA GTPase (Ras homolog gene family) is expressed at the growth cone and causes constitutive inhibition of axon regrowth in the CNS (Dergham et al., 2002). RhoA-ROK is also functional in the PNS and impedes axonal
regeneration following transection injuries (Cheng et al., 2008). To overcome such inhibition, growth cone cAMP signaling appears to play a facilitatory role (Lohof et al., 1992).

In the distal stump, both pro-inflammatory chemokines such as MCP-1 and MIP1α (macrophage inflammatory protein) and anti-inflammatory cytokines TGF-β1 and IL-10 show a biphasic response at days 1 and 14 following injury (Perrin et al., 2005). The inhibitory extracellular matrix is degraded by the matrix metalloproteinases (MMPs) in particular MMP-2 and 9, essential for the later axonal growth into the distal segment (Ferguson and Muir, 2000). Another MMP (MMP-3) has a significant role in motor endplate remodeling by preventing degradation of the motor end plate and thereby improving muscle target reinnervation following peripheral nerve injury (Chao et al., 2013). Further in the distal stump, axonal regeneration is supported by the SCs lining the denervated endoneurial tubes (Zochodne, 2008b). The maturation and remyelination of axons is critical for recovery from peripheral nerve injury. SCs execute remyelination of regenerated axons, and the key regulation of the myelination process begins when regenerating axons make contact with SCs, which then leads to the interaction of neuregulin-1 (NRG1) (located on the axon membrane) and ErbB receptor tyrosine-protein kinase on the SC membrane (Corfas et al., 2004; Nave and Schwab, 2005). Other pro-myelin molecules include transcription factors such as Sox-10, Krox-20 and Oct-6 (Patricia, 2007). Additional factors include Par-3, BDNF and GDNF, as well as the physical size of the axon (Hoke et al., 2002; Nave and Schwab, 2005; Patricia, 2007).

1.4.2 Regenerative events in the cell body

Unlike in the CNS, some regeneration in the PNS is possible. Following injury, a synchronized series of biochemical and molecular events begins, which are necessary for
successful regeneration to occur. In 1892, Franz Nissl observed morphological changes occurring in motor neurons following extraction of the facial nerve from its canal (Nissl, 1892). Chromatolysis, or a change in the staining characteristics of the cell body after staining and fixation, develops in the cell bodies of DRG sensory neurons or spinal cord motor neurons after axotomy. It involves migration of the nucleus to the periphery of the cell body and break up and dispersion of the endoplasmic reticulum, among other morphological changes (Lieberman, 1971b). The cascades of morphological and molecular changes in the neuronal cell body in response to injury represent the conversion of a neuron from maintenance/transmitting mode to a regenerative mode (Lieberman, 1971a; Fu and Gordon, 1997). For example, sensory neurons upregulate tubulin, actin, and the growth associated protein 43 (GAP 43). Endogenous brain derived neurotrophic factor (BDNF) appears to be necessary for the induction of regeneration associated gene (RAG) expression in injured sensory and motor neurons (Tetzlaff et al., 1989; Strittmatter et al., 1994). Motor neurons in the lower spinal cord do not die after axotomy, but a small population (approximately 10-20%) of sensory neurons do (Groves et al., 2003). One of the postulated mechanisms is a loss of trophic support from the distal nerve stump and the target organ, which are essential for the survival of neurons (Schmalbruch, 1987).

Signaling pathways such as the mitogen-activated protein kinase (MAPK), extracellular-signal-regulated kinase (ERKs) and e-Jun N-terminal kinase (JNK) pathways are activated at the injury site and retrogradely travel back to the cell body to activate intrinsic mechanisms of regrowth (Hanz et al., 2003). One such pathway is the dual leucine zipper kinase (DLK) pathway (Fan et al., 1996). DLK pathway has been demonstrated to be essential for axonal regrowth in C. elegans, Drosophila and recently in mice (Hammarlund et al., 2009a; Miller et al., 2009). DLK
is a MAP3K which, through its intermediate MAPKK, activates JNK and p38 MAPK (Fan et al., 1996; Gallo and Johnson, 2002).

The intrinsic intensity of activation by injury signals varies between neurons and influences whether successful regeneration will occur. The intrinsic growth state of a neuron depends on the extent of injury, type of neuron, age and location of the injury. More severe injuries, injuries more proximal or closer to the cell body and injuries in a younger age lead to larger changes in the cell body response (Seijffers et al., 2007). In the PNS, not all of the axons are able to regenerate through permissive SC endoneurial tubes, indicating that the intrinsic growth capacity of these neurons is compromised. Intrinsic neuronal mechanisms engage upregulation of regeneration-promoting molecules, which include but are not limited to upregulation of the cAMP levels, increased transcription of RAGs and cytoskeleton molecules, translation of cytoskeleton proteins by upregulated ribosomal proteins s6k (ps6k) and others (Neumann et al., 2002; Seijffers et al., 2007; Park et al., 2008; Zou et al., 2009; Abe et al., 2010; Christie and Zochodne, 2013). These mechanisms are required to stay active for longer durations for successful regeneration and target reinnervation to occur. On the contrary, molecules such as PTEN, RhoA and others dampen these intrinsic growth capabilities (Cheng et al., 2008; Christie et al., 2010). These are described below in detail.

The changes in neurons also accompany striking alterations in adjacent satellite glial cells, which wrap around sensory neurons in the DRG, proliferate in response to injury and become hypertrophic and upregulate expression of GFAP. These changes occur because of a continuous reversible signaling exchange between neurons and satellite cells (Zochodne, 2012).
Peripheral nerve regeneration can be divided into the following major events: Wallerian degeneration, axon regeneration/growth, and axon reinnervation. The SC proliferation and Bands of Bungner are also illustrated. The neuronal nuclei displaced to periphery and hypertrophic perineuronal satellite cells are also visible. Reproduced from (Zochodne, 2008b).

**Figure 1.7 Schematic of the events in peripheral nerve regeneration.**
1.4.3 Regenerative events in the proximal stump

Immediately following injury, the tip of the proximal nerve stump seals to avoid leakage of structural proteins and organelles travelling though axoplasmic transport. This leads to swelling of axons at the tip. This proximal nerve stump, attached to the cell body, sprouts growth cones within a few hours (Rotshenker, 1981). The initial growth cone formation occurs independent of the cell body, in part by local transport of cytoskeleton proteins and from local protein synthesis, and is crucial for axonal regeneration and the growth cone response (McQuarrie and Lasek, 1989; Willis and Twiss, 2006). In the next 1-2 days, axon elongation begins with a continuous supply of anterograde transport of structural proteins and organelles necessary for growth (Jiang and Schuman, 2002; Gumy et al., 2010). Growth cones continuously explore the regenerating microenvironment and respond to stimulatory or inhibitory molecular cues. At the leading edge of a growth cone, a continuous assembly and disassembly of actin filaments leads to extension of filopodia and growth cone motility. The growth cone is guided or supported by molecules produced by SCs as well as the extracellular matrix proteins, laminin and fibronectin (Gallo and Letourneau, 2004). Sprouts from a single parent axon advance in the distal nerve as a “regenerating unit” (Witzel et al., 2005).

Proximal segments of transected peripheral nerve trunks also undergo degeneration as far retrogradely as the first two nodes of Ranvier proximal to the lesion site (Friede and Bischhausen, 1980). This "die-back" degeneration is thought to occur because of an influx of calcium and activation of calcium-associated proteases (Cajal, 1928). The variability in the speed of axonal regeneration is known as staggered regeneration. Axonal regeneration is initially slow after what is known as its “latent period”, and thereafter reaches to a constant rate of elongation
(Sjoberrg and Kanje, 1990). The rate of regeneration varies between species, and in humans it is considered 1mm/day under ideal circumstances, a rate that is optimistic in many injury scenarios (Gutmann et al., 1942; Brushart, 2011). In some cases, regenerating axons form a neuroma, where misdirected axons swirl through and around connective tissue, presumed to be secondary to the lack of distal guiding SCs (Zochodne, 2008b).

Axons in the PNS regenerate more successfully after crush injuries than transection injuries because new axon sprouts can regenerate into Bands of Bungner. Bands of Bungner are basement membrane tubes surrounding previously degenerated axons that are associated with proliferating SCs in the distal stump (Chen et al., 2005). In transection injuries, regeneration is limited because the intact nerve trunk is under tension and transection leads to the retraction of distal and proximal stumps with a gap between them. The growth of axons from the proximal stump in the transection injury model is also often misdirected. This mismatched and misdirected reinnervation contributes to poor behavioural recovery (Witzel et al., 2005; McDonald et al., 2006). Since transection nerve injury is a serious and common clinical problem that causes complete axotomy of the nerve and involves severe constraints on regrowth, I have chosen this model to investigate the effect of extracellular ES and its influence on nerve regeneration.

Later on, axon regeneration is supported by the extracellular matrix (ECM) and neurotrophins. Laminin, an ECM protein, and its receptors integrins and dystroglycans, are expressed in intact nerves and upregulated following nerve injury in the PNS (Doyu et al., 1993; Patton et al., 1999). Laminin acts as a substrate to direct axonal regeneration (Doyu et al., 1993). Neurotrophic factors interact with cell surface receptors and play a role in neuronal survival and growth following injury (Miyata et al., 1986).
1.5 Neurotrophins and Peripheral Nerve Regeneration

Neurotrophins are a family of secreted proteins that induce survival, development, and differentiation of neurons, both central and peripheral (Huang and Reichardt, 2001; Sofroniew et al., 2001; Boyd and Gordon, 2003). The family of neurotrophins includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5). Neurotrophin-6 (NT-6) and neurotrophin-7 (NT-7) are additional neurotrophins. Trk and p75 are high and low affinity-binding receptors for neurotrophins respectively (Fu and Gordon, 1997; Friedman and Greene, 1999; Barrett, 2000). Classically, NGF binds to TrkA receptors, located mainly on sympathetic and small sensory nociceptive neurons. BDNF and NT-4/5 bind to TrkB receptors expressed primarily on motor neurons and medium sized sensory neurons in DRGs. NT-3 binds to TrkC receptors present on large caliber sensory neurons (Averill et al., 1995). p75 can bind to all of the neurotrophins and is expressed in approximately 79% of sensory neurons (Figure 1.8) (Funakoshi et al., 1993; Barrett and Bartlett, 1994; Zochodne, 2008b)

During development, embryonic neurons are dependent on NGF for survival and growth. Growing axons respond to many attractive and repulsive cues during development and are guided to their targets by gradients of Netrin-1, Shh, Ephrins, VEGF and other cues (Charron et al., 2003). In addition to survival signals, NGF also plays a role in guiding growth cone behaviour during development and peripheral target innervation. In adults, NGF expression is upregulated in the injured nerve, SCs, macrophages and mast cells. NGF directionally attracts the growth cones of injured embryonic and adult sensory neurons (Webber et al., 2008).
Injured neurons synthesize many neurotrophic factors, including NGF (Ernfors et al., 1989), BDNF, NT-3 (Schecterson and Bothwell, 1992; Kobayashi et al., 1996) and CNTF (ciliary neurotrophic factor) (Rabinovsky et al., 1992). All of these neurotrophins are also upregulated in the distal stump after injury and return to baseline after nerve regeneration, which suggests their potential role in the regenerative process. Additionally, the Trk receptors are upregulated in axotomized neurons. For example, TrkB and the low affinity p75 receptors are upregulated in axotomized motor neurons (Raivich and Kreutzberg, 1987; Verge et al., 1989; Koliatsos et al., 1991). Neurotrophins have autocrine and paracrine effects on neurons and nonneuronal cells through growth promoting pathways (Figure 1.8) (Verge et al., 1996; Friedman and Greene, 1999; Gavazzi et al., 1999)
Figure 1.8 Schematic of the type of peripheral neurons and analogous neurotrophins.

Sympathetic and small nociceptor neurons express TrkA receptors that respond to NGF. Motor and mixed afferent fibers express TrkB receptors and respond to BDNF and NT-4/5. NT-3 acts on TrkC receptors present on large diameter afferents. Reproduced from (Zochodne, 2008b).
1.5.1 Neurotrophin signaling pathways

Neurotrophins act through three classical signaling pathways: PI3-AKT, Ras-raf-ERK and SNT-PLC pathway (Bonni et al., 1999; Kaplan and Miller, 2000; Boyd and Gordon, 2003; Chao, 2003; Reichardt, 2006). One of the major pathways of neurotrophin signaling is the extracellular signal-regulated kinase, ERK or MAPK pathway (Bonni et al., 1999; Reichardt, 2006; Hammarlund et al., 2009b). Neurotrophins act on tyrosine kinase receptors and activate Ras, which in turn activates Raf and initiates a number of intracellular phosphorylation events to activate MEK1 and MEK2, and subsequently phosphorylates ERKs, particularly ERK1 and ERK2. MEK is then transported to the nucleus and activates c-jun and c-fos transcription factors, which in turn, phosphorylate cAMP response element-binding protein (CREB) (Bonni et al., 1999; Kaplan and Miller, 2000; Reichardt, 2006). This whole cascade has diverse cellular impacts depending on the ligand, and later the intensity and extent of signaling, to alter the levels of transcription factors, which are essential for nerve regeneration (Bonni et al., 1999; Kaplan and Miller, 2000; Reichardt, 2006; Hammarlund et al., 2009b).

The SNT-PLC pathway, which is similar to G-protein coupled receptor (GPCR) signaling, operates through the activation of inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) after neurotrophins bind to a receptor tyrosine kinase. Activation of this pathway leads to a rise in intracellular calcium and an activation of protein kinase C (PKC). Higher intracellular calcium leads to phosphorylation of a calcium response element binding protein-dependent mechanism for nerve growth (Bibel and Barde, 2000; Kaplan and Miller, 2000; Reichardt, 2006).

A major and crucial growth factor signaling occurs through the PI3K-Akt pathway, which is essential for survival, proliferation and differentiation of neurons (Soltoff et al., 1992).
Phosphoinositide 3-kinase (PI3K) converts phosphatidylinositol 3,4-biphosphates (PIP2) to phosphatidylinositol 3,4,5-triphosphates (PIP3). PI3-K is a second messenger necessary for the translocation of Akt to the cell membrane where it is phosphorylated (serine/threonine kinase) and activated by phosphoinositide-dependent kinase (PDK) 1 and PDK2 (Chao, 2003; Hemmings and Restuccia, 2012). Activated or phosphorylated Akt activates several signaling cascades essential for cell survival, differentiation and outgrowth through phosphorylation of downstream proteins such as mTOR, GSK3β, FOXO, BAD, and others (Soltoff et al., 1992; Kaplan and Miller, 2000; Huang and Reichardt, 2001; Chao, 2003).

1.5.2 Role of the PI3-K pathway in neuronal survival and regeneration

PI3-K was first recognized as a mediator of neurotrophin-mediated survival of embryonic neurons. Crowder and Freedman (1998) demonstrated that NGF treatment of neurons activates the Akt signaling cascade, and pharmacological inhibitors such as LY294002 and wortmannin block this effect. These results established that the PI3-K pathway is necessary and sufficient for the survival of NGF dependent sympathetic neurons. Similarly, sympathetic neuron survival and axonal outgrowth are activated by PI3-kinase and MEK signaling pathways (Atwal et al., 2000). In keeping with these findings, the responsiveness of growth cone guidance of Xenopus spinal neurons by NGF also depends on the PI3-K signaling (Ming et al., 1999a). Akt is a critical mediator of neurotrophin-dependent survival of neurons during development. In cerebellar neurons, IGF-1 promotes survival with the activation of the serine-threonine kinase Akt and the p70 ribosomal protein S6 kinase (Dudek et al., 1997). The PI3-K pathway has been shown to be crucial for the outgrowth of injured neurons and for successful nerve regeneration to occur
Motor neuron survival and axonal regeneration were accelerated in constitutively active Akt-overexpressing neurons following nerve transection (Namikawa et al., 2000). In vitro, the PI-3K pathway was required for neurite outgrowth of PC12 cells (Kimura et al., 1994) and adult sensory neurons (Kimpinski and Mearow, 2001; Jones et al., 2003b).

One of the major signaling pathways downstream of Akt activation involves the inhibition of GSK3β (Glycogen Synthase Kinase), which is identified as a key mediator of glycogen metabolism. Inactivated by phosphorylation, GSK3β mediates a variety of cellular responses, including survival through growth factors, cell fate specification during embryonic development, cell division, apoptosis and growth cone rearrangement (Cohen and Frame, 2001). Kim et al. (2006) reported that different isoforms of GSK3β have differential roles and act as a convergent point for axon growth pathways. In neurons, growth factors inactivate GSK3β by phosphorylation of Akt which leads to survival and growth (Crowder and Freeman, 1998; Cohen and Frame, 2001). GSK3β causes growth cone retraction and a subsequent decrease in outgrowth of sensory neurons (Eickholt et al., 2002; Jones et al., 2003a). GSK3β inhibition enhanced the neurite outgrowth of young DRG neurons and showed a synergistic effect with neurotrophins such as NGF and IGF-1 (Jones et al., 2003a).

Another important pathway downstream of Akt phosphorylation is the activation of mammalian target of rapamycin (mTOR), directly by phosphorylating and inactivating tuberous sclerosis protein 2 (TSC2) (Vander Haar et al., 2007). mTOR substrates include the eukaryotic translation initiation factor 4E binding protein 1 (4EBP1), and ribosomal protein S6 kinase (S6K), which, in turn, phosphorylates the ribosomal protein (ps6k), and promotes protein synthesis and cellular proliferation (Inoki et al., 2003; Hemmings and Restuccia, 2012). The
forkhead transcription factor, FOXO1 controls a variety of cellular processes including glucose metabolism, DNA repair, and apoptosis, among others, through regulation of the expression of relevant genes (Manning and Cantley, 2007). Activated Akt phosphorylates FOXO1 and thereby prevents its transcriptional activation. Phosphorylated Akt inhibits cell apoptosis by activating IκB kinase, and inhibiting BAD (Bcl-2-associated death promoter) (Datta et al., 1997; Hemmings and Restuccia, 2012).

1.5.3 PTEN

PTEN (phosphatase and tensin homolog deleted on chromosome ten) acts as a negative regulator of the PI3-K pathway by dephosphorylation of PIP3 and thereby decreasing activation of Akt and its downstream pathway (Leslie et al., 2005; Carracedo and Pandolfi, 2008). PTEN was first identified as a tumor suppressor, and is often mutated in several types of malignancy (Li et al., 1997). PTEN homozygous null mice are embryonic lethal and heterozygotes show increased tumor frequency (Li et al., 1997). PTEN is localized in both the cytoplasm and nuclei of neurons (Christie et al., 2010).

1.5.3.1 The role of PTEN in nerve regeneration

There is sufficient evidence and literature supporting the concept that a diminished intrinsic regenerative capacity of mature neurons (in PNS and CNS) is one of the major factors leading to regeneration failure. Removal of extracellular inhibitory cues and addition of growth factors was not sufficient for the desired regeneration outcomes (Dergham et al., 2002; GrandPre et al., 2002; Niederost et al., 2002; Zheng et al., 2003). PTEN is a key intrinsic neuronal
mechanism for regulating growth (Christie et al., 2010; Liu et al., 2011). Deletion of PTEN in corticospinal neurons enhanced regeneration of injured corticospinal axons past a spinal cord lesion (Liu et al., 2010). pS6k levels were reduced after injury in retinal ganglion cells, and silencing of PTEN or TSC1 reactivated the mTOR pathway, leading to extensive axon regeneration (Park et al., 2008). Additionally, PTEN in combination with inflammation and cAMP activated the PI3-K and MAPK signaling pathways and thereby augmented regeneration of retinal ganglion cells (Kurimoto et al., 2010). In the PNS, PTEN inhibition enhanced axonal regeneration and neurite outgrowth of adult sensory neurons (Christie et al., 2010).
Figure 1.9 Schematic of the PI3-K signaling pathway.

Neurotrophins/growth factors bind to tyrosine kinase receptors and activate PI3-K. PI3-K is a lipid kinase and generates PIP3 from PIP2. This leads to the translocation of Akt to the plasma membrane. Akt is then phosphorylated and activated by PDK1 and PDK2. Activation of Akt controls many cellular processes such as cell proliferation and survival. Two of the most studied pathways in nerve regeneration: mTOR and GSK3β are downstream of pAKT. PTEN inhibits the activation of Akt. Reproduced from (Porta and Figlin, 2009).
Diabetes mellitus (DM) is one of the most prevalent diseases in humans and has reached an endemic proportion affecting around 285 million people globally (Danaei et al., 2010). Neuropathy is the most common clinical complication associated with diabetes in 50% of patients (Dyck et al., 1993). Diabetic neuropathies are a group of nerve disorders that render damage to the PNS. Diabetes poses major hurdles to nerve regeneration and regeneration outcomes are severely compromised in diabetes. Diabetes targets multiple steps of the nerve regeneration cascade – the onset of axonal outgrowth (Bisby, 1980), axonal transport (Bisby, 1980), elongation of axons, maturation of axons (Kennedy and Zochodne, 2000) and reinnervation of the target organ (Polydefkis et al., 2004). Many associated mechanisms are postulated for this neuroregenerative failure in diabetics. These include reduced support by partnering SCs, microangiopathy, decreased blood supply to the nerve and the ganglia, a reduced inflammatory response in the distal segment, and decreased neurotrophic support (Ekstrom et al., 1989; Kennedy and Zochodne, 2000, 2002; Yasuda et al., 2003; Kennedy and Zochodne, 2005a). Other mechanisms for neuroregenerative failure include metabolic abnormalities, oxidative stress, decreased PKC subunit activation (Nakamura et al., 1999), abnormal activation of the RAGE (receptor for advanced glycation end products) pathway (Toth et al., 2008) and insulin resistance, a phenomenon recently identified in sensory neurons (Singh et al., 2012a). Injury-related rises in nerve blood flow are attenuated in diabetes (Kennedy and Zochodne, 2002) resulting in a less permissive regenerative microenvironment (Ebenezer et al., 2011). Wallerian degeneration is delayed with the late recruitment of macrophages and activation of SCs in injured diabetic nerves (Conti et al., 1997). Clinically, Sima et al. (1988) reported a decrease in
number of regenerating profiles following sural nerve biopsy in diabetic patients with sensory polyneuropathy.

Electrical stimulation (ES) has the potential to increase the intrinsic growth capacity of sensory and motor neurons (described below in detail), and its role in peripheral nerve injuries under the setting of diabetes is unknown. It is of interest to know whether ES might improve impaired regeneration in diabetic nerves, a more challenging but clinically relevant scenario. I investigated an electrical stimulation paradigm (detailed in Chapter 2) in cultured adult diabetic sensory neurons and in an animal model of chronic diabetic neuropathy after focal nerve injury and measured: regeneration and maturation of axon profiles in the distal nerve stump, reinnervation of the skin target organ, and electrophysiological and behavioural improvement (Chapter 5).

1.7 Electronic Modification of the Regenerative Microenvironment

The impact of local electric fields on wound healing has been recognized since the 18th century. The endogenous electric fields in wounds act as directional cues for cell migration to wound healing. Externally applied electrical fields enhances the migration of epithelial cells and repair epidermal wounds (Zhao, 2009). There are several other mechanisms reported behind ES-induced wound healing but they are beyond the scope of this thesis (Robinson, 1985; Kloth, 2005). More recently, Song et al., using an in vivo rat corneal model, demonstrated that a wound-induced electric field controls nerve sprouting, the direction of nerve growth and the rate of epithelial wound healing (Messerli and Graham; Song et al., 2004). Many studies showed that ES prevents atrophy of denervated muscle and maintains their contractile properties (Mokrusch
et al., 1990; Al-Amood et al., 1991). Despite a long history of clinical and preclinical use of ES in enhancing receptivity of a muscle target to regenerating axon reinnervation following peripheral nerve injuries, laboratory investigations examining direct impact of ES on axon regeneration, were lacking until a decade ago (Pette and Vrbov, 1992; Carraro et al., 2005).

1.7.1 ES in development

Intrinsic electrical activity has been documented to have an important role in the early development of the nervous system and to modulate cell differentiation, proliferation and migration. For example, spontaneous electrical activity is present during early development of the spinal cord, before the formation of synaptic connections and likely contributes to their progress (Spitzer, 2006a). Embryonic (E4) chick spinal cord neurons show rhythmic episodes of spontaneous activity and express cholinergic neurotransmitters, which modify these patterns of activity (O’Donovan, 1999; McLaughlin et al., 2003; Borodinsky et al., 2004). Mouse neural crest cells in culture isolated from the E9.5-E10 stage exhibited spontaneous calcium transients (Carey and Matsumotoa, 1999) that were crucial for the neuronal differentiation (Careya and Matsumoto, 2000). The mechanisms for generating spontaneous calcium transients depended on IP$_3$R-dependent calcium excitability of the endoplasmic reticulum and calcium element-binding protein (CREB) (Carey and Matsumotoa, 1999; Careya and Matsumoto, 2000). Spontaneous activity might play a role in axon guidance in the development of the spinal cord. For example, the netrin-1 response is dependent on intracellular calcium and cAMP levels in growth cones of Xenopus neurons. Blocking of cAMP by a specific competitive cAMP analog, Rp-cAMP, or inhibiting the cAMP-dependent protein kinase A (PKA) resulted in a repulsive growth cone
Intracellular calcium levels were sufficient to abolish both attractive and repulsive behaviours of netrin-1, indicating that calcium is upstream of this attractive and repulsive signaling pathway (Zhang and Poo, 2001).

### 1.7.2 Role of electrical stimulation in peripheral nerve regeneration

ES has been used to stimulate denervated muscle to reduce muscle atrophy after peripheral nerve injury. The idea for applying ES directly on the peripheral nerves may have originated from a study conducted by Hoffman in 1952. The author reported that ES of the spinal cord or whole nerve directly resulted in accelerated sprouting of intact axons and reinnervation of the partially denervated muscles (Hoffman, 1952). Several studies were published later between 1970-1990 exploring the impact of ES on injured nerves and muscle and reported benefits in peripheral nerve injuries in rats and dogs (Maehlen and Nja, 1982; Maier et al., 1982; Nix and Hopf, 1983; Román et al., 1987; Pomeranz and Campbell, 1993; Rozman et al., 2000). Direct stimulation of injured peripheral nerves was first investigated by Nix and Hopf who demonstrated benefits in muscle electrophysiology and also on twitch force and tetanic tension in the soleus muscle following crush of its nerve supply (Nix and Hopf, 1983). During the last decade (2000-2010), Gordon, Brushart and colleagues, and other labs reported and confirmed the role of a remarkably specific paradigm of ES (low frequency, 20 Hz at supramaximal [motor] voltage, 3 V for 1 h immediately after injury) in augmenting peripheral nerve regeneration (Al-Majed et al., 2000a; Brusht et al., 2002; Ahlborn et al., 2007; Gordon et al., 2008). Different voltage and frequency (lower and higher) paradigms with longer durations were evaluated but a paradigm of 20 Hz for 1-hour duration immediately after nerve injury was found to be superior.
(Al-Majed et al., 2000a; Brushart et al., 2002; Ahlborn et al., 2007; Gordon et al., 2008). Now, there is mounting evidence at the whole nerve trunk tissue level, that extracellular nerve trunk stimulation dramatically and specifically benefits motor and sensory axon regeneration. The axon outgrowth across a surgically repaired site is one of the limiting factors for successful reinnervation because this delay decreases the intrinsic capacity of injured neurons to regenerate their axons and simultaneously reduces the competence of SCs to guide axons to the denervated targets. Al-Majed et al. (2000a) measured this time delay in crossing the repair site and reported a dramatic improvement with the application of ES: axons were now able to cross the repaired surgical injury site in just 3 weeks compared to 8-10 weeks in sham stimulation. ES significantly enhanced the number of backlabeled motor neurons regenerating at time points from week 2 to 8 (Gordon et al., 2007). In a separate study, a duplicate stimulation paradigm (20 Hz, 1 h) also significantly increased the number of sensory neurons that regenerated axons beyond the repair site 4 days after injury and stimulation of the femoral nerve. The benefit of stimulation was correlated with a significant increase in the expression of GAP-43 mRNA in the regenerating neurons 2 days post-repair (Geremia et al., 2007). A low 20 Hz frequency is close to the physiological frequency of hindlimb motor neuron discharge (Hoffer et al., 1987). In motor neurons, ES applied at 20 Hz for 1 h was equally beneficial as a continuous stimulation for a 2 week period (Al-Majed et al., 2000b) compared to sensory neurons where ES for longer than 1 h was less effective (Geremia et al., 2007). An identical stimulation paradigm, involving 20 Hz stimulation for 1 h, significantly increased axon outgrowth of central primary afferent terminals into the dorsal columns of the spinal cord (Udina et al., 2008).

