# ELECTRICAL STIMULATION OF DENERVATED MUSCLE

# ELECTRICAL STIMULATION OF DENERVATED MUSCLE

By

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## Abstract

Functional recovery following peripheral nerve injuries is poor due to muscle atrophy and fibrosis being major contributing factors. Electrical muscle stimulation has been used for decades in some capacity to treat denervation related muscular changes. The research presented in this thesis explores a new stimulation paradigm and its effects on short and long term muscle denervation.

The first part of this work describes the new stimulation paradigm and the design and development of the stimulator used to deliver this paradigm. The paradigm involved daily 1-hour stimulation sessions featuring 600 contractions at high stimulus frequencies (100 Hz) and low pulse durations (200 μs). To test the device and paradigm, a pilot study involving muscle stimulation throughout a one month period of denervation in rat lower limb muscles was carried out. The results showed that this short but intense stimulus session significantly reduced the rate of muscle atrophy compared to animals that did not receive stimulation. Furthermore, muscle weight and consequently muscle force were also significantly greater.

The stimulus paradigm was then used to investigate muscle that was denervated and immediately repaired. Ideally, immediate nerve repair following nerve injuries produces the best outcome. One month of electrical muscle stimulation following nerve repair enhanced this outcome through significant increases in muscle weight and force. Additionally, contrary to many previous studies, the stimulus paradigm had no negative effects on reinnervation. Taken together, electrical muscle stimulation can provide significant improvements over the best case scenario of immediate nerve repair.

The third part of this work investigated the use of chronic electrical muscle stimulation throughout three months of denervation and the impact on reinnervation. Results showed that reinnervation in chronically stimulated animals were no different than animals that were denervated and immediately repaired.

The last part of this work combined the use of electrical muscle stimulation with sensory protection in chronically denervated muscle. Sensory protection involves suturing a sensory nerve to protect a muscle during denervation and was shown in previous studies to reduce muscle atrophy, preserve muscle spindles and the structure of the distal nerve stump. The results showed significantly greater muscle weights and force in the combined treatment compared to the individual treatments alone. Reinnervation in these animals was as good as those that were immediately repaired. This suggests that contractile support combined with sensory protection may provide superior functional outcomes in chronically denervated muscle.

The findings presented in this thesis provide new evidence for the use of short duration daily electrical muscle stimulation immediately following nerve repair or throughout long term denervation. Evidence for a new therapy, muscle stimulation with sensory protection, is also presented and shown to provide superior functional outcomes compared to either therapy alone. The contributions made in this body of work may provide clinicians with evidence to pursue clinical use of the outlined strategies and ultimately help patients optimally recover from peripheral nerve injuries.

## Acknowledgements

There are many people who played an important role during my graduate studies helping shape me as a researcher and an educator.

To my supervisor Dr. Hubert de Bruin, you have been instrumental in helping me get to where I am today. Your patience, good nature, knowledge and passion, have inspired me to become a better engineer and academic. You made my experience at McMaster a truly enjoyable and unforgettable one.

To my co-supervisor, Dr. Margaret Fahnestock, you opened up my eyes to what being a scientist is like. When I started my PhD I was a naïve engineer with little background in publishing papers and neuroscience. Your attention to detail and scientific rigor have allowed me to develop a skill set as a researcher and scientist.

I must also thank my committee members Dr. James Bain and Dr. Ian Bruce. Dr. Bain, although our chats were limited to a few they were always meaningful and full of enthusiasm and ideas. You would always pose great questions during our meetings and I would come away from them full of motivation to tackle the next problem. Dr. Bruce, you have taught me as an undergraduate and graduate student and introduced me to aspects of biological modeling. Although mathematical modeling is not my forte, I've come to appreciate the complexity and importance that it plays in helping understand physiological systems.

To my teaching mentor, Dr. Alexander Ball, you have really made learning and teaching anatomy one of the best experiences I have had in university. You have also provided invaluable advice on my career and other aspects of my life. I am definitely

going to miss popping into your lab and leaving hours later after having long discussions with you and your students.

The work in this thesis was a result of a collaboration of a few people, one of whom I could not have done without. Michael Holmes, thank you for being the surgical aspect of our team and providing me with advice ranging from science to life. Our discussions while working have always been enjoyable and the tips and tricks I have learned from you will not be forgotten. I also want to thank Mary Susan Thompson for bringing the histological aspect of the project together and Juan Pablo Lopez for launching the project off the ground at a fast pace. Pablo, it is too bad you had to leave McMaster but your passion and drive for science along with your care about the work we did here helped fuel it along until the end.

My penultimate thank you goes to my family. My parents have always been supportive of the decisions I have made and encouraged me to continue to learn and pursue higher education. You have instilled in me the importance of hard work and never giving up. More specifically, Dad, you always said, “Stay in school! Don’t leave, you don’t want to work for somebody else in a 9 to 5 job.” Well, I took your advice and stayed in school until the end and enjoyed every minute of it. Now it is time to make a career out of it.

Finally, I need to thank my best friend and wife Monika. She has been with me since the beginning of this academic journey supporting every decision I made and encouraging me to strive for success. Your love and support have made this ride a lot less bumpy than it could have been, thank you.

# Table of Contents

Abstract ..... iii

Acknowledgements ..... v

List of Figures ..... x

Preface ..... xii

Chapter 1: Introduction ..... 1

1.1 Background ..... 1

1.2 The Healthy Muscle ..... 1

1.3 The Healthy Peripheral Nerve ..... 3

1.4 Motoneuron Physiology ..... 5

1.5 Motor Units ..... 10

1.6 Muscle Fiber Types ..... 11

1.7 Muscle Contractile Properties ..... 14

1.8 Denervation ..... 15

1.8.1 Effects on Peripheral Nerve ..... 16

1.8.2 Effects on Muscle ..... 17

1.9 Treating Denervated Muscle ..... 20

1.9.1 Electrical Muscle Stimulation ..... 21

1.9.2 Support by Soluble Factors ..... 31

1.9.3 Sensory Protection ..... 34

1.10 Objective of This Thesis ..... 40

1.10.1 Hypothesis....40
1.11 References....42
Chapter 2: Instrumentation....53
2.1 Overview....53
2.2 Electromyography (EMG) Amplifier Design Requirements....53
2.2.1 EMG Hardware Implementation....54
2.3 Force Transducer Amplifier Design Requirements....58
2.3.1 Force Transducer Amplifier Hardware Implementation....58
2.4 Electrical Muscle Stimulator Design Requirements....63
2.4.1 Stimulator Software....63
2.5 References....70
Chapter 3: Paper One - A New System and Paradigm for Chronic Stimulation of Denervated Rat Muscle....71
Chapter 4: Paper Two - Electrical muscle stimulation following immediate nerve repair reduces muscle atrophy without affecting reinnervation....94
Chapter 5: Paper Three - Determining the effects of electrical stimulation on functional recovery of denervated rat gastrocnemius muscle using motor unit number estimation....121
Chapter 6: Paper Four - Combined sensory protection and electrical stimulation of denervated rat skeletal muscle improves functional recovery....137
Chapter 7: Conclusion and Future Work....171

7.1 Conclusion ..... 171

7.2 Recommendations for Future Work ..... 174

7.3 References ..... 177

# List of Figures

**Figure 1-1** The organizational structure of a muscle ..... 2

**Figure 1-2** Cross-section of a peripheral nerve ..... 4

**Figure 1-3** A cross-section of the spinal cord ..... 5

**Figure 1-4** Summary of the entire process of muscular contraction and relaxation ..... 7

**Figure 1-5** Different states of the acetylcholine channel ..... 8

**Figure 1-6** End plate potential graph ..... 9

**Figure 1-7** Twitch force responses from different types of muscles in human ..... 12

**Figure 1-8** Tension profile for a typical twitch response ..... 14

**Figure 1-9** Progression of a tetanic response ..... 15

**Figure 1-10** A timeline of electrical muscle stimulation research ..... 30

**Figure 1-11** A timeline of trophic support research ..... 39

**Figure 2-1** A block diagram of the EMG amplifier design ..... 54

**Figure 2-2** Schematic of the electromyography amplifier. ..... 56

**Figure 2-3** Printed circuit board 3D renderings of the electromyography amplifier ..... 57

**Figure 2-4** Block diagram of the force transducer amplifier design ..... 58

**Figure 2-5** Schematic of the force transducer amplifier ..... 61

**Figure 2-6** Printed circuit board 3D renderings of the force transducer amplifier ..... 62

**Figure 2-7** Schematic of a single channel of the muscle stimulator. ..... 65

**Figure 2-8** Schematic of the 5 channel breakout box ..... 66

**Figure 2-9** Printed circuit board 3D renderings of the muscle stimulator ..... 67

**Figure 2-10** Printed circuit board 3D renderings of the 5 channel breakout box ..... 68

**Figure 2-11** Software flow chart for the stimulator LabVIEW program .....69

# Preface

The work described in this thesis is presented as a "sandwich" thesis which consists of a series of four thematically related but distinct articles, two of which have been peer reviewed and published and two that have been submitted for peer review. Because of the interdisciplinary nature of the studies undertaken, the work presented is not the result of a sole author but collaborative effort of a number of people. As the doctoral thesis candidate, I have outlined below the contributions that I, the other authors, and contributors have made to each of the published or submitted articles.