The ultimate goal of successful axonal regeneration is to translate new approaches into functional benefits. Studies examining long-term electrophysiological recovery and functional
benefits after ES have been lacking. Nix and Hopf reported improvement in soleus muscle twitch force in rabbit with ES (Nix and Hopf, 1983). Pockett and Garvin (1985) showed an earlier arrival of the toe-spreading reflex with ES proximal to the crushed sciatic nerve (Pockett and Gavin, 1985). In a recent study, Ahlborn et al. (2007), using the femoral nerve injury model suggested that ES is associated with advanced recovery of knee extension in mice (Ahlborn et al., 2007). Carpal tunnel syndrome is a common clinical problem and a cause of peripheral nerve dysfunction; chronic median nerve compression during this syndrome induces dramatic changes in SCs: apoptosis, proliferation, higher expression of c-Jun, Krox-20, iNOS and others (Gupta et al., 2003; Gupta and Steward, 2003; Pham et al., 2009). Surgical decompression is used for treatment of chronic compressions and is not sufficient to restore function. Chan and Gordon (2010b) completed the first randomized clinical trial of ES in these carpal tunnel patients, and the results appear striking and promising. In essence, ES of median nerves in patients immediately after carpal tunnel decompression surgery increased motor axonal reinnervation, as measured by MUNE (motor unit number estimation), and recovery of conduction velocity in the compressed nerve segment. This study also indicated a decrease in the latent period for axons to cross the surgical repair site with ES in humans.

1.7.3 ES and CNS regeneration

There are several reports of ES on CNS regeneration, mainly examining spinal cord and optic nerve injuries. Borgens et al. (1981) reported that steady electric current applied across the completely severed spinal cord of the larval lamprey enhanced regeneration of the reticulospinal neurons. Growth cones were visible mainly in the stimulated axons. Following this, several other
researchers analyzed the impact of functional ES on spinal cord regeneration in vertebrates and mammals (Herman et al., 2002; Ragnarsson, 2007; Hamid and Hayek, 2008). Intermittent pulse ES (100 mV/mm to 500 mV/mm) promotes neurite regeneration in goldfish retinal explants (Yi-Ting et al., 2012). Neurite outgrowth of PC12 cells was enhanced by using an electrically conducting polymer and ES (Schmidt et al., 1997). Yuka et al. (2008) showed that early regenerative events were impacted by a short ES at 20 Hz immediately after optic nerve transection and this was associated with an increase in the number of surviving retinal ganglion cells (RGCs). Shorter durations of stimulation or stimulation before transection injury were not effective (Yuka et al., 2008). The mean number of regenerating axons significantly increased at 250 µm distal from the lesion site and an increase in IGF-1 immunoreactivity was also observed in retinas treated daily with transcorneal ES (Tagami et al., 2009). Overall there is substantial evidence demonstrating the beneficial effects of ES on CNS regeneration.

1.7.4 ES and axonal specificity

Misdirection of regenerating axons after peripheral nerve injuries complicates the functional outcome. Specificity of regeneration or preferential motor reinnervation (PMR) is the term coined by Thomas Brushart describing that after injury the regenerating motor axons of a mixed nerve preferentially reinnervate distal muscle branches (Brushart, 1988). It is of enormous importance in recovery from motor axon injuries that they must regrow to the correct targets for proper reconnection (Brushart, 2011). ES strikingly enhanced PMR, and the number of motor neurons projecting to the muscle were higher (Al-Majed et al., 2000b). L2/HNK-1 is a carbohydrate present only in muscle and not in cutaneous branches, and is selectively
upregulated in regenerating motor axons indicating its specific role in PMR (Eberhardt et al., 2006). Eberhardt et al. (2006) found that HNK-1 expression was essential for motor behavioural recovery after peripheral nerve injury and was dependent on BDNF/TrkB signaling. ES of the injured femoral nerve caused a motor nerve-specific improvement in HNK-1 expression in wild type mice that was not observed in heterozygous BDNF or TrkB-deficient mice (Eberhardt et al., 2006). After nerve injury, motor neurons differentially express PSA (Polysialic acid), a finding that correlates with PMR. The ES paradigm upregulated PSA levels and the specificity of regenerating axons (Franz et al., 2008). Franz et al. (2013) reported that transvertebral stimulation applied at the cervicothoracic spinal cord following femoral nerve injury improved the specificity of motor reinnervation and upregulated PSA-NCAM in motor neurons. ES also restored the specificity of sensory axon regeneration (Brushart et al., 2005).

1.7.5 ES and associated mechanisms

The precise mechanisms of ES are not fully understood; evidence thus far suggests that it signals the parent cell body to reprogram the RAG output of neurons. This may involve the rapid synthesis of neurotrophic factors and their receptors, changing neurons into a more permissive growth mode (Gordon et al., 2003). ES induces upregulation of BDNF, NT-3/4, Trk receptors and other neurotrophins (Al-Majed et al., 2000a; Al-Majed et al., 2004; English et al., 2007). Both ES and BDNF treatments were effective and synergistic in enhancing sciatic nerve regeneration following crush injury in rats (Alrashdan et al., 2011). The cell body autonomous effect was evident from an elegant study conducted by Al-Majed et al. (2010) (2000b) in motor neurons; where tetrodotoxin (TTX) was applied to the proximal end of the injured femoral nerve
to block the backfiring of action potentials, and was thereafter followed by ES. The blockade of action potentials by TTX abolished the positive effects measured with ES: the number of regenerating axons and the specificity of motor axonal regeneration. Similarly, regeneration and the specificity of sensory axons were abolished with TTX application (Geremia et al., 2007). These studies are indicative of a cell body response associated with ES (Al-Majed et al., 2000b; Gordon et al., 2003; Geremia et al., 2007). A conditioning lesion involves a prior injury to a peripheral nerve that conditions the neurons to respond more robustly to a second experimental injury in either the CNS or PNS (McQuarrie and Grafstein, 1973). The effect of a conditioned lesion can be mimicked by cAMP analogues (Neumann et al., 2002). Udina et al., (2008) compared the 20 Hz ES paradigm with a conditioned lesion in increasing DRG axon outgrowth into the CNS after a dorsal column lesion and reported the impact of ES was less robust. The outcomes of peripheral stimulation on central outgrowth is attributed to the increase in intracellular cAMP levels in DRG neurons similar to that involved in a conditioning lesion (Udina et al., 2008). Overall, there is increasing evidence that upregulation of neurotrophins; particularly BDNF is one of the major postulated mechanisms behind enhanced regeneration of motor and sensory neurons. In this thesis, I also measured the expression of specific neurotrophins (BDNF) and RAGs (actin, β-tubulin, GAP-43) both in vitro in cultured adult sensory neurons and in vivo in regenerating DRG sensory neurons following injury to the sciatic nerve. I further analyzed the downstream neurotrophin-signaling pathways, and in particular focused on the survival and regenerative, PI3-K pathway. These results are depicted in detail in Chapter 4.

Intracellular calcium in the cell body and growth cone are related to enhanced neurite
outgrowth and growth cone extension. Calcium increases neurite outgrowth and maintains a balance between assembly and disassembly of the cytoskeleton by regulating actin filament stability (Lankford and Letourneau, 1989). In vitro, depolarization of sensory neurons leads to a rise in intracellular calcium and upregulation of gene expression and neurite outgrowth (Kocsis et al., 1994). Kater and Miller (1991) proposed an optimal level of calcium, adequate for neurite outgrowth. This optimum level of calcium varies widely between the types of neurons (Paul J. Torreano, 1997). Similarly in adult sensory neurons the critical level measured was 35 nM, above and below which neurite outgrowth was hampered (Al-Mohanna et al., 1992). Direct ES of cultured Müller cells increased the transcription of BDNF through the L-type voltage-dependent calcium channel (Sato et al., 2008). Transcorneal electrical stimulation increased the survival of RGCs and enhanced axon regeneration through upregulation of growth factors, IGF-1 and BDNF (Morimoto et al., 2005; Miyake et al., 2007; Sato et al., 2008). IGF-1 was increased immediately after 10 mA ES with a rise in calcium. IGF-1 transcription and calcium influx were suppressed by application of nifedipine, an L-type calcium channel blocker (Tatsuhiko et al., 2008). I performed a series of experiments including two-photon calcium imaging and pharmacological inhibitors to dissect the role of intracellular calcium on neurite outgrowth associated with ES; the results are discussed in detail in Chapter 4.

The literature largely indicates that ES induced benefits are localized at the cell body level but it is possible that nonneuronal cells such as SCs or fibroblasts, present locally at the injury site or in the proximal stump of injured nerve, are involved. For example, it has been reported that SCs in response to ES secrete higher NGF in a calcium dependent fashion (Huang et al., 2009). Koppes et al. (2011) tested DC electric stimulation in combination with SCs and concluded that the combination treatment may provide synergistic guidance cues for improved
axonal growth relevant to nerve injuries in the peripheral nervous system. I explored the involvement and association of SCs present at the injury site and perineuronal satellite cells in the regenerative response to ES. The results are detailed in Chapter 4.

During my thesis research period, I worked both in vitro and in vivo to further investigate the role of extracellular ES in peripheral nerve regeneration. In animal experiments, I used an ES paradigm developed by Gordon and colleagues (Al-Majed et al., 2000b) to study the cellular and molecular mechanisms involved in promoting neuronal regeneration, and characterized the regenerative potential of this therapeutic modality in a severe transection injury where early outgrowth can be mapped, and in an experimental model of diabetes, which has substantial and persistent deficit in nerve regeneration, followed by target reinnervation and functional recovery. In vitro, at the single cell level, I examined how ES alters the initiation of neurite outgrowth, the extension of neurites during regeneration, and their response to various pharmacological blockers. The potential mechanisms I explored in this thesis include: the impact on growth factor signaling with a focus on the PI3-K pathway, the involvement of intracellular calcium and the contribution of partnering SCs and perineuronal satellite cells. These are the crucial focal points of my thesis.

1.8 Hypothesis and Objectives

Several facets of the ES-related regenerative response have not been studied to date. For example, it is uncertain whether nerves with transection injuries, with a gap between the distal and proximal stump, experience a benefit from the approach. While clinically common, this severe injury requires an initial outgrowth of axons and their partnering SCs into an outgrowth
zone beyond the proximal stump but prior to entry into the distal stump. Whether ES plays a role in this critical early step in regenerative outgrowth is uncertain. Similarly, there is only limited information as to the long-term electrophysiological outcome after a sciatic nerve transection and whether target tissues undergo functional reinnervation. What is the impact of ES in a clinically challenging model such as diabetes where nerve regeneration is inadequate and severely compromised? The mechanisms of the benefits to nerve regeneration are also not completely known.

1.8.1 Overall hypothesis

*A specific extracellular electrical stimulation paradigm will promote axon regeneration following peripheral nerve injury.*

1.8.2 Objectives

**Objective #1:** To examine the impact of extracellular ES on early axonal outgrowth, maturation of axons, target reinnervation and functional recovery after sciatic nerve injury. The measurables would include: axon and SC outgrowth on day 7 and fluorescent live imaging of the regenerated bridge (week 1-4) in thy-1 YFP mice, the repopulation of distal nerves with myelinated axons, and backlabeled motor neurons in the spinal cord. Reinnervation of cutaneous and muscle targets will be measured by the presence of axons in the distal toepads, and electrophysiological recordings. Finally, the long-term behavioural impact of successful neural regeneration will be observed by mechanical and thermal sensitivity. The impact of sequential stimulation will also
be quantified to verify whether it could further enhance the impact of single ES on nerve regeneration.

**Objective #2:** To explore the potential cellular and molecular mechanisms of ES enhanced peripheral nerve regeneration. Experiments will take advantage of a microelectrode array (MEA) to deliver localized ES and measure changes in intracellular calcium with analysis of neurite outgrowth in the presence of a specific inhibitor of the PI3-K pathway. mRNA levels of growth factors, regeneration-associated genes and the nonneuronal response will be observed on *in vitro* cultured sensory neurons and *in vivo* DRGs following injury and ES.

**Objective #3:** To establish whether an ES paradigm increases peripheral nerve regeneration in an animal model of diabetic polyneuropathy. Outgrowth of cultured adult diabetic sensory neurons *in vitro*, repopulation of distal tibial nerves, reinnervation of the skin epidermis, functional electrophysiological measurements, and behavioural studies will be carried out.
Chapter Two: Experimental Procedures
2.1 Animals

Outbred adult 18-20 g mice; CD-1 (Charles River, Canada), Thy-1 YFP (B6.Cg-Tg,16Jrs/J) (Jackson Labs), db/db (BKS.Cg-Dock7m +/+ Leprdb/J) (Jackson Labs) and adult 120-150 g SD rats (Charles River, Canada) were used in the study. Animals were raised in sawdust covered flooring in a normal wake-sleep environment. The procedures were reviewed and approved by the University of Calgary Animal Care Committee in conjunction with guidelines from the Canadian Council of Animal Care (CCAC).

2.2 Regeneration Model and Electrical Stimulation (ES)

To address aspects of in vivo regeneration, we used a model of sciatic nerve transection and surgical repair spanned by a silicone chamber. Under isoflurane anaesthesia, and using aseptic techniques and operating microscopic magnification, the left sciatic nerve of mice was exposed and transected just distal to the sciatic notch with microscissors. Proximal and distal stumps were secured using 10–0 sutures into the ends of a silicon nerve chamber (ID/OD, 0.64/1.19 mm) leaving a gap of approximately 2-3 mm between stumps. The proximal sciatic nerves were immediately stimulated after repair by gently lifting the proximal stumps on bipolar electrode hooks, with a paradigm identical to that used in previous work: 20 Hz frequency of electrical pulses of 0.1 ms duration at the supramaximal voltage (3 V) for 1 h (Al-Majed et al., 2000b) (Figure 2.1). Control sham stimulation animals underwent electrode placement without ES for 1 h. During the 1 h stimulation, the injury site was covered with saline moistened gauze. The electrodes were subsequently removed, the wound was closed, and the skin was sutured. The
same stimulation paradigm was tested before injury to record the compound muscle action potentials (CMAPs) subcutaneously, confirming supramaximal stimulation of motor axons, from the motor point of tibial interosseous foot muscles using stimulation of the sciatic nerve at the notch.

In a separate set of experiments, for sequential stimulation protocol, normal CD-1 mice were divided into three groups: control (sham stimulation), single stimulation and double/sequential stimulation (n=5-7/group/time point). On the day of surgery, similar to single stimulation (mentioned above), extracellular ES (3 V, 20 Hz, 0.1 ms, 1 h) was applied immediately following nerve transection and repair, to both single and sequential stimulation group animals. Sham stimulation underwent similar transection injury and repair but received no stimulation. On day 5, sequential stimulation animals received another epoch of the same stimulation paradigm invasively at the sciatic notch, anatomically identified. On day 10, a group of mice were harvested for immunohistochemistry to analyze early axon outgrowth as described below.
Figure 2.1 Illustration of the method of stimulation.

Reproduced from (Al-Majed et al., 2000b).
2.3 Immunohistochemistry

Mice were perfused transcardially with phosphate buffered saline (PBS) followed with 4% paraformaldehyde. DRGs (L4-5) and sciatic nerve bridges within the regenerative conduit were removed through the proximal stump, bridge and distal stump as one contiguous sample. Tissue samples were fixed in modified Zamboni’s fixative (2% paraformaldehyde, 0.5% picric acid and 0.1% PBS) overnight at 4°C. Tissues were then washed in PBS five times, cryoprotected in 20% sucrose/PBS and left at 4°C overnight. After embedding in optimum cutting temperature (OCT) compound (Miles, Elkart, IN), 16 µm thick longitudinal sections of sciatic nerve and 10 µm transverse sections of DRGs were placed onto poly-L-lysine coated glass slides. The primary antibodies used were monoclonal anti-NF200 (heavy subunit of neurofilament, axons, 1:800, Sigma, St. Louis, MO), rabbit polyclonal anti-Glial Fibrillary Acidic Protein (GFAP) (SCs, 1:250; Dako Diagnostics, Mississauga, Canada), goat polyclonal anti-CGRP (1:200, Abcam, Toronto) and rabbit polyclonal PTEN (1:50; Santa Cruz). Primary antibodies were incubated at 4°C for 48 h. Slides were then washed with PBS and incubated with secondary antibodies for 1 h at RT. Secondary antibodies were sheep anti-mouse IgG Cy3 conjugate (1: 100, Sigma) or Alexa Fluor 488 goat anti-rabbit IgG (H + L) conjugate (1: 400, Cedarlane Laboratories, Hornby, ON, Canada), and sheep anti-goat IgG Cy3 (1:100, Jackson immunoresearch, USA). After further PBS washing, cover slips were mounted onto the slides with bicarbonate buffered glycerol (pH 8.6) and the slides were viewed with a fluorescent microscope (Zeiss, Axioskop, Zeiss Canada, Toronto, Canada). Negative controls included omission of primary antibodies or secondary antibodies on parallel sections (not shown). The primary antibodies are listed in detail in Table 2.1.
2.4 Analysis of Early Axon and Schwann Cell Outgrowth

For regeneration bridges examined following sciatic nerve transection and surgical repair through a silicone chamber with and without stimulation, 16 µm thick longitudinal sections of the early regenerative bridge were made and underwent double labeling with antibodies against NF-200 to label axons and GFAP to label activated SCs. As in previous work, profiles from the beginning of the proximal portion of the regenerative bridge (first separate high power field: 40X objective, each field was 270 µm in diameter) were counted in a line perpendicular to the direction of the bridge in fields starting left to right to encompass all portions of the nerve across its diameter, then proceeded distally until reaching the last field expressing NF-200 or GFAP (final distance varied depending on the intervention) (Chen et al., 2005). For each intervention, means and standard errors (SE) were then calculated for each group of mice.
Figure 2.2 Analysis of axon and SC outgrowth during early regenerative events.

Sciatic nerves from proximal stump to distal end including regenerative bridges were harvested as one piece for longitudinal sectioning. Regenerating axons (labeled with NF-200) and proliferating SCs (labeled with GFAP) in each field were counted, beginning from the proximal injury site through fields of the corresponding regenerating bridge. Reproduced with permission from (Kemp et al., 2009).
2.5 Analysis of Skin Epidermal Axons

Footpad skin samples were harvested 21 days after sciatic nerve crush with and without stimulation. Skin punch was used to harvest a few mm sample of plantar footpad. Samples were then fixed in 2% PLP [paraformaldehyde (2%), l-lysine and sodium periodate) for 18 h at 4°C and cryoprotected overnight in 20% glycerol/0.1 M Sorrenson phosphate buffer at 4°C. The immunohistochemical approach was identical to published techniques from our laboratory (Kennedy and Zochodne, 2005a; Kan et al., 2012). Skin sections were of 25 µm thickness and washed in PBS, 1% Triton X100, blocked in 10% goat serum (1% BSA, 0.05% NaN3, 0.3% Triton X100, 0.05% Tween20/1X PBS) for 1 h at room temperature (RT). Primary antibody PGP9.5 (rabbit polyclonal; 1:1000, Encore biotechnology, USA) was applied overnight at 4°C followed by goat anti-rabbit Cy3; 1:100 (Jackson immunoresearch, USA) secondary for 1 h at RT. Images were captured using an Olympus laser scanning confocal microscope equipped with epifluorescence (100X magnification; resolution at 512 × 512 and scanning step size 1 µm) and a Zeiss Axioskope fluorescent microscope. Epidermal fibers labeled with PGP9.5 were counted in five adjacent fields of six sections for a total 30 fields per mouse at each time point. Both vertical (trajectory approximately 90° to the surface of the skin) and total axon profiles were analyzed. The total epidermal area analyzed was traced and the area measured to calculate axon densities. All analyses were conducted with the examiner blinded to the identity of the samples being studied.
2.6 Analysis of Regenerating Myelinated Sensory and Motor Axons

Samples of sciatic nerves constituting proximal and distal stumps and the regenerative bridge connecting them were harvested at 21 days after transection and chamber placement with and without stimulation. The material was fixed in glutaraldehyde (2.5%) buffered in cacodylate (0.025 M) overnight, washed and stored in cacodylate buffer (0.15 M), then fixed in osmium tetroxide (2%), washed in graded alcohols then embedded in epon (Ohnishi et al., 1976). Transverse sections of 1 µm thickness were made through the approximate centre of the regenerative bridge and stained with toluidine blue. Sections of the regenerating bridge, approximately at the centre, were photographed under oil immersion microscopy (100X). At 21 days regenerating bridges normally contain a central ‘fascicle-like’ structure that contains most of the regenerating myelinated axons. This portion of the bridge was identified and photographs of fields were taken starting in the upper left hand corner, then to the right, and down in six non-overlapping fields. In each field, the numbers and caliber of unequivocal myelinated axons were then measured using an image analysis program offline (Scion image, Frederick, MD, USA). Final measurements included axon density, myelin thickness, number of myelinated axons and total area of the regenerative bridge that included myelinated axons. For each intervention, means and SE were calculated for each group.

2.7 Retrograde Labeling and Counting of Motor Neurons

On day 21, retrograde tracer was applied to the sciatic nerve, 5 mm distal to the regenerated bridge to trace the number of motoneurons regenerating after control sham stimulation or ES (n=7/group). The tracer used was fast blue (FB, 2%, Polysciences, Inc.)
Warrington, USA) and was selected because FB is easily endocytosed and effectively retrogradely transported and resides in the neuronal cell bodies for more than 8 weeks (Choi et al., 2002). After the sciatic nerve was exposed, isolated and transected 5 mm distal to the regenerated bridge, a small piece of Gelfoam® (Upjohn, Don Mills, Ontario, Canada) soaked in 2% FB was applied to the cut nerve end for 1 h. Following this, the Gelfoam® was removed and the residual FB was thoroughly rinsed out with sterile 0.9% saline. Animals were kept for a week after tracer application to allow the retrograde tracer to travel back to neuronal cell bodies. Harvested lumber spinal cords were cut (coronal sections) at 40 µm thickness on a freezing microtome. Sections were dried, washed in PBS and coverslipped. Each spinal cord section was visualized with the 40X objective under UV fluorescence at barrier filter of 580 nm and counted backlabeled motor neurons. The counting of any split neuron twice was corrected using a method originally described by Abercrombie (Abercrombie, 1946) to approximate total motor neurons.

Retrograde labeling was also conducted in a separate set of experiments, involving the sequential stimulation protocol. In another set of CD1 mice from all three: control (sham stimulation), single stimulation and sequential stimulation, a 3-5 mm piece of nerve distal to the regenerated bridge and close to the sciatic branching, was cut and injected a retrograde dye, fast blue. On day 28, a week after dye injection when dye travelled back to the motor neuron cell bodies, ipsilateral lumbar spinal cords were harvested for counting the number of backlabeled motor neurons (described above).
2.8 Functional Motor Recovery:

2.8.1 Electrophysiology

Multifiber motor conduction recordings were carried out in left sciatic–tibial fibers in mice anesthetized with isoflurane (Kennedy and Zochodne, 2000). Recordings were measured at near-nerve subcutaneous temperature of 37°C, maintained by a thermosensitive heat lamp. CMAPs were recorded subcutaneously from the motor point of tibial interosseous foot muscles in response to supramaximal stimulation of the nerve at the sciatic notch and the knee. Motor conduction velocity was calculated between the notch and knee stimulation sites. The experimenter was not blinded for electrophysiological recordings. At the end of study, tissues were harvested and muscle weights were recorded. Sensory nerve action potentials (SNAPs) and sensor conduction velocities were recorded antidromically by stimulating sensory nerves distally and recordings were made proximally at fixed distances, the sciatic notch and the knee.

2.8.2 Functional recovery of sensation

Mice underwent mechanical (von Frey filaments) and thermal testing. There were 5 min intervals provided between a total of three trials performed during the same day. To test mechanical sensitivity of the foot, withdrawal in response to a stimulus consisted of increasing amount of force using calibrated (4-26 g) von Frey monofilaments (Stoelting, Wood Dale, IL) applied to the plantar surface of the paw to a mouse habituated within a plexiglass cage that had holes in the flooring to allow application. Each paw was probed three times on the plantar surface with enough force to cause slight flexion of the monofilament. For testing the recovery of
thermal sensation, we used the Hargreaves apparatus (Hargreaves et al., 1988). In brief, a radiant heat source was applied individually to the middle of either hindpaw and the latency (sec) to withdrawal was measured. Three separate trials were performed for the withdrawal response. Mechanical and thermal testing was performed on identical days with an interval of at least 1 h between the two tests.

2.9 Long Term Nerve Regeneration and Target Reinnervation

Sciatic nerves of B6.Cg-Tg(Thy1-YFP)16Jrs/J mice were imaged using a fluorescent microscope (Stereo discovery V8, Zeiss Canada, Toronto, Canada) at every week for four weeks after transection and placement of a regeneration conduit connecting the distal and proximal stump. To quantify the size of the bridge, the length of its fluorescence at 83X was calculated. The regenerated bridges were examined for the presence of fluorescence from the tip of the proximal stump into the bridge to its farthest extent, using Adobe Photoshop CS2. For long-term reinnervation studies, mice were anaesthetized using pentobarbital (65 mg/kg,i.p.) and foot innervation was visualized under fluoroscopy as described above. Measurements included the presence of axons in the toepad, and in the digital and tibial nerve.

2.10 The Microelectrode Array (MEA)

A microelectrode array (MEA) and electrical connection system were all fabricated at the Advanced Micro/nanosystems Integration Facility (AMIF), University of Calgary. The electrical connection system to the MEA was an adaptation of the printed circuit board and zero insertion force connector (Dalton and Kaler, 2007). The connection system allowed individual or multiple
electrodes to be accessed. The fabricated MEA consisted of an 8×8 array of 30 µm diameter pads on the ends of 15 µm wide traces on a 5x5 cm glass slide. The array corner electrodes were not present, leaving 60 electrodes in total in the array. The electrode metal layers were deposited by sputtering and consisted of a 10 nm thick chrome adhesion layer, with 200 nm thick gold on top. The metal layers were then patterned by standard photolithography or femto second laser machining to form the array (Hayden and Dalton, 2010). The 30 µm microelectrode pads of the array were each spaced 100 µm apart from each other. The media supporting the cells was contained over the array by a 4 mm high, 24 mm diameter custom cut glass well, which was held in place by polydimethylsiloxane (PDMS).

2.11 In vitro Studies of Adult Sensory Neurons

The procedures for adult sensory neuronal cell culture were modified from Lindsay and Andersen et al (Lindsay, 1988; Andersen et al., 2000). Briefly, MEAs were coated with Poly-L-lysine (Sigma, St. Louis, MO, USA) overnight and next morning washed 3 times with the double-distilled (DD) water and coated with 0.01% Laminin (Sigma, St. Louis, MO, USA) for 3 h at 37°C. L4 and L5 DRGs were dissected from 6 week old rats, washed with Hank’s balanced salt solution (HBSS,) and dissociated by incubating in L15 medium containing 0.1% collagenase (90 min, 37°C) followed by trituration and then passage through a 70 µm mesh. To partially remove SCs, the cell suspension was loaded onto 15% BSA (Sigma, St. Louis, MO, USA) in L15 and spun at 900 rpm for 10 min. Three layers from centrifugation were formed, where the middle layer contained SCs and debris and was removed carefully. After washing, cells were plated in low density diffusely onto the MEA precoated with 0.01% poly-l-lysine and laminin in
DMEM/F12 medium containing N2 nutrient supplement (1:100). Twenty four hours later, a select group of the neurons were stimulated at 20 Hz, 200 mV continuously for 1 h in MEA using a Grass stimulator (West Warwick, RI, U.S.A.). One half of the MEA was used for the stimulation and other opposite half of the same MEA dish was used as a sham control, remote from the active electrode. One half of the nutrient media was changed daily.

In a separate set of experiments, the adult sensory neurons isolated from CD-1 mice were grown in MEA under five sets of conditions: (i) control (sham stimulation); (ii) control plus a PI3-K inhibitor (LY294002, 30 µM, Calbiochem, EMD chemicals, US); (iii ) ES (iv) ES plus LY294002 (30 µM); or (v) ES plus nickel (Ni²⁺, 100 µM); each for 1 day starting from the time of plating.

Sensory neurons grown on the MEA were viewed by phase contrast using an inverted microscope (Zeiss Axiovert 40 CFL, Zeiss Canada, Toronto, ON) at 24 h after electrical stimulation (48 h after harvesting). Photos were taken by DIC (40X, 6.5 cm = 100 mm) and included for analysis if not overlapped with other neurons or glial cells. The total neurite outgrowth, number of primary neurites (defined as processes extending from the soma), length of the longest neurite and number of branches of a primary neurite were analyzed and quantified by MetaXpress software (Molecular Devices).

### 2.12 Calcium (Ca²⁺) Imaging

To examine whether MEA stimulations induce changes in intracellular calcium concentrations ([Ca²⁺]ᵢ) in DRG neurons, we performed two methods: Fura-2 calcium ratiometric
experiments using a conventional wide field fluorescence microscope and time lapse imaging using high resolution two photon microscope with Xrhod1 AM dye.

**2.12.1 Conventional wide field fluorescence microscope**

The experiments were conducted on isolated DRG sensory neurons cultured on MEA following the same procedure as detailed above. The next day (18 h after plating), culture media was replaced with HBSS (Hank’s balanced salt solution) and neurons were loaded with the membrane permeable and ratiometric Ca\(^{2+}\) sensor Fura-2 AM (Molecular Probes, Carlsbad, CA) at 5 µM for 30 mins at 37°C. Next, neurons were rinsed with HBSS three times and imaging experiments were performed at RT in HBSS media. DRG neurons were then exposed to excitation wavelengths 340 and 380 nm using a LAMBDA DG4 high-speed wavelength switcher (Sutter Instrument, Novato, CA). Specifically, plated neurons were selected based on their locations on the desired electrodes and were stimulated with 200 mV-5V, 20 Hz using a Grass stimulator. The emitted fluorescence signal was collected at 510 nm by a Retiga Exi camera. Images were acquired with the Northern Eclipse software ionwave program (Empix Imaging, Canada) and the ratio of Fura-2 fluorescence intensity at 340 nm and 380 nm (reflection of intracellular Ca\(^{2+}\) levels) were demonstrated.

**2.12.2 Two photon calcium imaging**

Isolated DRG sensory neurons were cultured on MEA (as described above) and were loaded with the calcium indicator X-Rhod-1-AM (1 µM; 580/602 nm excitation/emission maxima). Neurons were then kept at 37°C for 30 min followed by three washes with HBSS. Imaging
experiments were performed at RT in culture media. MEAs containing DRG neurons were placed on the stage of a Nikon Eclipse E800 upright microscope and observed with a water-immersion dipping objective (60X 1.0NA, Fluor, Nikon, Japan). Selected neurons based on their locations on the desired electrodes were stimulated with 200 mV-5 V, 20 Hz. MEAs were connected with a Grass stimulator and desired voltages were applied simultaneously with the measurement of fluorescence signal from the particular selected neuron. DRG neurons were excited with approximately 200 fs pulses, 80 MHz repetition rate at a wavelength of 925 nm generated by a Ti:sapphire laser (Tsunami; Spectra-Physics Lasers) pumped with a 10 W solid state green Nd:YVO₄ laser (Millenia Xs; Spectra Physics). Two-photon excited images of the fluorescence signal were collected using a custom-modified Nikon D-Eclipse C1 confocal microscope (Nikon D-Eclipse C, Nikon Instruments Inc, Melville, USA). Emitted fluorescence was split into two channels through a dichroic reflector centered at 585 nm and filtered through 525 ± 25 nm bandpass and 590 nm long pass filters. The emission fluorescence of Xrhod1 was detected with 590 nm channel with a photomultiplier tubes (Hamamatsu R5929). Laser power was approximately 3 mW under the objective. Three-dimensional image data were imported into ImageTrak (written by Dr. Peter Stys; http://www.ucalgary.ca/stys/imagetrak) for visualization and analysis.

2.13 Immunocytochemistry

For the immunocytochemistry experiments, cells were fixed in 2% paraformaldehyde (PFA) for 2h at 4°C followed by washing in PBS for 5 min, treated with 10% goat serum (0.3% Triton X100/1X PBS) for 30 min at RT, then the primary antibody, anti-NF200 (1:800, Sigma, St. Louis, MO) was applied for 1 h at RT. The samples were then washed in PBS three times for
5 min each and secondary antibody applied: sheep anti-mouse (1:200; Sigma) at RT for 1 h. The blocking/antibody solutions contained PBS, 0.3% Triton X100 and goat serum 10%. The samples were then washed three times further in PBS (5 min each), mounted using DAPI vectashield (Vector laboratories, Burlingame, CA) and imaged.

2.14 Western Immunoblot

DRGs (L4-L5) and sciatic nerve samples and in vitro adult sensory neurons were harvested in the RIPA lysis buffer (Fisher Scientific, Ontario, Canada) containing protease and phosphatase inhibitors (Roche Diagnostics, Laval, Canada). The protein content of both fractions was measured using BCA protein kit (Thermo Scientific, Rockford, IL, USA). Thirty micrograms of total protein (with 5% loading SDS dye) were electrophoresed on 10% SDS-PAGE and then transferred on PVDF membrane in Tris-glycine-methanol buffer overnight at 4°C. After blocking (10% nonfat dry milk and 0.05% Tween-20 in Tris-buffered saline), membranes were incubated overnight with a polyclonal antibody to Akt, p-Akt, GSK-3β, pGSK-3β, PTEN (Cell Signaling Technology, Beverly MA). Tubulin or actin detected using mouse monoclonal antibodies (Santa Cruz) were used as a loading control in 2% BSA in TBS. The next day, after three rinses in TBST [Tris buffer saline with Tween 20 (0.1%)] for 10 min each, horseradish peroxidase-labeled (HRP) secondary antibodies, anti-rabbit IgG HRP and anti-mouse IgG HRP (Santa Cruz Biotechnology), were incubated with the immunoblot at 1:5000 dilution for 1 h at RT. Signal detection was performed by exposing the blot to enhanced chemiluminescent reagents (ECL, Amersham Oakville, ON, Canada) for 2 min. The blots were subsequently exposed on Hyperfilm (GE Healthcare, Buckinghamshire, UK) to capture the images of the bands. The film images were digitized and the difference between the treatments was measured.
using pixel intensity and the size of the band using Adobe Photoshop (Adobe, San Jose, CA). The values were represented as the percentage difference from the control, with control values assumed as 1.

2.15 Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted using Trizol reagent according to the manufacturer’s protocol (Invitrogen, Inc., Burlington, Ontario, Canada). Approximately 1 µg of DNAse 1-treated RNA was used to synthesize first strand DNA utilizing SuperScript II First-strand Synthesis Kit (Invitrogen, Inc). Random hexamers (50 mg) were utilized as per the manufacturer’s protocol. First strand DNA was then used for PCR reactions. Real time quantitative PCR was performed on the ABI prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). Each PCR mixture contained 1U Taq DNA polymerase mixture (Invitrogen, Carlsbad, CA), 3 mM MgCl₂, 2.5 µM concentrations of primers of interest (synthesized by the University of Calgary DNA Lab), and 4 µL of cDNA in a total volume of 30 µL. The PCR was run according to the following conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 30 sec and 60°C for 1 min. The purity of each amplicon was determined using melting curve analysis. All samples and standards were run in duplicate. The primer sequences used were designed with Primer Express 2.0 (Applied Biosystems, Foster City, CA) and are listed in Table 2.2. Quantification of amplified products was done on a cycle-by-cycle basis using the fluorescence signal generated by binding of SybrGreen I fluorophore (Invitrogen, Carlsbad, CA) to double-stranded DNA. The cycle number at which the fluorescence signal crossed a fixed threshold (threshold cycle, CT) with an exponential growth of PCR product during the linear phase was recorded. Relative
expression values were generated using the comparative CT method \((2^{-\Delta\Delta CT})\) where all genes of interest were standardized to expression of 18S.
Table 2-1 Antibodies and dilutions for immunohistochemistry and immunocytochemistry.

NF-200, 1:800 (mouse monoclonal; Sigma)
pIRβ, 1:500 (Y972; rabbit polyclonal, Abcam)
Akt, 1:1000 (rabbit polyclonal; Cell Signaling)
p-Akt, 1:1000 (Ser473; rabbit monoclonal, Cell Signaling),
ps6k, 1:1000 (mouse monoclonal, Cell Signalling)
GSK-3β, 1:1000 (27C10; rabbit monoclonal, Cell Signaling)
pGSK-3β, 1:1000 [(Ser9) (5B3); rabbit monoclonal, Cell Signaling)
anti-actin antibody, 1:1000, (mouse monoclonal; Santa Cruz)
PTEN, 1:50 (rabbit polyclonal, Santa Cruz)
CGRP, 1:200 (goat polyclonal, Abcam)
Anti-tubulin, 1:1000 (mouse monoclonal; Santa Cruz)
Cy3, 1:100 (sheep anti-goat; Jackson Immunoresearch)
Alexa Fluor 488, 1:400 (goat anti-rabbit IgG (H+L); Invitrogen)
Cy3, 1:100 (sheep anti-mouse; Sigma)
Table 2-2 Primer Sequences.

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<th>Gene</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
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<td>S6K</td>
<td>MF5'-AAATGCTGCTTCTCGTCTTGGA-3'</td>
<td>MR5'-ACCTTCCGAGCCAAAAGCTCT-3'</td>
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<tr>
<td>CGRP</td>
<td>MF 5’-CCCTTCTCCCTATGACAGGAAA-3’</td>
<td>MR 5’-CAGATAAGCCAGAACCATGC-3’</td>
</tr>
<tr>
<td>PTEN</td>
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<td>MR5'-TGAAACCTCCCCATGTGCTGAT-3'</td>
</tr>
<tr>
<td>18S</td>
<td>M F 5’-TCCCTAGTGATCCCGAGAAAGT-3’</td>
<td>M R 5’-CCCTTAATGGCGATGATAGCGA-3’</td>
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<tr>
<td>Tubb</td>
<td>3MF 5’-AAGGTAGCCGTGTGACATCC-3’</td>
<td>MR 5’-TGCTCCGAGATGCCTTTGA-3’</td>
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<tr>
<td>NF-200</td>
<td>M F5'-TACAGAAAGCTCTCGTGAAGGC-3'</td>
<td>M R5'-TTATGTGGCTGGATATGGAGG-3'</td>
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<td>GAP43</td>
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<td>MR 5’-CCTTATGAGCCTTTATCCTCCGG-3’</td>
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2.16 Analysis

Results were presented as mean ± SEM and two groups compared using an unpaired Student’s t-test (two-tailed unless indicated) and Fisher’s exact test (as indicated) for dichotomous variables. Three or more groups were compared with a one-way ANOVA with Post hoc Tukey’s comparisons. Statistical significance was accepted at the level of p < 0.05.
Chapter Three: Accelerated Axon Outgrowth, Guidance and Target Reinnervation Across Nerve Transection Gaps following a Brief Electrical Stimulation Paradigm
3.1 Abstract

Regeneration of peripheral nerves is remarkably restrained across transection injuries, limiting recovery of function. Strategies to reverse this common and unfortunate outcome are limited. Remarkably however, new evidence suggests that a brief extracellular electrical stimulation (ES) paradigm, delivered at the time of injury, improves the regrowth of motor and sensory axons.

In this work, we explored and tested this ES paradigm, applied proximal to transected sciatic nerves in mice, and identified several novel and compelling impacts of the approach. Using thy-1 YFP mice with fluorescent axons that allow serial *in vivo* tracking of regeneration, we measured morphological, electrophysiological and behavioural indices of nerve regrowth.

We show that ES is associated with a 30-50% improvement in several indices of regeneration: regrowth of axons and their partnered Schwann cells across transection sites, maturation of regenerated fibers in gaps spanning transection zones, and entry of axons into their muscle and cutaneous target zones. In parallel studies, we analyzed adult sensory neurons and their response to extracellular stimulation while plated on a novel microelectrode array construct designed to deliver the identical ES paradigm as used *in vivo*. ES accelerated neurite outgrowth, supporting the concept of a neuron autonomous mechanism of action.

Taken together, these results support a robust role of brief ES following peripheral nerve injuries in promoting regeneration. ES has a wider repertoire of impact than previously recognized and its impact *in vitro* supports the hypothesis that a neuron specific reprogrammed injury response is recruited by the ES protocol.
3.2 Introduction

Although there is potential for regeneration in the peripheral nervous system, realistic recovery from nerve injury is most often incomplete and frequently renders severe and irreversible disability (Fu and Gordon, 1997). Despite the advent of expert microsurgical repair, peripheral nerve regeneration is slow and only partial, depending on the extent of injury. Complete functional recovery requires that regenerating axons cross the injury site and then reconnect to the target organ, an outcome that is unfortunately uncommon (Bisby et al., 1995; Fu and Gordon, 1997).

There are intriguing consequences of local electric fields on wound healing, including nerve injury, that have been recognized for a number of years. Several laboratory findings, particularly those of the Gordon laboratory, have identified the role of brief electrical stimulation (ES) in enhancing aspects of regeneration of injured peripheral nerves (Nix and Hopf, 1983; Al-Majed et al., 2000b; English et al., 2007; Franz et al., 2008). ES accelerated axon regrowth of both sensory and motor neurons in an animal model of femoral nerve injury (Al-Majed et al., 2000b; Geremia et al., 2007). ES appears not to alter the rate of the regeneration but to accelerate axon outgrowth across the suture site (Brushart et al., 2002). Normally motor axons preferentially reinnervate motor branches of a mixed peripheral nerve (Brushart, 1988). Following 1 h of ES, both motor and sensory axons regenerate preferentially into appropriate distal nerve branches (Al-Majed et al., 2000b; Franz et al., 2008). The accelerated axon outgrowth was sufficient to accelerate injury repair in a randomized clinical trial of ES in patients undergoing carpal tunnel decompression of the median nerve (Gordon et al., 2010b).
Work to date therefore has provided solid rationale to explore novel facets of the brief ES paradigm, including its impact on severe nerve transection injuries in which growth must span a gap between the proximal and distal stump. While this form of injury is regrettably common, it is unclear whether ES plays a role in this critical early regenerative outgrowth of axons and its partnering Schwann cells (SCs): from the proximal stump, crossing the gap and into the distal stump to allow reconnection (Chen et al., 2005). Additionally, there is inadequate information as to the long term electrophysiological and behaviour outcome after a sciatic nerve transection and target tissue reinnervation. In this work, we addressed these key facets of the ES response as it relates to these particularly challenging aspects of transection injuries. We utilized thy-1 YFP expressing mice with fluorescent axons to serially trace their growth across gap injuries and their reinnervation of the digits and measured regrowth with morphological, electrophysiological and behavioral endpoints. In parallel work we asked whether an identical ES paradigm delivered to adult sensory neurons in vitro, using a microelectrode array (MEA), might model the early outgrowth of our in vivo experiments. The findings identified remarkable and robust actions of ES on all of our measures of regeneration across transection gaps and indicate that it operates in a neuron autonomous fashion.

3.3 Specific Hypothesis #1

ES increases the regeneration of peripheral (sciatic) nerve axons and their accompanying SC partners across transection gaps in vivo and later translate into improved target reinnervation and functional recovery.
3.3.1 Objectives

Objective #1: To examine the impact of ES on early axon outgrowth across nerve transection gaps. The measurables would be: axon and SC outgrowth on day 7 and fluorescent live imaging of the regenerated bridge (week 1-4). Experiments will use thy-1 YFP mice for the latter in which outgrowth of regenerating fluorescent axons can be mapped in the live animal (Bridge et al., 2009).

Objective #2: To determine the impact of ES on the repopulation of distal sciatic nerve with myelinated axons. Experiments will measure the number of myelinated axons in the distal nerve stump and backlabeled motor neurons in the spinal cord.

Objective #3: To examine whether sequential stimulation (two epochs of stimulation) could further enhance the impact of ES on nerve regeneration. The number of regenerating axons from the proximal stump at an early time point (day 10) followed by backlabeled motor neurons in the spinal cord (day 21) will be measured.

Objective #4: To establish whether ES-induced axon outgrowth results in reinnervation of cutaneous and muscle targets in sciatic nerve transected animals. The presence of fluorescent axons in the distal toepads, and muscle electrophysiological studies will be carried out.

Objective #5: To determine the long-term behavioural impact of successful neural regeneration observed with ES. Sensorimotor tests including mechanical and thermal sensitivity will be analyzed.
3.4 Materials and Methods

3.4.1 Animals, Regeneration model and ES

Outbred CD-1 mice (18-20 g) and B6.Cg-Tg(Thy1-YFP)16Jrs/J mice were used in this study. For mapping early nerve regeneration events using immunohistochemistry, 6 mice for both control sham stimulation were used. A model of sciatic nerve transection and surgical repair was used followed by ES of injured proximal nerves using a similar protocol (2 V, 20 Hz, 1 h). Detailed procedure is in Chapter 2.

3.4.2 Analysis of early axon and Schwann cell outgrowth

For regeneration bridges examined 7 days following sciatic nerve transection and surgical repair through a silicone chamber with and without stimulation, 16 µm thick longitudinal sections of the early regenerative bridge were made and underwent double labeling with antibodies against NF-200 to label axons and GFAP to label activated SCs (Chen et al., 2005). For each intervention, means and standard errors (SE) were then calculated for each group of mice (n=6, and 3 sections analyzed/animal/group). Detailed procedure is in Chapter 2.

3.4.3 Sequential stimulation

For the sequential stimulation experiment, normal CD-1 mice were divided into three groups: control (sham), single stimulation and double/sequential stimulation (n=5-7/group/time point). On day 10, a group of mice (n=5) was harvested for immunohistochemistry to analyze early axon outgrowth. The protocol is described in detail in Chapter 2.
3.4.4 Analysis of regenerating myelinated sensory and motor axons

Samples of sciatic nerves constituting proximal and distal stumps and the regenerative bridge connecting them were harvested at 21 days after transection and chamber placement with and without stimulation. Final measurements included axon density, myelin thickness, number of myelinated axons and total area of the regenerative bridge that included myelinated axons. For each intervention, means and SE were calculated for each group (n=8-10 animal/group).

3.4.5 Retrograde labeling and counting of motor neurons

On day 21, retrograde tracer was applied to the sciatic nerve, 5 mm distal to the regenerated bridge to trace the number of motor neurons regenerating after control sham stimulation or ES (n=7/group). The backlabeled motor neurons were counted using the method originally described by Abercrombie (Abercrombie, 1946). Another set of animals (n=6-7) for sequential stimulation studies, were harvested on day 28 for similar measurements (described in detail in Chapter 2).

3.4.6 Long term nerve regeneration and target reinnervation

Sciatic nerves of B6.Cg-Tg(Thy1-YFP)16Jrs/J mice (n=5/group) were imaged using a fluorescent microscope (Stereo discovery V8, Zeiss Canada, Toronto, Canada) at every week for four weeks after transection and placement of a regeneration conduit connecting the distal and proximal stump. Measurements included the presence of axons in the toepad, and in the digital and tibial nerve, examined every week from week 4 to week 12.
3.4.7 Functional motor recovery: Electrophysiology

Starting at 9 weeks after sciatic nerve transection and surgical repair with and without ES, multifiber motor conduction recordings were carried out in left sciatic–tibial fibers in mice (n=5/group) anesthetized with isoflurane (Kennedy and Zochodne, 2000). At the end of the study, tissues were harvested and muscle weights were recorded.

3.4.8 Functional recovery of sensation

Mice underwent mechanical (von Frey filaments) and thermal testing weekly from week 6 to week 12 following sciatic nerve transection and repair with a silicone conduit with and without ES (n=5/group), as described in Chapter 2.

3.4.9 Fabrication of the microelectrode array (MEA) and analysis of electrical fields

A microelectrode array (MEA) was fabricated to analyze the impact of our specific stimulation paradigm on harvested adult sensory neurons (Fig. 3.1a-c) (Dalton and Kaler, 2007; Hayden and Dalton, 2010). A numerical simulation was carried out using a finite element model in COMSOL Multiphysics package (COMSOL Inc, 2010). For convenience, the coordinates x, y were defined at the electrode surface, where electrodes were symmetrical about the x- and y-axis, as shown in Fig. 3.1c. A 3-dimensional conductive media model was used to simulate the electrical field between the plated neurons and the underlying electrodes. For more accuracy, the electrodes and their corresponding conducting traces were included in the simulation. The simulation was performed by applying an electrical dipole of 200 mV randomly selected at
electrode 87 and 0 V at electrode 86. The medium was 1X PBS solution of pH=7.4 and its conductivity was 1.55 S/m (Fig. 3.1).
The fabricated MEA consists of an 8×8 array of 30 μm diameter pads on the ends of 15 μm wide traces on a 5x5 cm glass slide as illustrated. The array corner electrodes were not present, leaving 60 electrodes in total in the array. The electrode metal layers were deposited by sputtering and consisted of a 10 nm thick chrome adhesion layer, with 200 nm thick gold on top. The metal layers were then patterned by standard photolithography or femto second laser machining to form the array. The 30 μm electrode pads of the array were each spaced 100 μm apart from each other (A), denotation of MEA (C). The media supporting the cells was contained over the array by a 4 mm high, 24 mm diameter custom cut glass well, which was held in place by polydimethylsiloxane (B). Simulation studies were performed to measure current density with blue representing the lowest and red indicating highest current density (D). Current density is shown in centerline at the electrode surface (E). Current density is higher in the left hand side (x<0, near the electrodes at which current was applied across, #86 and 87), and it reaches its maximum of about 800 A/m². The current density dramatically declines on the opposite side of the array (x>0).
3.4.10 *In vitro studies of adult sensory neurons*

The procedure for adult sensory neuronal cell culture is described in Chapter 2. Sensory neurons were viewed by phase contrast using an inverted microscope at 24 h after electrical stimulation. Photos were taken by DIC (40x, 6.5 cm = 100 mm) and included for analysis if not overlapped with other neurons or glial cells. In particular, neurons were examined for their proportion with initial process formation (neurite initiation), the percentage of neurons with at least one neurite process equal or longer than cell diameter in length, the number of neurites per neuron, and the longest neurite.

3.4.11 Analysis

Results were presented as mean ± SEM and groups compared using an unpaired Student’s t-test (two-tailed unless otherwise indicated) and Fisher’s exact test (as indicated) for dichotomous variables. Statistical significance was accepted at the level of p < 0.05.

3.5 Results

3.5.1 Impact of ES on early outgrowth following nerve transection and surgical repair

To determine the impact of electrical stimulation (ES) on initial steps during *in vivo* axon outgrowth, we examined both axon and SC elongation from the proximal stump of transected sciatic nerves into a conduit in which their repopulation could be evaluated. ES enhanced short-term (early axon and SC) outgrowth in regenerated bridges (Fig. 3.2). The length of the longest growing axons, identified as NF-200 immunoreactive profiles, was significantly longer in the ES
group compared to control non-stimulated mice (Fig. 3.2c). The total numbers of regenerated axons in the bridges were also significantly higher with ES (Fig. 3.2d), and evaluation of individual outgrowth zones were different between ES and unstimulated nerves (Fig. 3.2f). The regenerated axons in the stimulation group grew longer distances as compared to control (Fig. 3.2f). GFAP-immunoreactive activated SCs were also identified at longer distances along the regenerating bridge following ES (Fig. 3.2i). The overall numbers of GFAP labeled SCs summed in all fields examined (Fig. 3.2j) were not different from controls although they did appear to accompany the rise in axon outgrowth (Fig. 3.2k). Taken together these findings indicated that ES increased early outgrowth of axons beyond the transection zone.
**Figure 3.2 ES enhanced short-term regeneration of mouse sciatic nerve.**

Immunohistochemical detection of axons and SCs in longitudinal sections of regenerated sciatic nerve 7 days following transection injury with and without ES is illustrated. Representative images of regenerated axons and reactive SCs from the proximal injury point to the end of regenerated bridges that include axons (A,B and G,H). The fluorescence signal detecting axonal neurofilament (NF-200) indicates greater numbers of axons and a longer axonal length in ES nerves (B) versus unstimulated sham controls (A). As well, GFAP positive SCs appear to be more abundant and intense in response to ES (H) as compared to unstimulated controls (G). Quantification of the axons (NF-200) and SCs (GFAP). The length of the longest growing axon and distance from proximal repair site where activated SCs were detected were significantly (p<0.05) greater with ES (C and I). The overall numbers (total count of all axons in all fields examined distal to the proximal stump; see Methods) of axons detected were also higher (p<0.05) (D) but the overall numbers of GFAP labeled SCs in ES group were not significantly different from controls (J). The number of regenerated axons and activated SCs extending into each micro-field (270 µm) is shown in (E,F,K). The first field (270 µm each) was identified from the proximal stump of the regenerated bridge and the last field, at the tip of the regenerated bridge. ES enhanced nerve regeneration as indicated. Values are expressed as Mean ± SEM, n=6 (3 sections /animal), *, p<0.05 (Control vs. ES, Student’s t-test, one-tailed), scale bar=40 µm.
3.5.2 Sequential stimulation

The augmentation of early axon and SC outgrowth after a single stimulation paradigm led us consider that sequential stimulation might show additive benefits on regeneration profiles. Using an approach where we stimulated the injured sciatic nerve on day 0 (immediately followed by the injury) and subsequently on day 5 noninvasively, we analyzed the early regenerative events by counting the number of regenerating axons and the length of longest regenerating axon in the regenerative bridge on day 10. As expected, single stimulation enhanced the number of regenerating axons as well as the length of the longest regrowing axon (Fig. 3.3a-c). Strikingly, sequential stimulation increased the number of regenerating axons compared to single stimulation (Fig. 3.3e). The length of longest growing axon was not different between single and sequential stimulation (Fig. 3.3d). Sprouting is a widespread phenomenon in regenerating peripheral nerves and each parent axon may sprout almost 20 daughter axons (Diamond et al., 1987). Thus, increases in the number of outgrowing axons with sequential stimulation could be a result of a higher number of regenerating sensory or motor parent axons or multiple sprouts arising from individual parent axons. Labeling of axons with NF-200 could not differentiate between these. To answer this question, we took an advantage of a retrograde backlabeling technique using fast blue dye, which can travel retrogradely and label motor neurons in the anterior spinal cord. The number of motor neurons regenerating was not different between the stimulation paradigms, which suggested that the rise in axon profiles identified was accounted for increased sprouting of parent axons with sequential stimulation (Fig. 3.3f,g). mRNA levels of the pan-neuronal marker; PGP9.5 mRNA levels were significantly increased with both single and sequential stimulation (Fig 3.3h). Calcitonin gene-related peptide (CGRP), an indicator of
sprouting was further increased with the sequential stimulation protocol (Fig 3.3i). We concluded that sequential stimulation directs axon sprouting without improvement in regenerating motor neurons compared to a single stimulation paradigm. Additional studies in this thesis therefore focus on the impact of the single stimulation paradigm.
**Figure 3.3 Sprouting of axons with sequential stimulation.**

Representative images of longitudinal sections of the regenerated sciatic nerve 10 days following transection injury and immunostained with NF-200 (axons) are illustrated; sham stimulation (A), single stimulation (B) and sequential stimulation (C). A higher number of NF-200 stained axons was detected with single and sequential stimulation compared to unstimulated sham controls. The length of the farthest growing axon from the proximal tip in the regenerating bridges was significantly higher with single and sequential stimulation compared to sham stimulation (D). The numbers of outgrowing axons were also higher with single stimulation compared to controls and was further enhanced by sequential stimulation (E). The mean number of backlabeled motor neurons that regenerated their axons 3 weeks after injury and stimulation paradigms is represented in (F,G). Both single and sequential ES significantly increased the number of motor neurons regenerating but there was no difference between the stimulation paradigms. Relative expression of *PGP9.5* (H) and *CGRP* mRNA (I) in ipsilateral DRGs with single and sequential stimulation. *CGRP* mRNA levels were further increased with the sequential stimulation paradigm. Values are expressed as Mean ± SEM, n=5-7, *, p<0.05, Student’s t-test.
3.5.3 Regrowth of myelinated axons beyond the site of nerve transection

Given the enhancement of early axon and SC outgrowth beyond a nerve transection observed at one week after injury, we next examined regrowth at a later time point known to coincide with maturation and remyelination of regenerating axons (Chen et al., 2005). By day 21, 14 out of 14 animals harvested in the ES group had complete macroscopic regenerated bridges that connected the proximal and distal sciatic nerve stumps. While these bridges included axons, they were largely composed of connective tissue. In the non-stimulated control mice, only 8 out of 14 animals demonstrated completed bridges (Fig. 3.4a,b) [p=0.01, Fisher’s exact test]. Analysis of the axonal content of the central portion of the regenerated bridge at 3 weeks after injury and ES verified that there were significantly more axons: total numbers of axons were higher than control (Fig. 3.4e) but the mean diameter (µm) of the axons were not significantly different [(Con, 2.03 +/- 0.05, ES, 1.99 +/- 0.08), p=0.33] with a similar average g-ratio (ratio of axon to fiber diameter) [(Con, 0.63 +/- 0.01, ES, 0.56 +/- 0.07), p=0.14] (Fig. 3.4c-e). Axon density (#/mm²) was higher in the regenerated bridge [(Con, 2527 +/- 855; ES, 5146 +/- 734), p<0.05] with ES. The total area (mm²) of the regenerated bridge in ES appeared higher after ES, but the difference was not significant [(Con, 8248 +/- 2541, ES, 12681 +/- 1690), p=0.08]. The higher counts of mature myelinated fibers in the regenerated bridges correlated with the finding that in distal stumps, only ES mice had axons demonstrating early myelination. This finding suggested that in the control distal stump axons were less mature and nonmyelinated whereas larger more mature myelinated axons of the ES group could be identified. Overall, the findings indicated that the early single ES paradigm impacted not only initial outgrowth, but also the later repopulation with myelinated axons in the regenerating cable and distally.
Figure 3.4 Impact of brief ES at later regenerative time points.