Chapter 3: A New System and Paradigm for Chronic Stimulation of Denervated Rat Muscle

Michael P. Willand, Juan Pablo Lopez, Hubert de Bruin, Margaret Fahnestock, Michael Holmes, and James R. Bain

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In this manuscript all authors contributed to the design of the experiments. Michael Holmes (MH) and Juan Pablo Lopez (JPL) were responsible for conducting animal surgeries. I designed, tested, and built the electrical muscle stimulator and tested the stimulation paradigm on the animals. I also designed and built the restraining devices for all animals with consultation from MH and JPL. Daily animal stimulation sessions and functional endpoint testing were carried out by JPL and me. Preparation and staining of tissue used for histology was done by JPL. All tissue was examined using light microscopy and imaged by me. I carried out all data and statistical analysis along with

writing the first draft of the manuscript. All authors contributed to revisions of the manuscript.

Chapter 4: Electrical muscle stimulation following immediate nerve repair reduces muscle atrophy without affecting reinnervation

Michael P. Willand, Michael Holmes, James R. Bain, Margaret Fahnestock, and Hubert de Bruin

Submitted to Muscle & Nerve

In this manuscript all authors contributed to the design of the experiments. Michael Holmes (MH) was responsible for conducting animal surgeries. I carried out the daily electrical stimulation sessions. Endpoint testing was completed by MH and me. Tissue collection for histological purposes was conducted by Mary Susan Thompson (MST) and me. Histological staining was carried out by Matthew Macdonald and MST. All tissue was examined using light microscopy and imaged by me. I carried out all data and statistical analysis along with writing the first draft of the manuscript. All authors contributed to revisions of the manuscript.

Chapter 5: Determining the effects of electrical stimulation on functional recovery of denervated rat gastrocnemius muscle using motor unit number estimation

Michael P. Willand, Michael Holmes, James R. Bain, Margaret Fahnestock, and Hubert de Bruin

Published in Conference Proceedings of the Annual International Conference of the IEEE Engineering in Medicine and Biology Society. 2011, 2011:1977-80.

In this manuscript all authors contributed to the design of the experiments. Michael Holmes (MH) was responsible for conducting animal surgeries. Motor unit number estimation software was written by Jill Salvador and modified by me. I carried out the daily electrical stimulation sessions. Endpoint surgeries were completed by MH, and I carried out the final motor unit number estimates. All data and statistical analysis along with writing the first draft of the manuscript was completed by me. All authors contributed to revisions of the manuscript.

Chapter 6: Functional outcomes following sensory protection and electrical muscle stimulation in denervated rat skeletal muscle

Michael P. Willand, Michael Holmes, James R. Bain, Hubert de Bruin, and Margaret Fahnestock

Submitted to the Journal of Physiology

In this manuscript all authors contributed to the design of the experiments. Michael Holmes (MH) was responsible for conducting animal surgeries. I carried out the daily electrical stimulation sessions with help from Larrisa Schudlo and Christine Gabardo. Endpoint surgeries were conducted by MH. I conducted all endpoint measurements and data collection. Tissue collection for histological purposes was conducted by Mary Susan Thompson (MST) and me. Histological staining was carried out by Matthew Macdonald and MST. All tissue was examined using light microscopy and imaged by me. Histological image analysis was primarily conducted by me with some aid from Bhairavi Sivasubramaniam. All data and statistical analysis along with writing the first draft of the manuscript was completed by me. All authors contributed to revisions of the manuscript.

# Chapter 1: Introduction

## 1.1 Background

The purpose of this chapter is to outline the background about the anatomy and function of normal healthy muscle and then describe how losing a functional connection (denervation) with the nervous system impacts a muscle's structure and function. Strategies to minimize or reverse the changes associated with muscle denervation will then be discussed. Finally, the objective and hypothesis of this work will be presented.

## 1.2 The Healthy Muscle

The muscles in the human body are divided into three different types: smooth, cardiac, and skeletal muscle. Each of these muscle types differ in their structure, location, and function. This thesis focuses on skeletal muscle as this type is affected by damage to the peripheral nervous system.

Each skeletal muscle in our body is comprised of a number of smaller functioning units called muscle fibers or myocytes. These fibers are long cylindrically shaped cells that are comprised of even smaller units called myofibrils. These are further broken down into the functional units that generate force, called sarcomeres. It is the collective contraction of thousands of sarcomeres that creates force and allows us to move our limbs. When viewed under a microscope, sarcomeres provide the striated appearance that is often used to describe muscles. These striations come from the positioning of the thick and thin filaments that make up a sarcomere. The thick filaments are comprised of myosin and the thin filaments are comprised of actin. The interaction of these filaments

forms the basis of force production in the muscle. Figure 1-1 shows the structural organization of skeletal muscle from the macroscopic muscle fibers to the microscopic filaments.

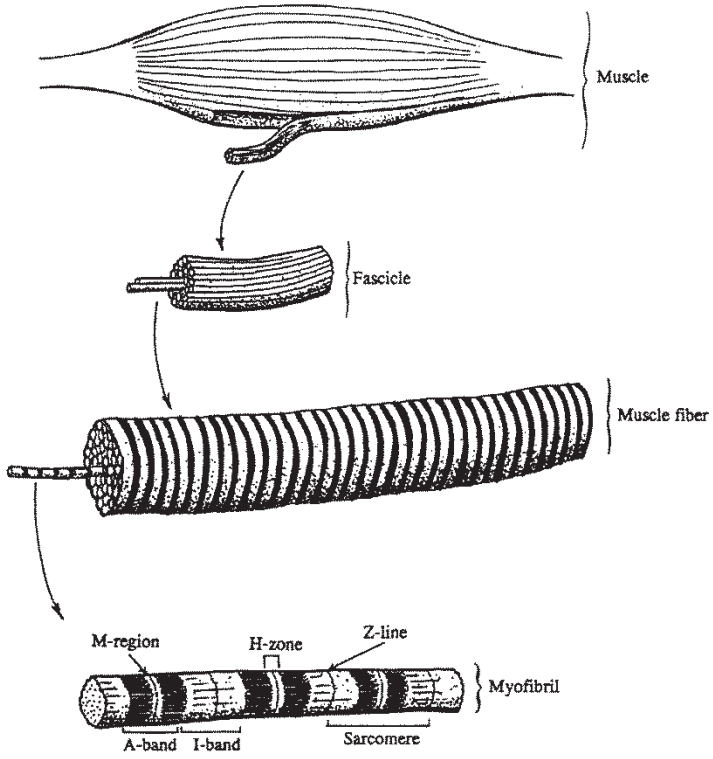


**Figure 1-1** The organizational structure of a muscle. From the macroscopic muscle as a whole to the individual components that make up a muscle (MacIntosh et al., 2006).

It is pertinent to note that skeletal muscle derives its name from the fact that the muscles connect to the skeleton. This connection is important as it is the connective tissue that translates the summated force from thousands of sarcomeres in order to facilitate movement at a joint. This organization starts with sarcomeres being arranged longitudinally to form a myofibril. The myofibrils are then packed together and surrounded by connective tissue called endomysium. These packed myofibrils represent a

single muscle fiber. Numerous muscle fibers are then bundled together and surrounded by perimysium to form a muscle fascicle. These fascicles are grouped together and wrapped by the last layer of connective tissue called epimysium. This last layer of connective tissue is continuous with the outer layer of bone connective tissue, the periosteum, and separates muscles from one another. All three layers of muscle connective tissue are connected to one another allowing for force to be summated from individual sarcomeres in order to produce the muscular contraction that we see (MacIntosh et al., 2006).

## 1.3 The Healthy Peripheral Nerve

In the normal functional human, peripheral nerves are the anatomical structures that connect our central nervous system (spinal cord, brain) to the structures that move our joints – skeletal muscles. Like the muscle, the peripheral nerve is comprised of various compartments. A cross section of a peripheral nerve is shown in Figure 1-2. The outermost layer is comprised of epineurium that surrounds a number of nerve fascicles. Larger nerves tend to have more fascicles than smaller nerves. Each fascicle is surrounded by perineurium and enclosed within the fascicle are numerous nerve fibers. Each nerve fiber contains an axon (either myelinated or unmyelinated) that is wrapped by a thin sheath of connective tissue called endoneurium (Flores, Lavernia, & Owens, 2000).