Macroscopic images of regenerated bridges after 3 weeks of nerve regeneration are illustrated. Note that the bridges from mice that underwent ES were of larger caliber (B) and appeared much thicker and robust than the unstimulated controls (A). Qualitative analysis of the central portion of the regenerated bridge at 3 weeks after injury and ES resulted in a greater bridge area (B) than sham stimulated controls (A), scale bar=5 mm. (C, D) Toluidine Blue-stained transverse semithin sections from the centre of the regenerative bridges harvested 21 days after sciatic nerve transection with (D) and without ES (C) with quantification of axonal number (E). Note that the cross section area of the bridge was larger (D), such that only a portion of the total number of myelinated axons is demonstrated in a field of identical size; total numbers of the axons were also higher (E) (p<0.05) with ES. Values are expressed as Mean ± SEM, n=8-10 animals/group, *, p<0.05, Student’s t-test, scale bar=10 µm
3.5.4 Numbers of motor neurons that regenerate axons

The number of sciatic motor neurons that regenerated their axons through the bridge between proximal and distal nerve stumps, with and without ES was evaluated 21 days after nerve repair using the retrograde tracer, fast blue. The total number of sciatic motor neurons in the anterior grey horn of the spinal cord that regenerated their axons was significantly greater with ES as compared to the numbers without ES (Fig. 3.5a). This outcome measure of nerve regeneration is consistent with our data of higher numbers of regenerating axons at the regeneration front within the chamber (Fig. 3.2d and 3.4e), confirming an impact of ES in promoting axon outgrowth after nerve transection (Brushart et al., 2002; Brushart et al., 2005).

3.5.5 Electrophysiological evidence of accelerated regrowth of motor axons

To evaluate the functional outcomes of accelerated axon outgrowth, we evoked supramaximal serial compound muscle action potentials (CMAPs) of tibial innervated muscles distal to the site of sciatic nerve transection and surgical repair. CMAPs reflect the number and maturation of motor axons that have reconnected to muscle endplates distal to the repair site. Recordings of CMAPs at week 10 identified earlier reappearance of CMAPs in mice that had undergone ES compared to unstimulated sham controls: 3 of 5 mice had recordable CMAPs compared to 1 out of 5 control mice. The mean amplitude of the CMAPs was higher in the ES than control groups at the 10 week time point (Fig. 3.5b). There was no significant difference in the motor conduction velocities between the groups (data not shown). Muscle weights that normally decline with denervation atrophy and recover with reinnervation (Midrio, 2006) were higher in the gastrocnemius muscles of the stimulated group of mice as compared to the sham
stimulated group, 12 weeks after sciatic nerve transection [(Con 0.10 +/- 0.03g; ES, 0.12 +/- 0.01 g), p=0.2]. Overall these findings demonstrated more rapid and complete motor reinnervation and muscle target reconnection after nerve transection when the proximal nerve stump was stimulated.
Figure 3.5 ES increased motor neuron regeneration.

The number of backlabeled motor neurons after 3 weeks of injury with and without ES are illustrated. Retrograde tracer was applied to the sciatic nerve, 5 mm distal to the conduit after control sham stimulation or ES. The mean number (±SEM) of backlabeled motor neurons that regenerated their axons (n=7) is indicated. Note that ES for 1 h significantly (p<0.05) increased the number of motor neurons regenerating (A). The amplitudes of the CMAPs recorded subcutaneously from the interosseous foot muscles were similarly higher (p<0.05) in the ES than control at 10 week time point (n=5) (B). Values are expressed as Mean ± SEM, n=5-7, *, p<0.05 (Control vs. ES, Student’s t-test, one-tailed).
3.5.6 Skin reinnervation

To evaluate the regrowth of axons with or without ES, we analyzed their serial growth in thy-1 YFP transgenic mice, in which a proportion of peripheral neurons and their axons can be identified noninvasively by virtue of their YFP fluorescence signal. Axonal repopulation of regenerating bridges with their proximal and distal stump was imaged under fluorescence microscope weekly starting from the day of surgery until week 4. To visualize regrowth in live animals, the sciatic nerve was temporarily exposed under anesthesia, the regenerate and proximal stumps imaged and the wound then repaired. Four weeks later, at a time point of 8 weeks after the initial injury and grafting, assessment of reinnervation of sensory end organs in the skin of the toes was initiated by assessing changes in axon specific fluorescence signal in these regions.

In the regenerating nerve, YFP labeled axons that formed bridges were more robust and had greater fluorescence intensity in ES than unstimulated control mice (Fig. 3.6a,b), indicating a higher content of YFP axons within them. When quantified at 4 weeks, the length of the regenerated bridge was significantly longer in the ES group compared to control (Fig. 3.6c). The mice whose repaired sciatic nerves were subjected to ES also had earlier foot and toepad skin reinnervation as compared to unstimulated controls. Although neither group had measurable reinnervation until week 7 (Fig. 3.6d,e), axons in ES treated mice were the first to be visualized in the digits and toepads (Fig. 3.6e). We quantified the number of digits and toepads reinnervated in each animal and in each group and the presence of axons in the tibial nerve of thy-1 YFP animals. Specific reinnervated toepads varied among individual mice. Reinnervation was identified one week later in the control-unstimulated mice. By 8 and 10 weeks, the number of toepads reinnervated was significantly higher in mice that had undergone ES. The reappearance
of axons in the digital nerve in the fingers of the foot after nerve regeneration was also elevated with ES at the 8 week time point after injury. The difference between the groups persisted until the end of the study (Fig. 3.6f-h).
Figure 3.6 ES improves long-term regeneration and reinnervation in YFP animals.

Fluorescent regenerated bridges in YFP animals after 4 weeks of injury with and without ES are illustrated (A and B). The dashed line indicates the outline of the silicon conduit placed to repair the transected proximal and distal sciatic nerve stumps. The arrow indicates the regenerated bridge in the conduit. YFP labeled axons that formed regenerative bridges were much more robust and brighter in ES (B) than in nonstimulated control mice (A) and correlated with a greater regeneration length (p<0.05) in the ES group quantified by measuring the length of fluorescence in the regenerated bridge (C). The photomicrographs in D, demonstrate the absence of nerve fiber reinnervating the toepad after 8 weeks of injury in controls (D) in contrast to the presence of nerve fibers after ES (E). ES mice had earlier foot and toepad skin reinnervation compared to unstimulated controls. Fluorescent microscopy images are from the tip of the hindpaw toe. On the y-axis is quantified mean number of the digits and toepads that were reinnervated/animal/group as determined from the presence or absence of YFP axons, and the presence of axons in the tibial nerve in thy-1 YFP animals (F, G and H). Note that at 8 and 10 week time point, significantly (p<0.05) higher numbers of toes were reinnervated after ES. Digits reinnervated were also significantly (p<0.05) greater at the 10 week time point. Values are expressed as Mean ± SEM, n=5/group, *, p<0.05, Student’s t-test, scale bar=40 µm
3.5.7 Functional recovery of sensation

To determine whether ES-induced accelerated axon regrowth is associated with improved sensory indices of behavior, we analyzed mechanical and thermal sensitivity of the distal hindpaw by recording the sensitivity of the sole of the foot to graded force delivered by Von Frey filaments (see Methods) and the latency (sec) to withdrawal of the foot to a radiant heat source applied to the hindpaw, respectively. This analysis was carried out serially after the sciatic nerve repair with and without ES. The sole of the foot in the mice of the ES group were more sensitive to mechanical and thermal stimuli from 6 weeks post-injury onward: the threshold force applied by the von Frey filaments to induce a withdrawal response and the latency for paw withdrawal after thermal exposure was lower in ES treated mice than unstimulated controls (Fig. 3.7a,b). For mechanical stimuli starting from week 9 onward, the animals in the ES group were closer to baseline and there was no significant difference between the sensitivity of the injured and normally innervated contralateral hindpaws, suggesting that this measure had normalized ipsilateral to injury. In contrast, in control mice the difference in sensitivity between the intact and injured side persisted (Fig 3.7a). For thermal stimuli the ES mice were more sensitive than the control mice at week 7 and 8. By 9 weeks the control mice had regained sensitivity and there was no longer a difference in their thermal sensitivity compared to ES mice (Fig. 3.7b). In conclusion, ES of the transected sciatic nerve led to more rapid return of sensory function indicating more rapid regeneration of sensory axons into their target zones.
Figure 3.7 Improved functional recovery with ES.

Sensitivity to the mechanical stimuli (grams of force applied) and the latency for paw withdrawal (sec) after thermal exposure was higher in the ES animals (A and B). The difference in mechanical sensitivity was sustained until 12 weeks of the study (A). Response to thermal stimuli was different between the groups at week 7 and 8 (B). Values are expressed as Mean ± SEM, n=5/group, *, p<0.05, Student’s t-test.
3.5.8 Early neurite outgrowth in vitro

Given the robust impact of a specific short duration ES protocol on early axon outgrowth in vivo, we asked whether a similar paradigm might alter direct neurite outgrowth of adult neurons in vitro. While stimulation of isolated neurons does not precisely replicate in vivo conditions of whole trunk stimulation in a live mouse, we duplicated the stimulation duration and frequency protocol as delivered in vivo, using a novel microelectrode array (MEA) construct specifically designed for this purpose. We first used a multiphysics package to model and simulate the current density generated by the MEA electrodes (COMSOL Inc 2010). As shown by the color intensity in Fig. 3.1d, strong current density was achieved closer to the two electrodes where the electrical dipole was applied, and the current density decreased rapidly with increasing distance. Fig. 3.1e shows current density in the centerline (x-axis) at the electrode surface (red dashed line in Fig. 3.1e). In this figure, we see that high current density was obtained within a distance of 4 mm in the left hand side (x<0, near electrodes 86 and 87), and it reached its maximum of about 800 A/m². Meanwhile, the current density was much smaller in the opposite side (x>0), and it dropped to nearly zero for x>2 mm. These findings verified that an applied ES paradigm would be limited to a localized area and involve only individual neurons.

Adult dorsal root ganglion (DRG) neurons were continuously stimulated using a 200 mV pulse signal with a frequency of 20 Hz and a pulse width of 1 ms for 1 h with the opposite, unstimulated portion of the MEA, where current density had declined to zero, being used as a control. Stimulation was applied 24 h after neurons were plated in culture dish, an early time point associated with only limited outgrowth in adult sensory neurons. Stimulation did not alter overall neuron viability. In particular, from 3 separate experiments, 120 neurons were analyzed...
for ES and 90 neurons for sham stimulation control (on the opposite side of the same dish, corresponding to areas with low to absent current density) for their proportion with initial process formation (neurite initiation), the number of neurites per neuron with neurite process equal or longer than cell diameter in length, and the longest neurite. At 24 h following ES, neurites were longer after ES (Fig. 3.8e). There were greater numbers of neurites per neuron (Fig. 3.8f) but the proportion of neurite bearing neurons were not different between the groups (Fig. 3.8g). Taken together, the in vitro studies provided confirmation that ES facilitates neurite outgrowth in adult sensory neurons in vitro indicating initiation of an acute plastic neuron growth response. The findings identified a direct impact on neuron behaviour and an outcome that was compatible with the enhanced outgrowth observed in vivo (Fig. 3.8 e-g).
Figure 3.8 Increased neurite outgrowth on MEA.

Isolated adult sensory rat neurons were grown for 24 h on a microelectrode array (MEA) coated with Poly-l-lysine and laminin. Representative images of isolated sensory neurons are shown before and 24 h (48 h in culture) after sham stimulation (A and B) and after 20 Hz, 1 h ES (C and D). Neurons were stimulated at 20 Hz, 200 mV continuously for 1 h with current applied between specific electrodes on the MEA and not on the opposite end of the MEA (with minimal current density, see Figure 3.1c,d) being used as a control (sham stimulation). At 24 h following ES the length of the longest neurites was significantly (p<0.01) greater after ES (E). There were borderline findings of higher (p=0.045, one-tailed t-test) numbers of neurites per neuron (F) but numbers of neurite bearing neurons were not different among the groups (G). Values are expressed as Mean ± SEM, n=3, *, p<0.05, Student’s t-test, scale bar=40 µm.
3.6 Discussion

This work indicates that a specific brief ES paradigm has a remarkable impact on both early events of peripheral nerve regeneration and later target reinnervation. The major and novel findings from this work were (i) ES accelerated outgrowth of axons and partnering SCs from the proximal stump of a complete sciatic nerve transection, into a regeneration conduit; the impact of ES does not require an adjacent distal stump and depends instead on events proximally; (ii) This early outgrowth translated into a more rapid reconstitution of the full bridge between the proximal and distal stump as judged by the reappearance of populations of YFP expressing regenerating axons; (iii) accelerated axon regeneration was associated with accelerated regrowth of other connective tissue constituents of the sciatic nerve stump that lend caliber to a regrown nerve segment; (iv) ES enhanced the recovery of electrophysiological properties of regrowing motor axons and enhanced repopulation of myelinated axons across the injury site with a higher number of motor neurons regenerating their axons; (v) ES was associated with more rapid axon reinnervation of the skin territories distal to the injury site and with recovery of mechanical and thermal sensitivity; (vi) a novel MEA designed as a substrate for harvested dissociated neurons at low density can provide localized stimulation; (vii) stimulation of adult sensory neurons \textit{in vitro} is associated with a rise in the length of neurite outgrowth.

3.6.1 ES enhances early axon and SC outgrowth beyond the site of a sciatic nerve transection

Axons and SCs are intimate partners in the regenerative microenvironment with active bidirectional molecular exchanges between them. In previous work, we have noted that this partnership is nearly invariant following nerve transection during the formation of new nerve
trunk bridges, and that prevention of SC proliferation dramatically impairs overall outgrowth (Hall, 1986; Chen et al., 2005; McDonald et al., 2006). SCs provide trophic support to the axons in the form of neurotrophins (NGF, BDNF, NT-3 and CNTF), neurotrophic factors and extracellular matrix proteins (Heumann et al., 1987; Boyd and Gordon, 2003; Chen et al., 2005; Hayworth et al., 2006). Axons provide several molecular cues like neuregulin to SCs for proliferation and myelination during regeneration (Guertin et al., 2005; Patricia, 2007; Toth et al., 2009). Growth of the axons following transection are also often misdirected, a problem not corrected by surgical apposition or placement of a conduit (Brushart, 1988). SCs provide important directional cues (Chen et al., 2005; Witzel et al., 2005).

Since it is unclear at what stage of the regenerative process ES might have an impact, we evaluated early axon and SC outgrowth from the proximal stump of transected axons. ES increased both the distance and numbers of outgrowing axons and concurrently the SCs associated with them. This is important because it indicates that ES generates a very early impact on the regenerative process and that there is proper fidelity of regrowth associated with axon partners. Should only nascent axon profiles have emerged from the proximal stump, their longer term viability would be uncertain. We also show that the impact of ES is not exclusively on SCs alone to encourage more robust leadership to their axon partners, but also impacts the intrinsic growth abilities of the neuron itself, resulting in outgrowth that is closely linked, as the case for the naïve state. Given this stable axon/Schwann cell partnership, the exchange sustains the regenerative effort. Evaluation of early outgrowth however is insufficient to predict what later bridge reconstitution, myelinated axon repopulation and eventual target reinnervation might be. For these questions we carried out other forms of analysis. Nonetheless, the findings indicated a robust ‘head start’ in the series of events required to allow regrowth.
3.6.2 Sequential stimulation increases axon sprouting

The robust impact of single stimulation on axon and SC outgrowth profiles led us to examine the impact of sequential stimulation after nerve injury: whether it would add any further benefit to staggered regeneration or axon outgrowth after nerve repair. We hypothesized that sequential stimulation would lead to better regeneration outcomes than single stimulation by further enhancing axon outgrowth. Indeed, a second stimulation epoch enhanced the benefit of the single stimulation paradigm by further increasing the number of outgrowing axons. The increase in number of NF-200 labeled axons could not differentiate between new growth of parent axons or sprouting from limited numbers of parent axons. To test for the possibility of sprouting, using a backlabeling approach, we quantified the number of regenerating motor neurons 3 weeks after injury and stimulation. Interestingly, the numbers of motor neurons regenerating were not different between the stimulation paradigms despite a higher number of neurofilament-stained axons in the regenerating bridge, which indicates enhanced sprouting of axons with sequential stimulation. This was further confirmed by an increase in mRNA expression of the sprouting-related genes, PGP9.5 and CGRP. Axon sprouting may be of benefit in conditions such as stroke where preserved or undamaged axons can sprout to their nearby territory and help to regain function (Hagg, 2006). Similarly, in peripheral nerve injuries, sprouting of motor axons enables them to reinnervate a higher number of muscle fibers, and creates more functional synaptic connections that enlarge their motor unit territory. This is valuable in regaining muscle function as it allows other denervated muscle to be reinnervated (Rafuse and Gordon, 1996). However, sprouting of axons may also cause hyperalgesia and increased pain sensation (Woolf and Mannion, 1999). Nonetheless, while increased sprouting
could be exploited to regain function these studies were beyond the scope of this work. Further studies in this thesis focuses on a single stimulation paradigm.

3.6.3 ES helps to reconstitute a regenerative bridge

An interesting and relatively unexplored facet of peripheral nerve regrowth involves how newly growing axons recruit other necessary cellular constituents to form a new nerve trunk. Reformation of a full nerve trunk over time after injury has been demonstrated for some years by Lundborg and colleagues (Lundborg, 1988). Our model was ideally placed to address this question because, from retraction of the proximal nerve stump, the gap between the proximal and distal stump requires regrowth of new tissue including axons, SCs, blood vessels, fibroblasts and others. We have already demonstrated above that SCs collaborate with axons during this enhanced outgrowth. Here we show that the connective tissue properties of the reformed bridges are more rapidly reacquired with ES. That they had an enhanced population of axons was demonstrated by their greater YFP intensity and area indicating greater axonal growth. Bridges were also more likely to be fully complete, beyond considerations of their axon investment alone, and those that were complete were larger in size.

3.6.4 Enhanced electrophysiological recovery, repopulation of myelinated axons and a higher number of motor neurons that regenerate axons

The electrophysiological studies used here specifically measured the CMAP and conduction velocity of regenerated nerves. The CMAP represents a summation of individual muscle action potentials at the motor endplate. Axonal loss results in loss or lower CMAP
amplitudes and reinnervated segments may have a higher latency in the segment of the examined nerve (Wilson et al., 1998). We found an earlier appearance of CMAPs with larger amplitude following ES. It has been recently reported that ES potentiates axonal regrowth and myelin maturation during peripheral nerve regeneration (Wan et al., 2010). We found that the regenerated bridges three weeks after injury and stimulation had greater repopulation by myelinated axons with greater nerve and axon area and axon density associated with ES. The appearance of the higher amplitude CMAPs is consistent with the finding of higher numbers of motor neurons that regenerated their axons to reinnervate target endplates. The higher number of myelinated axons in the regenerated bridges and distal stump that reinnervated target tissues earlier with higher numbers is also in agreement with the finding of earlier and larger CMAPs. CMAPs provide information about the numbers of regenerating motor axons that achieve target reconnection and it is important that enhanced CMAP recovery accompanied improved backlabelling of motor neurons. The enhanced regeneration of motor axons demonstrated by electrophysiology with ES however may have also translated into additional improved behaviour outcomes focused on motor function (Lu et al., 2009). While not carried out in this work, these might include paw grip strength and detailed analysis of gait, keeping in mind that gait analysis depends on both motor and sensory functional integrity. Detailed gait analysis, largely studied in rats, was beyond the scope of this work.
3.6.5 ES is associated with earlier skin reinnervation associated with more rapid recovery of sensory function

Functional recovery is frequently inadequate following injuries of the peripheral nerve trunk and regrowing axons must regenerate longer distances to reinnervate their denervated targets (Fu and Gordon, 1997). To address whether enhanced early outgrowth of axons and reformed bridges translates into better target reinnervation we used thy-1 YFP mice. These mice express spectral variants of GFP (yellow-YFP) at high levels in motor and sensory neurons, as well as in subsets of central neurons (Bridge et al., 2009). Axons are brightly fluorescent all the way to the terminals and expression is not normally detectable in nonneural cells. Mice expressing several spectral variants of fluorescent protein under control of a neuron specific thy-1 promoter have provided animals with specifically labeled neuronal, and in our case axon cohorts (Feng et al., 2000). While YFP does not necessarily label every peripheral neuron, there is no evidence that ES alters the proportion of expression among neurons (Groves et al., 2005; Witzel et al., 2005; English et al., 2007).

Following nerve injury the distal nerve trunk undergoes Wallerian or Wallerian-like degeneration over the course of time (Raff et al., 2002). When we began imaging our mice 4 weeks after injury and stimulation, axons were not visible in the toepad, digits and footpad. The newly regenerated fibers reappeared earlier in ES and the numbers of toepads and digits reinnervated were higher at every week time point. This earlier skin innervation was later translated into improved sensory recovery in the animals. Mice were less sensitive to thermal and mechanical stimuli after sciatic nerve transection but the recovery of function was improved with ES. The improved sensation to the mechanical and thermal stimuli was evident from week 6
onwards and there was a significant difference between the groups at 8 week time point. The early appearance of YFP axons and the later recovery of sensory function are consistent with the time for sensory afferents to effectively reinnervate and mature within the skin so as to transmit physiological sensory information. It is also likely that for behavioural responses to occur, sensation may not require a complete complement of reinnervated axons. The repopulations of the bridges, earlier appearance of the regenerated fibers and CMAPs with enhanced functional recovery all indicates the robust effect of the ES on nerve regeneration.

3.6.6 ES rapidly enhances neurite outgrowth, initiation and number in adult sensory neurons

We used a novel approach to stimulate individual neurons using a custom fabricated MEA. This provided a substrate for harvested dissociated neurons to receive localized stimulation and allowed stimulation either for a full cohort of neurons on a dish simultaneously or that of single isolated neuron on the electrode with other non-stimulated neurons serving as a control. In this way, we evaluated whether stimulation may be essentially neuron autonomous. This is qualified however by the fact that DRG cultures are rarely completely free of glial cells, and it is possible, but seems unlikely that these small groups of adherent cells triggered axon growth secondarily. The MEA allowed independent selection of the electrode or neuron to stimulate, the ability to plate a single cell on a desired electrode, and the capability of using the glass surface with coating of suitable substrates such as poly-l-lysine and laminin. The MEA was also well suited for other purposes such as stimulation of outgrowing axon while monitoring in real time, evaluating the impact of ES on the neuronal cell body or studying selective stimulation of remote axons to observe the local impact of stimulation.
We found greater neurite outgrowth, more neurons bearing neurites and more neurites per neuron 24 h after ES. Neurons bearing neurites were more numerous after ES indicating enhanced neurite initiation. These results are in agreement with *in vivo* findings of this study and earlier investigations in which greater numbers of motor and sensory axons crossed a nerve injury and repair site at an early time point in response to ES (Brushart et al., 2002; Geremia et al., 2007).

It is possible that ES not only upregulates the regeneration machinery at the cell body but also generates local changes at the level of regenerating axons and SCs. For example, it has recently been reported that SCs in response to ES secrete higher NGF in a calcium dependent fashion (Huang et al., 2009). Axons also possess protein synthesis machinery and are capable of translating mRNA localized in regenerating axons (Willis et al., 2005; Gumy et al., 2010). At the cell body level upregulation of RAGs, higher cAMP levels or intracellular calcium may be important in ramping up regenerative programs. In sensory neurons ES upregulates protein expression (Geremia et al., 2007), and increases intracellular cAMP (Udina et al., 2008). Rises in calcium also impact neurite outgrowth (Kocsis et al., 1994) through a Src-Ras signaling cassette involving a Ca\(^{2+}\)/calmodulin-dependent protein kinase IV pathway (Harper et al., 1994; Takemura et al., 2009). To demonstrate the link between the ES, higher intracellular Ca\(^{2+}\) levels and the enhanced neurite outgrowth that we have seen, further studies are required (Kocsis et al., 1994).

ES increased axon outgrowth of central primary afferent terminals into the dorsal columns of the spinal cord (Udina et al., 2008). Interestingly, the degree of accentuated axon outgrowth was comparable to that seen after a conditioning lesion (CL), an approach that elevates intracellular cAMP levels in DRG neurons among its mechanisms to accelerate
outgrowth (Udina et al., 2008). Though the exact mechanisms of ES actions are not fully understood, evidence thus far suggests that it signals the parent cell body to reprogram the regeneration-related gene (RAG) output of neurons (Al-Majed et al., 2000a; Geremia et al., 2007). This shift may involve the rapid synthesis of neurotrophic factors and their receptors changing neurons into a more permissive growth mode (Gordon et al., 2003). Indeed, the neurotrophin brain derived neurotrophic factor (BDNF) and its receptor trkB are rapidly upregulated in response to brief ES in regenerating motor and sensory neurons (Al-Majed et al., 2000a; Geremia et al., 2007), with BDNF being critical for the induction of a regeneration response in peripherally axotomized sensory neurons (Geremia et al., 2010).