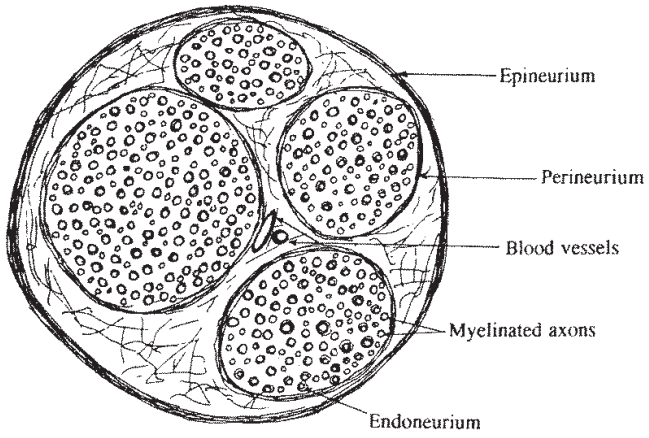


**Figure 1-2** Cross-section of a peripheral nerve showing four different fascicles with all associated connective tissue (MacIntosh et al., 2006).

Axons can be characterised as either motor or sensory depending on their action. Both types of axons can be surrounded by myelin, an electrical insulating material, produced by Schwann cells. The function of myelin is to increase the speed of neurotransmission. The origin of these axons depends on the type. Sensory axons arise from cell bodies found adjacent to the spinal cord in the dorsal root ganglion. The sensory cell bodies receive information from various peripheral sensory receptors that account for such sensations as touch, pressure, pain, and temperature. Another important sensory modality for proper muscle coordination is proprioception. Part of this sensation is provided by specialized receptors called muscle spindles that reside in the central part of a skeletal muscle. The spindles provide information on the changes of muscle length. Another proprioceptor found in series with skeletal muscle is the Golgi tendon organ. This receptor provides information on the changes of muscle tension. Together, the information from both Golgi tendon organs and muscle spindles is integrated by the central nervous system to calculate the relative position of a limb in space.

Motor axons have their cell bodies within the spinal cord, specifically in the ventral root. The collection of motor axon, cell body, and dendrites is termed the motoneuron (Tortora & Grabowski, 2003). Figure 1-3 shows the locations of the cell bodies of each type of neuron.

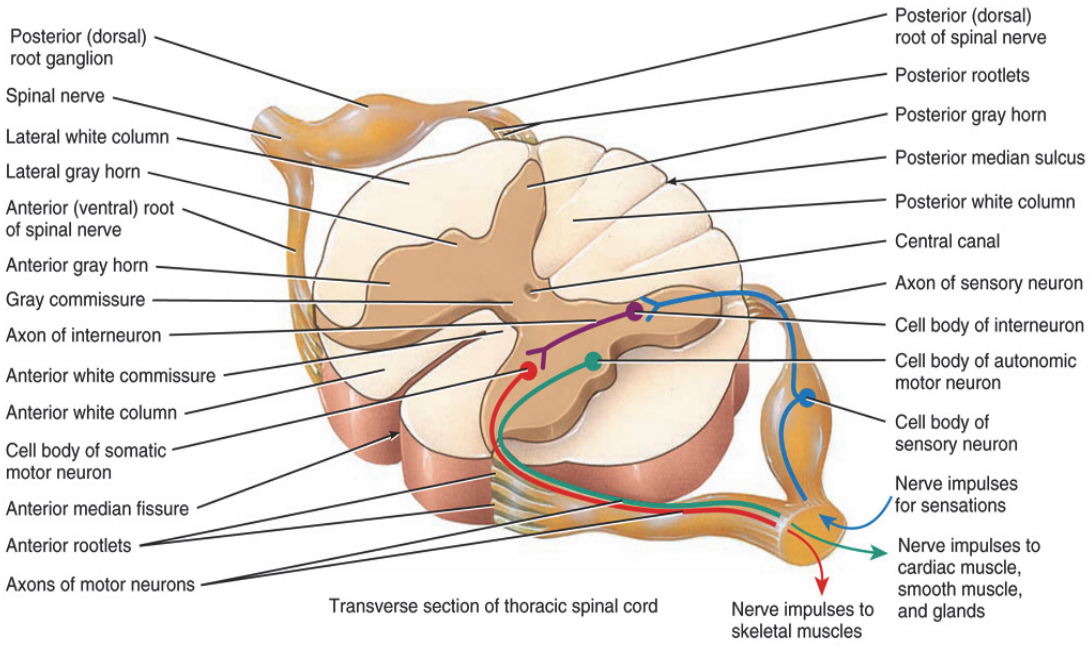


**Figure 1-3** A cross section of the spinal cord. The cell body of the motoneuron (red circle) originates in the ventral horn. The cell body of the sensory neuron (blue circle) originates outside of the spinal cord (Tortora & Derrickson, 2012).

## 1.4 Motoneuron Physiology

The role of the motoneuron is not only to quickly conduct electrical information to the muscle but also to maintain structure and cellular metabolism of itself and the connecting muscle fiber. This slower transportation system is called axoplasmic transport and provides a two way chemical communication system between nerve and muscle. It can be divided into two types based on direction, anterograde and retrograde transport.

Anterograde transport can be further divided up into a fast and slow transport system. The fast system typically transports metabolic products towards the muscle. These include mitochondria, vesicles, protein motors, neurotransmitters, and inhibitors of oxidative metabolism. The anterograde slow system moves components necessary for structural support such as microtubules, neurofilaments, actin, tubulin, and cytoplasmic enzymes (MacIntosh et al., 2006). Retrograde transport provides information to the cell body from the muscle. It also allows for muscle derived factors to be taken into nerve cell bodies to influence their survival, neurite outgrowth, or neurotrophin expression. These factors are known as neurotrophins and include brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5).

When the cell body of a motoneuron depolarizes it sends electrical information in the form of impulses or action potentials down toward the target muscle fiber. At the terminal end of the axon is a specialized structure that transduces the electrical signal into a chemical signal. This specialized structure is the neuromuscular junction. Action potentials that reach the neuromuscular junction are converted into a chemical message through the use of the neurotransmitter acetylcholine. This initiates the process of generating a contraction. The process is summarized in Figure 1-4.

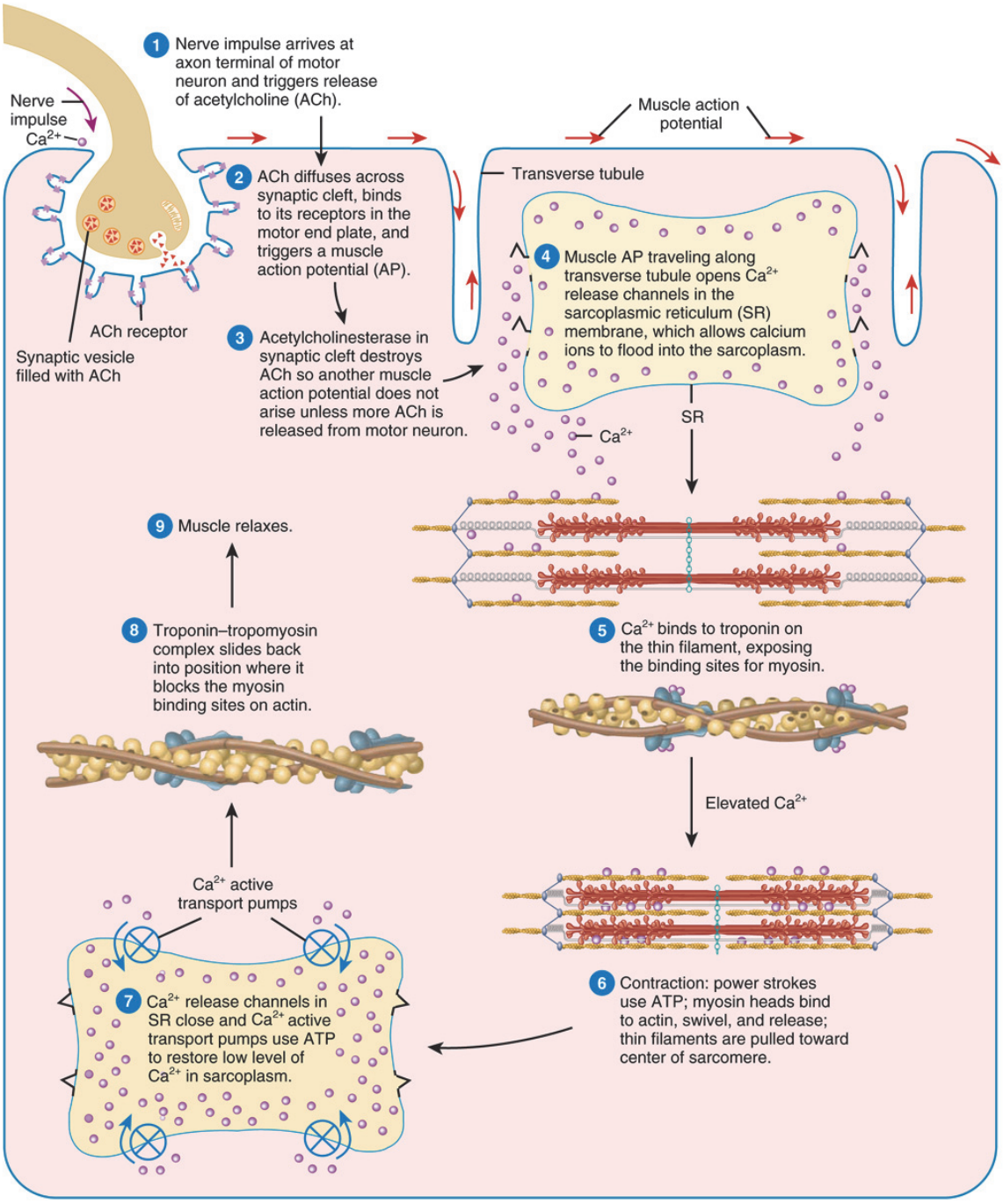


**Figure 1-4** Summary of the entire process of muscular contraction and relaxation (Tortora & Derrickson, 2012).

The acetylcholine that was released from the axon terminal flows into the synaptic cleft and binds to acetylcholine receptors located within the plasma membrane of the muscle fiber. Once bound to its receptor, a channel within the receptor opens to allow outflow of $K^+$ and inflow of $Na^+$ ions (Figure 1-5).

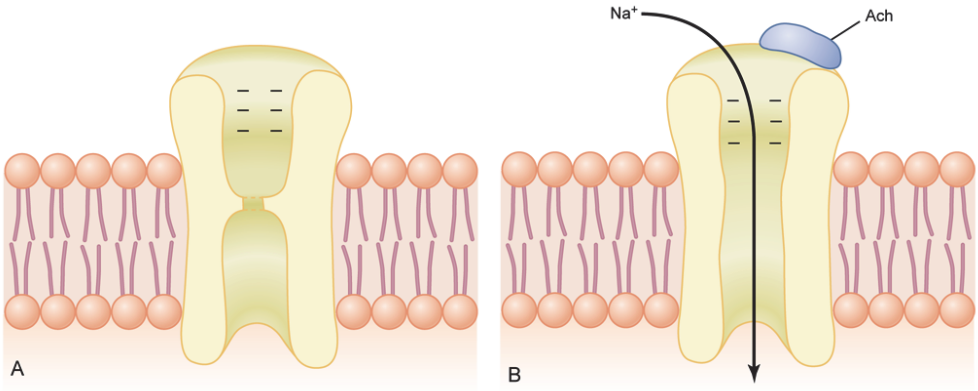


**Figure 1-5** Different states of the acetylcholine channel. The closed state is shown in A. When Ach binds to the receptor the channel opens (B) and allows an influx of sodium into the muscle (Guyton & Hall, 2006).

The opening of each channel contributes to a small depolarization of the muscle membrane as many more $Na^+$ ions enter the cell than $K^+$ ions leave the cell. This small depolarization is called the end-plate potential. When the end-plate potential is fully developed, it can cause the membrane potential to reach its firing threshold which results in voltage gated sodium channels opening. This leads to a massive influx of $Na^+$ ions flooding into the cell resulting in a muscle fiber action potential (Figure 1-6).

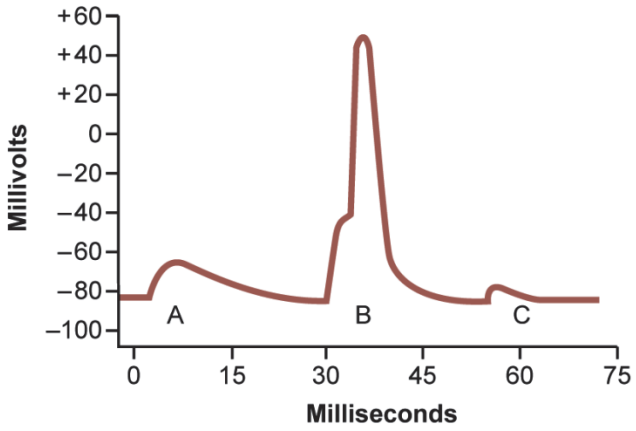


**Figure 1-6** When the end plate potential is too weak the muscle action potential fails to develop (A and C). However, when enough Ach is released to cause a sudden change in the membrane potential a full muscle action potential is realized (B) (Guyton & Hall, 2006).

The action potential then rapidly propagates throughout the muscle fibrils via intricate T-tubule pathways. This propagation is the beginning of the process called excitation-contraction coupling (Dulhunty, 2006). These tubules are continuous with the plasma membrane and transversely span the muscle fibrils allowing for rapid and synchronous depolarization. Surrounding the transverse component of the T-tubules are large sacs or cisterns called the terminal cisternae which contain $Ca^{2+}$ ions. The terminal cisternae extend longitudinally in the form of the sarcoplasmic reticulum. Together, the terminal cisternae and transverse tubule form an arrangement called a triad. When the action potential reaches the sarcoplasmic reticulum it causes release of $Ca^{2+}$ ions into the sarcoplasm that surrounds the myofibrils. $Ca^{2+}$ ions then bind to troponin which removes the inhibitory effect of tropomyosin. This allows for the active site on the actin filament to be uncovered and receptive to the heads of the myosin filament. As the heads attach to their new site they pull the actin filament forward. The heads then detach from the actin

filament with the aid of ATP and once again bind to a new site to further pull the actin forward. This is the basis for the sliding filament theory of force production. It is the summation of millions of these sliding filaments that results in the macroscopic forces that we see (Guyton & Hall, 2006; MacIntosh et al., 2006; Tortora & Grabowski, 2003).

## 1.5 Motor Units

Each motoneuron connects to multiple muscle fibers creating a functional group called a motor unit. The size of the motor unit, or number of muscle fibers connected by a single motoneuron, is determined by the function of the muscle. Smaller muscles typically require fine control of the structure and have fewer muscles per motoneuron. Examples include laryngeal muscles (2 to 3 fibers per axon) and muscles controlling the eyelid (10 to 20 fibers per axon). When the complexity or precision of the muscle action increases, the number of muscle fibers associated with each motor unit decreases. Conversely, larger muscles, such as those in the limbs, have many more muscle fibers per axon. This is because the force requirements are much larger in these muscles and fine control is not necessary. The biceps muscle in the upper limb can contain as many as 2000 to 3000 fibers per axon. The muscle fibers in each motor unit are not only associated with that particular motor unit but also interleave with other units in bundles of 3 to 15 fibers. This allows separate motor units to contract in a collaborative effort rather than in an individualistic manner (Guyton & Hall, 2006).

## 1.6 Muscle Fiber Types

Muscle fibers can be classified into two main categories based on their myosin content and contraction times: fast and slow. Fast muscles typically use a glycolytic metabolism and are white in appearance. Their contraction times are also very fast when compared to a slow muscle due to their extensive sarcoplasmic reticulum used for rapid release of $Ca^{2+}$ ions. Their role is to provide quick bursts of force in a non repetitive manner resulting in a muscle that fatigues very quickly. Slow muscles use an oxidative metabolism and are red in appearance due to the oxygen-binding pigment myoglobin. They are surrounded by a vast capillary network and have slow contraction times. Their primary role is in postural control where long repetitive contractions at low force are necessary resulting in a muscle that fatigues slowly (MacIntosh et al., 2006). Contraction times are derived from twitch force profiles where a single stimulus is delivered to the muscle and the corresponding force is measured. Figure 1-7 shows the differences in the twitch force profiles for various types of muscles in the human.

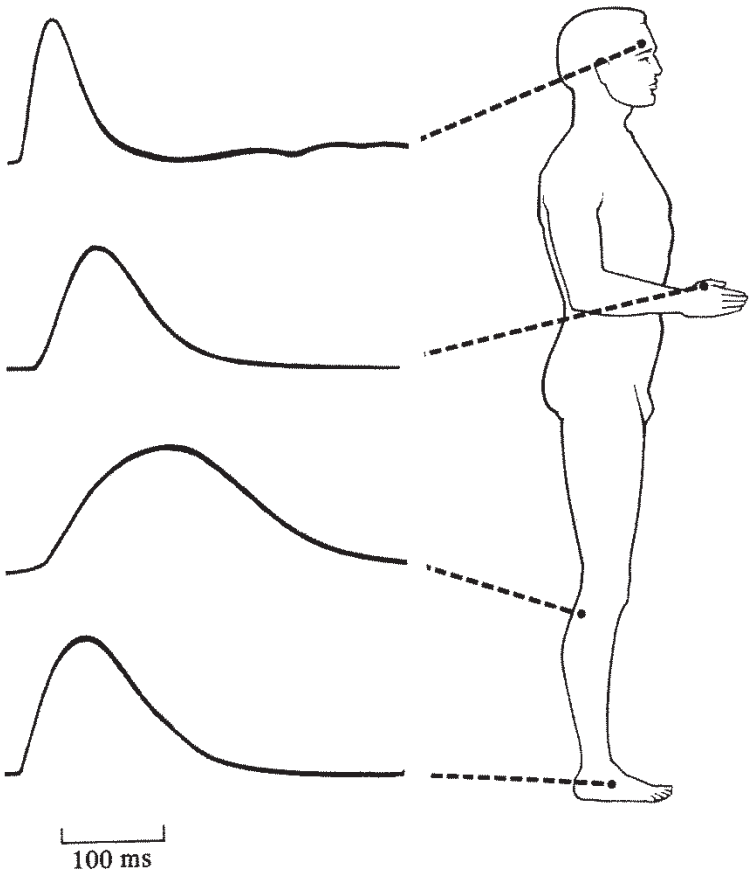


**Figure 1-7** Twitch force responses from different types of muscles in the human body. Ocular muscles produce very fast contraction and slow relaxation times. The soleus muscle in the limb produces a much longer contraction and relaxation time. These twitch characteristics provide information on the overall fiber type composition of the muscle (Reprinted, by permission, from A.J. McComas and H.C. Thomas, 1968, "Fast and slow twitch muscles in man", Journal of the Neurological Sciences 7: 304.).