3.7 Conclusions

In conclusion, I have found that ES enhances neurite outgrowth, neurite number and neurite initiation in vitro and improves structural and functional indices of both short-term and long-term regeneration in vivo in the setting of severe nerve trunk transection. In the next Chapter, I will explore potential mechanisms associated with the effectiveness of ES on peripheral nerve regeneration.
Chapter Four: Electrical Stimulation Facilitates Peripheral Nerve Regeneration through the PI3-K Signaling Pathway
4.1 Abstract

Brief extracellular electrical stimulation (ES) facilitates peripheral nerve regeneration, but its potential mechanisms are elusive (Chapter 3). Several laboratories have postulated upregulation of BDNF and its receptor TrkB and other regeneration-associated genes (RAGs) such as β-tubulin, actin and GAP-43 in motor and sensory neurons as underlying mechanisms (Al-Majed et al., 2000a; Geremia et al., 2007).

Using the advantage of our in-house designed microelectrode array we attempted to dissect the responsible mechanisms behind the effectiveness of ES on improving peripheral nerve regeneration. To assess the intrinsic growth state of neurons, isolated adult sensory neurons were stimulated with an ES paradigm (200 mV, 20 Hz, 1 h) and intracellular calcium levels were measured using two-photon microscopy. Alteration in neurotrophins and RAGs were assessed in cultured in vitro adult sensory neurons and in ipsilateral DRGs following a brief ES of injured sciatic nerves. The PI3-K neurotrophins signaling cascade was explored, particularly PTEN, an inhibitor of this pathway, in cultured sensory neurons in vitro and later in ipsilateral DRGs following sciatic nerve injury. Finally, the contribution of DRG perineuronal satellite cells was examined in the effectiveness of ES on peripheral nerve regeneration.

GAP-43 and β-tubulin expressions were enhanced in the ipsilateral lumbar DRGs with ES of injured sciatic nerves. Expression of markers of neuron-satellite cell interaction, CGRP, NRG and GFAP, were also increased in ipsilateral DRGs following ES. In vitro, sensory neurons demonstrated enhanced mRNA expression of the neurotrophin, BDNF. Cultured sensory neurons subjected to an ES paradigm, responded with a decrease in PTEN and a rise in pAkt expression with a concurrent reduction in GSK-3β levels. Adult sensory neurons in vitro had an increase in
intracellular calcium levels in response to ES. ES-induced neurite outgrowth was blocked by a PI3-K inhibitor (LY294002) but not by nickel (T-type calcium channel blocker) at 100 µM. Following ES of sciatic nerves in vivo, PTEN expression was reduced, and there was a rise in ps6k expression in ipsilateral DRGs. Overall, the nerve regeneration response to ES may include facilitation of the PI3-K signaling by PTEN suppression and increased neurotrophic support.

4.2 Introduction

Neurotrophins are a family of growth proteins that regulates neuronal survival, development and maintenance (Chao, 2003). Brain derived neurotrophic factor (BDNF) is a member of the neurotrophin family and binds to its receptors TrkB and the low-affinity neurotrophin receptor p75. Both of these receptors are expressed in motor and sensory neurons after axotomy and have an essential role in survival and peripheral nerve regeneration. Electrical activity regulates BDNF expression in neurons (Ghosh et al., 1994; Tongiorgi et al., 1997). ES-mediated peripheral nerve regeneration is correlated with enhanced expression of regeneration-associated genes (RAGs), BDNF and its receptor TrkB in motor (Al-Majed et al., 2000a; Al-Majed et al., 2004) and sensory neurons (Geremia et al., 2007). However, the impact of ES (electrical stimulation) on RAGs expression after sciatic nerve transection injuries, and in isolated sensory neurons in vitro where the neuron-autonomous impact can be analyzed, is unknown.

Roles of supporting cells, including Schwann cells (SCs) in peripheral nerves and perineuronal satellite cells in DRGs, are unknown in response to ES. However, some evidence suggests that glial cells could also respond to ES. For example, Stevens and Fields (2000) have
reported a rise in intracellular Ca\(^{2+}\) levels in SCs with low frequency ES. Similarly, axons stimulated for 30 min at 10 Hz in co-cultured DRG-SCs, induced c-fox and Krox-20 gene expression in SCs. An interesting observation by the same research group indicated that ES could change astrocytes from non-myelinating to myelinating cell phenotypes in the CNS (Ishibashi et al., 2006). Additionally SCs have been shown to synthesize NGF (nerve growth factor) in response to a similar ES paradigm that I used in my thesis, and changes in internal and external calcium levels were proposed as potential mechanisms (Huang et al., 2009).

Depolarisation augments intracellular calcium levels in sensory neurons (Cohan et al., 1987) which leads to growth cone extension (Kater and Mills, 1991) and neurite outgrowth (Kocsis et al., 1994). Calcium also plays an important role in axon pathfinding during development (Gomez and Zheng, 2006). Tongiorgi et al. (1997) showed that depolarisation by KCl induces upregulation of BDNF and TrkB mRNA in hippocampal neurons through L-type Ca\(^{2+}\) channels. Similarly, depolarisation-mediated calcium influx in cerebellar neurons was sufficient for NCAM- and L1-dependent neurite outgrowth. The neurite outgrowth was blocked by a reduction in extracellular calcium and with the intracellular calcium chelator BAPTA (Williams et al., 1992). Doherty et al. (1993) further demonstrated that L- and N-type calcium channels were responsible for NCAM-mediated neurite outgrowth. The inhibition of neurite outgrowth in cerebellar granular neurons by NG2 (a transmembrane chondroitin sulfate proteoglycan) was reversed by cAMP and an increase in intracellular calcium (Dou and Levine, 1997). In adult sensory neurons cultured in vitro, we observed a neuron-autonomous, rapid and robust activity-dependent increase in neurite outgrowth following ES (Chapter 3). In concurrence with the previous findings, we propose that changes in intracellular calcium might play a role in
this ES-induced neurite outgrowth and axon regeneration.

PTEN (phosphatase tensin homolog deleted on chromosome ten), described in Chapter 2 in detail, regulates the intrinsic growth capacity of adult neurons, and its deletion enhances regeneration of the CNS and peripheral neurons (Park et al., 2008; Christie et al., 2010; Liu et al., 2011). To our knowledge there is no report indicating that ES could impact tumor suppressors such as PTEN in the nervous system. However, some indirect evidence suggests that PTEN downregulation in the injured spinal cord modifies activity-dependent plasticity of the CNS neurons (Gang et al., 2012). RhoA is another substantial regenerative roadblock and is mainly present at the growth cone of central and peripheral axons (Luo et al., 1997). RhoA activation causes growth cone collapse and subsequently inhibits regeneration of the adult spinal cord (Conrad et al., 2005), CNS neurons (Lehmann et al., 1999), and peripheral sensory neurons (Cheng et al., 2008). Decreases in levels of regenerative roadblocks such as PTEN or RhoA may represent an important connection between molecular signals and the regenerative response observed with ES. The main goal of these experiments was to elucidate the potential mechanisms associated with the effectiveness of ES on peripheral nerve regeneration.

4.3 Specific Hypothesis #2

The PI3-K pathway and intracellular calcium are involved in accelerated axon outgrowth in vitro and in vivo.

4.3.1 Objectives

Objective #1: To determine whether ES enhanced peripheral nerve regeneration involves the PI3-K pathway. Experiments will determine the expression of a key member (PTEN) of this
pathway. Thereafter, analysis of neurite outgrowth in the presence of a specific inhibitor of PI3-K will be carried out.

**Objective #2:** To recognize the role of intracellular calcium in ES-induced neurite outgrowth of adult sensory neurons. Experiments will take advantage of a microelectrode array (MEA) to deliver localized ES and measure changes in intracellular calcium and neurite outgrowth.

**Objective #3:** To identify neurotrophins and regeneration-associated genes involved in ES-enhanced nerve regeneration. mRNA levels of regeneration-associated genes will be examined after sciatic nerve transection and the neurotrophin BDNF in cultured sensory neurons following ES. The nonneuronal (perineuronal satellite cells in DRGs) contribution to the ES-mediated regenerative response will be explored *in vivo* in ipsilateral DRGs following injury and ES.

### 4.4 Materials and Methods

#### 4.4.1 Animals, ES and immunohistochemistry

Outbred CD-1 mice were used in this study. To address changes in neurotrophins and perineuronal satellite cell markers in ipsilateral DRGs, the left sciatic nerve of mice was transected and the proximal and distal stumps were secured using 10–0 sutures into the ends of a silicon nerve chamber leaving a gap of approximately 2-3 mm between the stumps. The proximal sciatic nerves were immediately sham or electrically stimulated after repair using the same stimulation paradigm and methods described in Chapter 2. Three days after nerve repair and stimulation, mice were perfused transcardially, and DRGs (L4-L5) were harvested and fixed. Ten-micron thick sections were processed and stained with primary antibodies: anti-Glial Fibrillary Acidic Protein (GFAP), anti-CGRP (calcitonin gene-related peptide), PTEN and ps6k.
Secondary antibodies used were: sheep anti-mouse Cy3, goat anti-rabbit Alexa Fluor 488, and sheep anti-goat Cy3. The detailed immunohistochemistry protocol and list of primary antibodies are in Chapter 2.

4.4.2 In vitro studies of adult sensory neurons

The procedure for adult sensory neuronal culture is described in Chapter 2. Briefly, L4 and L5 DRGs were dissected from adult outbred CD-1 mice and were plated onto the MEA precoated with poly-L-lysine and laminin in DMEM/F12 medium containing N2 nutrient supplement and 10ng/ml NGF. The neurons were cultured under four different conditions: (I) control (sham stimulation); (II) ES only (III) ES plus a PI3-K inhibitor (LY294002, 30 µM, Calbiochem, EMD chemicals, USA); or (IV) ES plus nickel (Ni^{2+}, 100 µM). Eighteen hours after plating, treatments were applied and thereafter MEAs underwent either sham stimulation or ES (20 Hz, 200 mV, 1 h) using a stimulator (Grass Instruments, Warwick, USA). Sensory neurons were viewed by phase contrast using an inverted microscope at 24 h after ES. Final measurements included: the total neurite outgrowth, the length of the longest neurite and the number of branches of primary neurites.

4.4.3 Calcium (Ca^{2+}) imaging

To examine changes in intracellular calcium concentrations ([Ca^{2+}]_i) in DRG sensory neurons in response to ES applied through MEA, we performed two methods: Fura-2 calcium ratiometric experiments using a conventional wide field fluorescence microscope and time lapse
imaging using a two photon microscope. The experiments were performed 18 h after plating. The detailed procedure is in Chapter 2.

4.4.4 Western immunoblot

DRGs (L4-L5) and sciatic nerves were harvested 3 days after injury and stimulation. In vitro adult sensory neurons were harvested 24 h after stimulation. The samples were probed for: Akt, p-Akt, GSK-3β, pGSK-3β, PTEN, tubulin and actin antibodies. Horseradish peroxidase (HRP) labeled secondary antibodies: anti-rabbit IgG HRP and anti-mouse IgG HRP were used, and signals were detected by exposing the blot to enhanced chemiluminescent reagent. The film images were digitized and the difference between the treatments was measured using pixel intensity and band size using Adobe Photoshop. The values were represented as the percentage difference from the control, with control values designated as 1.0.

4.4.5 Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted and real time quantitative PCR was performed. The primers used and their sequence is detailed in Chapter 2. Relative expression values were generated using the comparative CT method (2^{-\Delta\Delta CT}) where the gene of interest was standardized to the expression of the housekeeping gene 18S.
4.4.6 Analysis

Results were presented as mean ± SEM and the two groups compared using an unpaired Student’s t-test (two-tailed unless otherwise indicated). Statistical significance was accepted at the level of p < 0.05.

4.5 Results

4.5.1 ES induces expression of neurotrophins and RAGs in ipsilateral DRGs

ES-mediated nerve regeneration is mainly attributed to upregulation of the growth factor BDNF and its receptor TrkB in motor neurons (Al-Majed et al., 2000a; Al-Majed et al., 2004) and to BDNF, regeneration/growth-associated protein, GAP-43, and cAMP in sensory neurons (Geremia et al., 2007); however, expression of RAGs and neurotrophins following ES of transected sciatic nerves has not been verified. Since we observed enhanced neurite outgrowth of isolated rat sensory neurons in vitro with ES (Chapter 3), we wondered whether neurotrophins such as BDNF and RAGs have any role in this plastic, neuron-autonomous regenerative response associated with ES. Consistent with our hypothesis, we noticed a robust rise in injury/regeneration-associated genes such as GAP-43 and β-tubulin in ipsilateral stimulated DRGs 3 days after the sciatic nerve transection and ES (Fig 4.1a,b). However, no changes in NF-200 mRNA levels were observed (Fig. 4.1c). Cultured sensory neurons subjected to an ES paradigm showed a significant upregulation of the neurotrophin BDNF and nonsignificant trend toward a rise in GAP-43 and β-tubulin transcript levels (Fig 4.1e-g). The less robust impact of ES on cultured sensory neurons in vitro on GAP-43 and β-tubulin expression may represent
possible involvement of other nonneuronal cells such as perineuronal satellite cells in DRGs
\textit{in vivo}, in producing the regenerative response of ES. We have also analyzed the levels of $Shh$
mRNA in DRGs with ES. Shh is a morphogen, critical for the nervous system development
(Charron et al., 2003; Yam et al., 2009), has an inconclusive role in CNS regeneration (Spencer
et al., 2004; So et al., 2006) and is expressed in SCs in the PNS (Hashimoto et al., 2008).
Recently, our lab has identified overexpression of Shh after sciatic nerve injury and has
suggested its beneficial role for nerve regeneration (Martinez, unpublished data). In this study,
we found enhanced expression of $Shh$ with ES in ipsilateral DRGs (Fig. 4.1d), and this indicates
a potential role in ES-mediated peripheral nerve regeneration.
Figure 4.1 ES enhances mRNA expression of RAGs in DRG neurons.

A-E: qRT-PCR expression of $\beta$-tubulin (A), GAP-43 (B), NF-200 (C) and Shh (D) in normal uninjured, and injured pooled DRG neurons with sham stimulation or ES. mRNA expression of $\beta$-tubulin, GAP-43, and Shh significantly increased with ES.

E-G: A significant upregulation of BDNF expression (E) and a nonsignificant trend in mRNA expression of $\beta$-tubulin (F), GAP-43 (G) in cultured sensory neurons in vitro was observed. n=3-4/group, values are represented as mean ± SEM, *p<0.05, Student’s t-test.
in vivo

A

B

in vitro

E

F

G
4.5.2 ES induces changes in the intrinsic growth state of neurons

There are several reports suggesting that depolarization augments calcium influx in neurons (Cohan et al., 1987; Kater and Mills, 1991); however, the ES paradigm I have used in this study has never been examined for its impact on intracellular calcium levels. We analyzed the alteration in intracellular calcium concentrations using MEA where we have the liberty to stimulate and measure calcium changes at the single cell level. At first, we demonstrated that sensory neurons grown on MEA are healthy and extend neurites with ES. During one hour of ES, no change in neuronal morphology was observed and neurons extended their growth cones. We also examined the growth cone turning response to ES by applying an electric field to a specific chosen electrode facing a growth cone approximately at 60-90° angle, however, after several attempts we could not detect any specific impact (data not shown). Within 18-24 hours of stimulation, neurons protruded new neurites from the cell bodies and the outgrowth was extensive (Fig 4.2a). We also assessed the response of ES on the neuronal membrane potential. With 200 mV at 20 Hz, applied through the MEA, sensory neurons demonstrated a minimal depolarization with no action potential being generated. Action potentials were observable at 7 V and higher (data not shown). Thereafter, we analyzed calcium influx in these neurons concurrently with ES. First, we measured calcium influx using a fura-2 calcium indicator with a conventional inverted fluorescence microscope equipped with a camera. We observed a rise in intracellular calcium but the response was of a smaller magnitude (Fig 4.2b,c). Later, we concluded that the signal we detected was suboptimal because of limitations of our system for recording from thick MEAs (500 µm). This led us to switch to a more sophisticated and advanced setup of using a two-photon microscope. Interestingly we observed a significant rise in
intracellular calcium levels in these neurons and the signal was reliable and consistent (Fig. 4.2d). Our neuronal stimulation paradigm of 200 mV led to a small but significant increase in intracellular calcium, but stimulation at higher voltages (2-5 V) was associated with a larger calcium influx (Fig. 4.2d-f). Neurons returned to their baseline calcium levels once we stopped stimulation and waited for a longer period, indicating that neurons were healthy and the rise in calcium was transient (data not shown). Our quantitation of the data revealed an overall significant (approximately 20%) increase in intracellular calcium upon ES (Fig. 4.2g). Another interesting finding was that neurons located on or closer to the electrode responded well with a larger calcium influx compared to the ones in the vicinity, as might be expected from spatial declines in current density.
Figure 4.2 Rise in intracellular calcium with ES.

A. Representation of a single isolated sensory neuron in culture on MEA before, during stimulation (200 mV, 2 V and 5 V at 20 Hz) and 18 h after ES. One hour of ES was well tolerated by the neuron, which was extending its growth cone within this time frame (arrow indicating the sprouting of the growth cone). Eighteen hours later, the growth was robust and new neurites were visible (arrowheads). B,C. The small rise in intracellular calcium was detected with a 200 mV stimulation paradigm observed using a conventional wide field fluorescence microscope from the neuron touching the electrode (arrow) (C). D. Example of a neuron undergoing ES and calcium measurement using two-photon microscopy. E,F. Representation of the quantification of two experiments showing a rise in intracellular calcium with a range of stimulation paradigms. G. The mean rise in intracellular calcium with the different stimulation paradigms. Values are represented as mean ± SEM. *p<0.05, Student’s t-test.
4.5.3 ES-associated upregulation of the PI3-K pathway in adult sensory neurons

The intrinsic growth state of neurons is potentiated by the upregulation of neurotrophins; contrarily, regenerative roadblocks such as PTEN and RhoA diminish these intrinsic growth capacities (Cheng et al., 2008; Christie et al., 2010; Park et al., 2010; Liu et al., 2011). PTEN inhibition augmented the regeneration of adult peripheral sensory neurons (Christie et al., 2010). Similarly inhibition of RhoA signaling enhanced axon outgrowth of transected peripheral axons (Cheng et al., 2008). The expression of these inhibitory molecules with extracellular ES is unknown. We addressed whether ES has any impact on these intrinsic growth regulators in vitro and in vivo. We observed significant downregulation of RhoA transcript in ipsilateral DRGs after ES (Fig. 4.3a). Similarly, cultured in vitro sensory neurons subjected to ES showed a nonsignificant reduction in RhoA mRNA levels (Fig. 4.3b). PTEN mRNA was also significantly downregulated after ES in cultured adult sensory neurons in vitro and in ipsilateral DRGs in vivo, indicating a robust impact of ES on PTEN expression (Fig. 4.4a and e). Using western blot analysis, we verified downregulation of PTEN protein expression in cultured sensory neurons exposed to ES (Fig. 4.4b-d) and this was associated with a rise in its downstream mediator pAKT, a convergent point of many cellular processes for growth and regeneration. Furthermore, a decrease in GSK-3β, a negative regulator of neuronal growth, was noted with a parallel increase in its inactive form, pGSK-3β, in cultured sensory neurons in vitro (Fig. 4.4c). Next we assessed PTEN expression in vivo in ipsilateral DRGs and sciatic nerves following crush injury with and without ES. We found a significant decrease in PTEN expression in both sciatic nerves and ipsilateral DRGs (Fig. 4.4f,g). Additionally, using an immunohistochemical technique we confirmed the decrease in PTEN expression in ipsilateral DRGs following ES (Fig. 4.4h).
Subsequently, we explored the downstream mechanisms linked to reduced PTEN expression that follow an ES paradigm. PTEN has been reported to regulate the mTOR pathway in injured retinal ganglion cells (Park et al., 2008). Our western blot analysis showed a trend towards an increase in expression of ps6K (phosphorylated ribosomal s6k protein) in ipsilateral DRGs following ES (Fig. 4.4i). Overall this significant impact of ES on PTEN is of importance given the substantial influence of PTEN on nerve regeneration. The decrease in PTEN expression may be a fundamental mechanism linked to ES-enhanced peripheral nerve regeneration.
Figure 4.3 Decrease in RhoA expression with ES.

A, B: RhoA mRNA expression in \textit{in vivo} DRGs. A. Transection injury was associated with a significant increase in RhoA expression in ipsilateral DRGs, consistent with previous findings (Cheng et al., 2008). This indicates that regenerative barriers such as RhoA are expressed during peripheral nerve regeneration and inhibit regrowth. The rise in RhoA expression in ipsilateral DRGs was significantly rescued by an extracellular ES paradigm, and a nonsignificant trend was observed in cultured sensory neurons \textit{in vitro} (B). n=3-4/group, values are represented as mean ± SEM. *p<0.05, Student’s t-test.
Figure 4.4 ES upregulates the PI3-K pathway in adult sensory neurons.

A: PTEN mRNA was significantly decreased in cultured adult sensory neurons following ES. B,C: Western immunoblot of PTEN, pAkt, pGSK-3β, and GSK-3β expression from adult sensory neurons exposed to an ES protocol. Note that PTEN and GSK-3β levels were reduced concurrently with an upregulation of pAkt and pGSK-3β expressions following an ES paradigm, compared to controls. D. Quantification of PTEN and pAkt band density normalized to actin or tubulin controls. E. qRT-PCR expression of PTEN mRNA was significantly downregulated in DRGs 3 days after injury and ES. F,G: Western immunoblot of pAkt and PTEN expression in DRGs (F) and sciatic nerves (G) 3 days following injury with and without ES. H. Immunohistochemical expression of PTEN in adult sensory neurons 3 days after injury and ES. PTEN intensity was reduced in DRG sensory neurons following ES. I. Western immunoblot of DRG neurons probed for ps6k expression with and without stimulation; actin is a loading control. Note: rise in ps6k expression in DRGs 3 days following injury and ES. Values are represented as mean ± SEM. *p<0.05, Student’s t-test, Scale bar=100 µm.
**In vitro**

**A**

Graph with bars showing normalized expression of PTEN in control versus ES conditions.

**B**

Western blots showing levels of PTEN, pAKT, GSK-3β, and Actin in control (Con) and ES conditions.

**C**

Western blots showing PTEN, pGSK-3β, and Tubulin in control (Con) and ES conditions.

**In vivo**

**D**

Graph showing protein expression levels of PTEN and pAKT in control and ES conditions.

**E**

Graph showing normalized expression of PTEN in control versus ES conditions.

**F**

Western blots showing PTEN and Actin in DRG sections for control (Con) and ES conditions.

**G**

Western blots showing PTEN and Actin in Sciatic Nerve sections for control (Con) and ES conditions.

**H**

Immunofluorescence images of PTEN in control (Control) and ES conditions.

**I**

Western blots showing ps6k and Actin in control (Con) and ES conditions.
4.5.4 ES-induced neurite outgrowth was blocked by a PI3-K inhibitor

The main goal of these experiments was to determine whether the PI3-K pathway and the intracellular calcium levels are involved in the ES-mediated regenerative response of cultured neurons in vitro. We first treated our primary sensory neuron cultures with BDNF, a well-known growth-promoting neurotrophin for TrkB-expressing sensory neurons, to confirm the integrity of our culture system. BDNF at 25 nM produced a significant increase in neurite outgrowth of adult sensory neurons compared to the control IgG (Fig. 4.5a,b). We further analyzed whether the PI3-K pathway is essential for the in vitro neurite outgrowth. Our treatment with a PI3-K inhibitor, LY294002 (25 µM) significantly reduced the neurite outgrowth in cultures, indicating the potential role of the PI3-K pathway in promoting neurite growth (Fig. 4.5c,d). Thereafter, we moved to an in vitro stimulation protocol using the MEA described above, and measured neurite outgrowth 1 day after ES. In accordance with our previous data (Chapter 3), neurite outgrowth was significantly enhanced with the ES paradigm compared to sham stimulation (Fig 4.6 a-d). The benefits of ES-induced neurite outgrowth were completely abolished in the presence of LY294002 (Fig 4.6e). Additionally, LY294002 treatment significantly reduced the length of the longest neurite and total number of branches per neuron (Fig. 4.6f,g). To dissect the role of intracellular calcium, we used nickel (100 µM) to assess its impact on neurite outgrowth. This concentration of nickel preferentially blocks low-voltage activated T-type calcium channels, widely expressed on small and medium sized adult sensory neurons in the DRG (Yusaf et al., 2001; Todorovic and Jevtovic-Todorovic, 2007; Iftinca and Zamponi, 2009). In preliminary experiments, we observed that nickel partly attenuated the rise in calcium inside the cell (data not shown). There was no impact of nickel treatment on ES-induced neurite outgrowth although
there was a trend towards a decrease in total neurite outgrowth and the length of the longest neurite but they were not significant (Fig. 4.6e,f). This preliminary observation suggests that low-voltage activated T-type calcium channels might not have a significant impact on neurite outgrowth in response to ES.
Figure 4.5 BDNF increases and a PI3-K inhibitor decreases neurite outgrowth of adult sensory neurons.

A. Total neurite outgrowth expressed as fold change was significantly increased with a 25 nM BDNF dose but not changed with control IgG peptide at the same dose. B. Examples of adult sensory neurons grown *in vitro* exposed to media only, control IgG, or 25 nM BDNF (*p<0.05, Student’s t-test, control vs. BDNF; n=3/group). C. Representative images of adult sensory neurons in culture in the presence of media only or LY294002. D. LY294002 showed significantly decreased neurite outgrowth. Values are represented as mean ± SEM. *p<0.05, Student’s t-test, n=3/group, Bar=50 µm.
Figure 4.6 A PI3-K inhibitor attenuated the increase in neurite outgrowth associated with ES.

A-D: Examples of adult sensory neurons grown in vitro on MEA exposed to media only (A), media + ES (B), media + ES + LY294002 (C) and media + ES + nickel (D). E-G: Total neurite outgrowth (E), length of the longest growing neurite and (F) total number of branches of a primary neurite (G), expressed as fold change compared to control. Values are represented as mean ± SEM. *p<0.05, Student’s t-test; n=3-4/group.
4.5.5 DRG perineuronal satellite cells contribute to the ES-mediated regenerative response

The relationship between axons and SCs is intimate and SCs support axon regeneration following injury (Chen et al., 2005). In Chapter 3, we described the presence of proliferating SCs up to longer distances in concert with a rise in axonal outgrowth in the regenerative bridge one week post-injury and stimulation. In this work, we observed a rising although nonsignificant trend in NRG mRNA, a potent inducer of SC proliferation (Wen et al., 1994), in stimulated sciatic nerve bridges, which may be correlated with the SC outgrowth in the regenerative bridge (Fig. 4.7a). Perineuronal satellite cells in the DRGs proliferate and upregulate the expression of fibroblast growth factor-2, glial cell line-derived neurotrophic factors, and neurotrophins such as NGF and NT-3 in response to peripheral nerve injury, and facilitate neuronal regeneration; however, the involvement of these nonneuronal cells in ES-associated peripheral nerve regeneration has not been explored (Gehrmann et al., 1991; Wen et al., 1994; Zhou et al., 1999). Therefore, we sought to further determine the possible involvement of perineuronal satellite cells in the ES-mediated regenerative response. Interestingly, we observed a rise in GFAP expression in the DRGs in the ES group (Fig 4.7b). Based on these findings and considering the earlier reports suggesting retrograde propagation of electrical signals back to the cell body (Al-Majed et al., 2000b); we focused our further studies on perineuronal satellite cells especially on the markers of neuron-satellite cell communication including CGRP, NRG, ErbB2 and ErbB3 (Chen et al., 2003). Peripheral nerve injury leads to increased expression of GFAP mRNA in perineuronal satellite cells (Fig. 4.7c). With ES, this was further enhanced by almost 7-fold indicating potential proliferation of perineuronal satellite cells in response to ES. In addition, ErbB2, ErbB3 and NRG mRNA expression were upregulated with nerve injury and further
increased significantly with ES (Fig. 4.7c). To explore additional signals for enhanced proliferation we evaluated CGRP, a peptide present selectively in small sensory neurons and a potent inducer of SC proliferation in peripheral nerves (Cheng et al., 1995; Li et al., 2004). CGRP mRNA was elevated in in vivo ipsilateral DRGs and a nonsignificant trend was present in vitro cultured sensory neurons upon ES (4.7 d,e). Additionally, using immunohistochemistry we established higher CGRP expression in ipsilateral DRGs in response to ES (Fig. 4.7f).
Figure 4.7 ES enhances neuronal satellite cell communication in DRGs.