Histochemical methods further divide the fast muscle fiber into various sub-types. The myosin ATPase staining method developed by Engel (Engel, 1998) identified different fiber types based on the amount of activity of the ATPase enzyme. Fibers with high activity stain dark when incubated at pH 10.4. These fibers are referred to as type II. Fibers that stain lightly at pH 10.4 are referred to as type I. Brooke and Kaiser furthered

this method by incubating the tissue at lower pHs resulting in type II fibers being divided up into three sub-types: A, B, and C (Brooke & Kaiser, 1970).

An important study by Burke (Burke, Levine, Tsairis, & Zajac, 1973) tied together the histochemical and contractile properties of a muscle. Through the stimulation of the ventral roots in a cat, Burke and colleagues showed that all muscle fibers belonging to the same motor unit are comprised of the same fiber type. This led to the current classification of motor units into three different types: slow (S); fast contracting, fast fatiguing (FF); and fast contracting, fatigue resistant (FR). Type S motor units stain weakly for ATPase (type I fibers), have slow contracting times, are resistant to fatigue and produce small forces due to the small number of fibers in each unit. Type FF motor units stain strongly for ATPase (type IIB fibers), have fast contracting times, are not resistant to fatigue and produce large force. The last type of motor unit, type FR, is intermediate in terms of its properties. Although the muscle stains strongly for ATPase (type IIA fibers), it is resistant to fatigue and has a fast contracting time. Force production is higher than type S units but lower than type FF. Further study into the differences between motor unit types revealed differences in axonal diameters (Burke et al., 1982; Ulfhake & Kellerth, 1982). Type S units have the smallest axonal diameters and type FF the largest, with type FR in between. This important finding correlates well with the fact that fast motor units which typically have many more muscle fibers have the largest axonal diameters.

## 1.7 Muscle Contractile Properties

As mentioned above, different muscle fiber types have different contractile properties. These properties can be characterized through visualization of the isometric twitch response. This response is elicited by fixing the muscle's length and providing maximal short duration single stimuli to the muscle or nerve. A typical twitch response and the identifying characteristic are shown in Figure 1-8. Contraction time is a measure of how fast a muscle reaches peak force. Half-relaxation time is a measure of the time it takes for a muscle to relax. Both of these properties provide insight into the type of muscle fibers (fast or slow twitch) that make up a muscle. On a macroscopic scale, whole muscle recordings of twitch force provide information on the overall fiber composition of the muscle (MacIntosh et al., 2006).

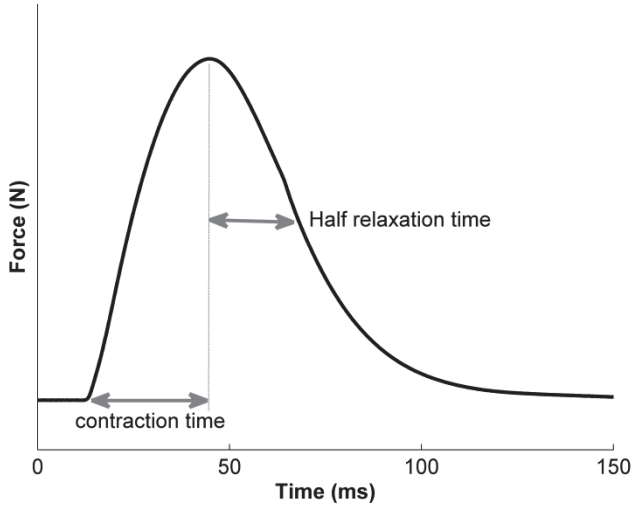


**Figure 1-8** Tension profile for a typical twitch response. The contraction time is defined as the time to reach peak force. Half relaxation time is defined as the time from peak force to half that value.

As the frequency of elicited twitch contractions increases there is a point where a new contraction occurs before the previous one has finished, leading to an increase in total force. When the frequency reaches a critical level the twitches fuse together to create a smooth muscular contraction. This is known as a tetanic contraction. Maximum muscle force is produced at frequencies just past the critical level (Guyton & Hall, 2006). Figure 1-9 shows the progression of fusion from twitch to tetanic responses.

The ratio of twitch to tetanic peak values, known as the twitch-tetanus ratio, provides information on the dynamics of $Ca^{2+}$ release and uptake from the sarcoplasmic reticulum as well as the biomechanical properties of the muscle and tendon (Close, 1972).

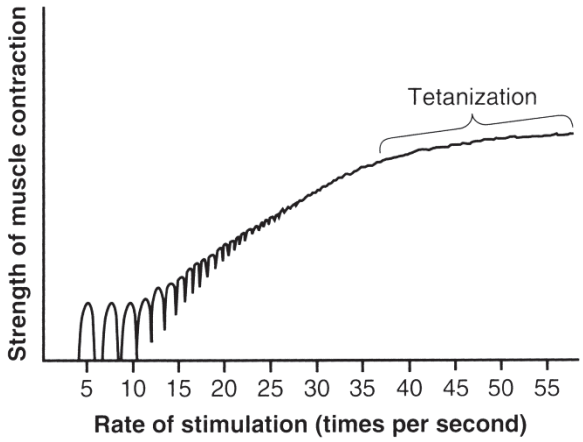


**Figure 1-9** Progression of a tetanic response. Note the gradual fusion of successive twitch responses as the frequency increases (Guyton & Hall, 2006).

## 1.8 Denervation

When peripheral nerves are cut or crushed during a sustained or acute injury, the muscles lose their connection with the central nervous system and become denervated. In order for full functional recovery to occur, the peripheral nerves must grow from the site

of injury until they reach the muscle and form functional neuromuscular junctions. This section will describe the effects of denervation on both the nerve and muscle.

### 1.8.1 Effects on Peripheral Nerve

When a nerve is sectioned into two parts, different changes occur in each of the parts. The section of nerve that is distal to the injury, called the distal stump, undergoes a process termed Wallerian degeneration. This process was first described by Waller in 1850 after he observed changes to the nerve stump following transection of cranial nerves in frogs (Waller, 1850). This process involves the breakdown of the cytoskeleton and degeneration of the axon. As early as 5 minutes following nerve transection the distal stump undergoes short distance (hundreds of microns) acute axon degeneration (Kerschensteiner, Schwab, Lichtman, & Misgeld, 2005). Sealing of the distal stump follows and retraction of the myelin sheath occurs at the nodes of Ranvier. Axoplasmic transport still occurs during this time, as bulging at the nodes of Ranvier and the sealed end of the distal stump have been observed (Waxman & Kocsis, 1995). Over the next 24-72 hours there is a latent period of degeneration. During this time, the distal stump remains electrically conductive and able to propagate action potentials towards the muscle when electrically stimulated (J. T. Wang, Medress, & Barres, 2012). Once the latent phase is over, the axon rapidly fragments. As the axon breaks down into its cytoskeletal components, the myelin sheaths also disintegrate and a proliferation of Schwann cells and macrophages aid in removing the debris (MacIntosh et al., 2006). Schwann cells also play an important role in nerve regeneration. They release a number of neurotrophic factors that promote neurite outgrowth and survival. These factors include

nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), and neurotrophin-4 (NT-4) (MacIntosh et al., 2006).

As the distal stump undergoes Wallerian degeneration, the other end of the nerve, called the proximal stump, undergoes changes as well. Just as in the distal stump, the terminal end is sealed almost immediately following transection. Axoplasm and other material form a swelling of the stump. Less than 24 hours after transection, the swelling further distends and growth cones become visible. New growing axons then penetrate this growth cone and elongate towards the distal stump. These axons can branch many times as well as sprout from the nodes of Ranvier that are proximal to the terminal end of the nerve (Fawcett & Keynes, 1990). Schwann cells play an important role in regeneration at the proximal stump. Besides providing trophic signals that guide branching and growth towards the distal stump, Schwann cells also preserve the distal stump by creating a band of cells (bands of Bungner) that surrounds the endoneurial tube (MacIntosh et al., 2006). When proximal axons reach this tube they are guided towards the muscle. Initially, the new axons lack myelin but over time they become myelinated. However, the proximal stump still undergoes degenerative changes (MacIntosh et al., 2006). It was found that if the new axons do not reinnervate muscular targets they start to atrophy (Gordon, Gillespie, Orozco, & Davis, 1991). Larger axons were found to atrophy the most, and some lose their structural shape and collapse (Gillespie & Stein, 1983).

### 1.8.2 Effects on Muscle

One of the most stark visual changes in a muscle that loses its functional connection is the rapid decrease in size, called denervation atrophy. In humans, muscle

fiber size can diminish by as much as 50% following 2-3 months of denervation (Aird & Naffziger, 1953). Muscle fibers in rodents decrease at an even faster rate. It is not uncharacteristic for atrophy in rats to reduce muscle fiber size by 50% in only 2 weeks time (Ohira, 1989). There is evidence that a loss of contractile activity in a muscle is at least partially the cause of this atrophy. Studies that used impulse conduction blocking chemicals (such as tetrodotoxin) in healthy muscles showed denervation-like changes that were no different from a muscle that had its nerve severed (Buffelli, Pasino, & Cangiano, 1997; Pasino et al., 1996). Atrophy is followed by two distinct phases, acute degeneration and fibrotic dedifferentiation (Tower, 1935) . In these two latter phases, not only are the muscle fibers atrophied but some of them undergo irreversible changes resulting in necrosis of the fiber. Mononuclear cells infiltrate the area of the necrotic fiber and phagocytose the debris. Fat cells eventually replace areas of degenerated muscle fibers (MacIntosh et al., 2006). Muscle spindles are also affected by denervation. The intrafusal muscle fibers undergo atrophy much like extrafusal (main) fibers and the intracapsular space within the spindle disappears (Elsohemy, Butler, Bain, & Fahnestock, 2009). Interestingly, atrophy does not affect fast and slow muscle equally. In the rat, slow muscles, such as soleus in the lower limb, undergo atrophy much more quickly and to a larger extent than fast muscles like the extensor digitorum longus in the lower limb (Al-Amood, Lewis, & Schmalbruch, 1991; Wroblewski, Edström, & Jakobsson, 1989). It has been suggested that the differences in rate of atrophy between types of muscles is caused by the natural length of the muscle or the amount of passive stretch it undergoes (Cotlar, Thrasher, & Harris, 1963).

Muscle fiber type changes also occur during denervation. However, various animal species exhibit different types of changes. In the guinea pig and rat there is a tendency for slow muscle fibers (type I) to change to fast muscle fibers (type II) (Karpati & Engel, 1968). In the rabbit, both muscle types progress towards type I fibers (Jaweed, Herbison, & Ditunno, 1975). Interestingly, muscles that have a mixed fiber type but are primarily fast, such as the diaphragm or gastrocnemius, transform into almost an exclusively fast type muscle (Carraro, Catani, & Dalla Libera, 1981).

Changes to ultrastructural components of the muscle following denervation have also been described. Various organelles within the muscle all show signs of atrophy. These organelles atrophy at very similar rates as the entire muscle fiber (Stonnington & Engel, 1973). Rates of atrophy are no different between fast and slow muscle types. However, both mitochondria and the sarcoplasmic reticulum first enlarge before atrophying (MacIntosh et al., 2006). The enlargement of the sarcoplasmic reticulum would have an impact on excitation-contraction coupling. It was found that the enlargement is due to an increase in $Ca^{2+}$ uptake (Brody, 1966; Germinario et al., 2002). This enlargement leads to greater amounts of $Ca^{2+}$ released into the sarcoplasm. When combined with a slower $Ca^{2+}$ reuptake, these changes support the hypothesis that early (2-4 days) changes in contractile properties following denervation are due to changes in the sarcoplasmic reticulum (Midrio, Danieli Betto, Betto, Noventa, & Antico, 1988).

The contractile changes mentioned usually represent a slowing in the contraction speed that is more evident in fast muscles than slow muscles (Lewis, 1972). Twitch and tetanic forces are also diminished due to atrophy of the muscle fibers. The twitch-tetanus

ratio increases and is likely caused by changes to the muscle membrane properties (Finol, Lewis, & Owens, 1981). Indeed, the muscle membrane undergoes a number of changes. The first change that occurs within hours of denervation is a drop in resting membrane potential caused by inhibition of the $Na^+$-$K^+$ pump and a decrease in membrane permeability to $K^+$ (Albuquerque & Thesleff, 1968; Bray, Hawken, Hubbard, Pockett, & Wilson, 1976). Shortly after, a transient increase of tetrodotoxin (TTX) resistant sodium channels appears in the muscle (Harris & Thesleff, 1971). These channels peak 3-5 days after denervation in rodents and then decline (Lupa, Krzemien, Schaller, & Caldwell, 1995). It was found that these new TTX-resistant channels are embryonic forms of mature sodium channels (Kallen et al., 1990; Trimmer, Cooperman, Agnew, & Mandel, 1990). Lastly, the muscle develops increased sensitivity to acetylcholine resulting from the expression of new acetylcholine receptors which can be detected approximately 24 hours after denervation (Axelsson & Thesleff, 1959).

## 1.9 Treating Denervated Muscle

Prolonged muscle denervation greatly affects functional recovery. As the length of denervation increases, the muscle becomes less receptive to new axons due to its atrophy and fibrotic tissue proliferation (Irintchev, Draguhn, & Wernig, 1990). An important study by Gordon and Fu examined the effects of different denervation times followed by nerve repair on functional recovery in rats. They concluded that long term denervation can account for a 90% reduction in the number of functional motor units (Fu & Gordon, 1995). The gold standard clinical treatment is to immediately repair a severed nerve. However, if this cannot be accomplished then other treatments need to be pursued to keep

the muscle healthy over the period of nerve regrowth. A great deal of literature has been written about different treatments for denervated muscle. Traditionally, they fall into two independent groups: neuromotor or neurotrophic treatment. The former bases treatment on providing electrical impulses to the muscle to simulate what the normal nervous system would provide. The latter group bases treatment on the premise that the axon does not only provide electrical impulses to maintain a muscle but also provides chemicals that act on the tissue independent of electrical activity. These two treatments will be discussed at length followed by a relatively new treatment called sensory protection, which has shown to be effective at improving functional recovery.

### 1.9.1 Electrical Muscle Stimulation

The use of electrical stimulation as a therapy is not something new. It was used as a medical treatment over 2000 years ago when one of the first Roman physicians, Scribonius Largus, recommended the use of torpedo fish, which provide electric shocks, to treat such minor ailments as headaches and joint pain (McNeal, 1977). It has also been used to increase muscle mass, strength and aerobic capacity (Banerjee, Caulfield, Crowe, & Clark, 2005).

The first reported use of electrical stimulation of denervated muscle was carried out by Reid in 1841 (Reid, 1841). Back then, the major argument was, what controls contractility of muscle. Most scientists hypothesized that muscle cannot contract independently of the nerve and this was disproven by Reid in his report. The experiments were carried out in frogs, similar to where Galvani started when he discovered bioelectricity. Daily galvanic stimulation (similar to using a battery) was applied to limb

muscles that were centrally denervated and after 2 months Reid noticed no difference in muscle size between treated and control limbs. Although Reid showed positive effects of electrical stimulation, the controversy over this treatment was just beginning.

In the early 1900s, Langley and Kato investigated the use of galvanic stimulation to reduce fibrillation potentials that occur during denervation (Langley & Kato, 1915). The hypothesis at that time was that muscle atrophy was due to fibrillation causing the muscle to fatigue. Additional stimulation provided electrically would be of no benefit. Langley and Kato concluded that galvanic stimulation did not prevent fibrillation and benefits to muscle weight were minimal. However, they stated that the surface stimulation they were using may have been too weak to forcefully contract the muscle. In a review paper a few years later, Langley and Hashimoto showed that much stronger galvanic stimulation can stop fibrillation but does not prevent atrophy (Langley & Hashimoto, 1918). In 1920 a study by Hartman in Toronto investigated daily galvanic stimulation in rabbit limbs that had their sciatic nerves crushed. The treatment involved 15 minutes of galvanic current delivered at 1 Hz. Hartman concluded that electrical stimulation was of no benefit (Hartman & Blatz, 1920). He also stated that the currents used only contracted superficial muscle fibers.

It was not until the 1930s that Fischer stated the obvious, that weak currents may be the problem causing electrical stimulation to be ineffective. Fischer repeated the previous experiments using 12-20 minutes of daily faradic or galvanic stimulation in denervated rat gastrocnemius and soleus muscles (Fischer, 1939). The difference between faradic current and galvanic current is that faradic is alternating at a predetermined

frequency whereas galvanic is direct current. Fischer updated the way galvanic current is delivered using a switching apparatus to produce frequencies of stimulation between 8 and 20 Hz. He concluded that vigorous contractions produced using strong currents can markedly reduce muscle atrophy. Fischer also investigated the optimal time to start electrical stimulation. He stated that to obtain maximal benefit of the treatment it must be started immediately after denervation, a point that was confirmed by others (Gutmann & Guttmann, 1944). Solandt et al. investigated the use of faradic, galvanic, and 25 and 60 Hz sinusoidal currents on denervated rat and soleus muscles (Solandt, DeLury, & Hunter, 1943). They concluded that 25 Hz sinusoidal current produced the best results in terms of minimizing atrophy and this was supported by others (Grodins, Osborne, Johnson, Arana, & Ivy, 1944). Despite the literature using strong currents in reducing atrophy the negative effects reported for weaker stimuli have added controversy and delayed adoption of electrical stimulation for clinical use.