A. *NRG* mRNA expression in sciatic nerves following sham stimulation and ES. B. Western immunoblot labeled with GFAP in DRGs with and without ES. C. qRT-PCR expression of *GFAP, ErbB2, NRG* and *ErbB3* mRNA in ipsilateral DRGs. All of these markers were enhanced in DRGs with ES of injured sciatic nerves. D,E: Expression of *CGRP* mRNA was significantly increased in DRGs (D) and a nonsignificant trend was observed in isolated cultured sensory neurons with an ES paradigm (E). F. Representative images of CGRP expression in DRGs with and without ES. Values are represented as mean ± SEM. *p<0.05, Student’s t-test; n=3-5/group.
4.6 Discussion

In this study we investigated the potential mechanisms associated with the benefits of electrical stimulation on peripheral nerve regeneration. Important findings from this work are: ES induces (1) upregulation of regeneration-associated genes including GAP-43 and \( \beta \)-tubulin in ipsilateral DRGs and the neurotrophin BDNF in cultured sensory neurons \textit{in vitro}, (2) changes in the intrinsic growth state of neurons with a rise in intracellular calcium levels (3) downregulation of regenerative roadblocks, PTEN and RhoA (4) upregulation of the PI3-K pathway and particularly rises in pAkt \textit{in vitro}, (5) enhanced neurite outgrowth which could be blocked using a PI3-K inhibitor, (6) the possibility of enhanced communication between neurons and perineuronal satellite cells with an upregulation in CGRP, GFAP, NRG, ErbB2, and ErbB3 mRNA in DRGs.

4.6.1 Upregulation of RAGs in sensory neurons in response to ES

Peripheral nerve injury induces upregulation of the immediate early genes such as c-jun and c-fos, the regeneration/growth-associated genes, mainly GAP-43, neurotrophic factors including BDNF and NT-3/4, and cytoskeletal proteins such as tubulin and actin in the neuronal cell body (reviewed by Tetzlaff et al., 1989; Herdegen et al., 1997; Boyd and Gordon, 2003). Increases in tubulin and GAP-43 expression are directly correlated with peripheral nerve regeneration (Strittmatter et al., 1994). BDNF has been shown to promote survival of the motor neurons and the TrkB expressing population of sensory neurons \textit{in vivo} (Yan et al., 1992; Geremia et al., 2010). Local application of BDNF enhances sprouting in CNS axons and improves regeneration of axotomized motor neurons (Boyd and Gordon, 2002) and sensory
neurons (Geremia et al., 2010).

In this study, we used a sciatic nerve transection and surgical repair model, and conducted analysis at day 3 after repair and stimulation. We intentionally selected a 3 day time point for assessing RAG expression because of the temporal expression of these genes after injury, and of the previous findings indicating that ES promotes the early outgrowth of axons across the repair site within first 3-4 days (Al-Majed et al., 2000b) and we have also observed higher axonal outgrowth at day 7 (Chapter 3). We noticed a significant rise in RAGs including \(\beta\)-tubulin and GAP-43 in ipsilateral DRGs following sciatic nerve transection and stimulation. These results are consistent with previous findings from a femoral nerve injury model (Al-Majed et al., 2004). All these findings including ours, were carried out using in vivo models, which involve several cell types other than neurons that are the likely targets of ES (Al-Majed et al., 2000a; Geremia et al., 2007). Our in vitro culture system was well placed to dissect the individual response of neuronal and nonneuronal cells to ES. Increased BDNF and RAG expression in cultured sensory neurons and its correlation with higher neurite outgrowth (Chapter 3) suggests that ES could directly act on isolated neurons (Singh et al., 2012b). An important caveat was that the in vitro impact on isolated neurons was of lower magnitude compared to the robust impact observed in DRGs suggesting that the ES-mediated responses may also involve other nonneuronal cells. SCs in the peripheral nerves and perineuronal satellite cells in the DRGs are the two most closely associated cells to axons and neurons respectively. Therefore, we next investigated the possible contribution of these cells in the regenerative response to ES.
4.6.2 Involvement of perineuronal DRG satellite cells in mediating ES response in peripheral nerve regeneration

The basis of the hypothesis that perineuronal satellite cells are contributing to ES enhanced nerve regeneration was developed when we analyzed axon and SC outgrowth in regenerative bridges. In concert with axonal outgrowth, higher numbers of SCs were observed up to longer distances in the regenerative bridge, demonstrating a possible proliferation response with ES (Chapter 3). This hypothesis is further strengthened by the previous reports indicating upregulation of gene expression in SCs in response to ES (Stevens and Fields, 2000) and the SC dependent release of NGF following ES (Huang et al., 2009). Interestingly, in this work, we found an upregulation of GFAP mRNA expression in ipsilateral DRGs suggesting a possible involvement of perineuronal DRG satellite cells in the ES-mediated regenerative response. The communication between DRG neurons and tightly wrapped perineuronal satellite cells is intimate and bidirectional (Gehrmann et al., 1991; Zhou et al., 1999). Perineuronal satellite cells proliferate in response to axonal injury and support neurons during regeneration (Wen et al., 1994; Zhou et al., 1999; Chen et al., 2005). In this study, we observed a rise in the expression of NRG and CGRP, possibly secreted by neurons, and these are potent regulators of SC proliferation in sciatic nerves and perineuronal satellite cells in DRGs (Chen et al., 2003; Hayworth et al., 2006; Toth et al., 2009). We also noticed an upregulation of ErbB2 and ErbB3 receptors (on which NRG acts as a ligand), expressed on satellites cell, which are critical for mediating the neuron-satellite cell communication and possibly phenotypic changes such as pain (Marchionni et al., 1993; Chen et al., 2003). How these changes have occurred is not known at this stage but possibly includes retrograde travel of action potentials back to the neuronal cell
body. This could potentially enhance the neuron and neighbouring satellite cell response to injury and ES. Not examined here, blocking action potentials with tetrodotoxin (TTX) during the ES protocol followed by the expression analysis of these markers would be the next logical experiment, to verify the retrograde response of ES to the cell body, an approach already verified to impact the ES regenerative response (Al-Majed et al., 2000b; Geremia et al., 2007).

4.6.3 Alteration in the intrinsic growth state of sensory neurons with ES

The increase in neurotrophin BDNF and regeneration-associated gene expression in cultured sensory neurons in response to ES suggests that ES impacts the neuronal cell body. Additionally, TTX blocking ES regenerative response experiments indicated the same (Al-Majed et al., 2000b; Geremia et al., 2007). We have also noticed an increase in neurite outgrowth in isolated neurons following ES (Chapter 3). These experiments indicated that direct membrane depolarization through ES has a role to play in this response. Blocking calcium influx attenuated upregulation of immediate early genes such as c-fos (Sheng et al., 1993), intracellular cAMP (Ming et al., 2001; Udina et al., 2008), and finally neurite outgrowth (Kocsis et al., 1994). We analyzed sensory neurons in response to ES amid a possibility that this would lead to enhanced neurite outgrowth through a rise in intracellular calcium and downstream signaling. We found a small but significant rise in intracellular calcium in isolated sensory neurons in response to the stimulation paradigm we used in neurite outgrowth studies. Calcium studies were carried out 18-24 hours after plating; a time point that coincides with our neurite outgrowth assay protocol. Kater et al. (1987) proposed a “set hypothesis” suggesting a small window of calcium appropriate for the growth. Any changes below and above this were associated with either a smaller or no response. Specifically, a larger increase in calcium inside the cell causes
excitotoxicity and neuronal damage (Cohan et al., 1987; Kater and Mills, 1991). This suggests that the signals we observed with our stimulation paradigm were close to the range reported to impact growth.

Calcium entering through the voltage-gated calcium channels (VGCCs) can regulate downstream physiological responses including neurotransmitter release, shaping of the action potential firing pattern, and calcium-dependent gene expression (Cohan et al., 1987; Williams et al., 1992; Kocsis et al., 1994; Rusanescu et al., 1995; Ghosh-Roy et al., 2011). Several types of calcium channels are expressed on the DRG sensory neurons including T-, N-, P/Q- and L-type and play an important role in maintaining the normal physiological functions, mediating pain processing and impacting neurite outgrowth (Lankford and Letourneau, 1989; Perez-Reyes et al., 1998; Yusaf et al., 2001; Todorovic and Jevtovic-Todorovic, 2007; Iftinca and Zamponi, 2009). To further assess the involvement of calcium in ES-induced neurite outgrowth, we used nickel as a blocker (Zamponi et al., 1996). Nickel has been generally considered a selective blocker of low-voltage activated T-type calcium channels, and at higher concentrations it could also inhibit R-type calcium channels (Zamponi et al., 1996; Kang et al., 2007). Low-voltage activated T-type calcium channels are exclusively expressed on small and medium diameter sensory neurons and have been studied for their role in sensory perception and nociception (Perez-Reyes et al., 1998; Yusaf et al., 2001; Bourinet et al., 2004; Todorovic and Jevtovic-Todorovic, 2007; Iftinca and Zamponi, 2009). The improvement observed in the mechanical and thermal sensation in animals following injury and ES (Chapter 3), and given the role of T-type channels in pain sensation, led us to consider the involvement of T-type voltage gated channels in ES-enhanced neurite outgrowth. Using nickel as an inhibitor, we nonetheless noted improvement in neurite outgrowth with ES implying that low-voltage activated T-type calcium channels might not be the
responsible mechanism. However, this experiment was not well placed to examine the role of all VGCCs because nickel at the concentration we used (100 µM) is not effective in blocking high voltage-gated calcium channels (VGCCs) such as L-, N- and P-type (Hofmann et al., 1987; Kang et al., 2007). Use of cadmium to block high VGCCs in addition to nickel, may further explain precisely the role of VGCCs in the ES-mediated neurite outgrowth (Hofmann et al., 1987). Particularly, an L-type channel with slow activation and inactivation kinetics (Perez-Reyes et al., 1998), has been suggested as the key VGCC involved in neurite outgrowth, and blocking this channel reduces neurite outgrowth (Robson and Burgoyne, 1989; Doherty et al., 1993). Calcium imaging and a neurite outgrowth assay in the presence of a dihydropyridine such as nifedipine (5 µM) could address the potential involvement of L-type calcium channels in the ES-mediated regenerative response (Hofmann et al., 1987; Collins and Lile, 1989; Helton et al., 2005). Nonetheless, nickel at the concentration we used does not appear to be responsible for ES-induced neurite outgrowth, and in order to dissect out the link between the observed rise in intracellular calcium and neurite outgrowth associated with ES, additional studies using a variety of pharmacological blockers targeting specific VGCCs are required.

4.6.4 ES reduces intrinsic barriers of peripheral neuron outgrowth

Intracellular inhibition of growth is one of the potential mechanisms for confining outgrowth of peripheral neurons and mediating the limited recovery following peripheral nerve injuries (Christie et al., 2010). PTEN and RhoA are two important molecules expressed at the time of regeneration that impede the desired regrowth. RhoA, although not explored in detail here, is present and expressed in the central and peripheral axon growth cone (Cheng et al.,
Many extrinsic inhibitory molecules converge on RhoA kinase and limit the growth of central axons (Niederost et al., 2002; Conrad et al., 2005). In peripheral neurons, RhoA restricts regeneration of transected axons (Cheng et al., 2008). Interestingly, in this study, we observed a significant decrease in its expression in response to ES. Udina et al. (2008) previously reported that ES enhances cAMP levels in sensory neurons and resembles a pre-conditioning injury. cAMP inhibits RhoA and facilitates outgrowth of injured axons. The decrease in RhoA could be secondary to an increase in cAMP levels (Laudanna et al., 1997; Dong et al., 1998).

Additionally, we observed a robust decrease in PTEN expression, which inhibits the PI3-K/Akt signaling pathway, a critical survival and regenerative pathway in peripheral neurons downstream of neurotrophins (Hemmings and Restuccia, 2012). Nusser et al. (2002) reported that PI3-K activation increases Rac1 activity and causes transient inactivation of RhoA during early axon outgrowth. Using PI3-K inhibitors, LY294002 and wortmannin, the authors further suggested the involvement of the PI3-K in NGF-induced RhoA translocation (Nusser et al., 2002).

PTEN inhibition was associated with enhanced regeneration of retinal ganglion cells by phosphorylation and activation of ribosomal S6k and mTOR (Park et al., 2008). We also found a similar rise in ps6k expression in peripheral sensory neurons in response to ES. Christie et al. (2010) showed that PTEN is widely expressed in injured neurons, axons and SCs. Transient inhibition of PTEN using siRNA leads to facilitation of peripheral nerve regeneration with upregulation of pAkt and pGSK-3β (Christie et al., 2010). In this work, with the application of ES, we also observed a concurrent increase in pAkt and a reduction in GSK-3β, both convergent onto growth cone extension and axonal outgrowth (Kimpinski and Mearow, 2001; Eickholt et al., 2002). Benefits of ES were interrupted with LY294002, a pharmacological inhibitor of the PI3-
K. Neurite outgrowth, and the number of processes and branches per neuron, were all reduced significantly in the presence of LY294002. This suggests that the PI3-K pathway is crucial for regeneration of peripheral neurons and that ES targets this pathway to boost the regenerative machinery. Based on this work, we suggest that ES promotes a pro-survival PI3-K pathway in two ways: by producing higher levels of growth factors such as BDNF, which can stimulate this pathway; and also by reducing endogenous inhibitors of this pathway, such as PTEN.

4.7 Conclusions

In this Chapter, I showed that ES-mediated peripheral nerve regeneration includes: increased expression of RAGs such as β-tubulin and GAP-43 in DRGs and the neurotrophin BDNF in cultured sensory neurons; activation of the PI3-K signaling pathway primarily through a decrease in PTEN expression; and enhanced communication between DRG neurons and perineuronal satellite cells. The results also indicate that the ES-mediated regenerative response is associated with a rise in neuronal calcium concentration that may not involve the low-voltage activated T-type calcium channels. In the next Chapter, I will explore the impact of ES in a challenging animal model of diabetes with severe regenerative deficits.
Chapter Five: Electrical Stimulation Promotes Peripheral Nerve Regeneration in an Animal Model of Diabetic Neuropathy
5.1 Abstract

Peripheral nerve regeneration is compromised in diabetes mellitus. As detailed in Chapters 1 and 3, a short extracellular electrical stimulation (ES) in nondiabetic nerves, applied immediately after nerve injury augments axon regeneration, target reinnervation and functional benefits (Al-Majed et al., 2000b; Singh et al., 2012b). The potential impact of this intervention in injured diabetic nerves, a common but more challenging scenario, is uncertain. The present study explored the impacts of ES on functional recovery of injured sciatic nerves of diabetic mice.

In this work, we used STZ-induced type 1 diabetic mouse model. First, based on our mechanistic data from Chapter 4, we analyzed expression of PTEN in diabetic sensory neurons. Following this, a cohort of 2-month type 1 diabetic mice was divided into ES and sham stimulation groups following sciatic nerve crush. ES (3 V, 20 Hz, 1 h) or sham stimulation was applied proximally to the injured nerves. Nerve conduction and behavioural (response to noxious mechanical and thermal stimuli) studies were carried out on day 0 (before) and 14 and 28 days post nerve injury. In separate experiments in vitro, the neurite outgrowth of isolated adult diabetic sensory neurons was measured following an ES paradigm (200 mV, 20 Hz, 1 h) applied through a microelectrode array.

Diabetic mice were hyperglycemic and gained less weight than nondiabetic controls. Motor and sensory conduction velocities were significantly reduced in diabetic mice compared to nondiabetics. Diabetic animals were hypoalgesic to thermal and mechanical stimuli. PTEN expression was increased in diabetic DRGs as measured by qRT-PCR, western immunoblotting and immunohistochemistry. In vitro, neurite outgrowth was higher in stimulated diabetic sensory neurons than controls. Focal sciatic nerve injury was associated with a loss, then gradual
recovery of SNAP and CMAP amplitudes during regrowth, but these recovered less well in diabetics, as did their associated sensory and motor conduction velocities. Application of ES improved the recovery of CMAPs and motor conduction velocities at 28 days following injury in diabetics. The density of regenerating intraepidermal fibers and tibial myelinated axons were higher after ES in diabetics. Overall, extracellular ES facilitates robust nerve regeneration in a diabetic animal model, despite its inherent limitations in regeneration.

5.2 Introduction

Diabetes mellitus is one of the most prevalent diseases of the modern world (Danaei et al., 2010). Diabetes targets multiple organs including the nervous system and causes degeneration of both sensory and motor neurons (Amos et al., 1997; UKPD Group, 1998; Eppens et al., 2006). Peripheral nerve injuries are unfortunately very common and despite the advancement in surgical repair, patients often suffer from life-long disabilities (Kelsey, 1997; Stoll and Muller, 1999). These injuries are further complicated in diabetic nerves owing to neuroregenerative failure. Diabetes generates a ‘double hit’, in which diabetic neurons and axons that are already targeted by a degenerative process, have a poor regenerative response to additional injury (Kennedy and Zochodne, 2005b). Numerous mechanisms of regenerative failure have been proposed in diabetic nerves that include: microangiopathy, impaired Wallerian and Wallerian-like degeneration in the distal stump, insufficient support from partner SCs and reduced trophic factor availability (Greene et al., 1988; Bradley et al., 1995; Kennedy and Zochodne, 2000, 2005b; Ebenezer et al., 2011). Moreover, slower nerve regeneration was also observed in diabetic patients without features of neuropathy, indicating that diabetes impairs neuroregeneration at early stages.
Despite several mechanisms of neuroregenerative failure that have been identified; only a small number of them offer strategies for nerve repair.

PTEN acts as a regenerative brake and negative regulator of the PI3-K pathway, an essential pathway for cell survival, proliferation and regeneration (Hemmings and Restuccia, 2012). As discussed in Chapter 2, inhibition of PTEN enhances regeneration of central and peripheral neurons (Park et al., 2008; Christie et al., 2010). PTEN expression in diabetic neurons has not been previously described. We hypothesized that intrinsic growth capabilities of diabetic sensory neurons are impaired, and PTEN might be implicated in neuroregenerative failure. Finally, we exploited ES as a therapeutic approach to heighten the intrinsic growth capabilities of these neurons and evaluated whether its potential benefit might be linked to PTEN.

As discussed in Chapters 1 and 3, a short extracellular ES applied immediately after nerve injury augments nerve regeneration and enhances intrinsic growth capabilities of sensory neurons (Chapter 4) (Al-Majed et al., 2000b; Gordon et al., 2010b; Singh et al., 2012b). The role of ES on peripheral nerve regeneration in the settings of diabetes is unknown. This study explored the potential impact of ES on neurite outgrowth in cultured adult diabetic sensory neurons \textit{in vitro} and its benefits on \textit{in vivo} axon regeneration of injured diabetic peripheral (sciatic) nerves.

\subsection*{5.3 Specific Hypothesis \#3}

\textit{An extracellular ES paradigm can overcome challenging regenerative environment associated with diabetes and promote axon regeneration of diabetic nerves.}
5.3.1 Objectives

Objective #1: To determine the expression of PTEN in both normal and injured diabetic DRG neurons. Experiments will examine mRNA and protein expression in diabetic and nondiabetic DRGs before (uninjured) and following sciatic nerve crush.

Objective #2: To establish whether ES increases neurite outgrowth of cultured adult diabetic sensory neurons in vitro.

Objective #3: To examine whether ES enhances axonal regeneration and target reinnervation following sciatic nerve crush in diabetics. Experiments will measure the number of myelinated axons in the distal nerve and the reinnervation of cutaneous skin targets.

Objective #4: To determine whether ES improves functional and behavioural outcomes following peripheral nerve injury in diabetics. Motor and sensory electrophysiological recovery and behavioural recovery of sensation will be carried out following sciatic nerve injury and ES.

5.4 Materials and Methods

5.4.1 Animal model of diabetes

Outbred CD-1 mice of body weight 18-20 g (Charles River, Canada) and db/db (BKS.Cg-Dock7m +/+ Leprdb/J) (Jackson Labs), a model of type 2 diabetes, were used in the study. CD-1 mice were made diabetic (type 1) by intraperitoneal streptozotocin (STZ, sigma, USA) injected over three days at the doses of 85, 70 and 55 mg/kg. STZ was dissolved in the citrate buffer (pH=4.5). Control non-diabetic CD-1 mice received intraperitoneal injections of citrate buffer only (pH=4.5). Diabetes was confirmed using measurement of blood glucose and
body weights at 1 and 2 months after STZ injections. Blood glucose was measured using a glucometer (Ultra One, Life First, USA).

**5.4.2 PTEN expression**

Detailed immunohistochemistry methodology is provided in Chapter 2. L4-L5 DRGs were harvested from control and STZ injected CD-1 mice at two months of diabetes, and db/db mice at 8 weeks of age. Ten micrometer thick sections were placed onto poly-L-lysine coated glass slides. Primary antibodies applied were PTEN and ps6k and incubated at 4°C for 24 h. The secondary antibodies used were: anti-mouse Cy3 or Alexa Fluor 488 goat anti-rabbit. The slides were viewed with a fluorescent microscope (Axioscope, Zeiss Canada). Negative controls included omission of primary antibodies on parallel sections (not shown). Pixel intensity measurements and analysis were performed using Adobe Photoshop CS4 (Adobe Systems). For semi-quantitative analysis of PTEN expression in neurons, they were divided into four categories of arbitrary intensity levels: >100 (arbitrary units) were considered positive, medium (100-150), strong (150-200) and very strong (>200). Measurements included the total number of neurons in each category. A total of 3 sections per mouse were analyzed (approximately 250–500 neurons/mouse). For neuronal size analysis, luminosity and size of each neuron from n=3 DRG sections/group (approximately 400-500 neurons from each of control and diabetic group) were measured provided they were approximately centrally sectioned, as indicated by DAPI nuclear staining. Luminosity and size was measured using Image J (NIH software). Neuronal size and their respective luminosities were plotted on a graph by subdividing neurons into a pool of >15 µm, 15-20 µm, 20-30 µm, 30-40 µm and >40 µm based on their size.
5.4.3 Sciatic nerve crush

Under isoflurane anaesthesia, and using aseptic techniques, the left sciatic nerve of type 1 diabetic mice (n=7/group) was exposed and crushed just distal to the sciatic notch with sterile forceps for 15 sec. The same experimenter performed the crush to maintain consistency between the animals. Sciatic nerves proximal to the crush site were immediately stimulated using bipolar electrode hooks and the same stimulation paradigm that is detailed in Chapter 2. Control sham stimulation animals underwent the same procedure but the stimulator was not switched on.

5.4.4 Analysis of regenerating myelinated sensory and motor axons

Tibial nerve samples distal (~10 mm) to the crush site were harvested at 21 days post nerve injury with and without stimulation. The detailed method is described in Chapter 2. The tibial nerve sections were photographed under oil immersion microscopy (100X) in several non-overlapping fields to cover the entire nerve area. In each field, the numbers and caliber of unequivocal myelinated axons were then measured. Final measurements included axon density, number of myelinated axons and axon diameter (Scion image, Frederick, MD, USA).

5.4.5 Electrophysiology

Multifiber motor and sensory conduction recordings were carried out in left sciatic–tibial fibers in mice anesthetized with isoflurane at day 0 (basal, before injury) and 14 and 28 days post nerve crush as described in Chapter 2 (Kennedy and Zochodne, 2000).
5.4.6 Immunohistochemistry and analysis

Footpad skin samples were harvested at 21 days after sciatic nerve crush with and without stimulation following the same protocol described in detail in Chapter 2. The immunohistochemical approach was identical to published work (Kennedy and Zochodne, 2005a; Kan et al., 2012). Skin sections of 25-μm thickness were incubated with rabbit PGP9.5 primary antibody followed by goat anti-rabbit Cy3. Images were captured using an Olympus laser scanning confocal microscope. Epidermal fibers labeled with PGP9.5 were counted in five adjacent fields of six sections for a total 30 fields per mouse at each time point. Both vertical (trajectory approximately 90° to the surface of the skin) and total axon profiles were analyzed. The total epidermal area analyzed was traced and the area measured to calculate axon densities.

5.4.7 Functional recovery of sensation

Mice underwent mechanical (von Frey filaments) and thermal (Hargreaves) testing at day 0 (before crush injury), 14 and 28 days following sciatic nerve crush according to the detailed protocol in Chapter 2. Three separate trials were performed for the withdrawal response. Mechanical and thermal testing were performed on identical days with an interval of at least 1 h between the two tests.

5.4.8 In vitro studies of adult sensory neurons

DRGs (L4-L5) were dissected from wild type and STZ-induced diabetic mice after two months duration. The detailed procedure for adult sensory neuronal cell culture is described in Chapter 2. Isolated DRG neurons were plated in low density onto the precoated MEA containing
growth medium similar to that described in Chapter 2 with an addition of 10 ng/mL NGF (Invitrogen). Diabetic sensory neuronal cultures were maintained in 30 mM glucose in media. Twenty-four hours later, MEAs underwent either sham stimulation or ES (200 mV, 20 Hz, 1 h) using a Grass stimulator (West Warwick, U.S.A.). Sensory neurons were viewed by phase contrast using an inverted microscope (Zeiss Canada, Toronto) at 24 h after ES (48 h in culture). Images were taken and total neurite outgrowth, number of primary neurites (defined as processes extending from the soma), length of the longest neurite and the number of branches of a primary neurite were analyzed and quantified by MetaXpress (Molecular Devices).

5.4.9 Western immunoblot

DRGs (L4-L5) and sciatic nerves from adult wild type and 2 month type 1 diabetics were harvested before (day 0, basal) and 3 and 6 days post nerve crush injury in RIPA lysis buffer containing protease and phosphatase inhibitors (Roche Diagnostics). The detailed protocol is in Chapter 2. Briefly, the blots were labeled with PTEN and ps6k antibody and actin was used as a loading control. Secondary antibodies used were anti-rabbit IgG HRP and anti-mouse IgG HRP. A Table of antibodies used is provided in Chapter 2. The protein signal was detected by exposing the blot to enhanced chemiluminescent reagents (Amersham, Canada). The film images were digitized and the difference between the treatments was measured using pixel intensity and the size of the band using Adobe Photoshop (Adobe, San Jose, CA). The values were represented as the percentage difference from the control, with control values assumed as 1.
5.4.10 Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The detailed protocol is described in Chapter 2. Total RNA was extracted using Trizol reagent from control and diabetic DRGs before (basal, day 0) and day 3 and 6 after crush injury and first strand DNA was synthesized utilizing SuperScript II First-strand Synthesis Kit (Invitrogen, Inc.). The primer sequences used were designed with Primer Express 2.0 (Applied Biosystems, Foster City, CA) and are listed in Table 2.2 in Chapter 2. Relative expression values were generated using the comparative CT method \(2^{-\Delta\Delta CT}\) where all the genes of interest were standardized to expression of the housekeeping gene 18S.

5.5 Analysis

Results were presented as mean ± SEM and two groups compared using an unpaired Student’s t-test (two-tailed unless otherwise indicated). Statistical significance was accepted at the level of \(p < 0.05\).