The next four decades produced the majority of the literature in the field. Most of these studies investigated the natural pattern of electrical discharge in fast and slow muscles and effects of stimulation on contractile, metabolic, and histological properties of the muscle. A landmark study was published by Buller, Eccles, and Eccles in which a nerve innervating a slow muscle was crossed with a nerve innervating a fast muscle (Buller, Eccles, & Eccles, 1960). This cross-union produced muscles with properties similar to the original muscles, demonstrating a phenotypic influence of motor nerves on muscle. This work was furthered by Salmons and Vrbová where they stimulated electrically quiescent soleus muscle, tibialis anterior, and extensor digitorum with a slow

pattern (Salmons & Vrbová, 1969). They showed that a continuous slow pattern being delivered to a normally innervated fast muscle can change the phenotype to one that resembles a slow muscle. Similarly, stimulating an electrically silent slow muscle with a slow pattern can prevent the natural shift towards a fast phenotype. This work demonstrated that stimulation with a pattern that resembles normal activity can maintain muscle phenotypes. Lømo and colleagues extended this work by stimulating denervated soleus muscle at both fast (100 Hz) and slow (10 Hz) frequencies with a daily mean frequency of 2 Hz in both cases (Lømo, Westgaard, & Dahl, 1974). The reason to stimulate denervated muscle was to remove any effects an intact nerve can have on contractile properties of the muscle. Even though the stimulation patterns were not representative of physiological motor drives, it still managed to change the contractile properties of a muscle. When stimulated at 100 Hz, the soleus had properties similar to a fast muscle. Slow frequency stimulation at 10 Hz maintained the soleus muscle as a slow type. In both cases, muscle atrophy was significantly reduced. However, the conclusion that frequency of stimulation can modify contractile properties was not supported by others (Al-Amood, Finol, & Lewis, 1986; Termin, Staron, & Pette, 1989). Al-Amood and Lewis investigated the use of constant frequency (40 Hz) stimulation with differing stimulus intervals (60-600 s) and the use of constant intervals with varying frequency (10-100 Hz) and number of pulses on denervated rat soleus (Al-Amood & Lewis, 1987). They concluded that contraction times stayed the same regardless of the frequency used but confirmed that slow continuous stimulation at 10 Hz can prevent changes in denervated soleus muscle. Based on their own observations and those of others (Eerbeek, Kernell, &

Verhey, 1984; Sreter, Pinter, Jolesz, & Mabuchi, 1982), Al-Amood and Lewis stated that it is the time between contractions rather than the frequency that can alter contractile properties of muscles.

Although most researchers tried to use stimulation patterns that mimicked the natural firing frequency, there was no conclusive quantitative data of what the firing frequency is *in vivo*. Hennig and Lømo solved this problem by examining firing patterns in a number of different motor neurons in rat fast and slow rat muscle over a 24 hour period (Hennig & Lømo, 1985). They quantified the number of impulses each type of motor unit produced along with the firing frequency. The major conclusion was that slow muscle fibers have a continuous motor drive firing, receiving almost 500,000 impulses per day, whereas fast fatiguing fibers have very brief and short motor drive, receiving up to 11,000 impulses per day. These results allowed Eken and Gunderson to use this information to test a new pattern of stimulation on fast muscle that resembles physiological activation of the muscle (Eken & Gundersen, 1988). Their triplet pattern, which featured three pulses with the first two separated by 5 ms and the last two by 10 ms, preserved normal contractile properties in fast muscle.

By the late 1980s there was no doubt that electrical stimulation can change or preserve muscle properties. Advances in miniaturization led to microelectronics which allowed the development of a number of implantable stimulators for animal studies (Dennis, 1998; Dennis, Dow, & Faulkner, 2003; Jarvis & Salmons, 1991; Lanmuller et al., 2005; Loeb, Peck, Moore, & Hood, 2001). This facilitated the adoption of continuous 24 hour stimulation paradigms. Williams has shown both in animals and humans that

when using a commercial implantable stimulator, a 24 hour stimulation paradigm was able to drastically reduce muscle atrophy (Nicolaidis & Williams, 2001; Williams, 1996). More recently, Dow and colleagues investigated 24 hour stimulation on denervated rat extensor digitorum muscle and showed that the use of a high frequency pattern (100 Hz) was effective at maintaining muscle mass at levels no different than a control muscle (Dennis et al., 2003). The same group then investigated the number of contractions to maintain muscle mass and force using the same 100 Hz pattern (Dow et al., 2004). They found that 200 to 800 contractions per day maintained muscle weight and force, and in some cases hypertrophied muscle fibers. To extend previous work by Al-Amood and Lewis, Dow and colleagues then investigated the distribution of rest periods for a various number of daily contractions (Dow, Faulkner, & Dennis, 2005). They concluded that stimulation can be compressed into 4 hour work sets and be effective at maintaining mass and force if 200-800 contractions per day are delivered. When 100 contractions were used in one day with a rest period of 20 hours, the results were similar to a denervated muscle with no stimulation. Although 24 hour chronic stimulation was shown to be beneficial at maintaining mass and force, a paradigm that does not require continuous stimulation would be more beneficial for treating denervated muscle clinically. This is because 24 hour paradigms require costly implantable stimulators and may provide patients with discomfort as stimulation continues throughout the day.

Ashley and colleagues investigated various patterns of stimulation delivered to denervated muscle for only one hour per day (Ashley et al., 2008). However, their model allowed the onset of denervation changes to occur over a 10 week period before

stimulation was started. This made it difficult to compare results to previous studies where stimulation is normally started immediately following nerve transection. Nevertheless, Ashley and colleagues showed that as little as 24,000 impulses per day delivering 1200 contractions daily significantly increased force and weight. These results suggest that in both rabbit and rat (Dow et al., 2004) a small number of daily contractions and impulses delivered over a short period of time can facilitate recovery of muscle mass and force.

The majority of the previously discussed studies investigated electrical stimulation on denervated muscle without examining functional recovery following a nerve reconnection. Functional recovery is typically defined by muscle weight, force, contractile properties, reinnervation, and histological results. Interestingly, the use of a 24 hour paradigm during 3.5 months of denervation in rat muscle prior to reinnervation was shown to have no benefit on functional recovery (Dow, Cederna, Hassett, Dennis, & Faulkner, 2007). This was in contrast to work by Williams and Marqueste and colleagues where electrical stimulation had positive effects on functional recovery (Marqueste et al., 2006; Nicolaidis & Williams, 2001; Williams, 1996). However, the studies with positive results all continued to stimulate the muscle once functional reinnervation was thought to have taken place, potentially confounding the results (Dow et al., 2007). More recently, Gigo-Benato and colleagues electrically stimulated rat tibialis anterior muscle following sciatic nerve crush (Gigo-Benato et al., 2010). They concluded that stimulation has detrimental effects on functional recovery in that muscle weights and cross sectional area were decreased. They stated that electrical stimulation should not be used prior to

reinnervation. However, these results were questioned as only 20 contractions were produced during stimulation sessions that occurred every 48 hours (Salmons, 2011). The negative results may be attributed to low levels of stimulation.

Effects of electrical stimulation on reinnervation have also been investigated. There are many reports that show electrical stimulation prior to and continued after reinnervation has no adverse affects (Cole & Gardiner, 1984; Eberstein & Pachter, 1986; Sebille & Bondoux-Jahan, 1980; Valencic, Vodovnik, Stefancic, & Jelnikar, 1986). The muscle endplates from short-term stimulated animals are closer in appearance to control muscles than those animals that did not have stimulation (Eberstein & Pachter, 1986; Sebille & Bondoux-Jahan, 1980). Similarly, Jansen and colleagues showed that stimulation following nerve crush does not affect axonal regrowth and reinnervation (Jansen, Lomo, Nicolaysen, & Westgaard, 1973). However, there is a growing body of evidence stating that chronic stimulation suppresses reinnervation. Hennig reported that a delayed onset of electrical stimulation following cryo-freezing of the sciatic nerve in rats reduces reinnervation as judged by twitch force ratios (Hennig, 1987). Ironton and colleagues reported that stimulation immediately following partial denervation in mice reduces the number of terminal sprouts being formed but not the number of nodal sprouts (Ironton, Brown, & Holland, 1978). This was confirmed more recently by Love et al. where they found that electrical stimulation affects the processes grown by terminal Schwann cells (Love, Son, & Thompson, 2003). These processes aid in promoting terminal sprouting in partially denervated muscle. They concluded that stimulation may be providing the Schwann cells with a chemical cue to inhibit sprouting, supporting the

hypothesis that denervated muscle is sending out diffusible signals or trophic factors (Brown, Holland, & Hopkins, 1981; van Mier & Lichtman, 1994).