5.6 Results

5.6.1 Diabetic polyneuropathy (DPN) model

The STZ-induced type 1 diabetic mouse model is well characterized for the features of diabetic polyneuropathy (DPN) (Yagihashi, 1995; Kalichman et al., 1998; Sullivan et al., 2007). In this study, diabetic mice injected with STZ were severe hyperglycemic within 2 weeks of injection and elevated glucose levels were maintained throughout the study (Fig. 5.1a). Two months following diabetes induction, diabetic mice were lower in body weight compared to wild
type (WT) controls (Fig. 5.1b). In accordance with previous reports, the diabetic animals at 2 months of diabetes developed features of neuropathy: motor and sensory conduction velocities were reduced (Fig. 1c,d), compound muscle action potential (CMAP) amplitudes were lower (Fig. 5.1e), and abnormal sensation was evidenced by development of thermal and tactile hypoalgesia (Fig. 5.1g,h) (Yagihashi, 1995; Kalichman et al., 1998; Sullivan et al., 2007).
Figure 5.1 Characterization of chronic animal model of diabetic polyneuropathy.

A,B: Blood glucose levels 2 months after STZ injection were significantly higher (A) and the gain in body weight was smaller in diabetics than WT controls (B). C-E: Electrophysiological recordings of control and diabetic nerves. Motor and sensory CVs and CMAP amplitudes were significantly reduced in 2-month old diabetics (C-E) with no significant change in SNAP amplitudes being noted (F). G,H: diabetic animals were hyposensitive to mechanical (G) and thermal stimuli (H). A total of 10 WT and 26 type 1 diabetic animals were analyzed. Values are represented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, Student’s t-test.
5.6.2 Expression profile of PTEN, a potential growth barrier, in diabetic DRGs

The expression of PTEN within uninjured and injured adult DRGs and peripheral axons has been examined (Christie et al., 2010), however its expression in diabetic sensory neurons and nerves has not been analyzed. Our goal was to determine expression of PTEN mRNA and protein in diabetic and WT DRGs within uninjured adult DRGs and following a sciatic nerve injury. PTEN mRNA levels were substantially higher in diabetic DRGs compared to wild type controls (Fig. 5.2a). Similarly western blot analysis showed higher expression of PTEN in diabetic DRGs (Fig 5.2b,c). The observed rise in PTEN can result from neuronal and nonneuronal cells; to confirm that upregulation involved neurons, we utilized an immunohistochemical approach and assessed PTEN expression in diabetic and nondiabetic DRGs (Fig. 5.2d) We quantified the neuronal luminosity (described in methods), and found that in type 1 diabetes, PTEN expression was present in higher numbers of sensory neurons with greater luminosity intensity (Fig. 5.2f). There was a nonsignificant trend towards increased PTEN protein expression in DRGs of the db/db, type 2 diabetic animal model. For future studies, we used type 1 diabetic models thereafter. We previously reported that PTEN expression is selectively higher in small sensory neurons (Christie et al., 2010). We hypothesized that the presence of a higher number of PTEN positive neurons in diabetes comes from an unexpected gain-of-expression of PTEN in small and medium size neurons under the diabetic environment. Supporting this idea, we found higher numbers of medium to large size PTEN positive neurons in diabetic mice (Fig 5.3a). Next, we wondered how PTEN expression is altered after nerve injury in control and diabetic DRGs and whether the observed upregulation in diabetics is maintained following injury. Consistently in diabetics, higher expression of PTEN mRNA persisted 3 days after sciatic nerve injury (Fig 5.2f). Similarly, protein expression in diabetic DRGs was higher on day 3 and 6 after sciatic
nerve injury compared to corresponding controls (Fig. 5.2g). PTEN expression was also upregulated in nondiabetic controls indicating that regenerative brakes were present to diminish nerve regeneration following injury. PTEN inhibition enhances axonal regeneration in CNS neurons by upregulation of its downstream mediator, ps6k ribosomal protein, a molecule essential for protein synthesis and growth cone formation (Park et al., 2010). We observed significantly lower levels of ps6k in diabetic sensory neurons compared to wild type controls with both western blotting and immunohistochemistry (Fig. 5.3b,c). Overall these findings led us to hypothesize that regeneration deficits in diabetes may be attributed to higher PTEN expression, which in turn may lead to a decreased intrinsic growth state of neurons.
Figure 5.2 PTEN expression was elevated and extensive in adult diabetic sensory neurons.

A. PTEN mRNA transcripts in wild type (WT) and diabetic DRGs indicating higher expression of PTEN in diabetics. B. Western immunoblots labeled with PTEN in WT and diabetic DRGs before (basal, day 0) and 3 and 6d after sciatic nerve injury. Actin is used as a loading control. C. Quantification of protein data from B. Note: a robust rise in basal expression of PTEN protein in diabetic DRGs compared to nondiabetics. D. Examples of adult sensory neurons from WT, STZ-induced type 1 diabetic, and db/db, type 2 diabetic DRGs labeled with PTEN (green). Note that PTEN is expressed in a larger number of neurons with higher luminosity in diabetics. F. Quantification of neuronal luminosity (described in the methods section). Note a shift in the percentage of neurons with higher luminosity intensity specifically in type 1 diabetes compared to controls. G. PTEN mRNA levels in WT and diabetic DRGs 3d after sciatic nerve injury. H. WT and type 1 diabetic DRGs immunostained with PTEN (green), before (basal, day 0) and 3 and 6d after sciatic nerve injury. For each of immunohistochemistry, western blotting and qRT-PCR experiments, n=3-5 animals per group/time point was used. Values are represented as mean ± SEM. *p<0.05, Student’s t-test, Scale bar =50 µm.
Figure 5.3 PTEN is expressed in small to medium and large sized diabetic sensory neurons with a concurrent decrease in its downstream target, ps6k.

A. Neuronal size analysis from WT and type 1 diabetic sensory neurons indicates a wider expression of PTEN. PTEN expression is significantly higher in smaller neurons with concurrent expression in medium to large caliber sensory neurons in diabetics. B, C: Marked decline in ps6k protein in adult 2 month diabetic DRGs and littermate controls analyzed using western blot (B) and immunostaining (C). Note: ps6k is mainly expressed in sensory neurons. For size analysis, n=3 DRG sections/group (approximately 400-500 neurons were analyzed from each of diabetics and nondiabetics). Values are represented as mean ± SEM. *p<0.05, Student’s t-test, scale bar =50 µm.
5.6.3 Enhanced neurite outgrowth of adult diabetic sensory neurons in response to ES

We observed in Chapter 3 that ES enhances neurite outgrowth of nondiabetic adult sensory neurons isolated from rats; however, the impact of ES on diabetic neurons has not been examined. At first, we wondered whether sensory neurons harvested from a challenging diabetic environment poses a significant difficulty in extending their neurites during \textit{in vitro} conditions with the maintenance of high glucose levels in the growth media. In our study, diabetic neurons were similar to nondiabetics in terms of baseline neurite outgrowth properties (data not shown). Adult sensory neurons grown on MEA were electrically stimulated 24 h after seeding and neurite outgrowth was measured 24 h later (48 h post seeding). As described in Chapter 3, ES significantly enhanced the neurite outgrowth and number of processes per neuron in cultured adult nondiabetic sensory neurons. ES with a similar stimulation paradigm significantly enhanced neurite outgrowth and the number of branches per neuron in cultured diabetic neurons (Fig 5.4a-d). ES was also associated with a nonsignificant trend toward improvement in the length of the longest regenerating neurite and number of processes per neuron (Fig 5.4e,f). Increase in neurite outgrowth of diabetic neurons with a direct neuronal stimulation supports the idea of a neuron autonomous impact of ES.
Figure 5.4 ES increases neurite outgrowth of adult diabetic sensory neurons.

A, A’, B, B’: Examples of adult diabetic sensory neurons grown in vitro on MEA with and without ES. In each image, an inset is shown with an enlarged neuron and its processes. Stimulated neurons have more robust outgrowth. C-F: Quantification of total neurite outgrowth (C), branches per neuron (D), longest neurite length (E) and processes per neurons (F). ES was associated with a significant increase in neurite outgrowth and number of branches per neuron and trended toward a nonsignificant improvement in the number of processes per neuron and length of longest regenerating neurite. Values are represented as mean ± SEM from n=3 separate in vitro experiments. p<0.05, unpaired Student’s t-test.
5.6.4 ES improved functional electrophysiological recovery

ES significantly improved CMAP amplitudes in nondiabetics after sever transection injuries (Chapter 3). Previous findings also indicated that a brief ES paradigm enhances the muscle target reinnervation (Gordon et al., 2007; Gordon et al., 2008; Gordon et al., 2010b) and the skin epidermis reinnervation (Chapter 3). Similarly, ES was associated with an improvement in behavioural pain sensitivity in nondiabetics (Chapter 3); however all these measurements are not assessed in diabetic nerves, where axons are already undergoing a degenerative process (Kennedy and Zochodne, 2000, 2005a). The goal of these experiments was to analyze electrophysiological and behavioural recovery in diabetic nerves following crush injury and ES. ES had no impact on blood glucose and body weights of control and diabetic animals (Fig 5.5a,b). Electrophysiology and behavioural studies were conducted at day 0 (basal) and 14 and 28 days post nerve crush. We were not able to record either motor or sensory potentials at day 14 indicating ongoing denervation of muscle and skin targets at that time. Pre-injured CMAP amplitudes were lower in diabetic mice compared to citrate controls (Fig 5.6a). Crush injury to sciatic nerves abolished CMAP amplitudes and motor conduction velocities that recovered slowly. The recovery was slower than concurrent nondiabetics studied in a separate work (data not shown). ES significantly rescued these deficits but values were still lower compared to pre-injury levels (Fig 5.6a,b). SNAPs were also lower in diabetics and were similarly interrupted by crush injury followed by slow recovery. ES was associated with a nonsignificant trend towards improvement in the SNAP amplitude and conduction velocity (Fig 5.6c,d). In sensory behaviour testing, before injury, diabetics were hyposensitive for both thermal and mechanical sensitivity. Nerve injury had a varied impact: diabetic animals trended toward hypersensitivity to mechanical
sensation and decreased responsiveness to thermal stimuli. Sciatic lesions are not associated with complete loss of sensation in the hindlimbs of rodents, likely because of compensatory function in neighbouring nerve territories (Zochodne lab, unpublished data). On day 28, ES had a significant impact on mechanical pain sensation, animals were responding at lower threshold compared to sham stimulation. These changes might be attributed to enhanced target reinnervation or sprouting of the axons locally in response to ES. ES had no significant impact on thermal pain sensitivity (Fig 5.6e,f).
Figure 5.5 ES has no impact on blood glucose and body weights of diabetic mice.

A, B: Blood glucose and body weight levels before injury (2 months diabetics) and 28 days post nerve crush injury with and without diabetes. No change in blood glucose levels and body weight gain was noted with ES. n=5-7 animals/group. Values are represented as mean ± SEM.
A

Blood Glucose (mmol/L)

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<td>28 days after crush injury</td>
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Diabetics

B

Body Weight (g)

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<td>Baseline (before injury)</td>
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<tr>
<td>28 days after crush injury</td>
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Diabetics
Figure 5.6 Electrophysiological recovery in diabetics with ES.

Electrophysiological parameters (CMAP and SNAP amplitudes, sensory and motor conduction velocities) were not measurable at day 14 post nerve crush indicating a loss of these parameters after nerve injury and then slowly regained over the time with axon regeneration and target reinnervation. This also suggests a severe failure of regeneration in diabetics and continuing denervation of target organs at that time. A: CMAPs before (day 0) and 28 days after injury. Preinjured CMAP amplitudes were lower in diabetics compared to citrate controls. CMAP amplitudes were abolished following sciatic nerve injury, which recovered slowly in diabetics. ES significantly rescued this fall in amplitude but remained lower than preinjury values. B: Motor conduction velocities were also interrupted with sciatic nerve crush and gradually improved. ES was associated with significant improvement at day 28 following injury. C: Preinjured SNAPs (day 0) were lower in diabetics than controls and abolished after nerve injury (day 14, not measurable) and then slowly recovered at 28 days after injury. ES had no significant impact on SNAP amplitudes. D: Sensory CVs before (day 0) and 28 days post injury. Sensory conduction velocities in regenerating sensory axons were interrupted after nerve injury and similar to the SNAP amplitudes, ES had no significant impact on sensory conduction velocities at day 28 after injury. Values are represented as mean ± SEM. *p<0.05 Student’s t-test, n=6-8 animals/group.
5.6.5 **ES increased the number of regenerating myelinated axons in tibial nerves**

In Chapter 3, we reported that ES resulted in an increased number of regenerating myelinated axons beyond a nerve crush zone 3 weeks after injury. This early maturation of regeneration was associated with enhanced target reinnervation and functional improvements. After taking into consideration this robust regeneration in normal mice, we explored whether a similar stimulation paradigm has benefits in a more challenging regeneration model such as that associated with diabetes. We observed a reduction in the number of axons repopulating the distal tibial nerve in diabetics three weeks after injury (Fig 5.7c). ES demonstrated a significant increase in myelinated axon counts in the distal stump of regenerating tibial nerves compared to sham stimulation (Fig 5.7a-c). Mean axonal diameter representing axonal caliber and maturation of regeneration trended toward larger values in the ES group animals compared to sham stimulation (Fig 5.7a,b,d). This robust improvement in axonal regeneration may have been important in supporting later target reinnervation in the skin and muscle.

5.6.6 **ES is associated with greater epidermal reinnervation**

After injury, the distal part of the nerve undergoes degeneration and new axons are required to regrow and reach skin targets in the footpad to repair sensation. This typically takes 3-4 weeks after crush injury and approximately 7-8 weeks after transection injury (Kennedy and Zochodne, 2000; Polydefkis et al., 2004). In nondiabetic thy-1 YFP mice, with in vivo live animal imaging, we observed an earlier and enhanced arrival of cutaneous axons in the ipsilateral toepads following sciatic nerve transection and stimulation (Chapter 3). We explored whether a similar ES approach could increase epidermal reinnervation in diabetic footpads after nerve injury,
which already have deficits in the intraepidermal nerve fiber density. We analyzed vertical (perpendicular to the surface of skin) and total axons in the epidermis of diabetic animals after injury, with and without ES. As expected, the numbers of axons reinnervating 3 weeks after sciatic nerve injury were trended towards lower values in diabetics, indicating regeneration deficits (Fig 5.7d-g). ES of injured diabetic nerves rescued this deficit and both vertically directed and total epidermal axons were significantly increased with ES 3 weeks after injury (Fig 5.7d-g)
Figure 5.7 Augmented repopulation of myelinated axons in the distal tibial nerve and improved skin target reinnervation in response to ES.

A, B: Representative images of semi-thin sections of regenerating distal tibial nerves (10 mm from the injury site) harvested 21 days post sciatic nerve crush from the diabetic mice, with and without ES. C: Quantification of total myelinated axons. The numbers of regenerating myelinated axons were significantly lower in diabetic animals indicating a severe regeneration deficit. The wild type (WT) control values are from nondiabetic mice studied concurrently in a separate work. ES enhanced the number of regenerating myelinated axons in distal tibial nerves. D: Mean axon diameter of myelinated axons. This was calculated by measuring axonal caliber of each counted myelinated axon. ES has a nonsignificant trend towards improvement in axon diameter. E,E’,F,F’: Representative images of diabetic footpads harvested 21 days post nerve crush with and without ES. Newly regenerating terminal sensory axons crossing the dermis-epidermis border and reinnervating the skin epidermis are visible. G,H: Quantification of the vertical skin fiber density and total skin fiber density. ES significantly improved reinnervation outcomes in diabetic skin. Values are represented as mean ± SEM. *p<0.05 Student’s t-test, n=5-7 animals/group, Scale bar = 20 µm.
5.7 Discussion

The major findings of this work are: 1) diabetic mice demonstrate features of diabetic neuropathy including abnormalities in electrophysiological measurements (decrease in sensory and motor conduction velocities, reduced CMAP amplitudes) and decreases in mechanical and thermal sensitivity; 2) diabetic mice had severe defects in their neuroregenerative response as evidenced by reduced motor and sensory conduction velocities and amplitudes after injury, reduced reinnervation of the epidermis, and impaired repopulation of myelinated axons; 3) ES was associated with a robust rise in neurite outgrowth and number of branches in adult diabetic sensory neuronal cultures in vitro; 4) ES significantly improved motor electrophysiological recovery (improved motor conduction velocity and CMAP amplitudes and) and there were trends towards improvement in sensory electrophysiology; and 5) there was enhanced repopulation of distal tibial nerve segments and reinnervation of target skin organ in diabetic mice with ES.

5.7.1 Chronic diabetic neuropathy and regeneration deficits

The mouse model of diabetic neuropathy used in this work closely resembled human diabetic neuropathy with features that include conduction slowing, loss of sensation, and loss of epidermal axons (Yasuda et al., 2003; Zochodne, 2008a). Diabetic neuropathy has initial features of a reversible metabolic abnormality but later on irreversible structural and functional malfunction arises (Ziegler, 2011). In our model, blood glucose levels remained high throughout and there was no spontaneous recovery during the study period. We found significantly impaired motor and sensory conduction velocity. Decreases in CMAP amplitudes were consistent with the distal degeneration of axons in diabetic nerves (Kennedy and Zochodne, 2000, 2005a). Axon loss
is more prominent in sensory terminals and is targeted first during diabetes (Dyck et al., 1986). Behavioural deficits with reduced thermal and mechanical sensitivity parallel this loss (Zochodne, 2008a; Ziegler, 2011).

Neuroregenerative deficits are prominent in diabetics. Superimposed focal injuries on degenerating diabetic axons have significant bearing on axon regeneration (Kennedy and Zochodne, 2000). PTEN is a regenerative roadblock that influences the intrinsic growth capabilities of retinal ganglion cells (Park et al., 2008) and peripheral sensory neurons (Christie et al., 2010). Surprisingly, the levels of PTEN mRNA and protein were substantially higher in diabetic sensory neurons, a novel finding. Furthermore, levels of ps6k, a protein necessary for neuronal protein synthesis and downstream of PTEN, was significantly reduced in diabetic DRGs, similar to that observed in retinal ganglion cells after injury (Park et al., 2008). Recently, it has been shown that a PTEN mutation is associated with insulin resistance in patients (Pal et al., 2012) and activation of the PI3-K pathway through deletion of PTEN in the skeletal muscle protects animals from developing insulin resistance (Wijesekara et al., 2005). Additionally, deletion of PTEN in adipose tissue improves insulin sensitivity in mice (Kurlawalla-Martinez et al., 2005). These studies indicate a potential role of PTEN in diabetes and its progression, but do not relate specifically to its neurological or other complications. Our observation of enhanced expression of PTEN with a simultaneous reduction in ps6k expression in adult diabetic sensory neurons suggests that the PI3-K pathway may be compromised during diabetic neuropathy, and could be one of the potential mechanisms behind reduced intrinsic growth capabilities and the development of neuroregenerative failure. In Chapter 4, we observed that ES has a potential to improve intrinsic growth capabilities in nondiabetics: mechanisms include upregulation of neurotrophins, especially BDNF (Chapter 4 and Geremia et al., 2007), regeneration-associated
genes (Chapter 4 and Al-Majed et al., 2004) and cAMP (Udina et al., 2008), rises in intracellular calcium, and decreases in PTEN and RhoA (Chapter 4). Based on these observations, in this work, I used ES as a modality to improve axonal regeneration in a diabetic model.

5.7.2 ES enhanced neurite outgrowth of diabetic sensory neurons

Neurite outgrowth of adult type 1 diabetic sensory neurons was comparable to control values. We have observed similar results in the past with type 1 diabetic mouse and rat models in accordance with previous literature (Sango et al., 1995). However, Luo et al. (2002) reported impaired neurite outgrowth of diabetic neurons whereas Saito et al. (1999) and Sango et al. (2002) showed instead improved neurite outgrowth of diabetic DRGs. Not all of these studies may have applied appropriate concentrations of glucose in their media to match in vivo and in vitro exposure before testing. Although no difference in baseline outgrowth was observed, the impact of ES was robust on diabetic neurons and significantly enhanced neurite outgrowth and number of processes per neuron. These results demonstrated a neuron autonomous impact of ES originating at the cell body level.

5.7.3 Enhanced repopulation of tibial nerves with myelinated axons and accelerated target reinnervation

The morphometric analysis identified severe regenerative constraints in diabetic animals. The numbers of regenerating myelinated axons in the distal tibial nerves were significantly lower in diabetics compared to nondiabetics. This was in accordance with previous reports indicating similar decrease in regenerative myelinated axons after crush and transection injuries (Kennedy
and Zochodne, 2000). With ES, distal nerve segments were repopulated with elevated number of myelinated axons. In nondiabetics, we observed a similar rise in myelinated axons with a higher number of regenerating backlabeled motor neurons in the spinal cord (Chapter 3). Significant benefits of an extracellular ES in both nondiabetics with severe transection injuries and in a regeneration deficient diabetic animal model indicate a robust impact of ES in peripheral nerve repair. For successful regeneration to occur, enhanced axonal regeneration should later translate into target reinnervation and subsequent behavioural recovery. Decreased intraepidermal nerve fiber density (IENFD) is a hallmark of diabetic neuropathy in animals and humans (Polydefkis et al., 2001). Reinnervation is frustratingly slow after nerve injury and is additionally aggravated by superimposed diabetes (Kennedy and Zochodne, 2000; Polydefkis et al., 2004). In Chapter 3, with ES, we observed an enhanced and earlier cutaneous target reinnervation following severe transection injuries in thy-1 YFP animals. In this work, in diabetic animals, application of ES was associated with a rise in the density of reinnervating epidermal axons after crush injury. While we did not analyze subclass distinctions among these regrowing axons, we instead analyzed functional and behavioural recovery with and without stimulation in diabetics.

5.7.4 Electrophysiological recovery and functional outcome

The present study showed an overall significant improvement in motor nerve conduction velocity and amplitudes and a nonsignificant trend towards improvement in sensory electrophysiological measurements. The increase in CMAP amplitude was associated with an increase in the number and caliber of myelinated axons in distal nerve segments. During regeneration the maturity of axons with an increase in axonal diameter is coupled with enhanced
myelination (Waxman, 1980). Our finding of a higher motor conduction velocity likely indicates enhanced myelination of regenerating axons (Patricia, 2007; Zochodne, 2008b). Sensory fibers are degenerated early in diabetes and are severely involved, for example, sensory afferent in the epidermis are lost during the pre-diabetic state (Polydefkis et al., 2004; Zochodne, 2008a). Provided that sensory neurons are already degenerated, nerve injury added further damage to these fibers (Kennedy and Zochodne, 2005b). More advanced sensory axon pre-existing damage with superimposed injury may be one of the plausible explanations for the varying electrophysiological response.

Neuropathic pain is one of the most common symptoms of diabetic neuropathy (Dyck et al., 1993; Sima et al., 2005). Despite several experimental and clinical studies, the mechanisms underlying chronic diabetic pain are not fully unravelled (Colburn et al., 1997; Baron, 2000; Sima et al., 2005). Type 1 STZ-induced diabetic model consistently have features of hypoalgesia for both mechanical and thermal pain. The decline in sensitivity is in part through decreases in nociceptor fibers innervating the skin epidermis (Polydefkis et al., 2001). Nerve injury also is associated with overactivation of retained nociceptor fibers (Dyck et al., 1993). Diabetic animals showed partial symptoms of mechanical hypersensitivity 2 weeks after nerve injury, likely from a similar mechanism involving adjacent nerve territories. In these territories, collateral sprouting may contribute to a paradoxical enhancement of pain sensitivity (Jackson and Diamond, 1981; Diamond et al., 1987). We were not able to record any CMAPs and SNAPs at this time point likely indicating that a small number of regenerating axons reaching skin epidermis and muscle are enough however to transmit some sensory stimuli. It is known that newly regenerating axons have smaller internodes and show lower conduction velocities than uninjured axons but despite these structural differences they may be functional and transmit sensory information (Vizoso and

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Electrically stimulated injured nerves had significant increase in mechanical sensitivity to noxious stimuli at day 21. This was associated with an improved target reinnervation and partial electrophysiological sensory recovery. A nonsignificant decrease in thermal sensitivity observed in diabetics with crush injury was not totally unexpected, and may be attributed to poor reinnervation of small nociceptors in the epidermis by this time point, consistent with our previous observation in nondiabetics after transection injury (Singh et al., 2012b). No significant impact of ES was observed on thermal sensitivity.

Overall, in diabetic animals we observed a significant improvement in motor electrophysiology and an enhanced reinnervation of sensory axons in the skin with partial behavioural benefits. Based on these experiments, we are not able to conclude whether there are any specific differential responses of ES on sensory and motor axon regeneration. Further studies examining individual fiber populations through immunostaining or backlabeling techniques in addition to the detailed terminal sensory and motor behavioural analyses are essential.

5.7.5 Proposed mechanisms of improved nerve regeneration in diabetics

Although unexplored, we suggest an ES-induced enhanced intrinsic growth state of neurons as one of the potential mechanisms behind improved regenerative outcomes in diabetics. We have several reasons to suggest this. Firstly as observed in Chapter 4, ES is associated with a reduction in regenerative roadblocks such as PTEN and RhoA. We observed a substantial rise in PTEN mRNA and protein in diabetic sensory neurons, and in Chapter 3 we found a significant impact of ES in reducing its expression in nondiabetics. This observed impact in nondiabetics could potentially be extrapolated to diabetic neurons. Secondly, ES is associated with a rise in
intracellular calcium levels and it could promote pro-regenerative pathways (Chapter 4). Thirdly, ES upregulates neurotrophins in both motor and sensory neurons in several models of nerve injury and also enhances their signaling pathway (Chapter 4) (Al-Majed et al., 2000a; Singh et al., 2012b). Future studies delineating these mechanisms are warranted.

5.8 Conclusions

In this Chapter, I demonstrated that electrical stimulation is an efficacious treatment in improving peripheral nerve regeneration in a chronic animal model of established neuropathy associated with diabetes. Augmenting the intrinsic growth capacity of sensory neurons emerges as a valid approach to enhance nerve repair in diabetics.
Chapter Six: Overall Discussion
6.1 Introduction

This thesis examined the impact and mechanisms of electrical stimulation (ES) in rodent models of peripheral nerve injury including a severe transection injury model and a challenging diabetes disease model with limited regenerative capacity. ES had a robust impact on early regenerative outgrowth of axons and SCs following nerve transection. This early-enhanced outgrowth has later translated into improved target reinnervation and functional benefits. Most importantly, the mechanisms of ES-mediated enhanced regeneration were explored in this model. Our results revealed that ES activates the PI3-K pathway in sensory neurons through PTEN inhibition, augments the supply of the neurotrophin BDNF, upregulates regeneration-associated genes, and generates support from other nonneuronal cells. The robust impact of ES was appreciable even when analyzed during diabetes, which poses several neuroregenerative barriers to regrowth. Overall, the results I generated in this thesis suggest ES as a potential therapy for human peripheral nerve injuries. The present findings also raise several interesting questions and discussion topics: the possible role of ES on the growth cone and in the injury environment; the unexplored mechanisms of ES associated benefits; the impact of ES in the distal stump, particularly on proliferating SCs and other nonneuronal inflammatory cells; the impact of ES in chronic denervation injuries; ES as a strategy for CNS injuries; development of an axon-electronic interface; and finally the potential clinical uses of ES.
6.2 Challenges to Peripheral Nerve Regeneration and the Impact of ES on the Regenerative Cascade

6.2.1 Challenges to human nerve regeneration

Peripheral nerve regeneration is complex and involves a series of molecular and biochemical events that must occur to regain motor and sensory functions. One of the major limitations to human nerve regeneration is the long distances that axons must traverse to reach their target organs. Often human nerve injuries are compared to the experimental rodent models, where the rate of regeneration is faster and the distance axons need to travel is shorter, depending on the lesion site. The proposed 1 mm/day rate of regeneration in humans is correlated with a slower rate of axonal transport of cytoskeleton proteins and is optimistic in certain clinical injuries (Gutmann et al., 1942). Considering this best expected circumstance, injuries, for example, at the proximal brachial plexus level, would take approximately 500 days for axons to regenerate to their target muscles. This considerable delay is further complicated by chronic denervation of the distal nerve stump and target end organs. The delay for regenerating axons to cross the surgical repair site forms the major hurdle for achieving the desired rate of regeneration. In Chapter 3, I observed that ES decreases this latent period of regeneration by enhancing regenerative sprouting from the proximal stump. Proximal ES, after nerve injury as applied in this thesis, influences a number of facets of injury microenvironment including SCs, inflammatory cells, the extracellular matrix and growth cone dynamics.
6.2.2 Growth cones

Growth cones at the tip of regenerating axons continuously sample their environment and advance by rapidly changing direction and branching through actin and microtubule dynamics. They participate in the removal of materials or debris in the path of the regenerating axon (Siconolfi and Seeds, 2001). Growth cones express high affinity Trks and low affinity p75 receptors and respond to neurotrophic factors by allowing dynamic changes in their receptor expression pattern (Verge et al., 1990). There are several lines of evidence suggesting the role of ES in altering the intrinsic growth cone dynamics. In the developing nervous system, electrical activity modulates axon pathfinding and initial synapse formation (Zhang and Poo, 2001; Spitzer, 2006b; Hao et al., 2012). In cultured Xenopus neurons, ES converts the netrin-1 response from repulsive to attractive by influencing cAMP levels in the growth cone (Ming et al., 2001). The elevated concentration of calcium in the growth cone enhances the levels of cAMP and in turn stimulates PKA and mediates cathodal attraction in response to ES (Kater and Mills, 1991). ES also modulates Rho-GTPase activity including actin polymerization/depolymerization in growth cones in vitro. Moreover, neurotrophins bind to tyrosine kinase receptors and enhance the PI3-K signaling pathway in growth cones (McCaig et al., 2002). In a complex regenerative environment, sensory neurons respond to guidance cues, and it has been shown that injured adult TrkA-expressing sensory neurons in culture respond to and are attracted towards an NGF gradient, and this response may involve PI3-K pathway activation (Webber et al., 2008). Our studies along this line, to identify growth-cone turning of in vitro cultured neurons, in response to ES, were not successful. This could be attributed to multiple reasons, such as spreading of the electric field in MEA, insufficient voltages used in the study, presence of growth factors in the
media and others. In this thesis, I observed: activation of PI3-K signaling, a decrease in RhoA mRNA level and a rise in the intracellular calcium in response to ES—all converging to alter growth cone dynamics. Given that expression of PTEN, PI3-K and RhoA are altered in growth cones, concurrent with continuous calcium fluctuations, the ES-mediated response that we observed in the cell body could be effective at the growth cone level as well and may impact its physical dynamics. The analysis of growth cones in vivo in a complex injury environment is very challenging. However, all previous findings including the data presented in this thesis, strongly suggest that ES of injured proximal axons in vivo can significantly modify the behavior of growth cones.

6.2.3 The interstump gap and extracellular matrix

The formation of a full nerve trunk after transection injuries is critical and essential for successful nerve regeneration. Regenerating axons must navigate through the interstump nerve trunk in order to successfully reach the distal stump and reinnervate their appropriate end organs (Lundborg et al., 1982). The interstump nerve trunk is characterized by the presence of axons, SCs, blood vessels, connective tissue, extracellular matrix, macrophages, fibroblasts and other cell types (Thomas, 1966; Lundborg et al., 1982). The specific signals involved in reconstituting the bridge are obscure. Transection injury (performed in this thesis) leads to retraction of the proximal and distal stumps and thereby generates a gap in between, and is thus a perfect model for analysing the impact of any potential treatment on new nerve trunk formation. We observed efficient bridge reconstitution with our ES paradigm (Chapter 3). The newly formed bridges contained a higher number of axons and SCs. However, how other cell types such as blood...
vessels, resident and recruited inflammatory cells and connective tissues were impacted upon ES is unknown.

The ECM contains collagen, laminin and fibronectin and plays an important role in axon regeneration. For example, laminin enhances axon regeneration by binding to the cell surface integrin receptors (Giancotti and Ruoslahti, 1999). On the contrary, the ECM contains inhibitory molecules as well, such as CSPGs, and axon regeneration is further inhibited by the presence of glial scars in the ECM, at least in spinal cord regrowth (Bradbury et al., 2002). Interestingly Beaumont et al. (2009) in a preliminary study reported that combining chondroitinase ABC (to degrade CSPGs) and ES has no additional benefits. However, the study was conducted on a direct nerve repair model, where individual treatment was sufficient to demonstrate significant benefits and was not well placed to delineate the impact of ES on CSPGs or ECM. The interaction of neural surface receptors and ECM molecules is critical for the neuron-glia interactions during development and nerve regeneration. Several reports indicate that ES enhances this interaction. Electrical activity regulates the expression of calcium-dependent cell adhesion molecules such as N-cadherin in DRG neurons (Itoh et al., 1997). Pro-regenerative L1, N-CAM, laminin, and tenascin are up-regulated by denervated and proliferating SCs (Martini et al., 1988); ES also induces SC proliferation and enhances its interaction with axons (Chapters 3 and 4). ES has also been reported to induce secretion of type II collagen from chondrocytes (Brighton et al., 2008). Collectively, results from this thesis and previous findings indicate that ES may modulate ECM molecules during regeneration. Additional experiments examining outgrowth of isolated sensory neurons or DRG explants on an inhibitory substrate in vitro, and in vivo analysis of ECM molecules such as laminin and CSPGs in response to ES, could throw light on the potential impact of ES on these molecules.
6.2.4 Additional mechanisms of ES

Peripheral nerve regeneration involves multiple pathways and mechanisms. ES enhances the intrinsic growth state of neurons and thereby impacts many of these pathways. Several potential mechanisms for the effectiveness of ES on peripheral nerve regeneration were explored in this thesis: the PI3-K pathway, intracellular calcium levels, expression of neurotrophins and RAGs, and finally the involvement of perineuronal satellite cells. ES upregulates neurotrophins, particularly BDNF, which binds to its receptor TrkB and activates three major signaling pathways: Ras-MAPK, PLCγ and PI3-K. In this thesis, I observed an upregulation of the PI3-K pathway, but there could be a possible contribution of other growth factor signaling in the ES-induced regenerative response. For example, it was recently shown that motor neurons respond to ES with an induced calcium influx and upregulation of MAPK signaling (Wenjin et al., 2011).

The rise in intracellular calcium and its potential impact on neurite outgrowth in response to ES was assessed in Chapter 4. In this thesis, the impact of only one low-voltage gated calcium channel (VGCC) blocker, nickel, was examined, however to identify the relationship between the intracellular rise in calcium and the regenerative response, the specific involvement of each VGCC and the intracellular calcium stores needs to be analyzed. Using blockers of L-type calcium channels, such as nifedipine, would be of the most interest given their role in depolarization induced neurite outgrowth (discussed in Chapter 4) (Robson and Burgoyne, 1989; Doherty et al., 1993). The significance of internal calcium stores could be examined by using BAPTA to chelate the intracellular calcium stores. Specifically, two types of internal calcium stores, IP3-sensitive and caffeine/ryanodine-sensitive stores have been implicated in an activity-dependent release of neurotrophins and in response to physiological ES (Balkowiec and Katz,
2002; Khatib et al., 2004). Using pharmacological blockers such as U73122, a specific inhibitor of phospholipase C (to assess IP3 sensitive stores), and Dantrolene, a ryanodine receptor antagonist, the specific involvement of these stores could be observed (Huang et al., 2009). Calcium influx leads to upregulation of gene expression by acting on the CaM kinase-CREB transcription pathway. The calcium-CREB pathway is recognized to have a role in peripheral nerve regeneration and how this pathway is impacted by ES would be of interest to study (Ghosh-Roy et al., 2011). There are several other pathways including PLC, protein kinase A (PKA), and many kinases and phosphatases, which are downstream of calcium and have an influence on peripheral nerve regeneration, but have not been studied in relation to an ES paradigm.

Another avenue to consider is the local protein synthesis. Local protein synthesis within axons plays a significant role in axon regeneration (Zheng et al., 2001; Verma et al., 2005). Adult sensory and motor axons have all the machinery available (either transcribed locally or transported from the cell body) for the intra-axonal protein synthesis, which is critical for the initial growth cone formation after nerve injury (Zheng et al., 2001; Verma et al., 2005). In Chapter 3, I observed that ES decreases the latent period of initial axon regeneration across the surgical site, and a higher number of axons were evident up to longer distances in the regenerative bridge. Similarly, according to an earlier report, motor axons, in response to ES, crossed the surgical repair site in a much shorter time compared to controls (3 weeks vs. 8 weeks) (Al-Majed et al., 2000b). This rapid growth response suggests a possible role of ES in enhancing local protein synthesis. The PI3-K is upstream of multiple pathways necessary for protein synthesis in neurons (Ming et al., 1999b). The mTOR pathway is one of such candidate pathways, and our observation of upregulation of the PI3-K/mTOR pathway by ES (Chapter 4)
implies that ES may be mediating local protein synthesis during early axonal outgrowth (Abe et al., 2010). Furthermore, it has been shown that SCs transfer their own endogenous polyribosomes to injured axons during regeneration (Court et al., 2008). Given the role of ES in enhancing axon-SC signaling during regeneration (Chapters 3 and 4), it is interesting to speculate that the augmented bidirectional signaling also involves ribosomal transfer and in turn protein synthesis. Overall, all of these previous findings correlate with the indirect impact of ES on protein synthesis presented in this thesis and suggest that ES may modify the local protein synthesis in regenerating axons.

6.3 Future Directions

6.3.1 ES and the distal stump

6.3.1.1 Schwann cells

The results presented in this thesis show that ES could impact SCs in association with outgrowth of axons in the regenerative bridge and upregulates GFAP (marker of SC proliferation) expression in DRGs (Chapters 3 and 4). Additionally, enhanced expression of potent mitotic agents such as CGRP and NRG, which augment SC proliferation, was observed in concert with upregulation of ErbB2 and ErbB3 receptors in DRGs following ES. A previous study has reported enhanced production of growth factors by SCs in response to ES (Huang et al., 2009). Furthermore, increased expression of GFAP was observed in astrocytes following ES of the hypoglossal nerve (Hall et al., 1989). In this thesis I studied early axonal and SC outgrowth emerging from the proximal stumps of transected sciatic nerves in a regenerative
chamber wherein the proximal and distal stumps were apart; this was independent of the molecular changes occurring in the degenerating distal stump. SCs in the distal stump have extensive roles including phagocytosis of myelin and axonal debris, recruitment of macrophages, neurotrophic support to regrowing axons, and formation of Bands of Bungner (Stoll et al., 1989; Boyd and Gordon, 2003). It is likely that ES in the distal stump may enhance some of these SC responses, as observed in this work in the proximal stump, and warrants an analysis. SC migration is critical for nerve regeneration. Following axotomy, SCs migrate in both directions, proximal and distal, to support axonal regeneration (Chen et al., 2005). Similarly, migration of SCs to endoneurial tubes is essential for the formation of Bands of Bungner. Migration of many cell types such as macrophages, epithelial cells, fibroblasts and SCs has been reported in response to ES. Macrophages show ES-mediated pseudopodial activity during wound healing (Orida and Feldman, 1982; Li and Kolega, 2002). Human fibroblasts demonstrated directed migration in response to ES applied at 100 mV/cm (Sun et al., 2004). The impact of ES on SC migration has been shown recently in chick embryos, wherein the SCs showed a directional response to as low as a 3 mV mm$^{-1}$ electric field (McKasson et al., 2008). Extrapolating this evidence, it could be speculated that ES at the distal stump may enhance SC migration, a desired event for successful nerve regeneration.

Another interesting angle to this discussion is that the PI3-K pathway is critical for SC development and proliferation (Campana et al., 1999). The activation of the PI3-K pathway is essential for SC motility and survival through IGF-1 (Cheng et al., 2000; Higuchi et al., 2001). Interestingly, PTEN, the master regulator of this pathway, is widely expressed in the normal and injured SCs in sciatic nerves (Christie et al., 2010). A significant reduction in PTEN expression in DRGs and sciatic nerves in response to ES (Chapter 4) may result from both neuronal and
other resident non-neuronal cells such as SCs. How PTEN expression in SCs influences axon regeneration is unknown but its inhibition in SCs may contribute to enhanced nerve regeneration in response to ES. Overall, ES-mediated elaboration of the proliferation, migration and survival of SCs in parallel with facilitatory axon-glia interactions would aid trophic and guidance support to neighbouring regenerating axons.

6.3.1.2 Inflammatory cells

The primary role of inflammatory cells involves phagocytosis of myelin and axonal debris during Wallerian degeneration (George and Griffin, 1994). Activation of the PI3-K pathway in T lymphocytes maintains survival and is mainly responsible for the movement of these cells (Buckler et al., 2008). Similarly, macrophages utilize the PI3-K pathway for phagocytosis (Tamura et al., 2009). In this thesis, I showed that ES modulates the PI3-K pathway (Chapter 4); hence, it could be possible that upregulation of PI3-K signaling in these cell types may enhance Wallerian and Wallerian-like degeneration and debris clearance. ES is known to have a direct and indirect influence on inflammatory cells. For example, ES of afferent vagus nerve fibers induces IL-1β expression in the brain (Hosoi et al., 2000). In chronic injuries such as chronic denervation, macrophage infiltration is required for the priming of SCs to proliferate and secrete growth factors (Sulaiman and Gordon, 2002). One such candidate is TGF1β, and interestingly, the secretion of TGF1β is augmented in osteoclasts in response to ES (Zhuang et al., 1997), and although unexplored, a similar response could be expected in the distal stump following ES of chronically denervated distal stump. Therefore, ES of inflammatory cells in the distal stump could ultimately accelerate Wallerian or Wallerian like degeneration.
6.3.2 Chronic nerve injuries

Although chronic nerve injuries are clinically more relevant, laboratory investigations using chronic injury models are less common because of their extensive time frame. Chronic denervation of resident SCs in the distal nerve stump leads to a progressive decline in the regenerative potential of these cells. For example, in a chronic denervation model where a freshly cut tibial nerve was sutured to a chronically denervated common peroneal distal stump, the number of motor neurons regenerated decreased progressively with the duration of denervation (Fu and Gordon, 1995a). Thus, reduced support for regenerating axons in the distal stump accounts for the limited functional recovery as a function of time and distance. Similarly chronic axotomy, or prolonged separation of injured axons from their target organ, reduces their intrinsic growth capacity to regenerate (Fu and Gordon, 1995b). Human nerve injuries typically require a delay in repair; therefore studying the impact of a potential new therapy in a rodent model, immediately after injury, may not address the clinical problem adequately. Given that animal models demonstrate excellent regenerative capabilities compared to humans, it is therefore clinically relevant to analyze the impact of ES in a more severe, chronic model of nerve injury, where ES could be applied after a delay. One of the major contributing factors for progressive decline in the SC capacity to support is their reduction in secretion of neurotrophic factors such as GDNF (Hoke et al., 2002). Experimental evidence from in vitro cultures demonstrated that SCs produce higher levels of growth factors in response to a similar ES used in this thesis, indicating that ES could also benefit axons regenerating distally in chronic injuries (Huang et al., 2009). Additionally, early evidence by Chia-Chou et al. (2010) and Gordon et al. (2010a) submitted data), however, does suggest that delayed intervention may yet benefit nerve
regeneration, although further work is required. Given the findings in this thesis, the type of injury and animal models analyzed, and the identification of endpoints indicating an impact of the paradigm, we believe it is appropriate that future studies would now address more chronic injuries, including those with a significant delay between the injury and application of ES.

6.3.3 ES and CNS regeneration

The limited axonal regeneration in the CNS is primarily attributed to: an insufficient regenerative response of axotomized neurons, inhibitory proteins secreted by oligodendrocytes, glial scar formation and attenuated Wallerian degeneration in the distal stump. Interestingly, ES has an impact on all of these events directly or indirectly. In the CNS, neurons respond inadequately to the injury, and the upregulation of RAGs and growth factors is very limited and transient compared to the PNS. I observed in Chapter 4 that ES augments the intrinsic growth state of PNS neurons by upregulating growth factors, particularly BDNF, and RAGs in agreement with previous reports in sensory and motor neurons (Al-Majed et al., 2000a; Al-Majed et al., 2004; Geremia et al., 2007). ES also facilitates the intrinsic growth capabilities by reducing PTEN expression and upregulating the PI3-K pathway. PTEN deletion has already been shown to increase regeneration of the optic nerve and spinal cord (Park et al., 2008; Liu et al., 2010). Because of the increased intrinsic growth state of the CNS neurons by both of these above-stated mechanisms, and given the potential of ES to modulate the same, I contemplate that ES would also enhance axonal regeneration in the CNS.

Inhibitory molecules including myelin-associated glycoprotein, oligodendrocyte myelin glycoprotein (OMgp), and Nogo bind to Nogo-66 receptor (NgR) and cause growth cone
collapse through RhoA kinase (Mukhopadhyay et al., 1994; GrandPre et al., 2002; Niederost et al., 2002). Interestingly, I observed a reduction in RhoA mRNA levels in adult sensory neurons following ES. These results could be extrapolated to the CNS where RhoA is a significant barrier to growth. Therefore, it would be very interesting to examine the impact of ES on RhoA expression in CNS growth cones. Interestingly, ES has been reported to convert myelin-associated inhibitory responses to favourable ones in growth cones and it would be of tremendous importance to analyze similar responses to ES in CNS injuries (Ming et al., 2001). I presume that ES, by reducing RhoA signaling and decreasing the inhibitory effects of myelin on growth cones, would be an ideal strategy to improve regenerative outcomes in CNS injuries.

Developing neurons depend upon NGF for survival (Barde et al., 1980). In an adult DRG, approximately 10-20% of sensory neurons die after axotomy, whereas a large proportion of CNS neurons undergoes degeneration after axotomy (Schmalbruch, 1987). It is interesting to note that developing sympathetic neurons can survive without NGF by means of KCl-induced depolarization (Koike and Tanaka, 1991). There are several lines of evidence suggest that ES can act as neurotrophic factors for neuronal survival and in combination, can enhance growth factor responsiveness (Johnson et al., 1992; Goldberg et al., 2002). Interestingly, Goldberg et al. (2002) showed that electrical activity promotes survival and axonal regeneration by enhancing the neurotrophic responsiveness of retinal ganglion cells. A previous report showing massive death of retinal ganglion cells upon blockade of electrical activity further substantiates the role of ES on neuronal cell survival (Lipton, 1986). Thus it would be of great interest to analyze the impact of ES on the survival of CNS neurons following injury.

ES also modulates the response of nonneuronal cells in the PNS and it would be of interest to examine the similar impact of ES on oligodendrocytes and astrocytes in the CNS after injuries.
Finally, we observed that sequential ES could promote sprouting of injured peripheral nerve axons (Chapter 3) and it is interesting to postulate that similar studies can be pursued in the CNS. Clinically, this would be incredibly important because collateral sprouting of axons after CNS injuries could partially recover function (Raineteau and Schwab, 2001; Bareyre et al., 2004). Collectively, several relevant mechanisms that are detailed in this thesis in connection with ES and peripheral nerve regeneration could be examined in CNS injuries and would be of significance.

6.3.4 Identifying the subpopulations of DRG sensory neurons impacted by ES

PTEN is expressed ubiquitously in sensory neurons in a naïve state, but its expression is selectively elevated in small IB4-positive neurons (Christie et al., 2010). IB4-positive neurons have impaired axon regeneration in vitro and are postulated to have a compromised intrinsic regenerative capacity (Tucker et al., 2006; Leclere et al., 2007). Provided that PTEN negatively regulates the PI3-K pathway and is an intrinsic roadblock to growth, its high expression might be responsible for the reported regenerative failure of IB4-positive neurons. Does the ES dependent reduction in PTEN mediate the regenerative response selectively in these neurons? IB4-positive sensory afferents innervate the skin epidermis and mediate pain sensitivity (Vulchanova et al., 2001). A profound decrease in intraepidermal nerve fibers is one of the major causes of the sensory loss in diabetics (Polydefkis et al., 2001). Interestingly, we observed improved reinnervation of skin epidermis in diabetic and non-diabetic animals following ES (Chapters 3 and 5). It could be possible that the majority of these fibers are PTEN-positive and that the ES-dependent reduction of PTEN in these fibers has enhanced their regrowth in the complex milieu.
of the skin epidermis. This is an exciting avenue to explore, and the impact of ES on specific subpopulations of sensory neurons needs further attention to delineate the precise impact of this approach. This can be studied using several approaches. First, sub-populations of sensory neurons can be separated based on their surface receptor expression, using magnetic beads \textit{in vitro}, followed by their response to ES on neurite outgrowth. Second, regenerating axons in the skin and muscle could be backlabeled in the DRG using a retrograde tracer dye and subsequent co-labeling with a specific marker for each neuronal population such as IB4, CGRP, neurofilament or direct labeling regenerating axons in the skin epidermis. The latter approach is difficult because of challenging immunostaining of skin fibers especially IB4, which also labels keratinocytes. Preliminary experiments in our laboratory have demonstrated labeling regenerating profiles with a range of markers in the skin and I am in the process of characterizing the phenotypic profile of regenerating epidermal axons in response to ES. Overall, based on the results presented in this thesis, ES enhances regeneration of both motor and sensory axons; however, it would be of importance to identify the specific subpopulations responsible for the behavioural recovery in order to translate the knowledge to clinics.

6.3.5 \textit{ES-induced long-term motor recovery}

ES improves both sensory and motor axonal regeneration after axotomy. I observed behavioural recovery of sensation by measuring the response to mechanical and thermal stimuli (Chapters 3 and 5). However, the experiments focusing on long-term behavioural recovery of motor function are lacking. In Chapters 3 and 5, I observed ES-mediated enhanced regeneration and subsequent target reinnervation of motor axons to muscle targets through functional
electrophysiological analysis. The observed electrophysiological recovery normally translates into enhanced behavioural outcomes, however, this could not be extrapolated directly, because the behavioural recovery not only requires successful axon regeneration and target reinnervation but also involves proper synapse formation and reorganization of the cortical map in the brain (Lu et al., 2009). It would be important to analyze the impact of ES on the behavioural recovery of motor function using measurements such as paw grip strength and the rotarod test, to an extensive and detailed measurement such as kinematics analysis and gait analysis (Muir and Webb, 2000).

6.4 Axon-Electronic Interface

The impact of ES on decreasing the initial latent growth period of injured axons (Chapter 3) led us to consider that multiple stimulation of outgrowing pioneer axons in parallel with their growth rate in vivo, by interfacing with an electronic array may further reduce this time delay, and thereby improve functional outcomes. The development of an axon-electronic interface is a challenge as there are many hurdles to conquer. Chronic application of ES on peripheral nerves involves interactions of an electrical device with soft excitable tissue. An axon-electronic interface typically should be able to set up a communication between the electronics and nerve tissue and also be compatible with the adjacent soft tissue. The interface must be both efficacious and safe: having the ability to elicit the desired regenerative response, and at the same time without undesired side effects. On the same line, the MEA utilized in this thesis and our currently under development in vivo flexible stimulator (prototype is ready to use), both includes polydimethylsiloxane (PDMS), a biocompatible and ideal substrate for neural interfaces and has
a mechanical impedance close to that of soft tissues such as nerves (Guo et al., 2010; Meacham et al., 2010).

The observation that sequential stimulation mediates sprouting of axons without increasing the number of motor neurons is a debatable approach for nerve regeneration (discussed in Chapter 3). Al-Majed et al. (2000b) observed that the response to stimulation over 2 weeks was similar to the response observed in one hour. Thus it is controversial as to why multiple stimulation is needed using an interface. Our approach is to stimulate the tip of outgrowing axons in conjunction with their rate of growth. All of the earlier reports included stimulation of the proximal stump instead of stimulation of the tip of outgrowing axons, which I am proposing. Additionally, a biodegradable axon-electronic interface has many other advantages. First, later sequences of multiple stimulations could be provided noninvasively. Second, the interface could be manipulated for growth factor delivery. For example, growth factor such as NGF or BDNF could be slowly released from charged NGF or BDNF beads coated onto the electrodes (Lee et al., 2012). This will provide further additive benefits to nerve regeneration.

6.5 Clinical Significance of the Findings: Research from Bench to the Bedside

Animal studies, from others and the ones presented in this thesis, have demonstrated that ES has the potential to serve as a therapy for peripheral nerve repair. In the pilot clinical trial, Gordon and Chan (2010b) determined the impact of ES in patients with median nerve compression in carpal tunnel syndrome. These patients had a marked loss of motor axons reinnervating the thenar muscle. ES was applied immediately following carpal tunnel release surgery on the proximal median nerve using the same stimulation paradigm (20 Hz, 1 h) as
examined in this thesis. ES significantly improved axonal regeneration and target innervation as analyzed by the motor unit number estimation (MUNE). These results were striking as the MUNE was improved in a 6-8 month time period compared to the slower recovery in control group subjects after 1 year. These preliminary results are very encouraging (Gordon et al., 2010b). While the application of ES in a clinical setting is promising, there are several considerations that must be addressed prior to their use in humans.

Use of ES has many advantages over other nerve repair therapies. Any pharmacological or biological therapeutic approach usually has unwanted side effects associated with it. ES has an advantage – animal studies and preliminary human studies indicated that it is well tolerated and no undesirable side effects were observed. There could be a possibility that it activates other neuronal circuits in the spinal cord and higher brain regions and modifies the somatosensory cortical map for pain and other pathways. These parameters are complicated to measure and changes in it are secondary to the nerve regeneration process, even without any therapeutic modality. Next, the effective regenerative paradigm of one hour extracellular ES following nerve repair is an appealing addition to peripheral nerve repair. For example, patients can easily receive ES while they are undergoing nerve repair microsurgery. Despite all of these advantages, I would like to comment that, because successful nerve regeneration includes complex biochemical and molecular pathways, multiple intervention strategies might be more effective in humans.

Overall, while we believe that the approach will have wide applicability, more clinical evidence for its benefits will be required despite the promising data thus far. We recognize that the application of ES immediately after injury, would not translate into a number of clinical scenarios, where a significant delay in applying the approach would be expected. Future experiments aimed at analyzing the impact of ES on injuries, such as chronic denervation and
having a time delay between the injury and stimulation, would address the inherent delay that clinicians face during nerve repair.

6.6 Conclusions

1. ES enhances both short-term (early axon outgrowth) and long-term (increased number of myelinated axons and backlabeled motor neurons) regeneration indicating a robust and sustained impact on regeneration, even in severe transection injuries (Chapter 3).

2. ES showed earlier skin reinnervation associated with more rapid electrophysiological recovery and a regain of mechanical and thermal sensation (Chapter 3).

3. The mechanisms associated with the benefits of ES include: upregulation of the neurotrophin BDNF and regeneration-associated genes such as β-tubulin and GAP-43, activation of the PI3-K pathway through inhibition of PTEN, preliminarily noninvolvement of low-voltage gated T-type calcium channels, and a possible response from nonneuronal cells (Chapters 3 and 4).

4. ES rapidly enhances neurite outgrowth in adult sensory neurons isolated from normal and diabetic animals in vitro (Chapters 3 and 5).

5. ES improves peripheral nerve regeneration: motor electrophysiological recovery, repopulation of distal tibial nerves, and reinnervation of target skin organ, in a clinically relevant animal model of diabetes, with limited regeneration capabilities (Chapter 5).

In summary, the experiments presented here have demonstrated that ES could be a potential clinical therapy for improving the regeneration of peripheral nerve injuries.
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