Electrically stimulating denervated muscle has come a long way since Reid's first report in 1841. It is clear that electrical stimulation can change muscle contractile properties depending on the stimulation pattern used. New short term stimulation patterns along with continuous 24 hour patterns have shown promising results in reducing muscle atrophy. Although there are studies that claim stimulation to be ineffective, the protocols used typically do not elicit strong contractions or fail to provide an adequate number of daily stimuli. There still are concerns with the effects of stimulation on reinnervation and functional recovery. However, limited human trials have shown that long term electrical muscle stimulation can significantly improve functional outcomes (Kern, Salmons, Mayr, Rossini, & Carraro, 2005; Mödlin et al., 2005), making this an important treatment for denervated muscle. A limited representation of the outlined research is visualized in a timeline shown in Figure 1-10.

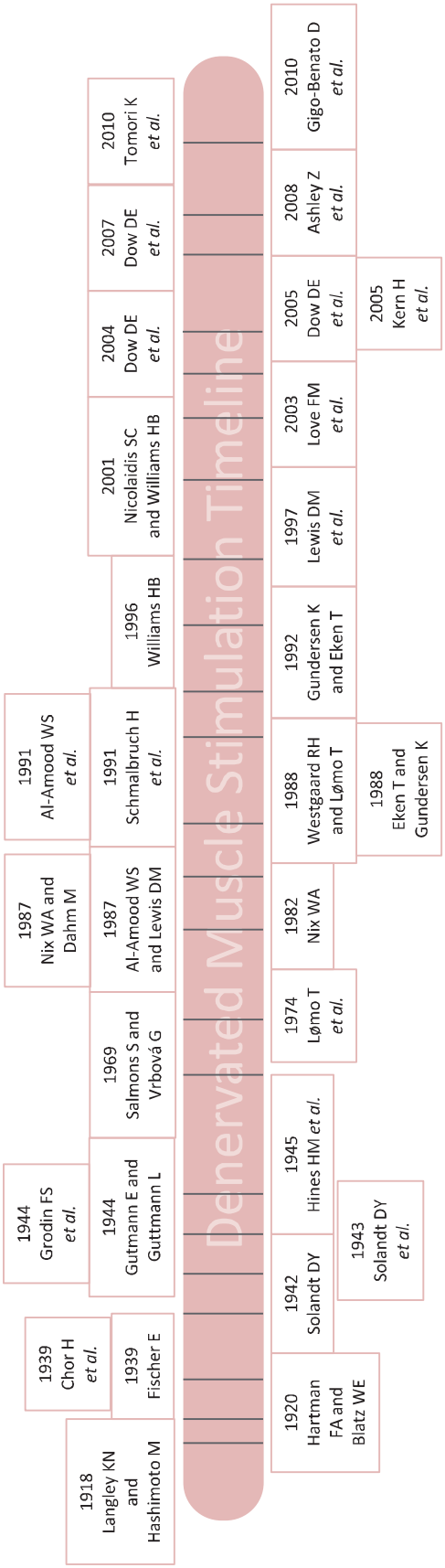


**Figure 1-10** A partial representation of published research where electrical muscle stimulation was investigated either to treat denervated muscle or to modify muscle contractile properties.

### 1.9.2 Support by Soluble Factors

In the 1930s Tower investigated whether peripheral nerve lesions were the sole source of denervation atrophy. She designed a clever study where the ventral roots of a spinal nerve were cut and compared that to sectioning of a peripheral nerve. She concluded that the degenerative changes were similar in both cases, ruling out any effect of sensory afferents on denervation atrophy (Tower, 1935). Furthermore, Tower extended this work by examining differences between denervated muscle (via peripheral nerve section) and disused muscle (via spinal cord section and deafferentation). Tower reported histological differences between the muscles and concluded that having an intact nerve provides trophic support to the muscle and that atrophy following denervation is a result of both a lack of contractile activity and trophic support (Tower, 1937). In the important study by Buller and colleagues where the innervation of fast and slow muscles was crossed, they suggested that differentiation of muscle type was due to trophic influences from fast and slow type motoneurones (Buller et al., 1960). This led to numerous studies investigating the role of trophic factors in determining muscle contractile properties. Lømo and colleagues disproved Buller's hypothesis by denervating the soleus muscle in the rat and electrically stimulating it with fast and slow paradigms. The contractile properties of the muscle changed according to the paradigm used demonstrating that it is activity that determines the contractile properties (Lømo et al., 1974). Other studies used conduction blocking agents like tetrodotoxin on intact nerves and showed that no differences were found in contractile and histological properties between conduction blocked muscles and denervated muscles (Buffelli et al., 1997). This gave further support

to the fact that muscle contractile properties are activity dependent and not trophic dependent. However, this conclusion was in contrast to Tower's work comparing denervated muscle and disused muscle. Further studies into spinal cord sectioned animals revealed that all impulses are not abolished when a spinal cord is severed (Eldridge, Liebhold, & Steinbach, 1981; Johns & Thesleff, 1961). Spontaneous discharges have been observed and this small amount of contractile activity may be enough to modify muscle properties (Lømo et al., 1974).

The use of exogenous treatments for denervated muscle has also been explored. Extract from sciatic nerves was used to promote growth of chick muscle in culture (Popiela, 1978). This work was extended by Davis and Kiernan by injecting sciatic nerve extract in denervated rat extensor digitorum longus muscle (Davis & Kiernan, 1980, 1981). They stated that myotrophic influence was responsible for 40% of the decrease in muscle weight and type IIB fiber cross sectional areas. The remaining 60% could be accounted for by muscle disuse.

As individual myotrophic factors were identified their roles in maintenance of muscle were investigated. Application of ciliary neurotrophic factor (CNTF) in denervated muscle was shown to decrease muscle atrophy and increase twitch and tetanic forces (Helgren et al., 1994; Huang, Wang, Hong, Wan, & Kang, 2002). Although synthesized by Schwann cells, with primary effects on neurons (Arakawa, Sendtner, & Thoenen, 1990), muscles also have receptors for CNTF and these are upregulated following denervation (Helgren et al., 1994). Other myotrophic factors that have shown positive results on reducing denervation associated changes are insulin-like growth factor-1 (IGF-

1) and leukemia inhibitory factor (LIF). IGF-1 was injected into denervated mouse gastrocnemius muscle throughout 8 weeks of denervation. Following this period, muscle weight and fiber diameter were similar to muscles of fully innervated controls. However, twitch and tetanic forces were approximately 50% of control values (Day et al., 2002). LIF has shown benefits in cell culture by stimulating myoblast proliferation and skeletal muscle regeneration following injury (Austin, Bower, Kurek, & Vakakis, 1992; Barnard, Bower, Brown, Murphy, & Austin, 1994). The effect of LIF on rat gastrocnemius muscle following nerve crush was reported by Finkelstein et al. (Finkelstein, Bartlett, Horne, & Cheema, 1998). They showed significant increase in muscle weight and fiber cross sectional area 8 weeks following crush and a single injection of LIF. Interestingly, type II fibers responded more favorably to LIF than type I fibers with increases of 83% versus 54% versus nerve crushed controls, respectively.

Neurotrophic factors such as brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), and neurotrophin-3 (NT-3) have been shown to be effective in promoting or maintaining innervation of skeletal muscle (Kucera, Ernfors, Walro, & Jaenisch, 1995; Lohof, Ip, & Poo, 1993; T. Wang, Xie, & Lu, 1995). Application of exogenous BDNF, GDNF, or a combination of the two at least 10 days following axotomy dramatically increased axonal outgrowth (Boyd & Gordon, 2003). More specifically, GDNF has been reported to be an important regulator in synaptic plasticity (Keller-Peck et al., 2001). Over-expression of skeletal muscle GDNF led to increased branching of motoneuron axons and the creation of multiple end plates leading to hyperinnervation (Zwick, Teng, Mu, Springer, & Davis, 2001).

The hypothesized role of trophic support in muscles has changed dramatically over the years. Initially, it was thought that contractile properties were controlled by chemical substances within the nerve. The advent of electrical stimulation has shown that it is the activity within the muscle that determines contractile properties. As molecular biology techniques were refined, specific neurotrophic and myotrophic factors have been identified. The role of each of these is becoming clearer, however the use of exogenous trophic factors clinically is still not a viable option as the administration, dosage, and side effects, are not completely understood. Another therapy that has shown promising results is sensory protection.

### 1.9.3 Sensory Protection

This treatment modality connects a sensory nerve to the distal stump of a denervated muscle in order to babysit it during the period of denervation. The use of sensory to muscle nerve grafts was investigated over 100 years ago (Langley & Anderson, 1904), and this early work showed that no motor connections were made. Weiss and Edds confirmed this finding and suggested that although sensory fibers can make contact with a muscle they do not prevent denervation atrophy or maintain contractile properties because they lack the “trophic action” or contractile support that motoneurones normally provide (Weiss & Edds, 1945). Gutmann independently came to similar conclusions and showed that sensory fibers do not form connections with the end plate region in the muscle (Gutmann, 1945).

These early negative results stymied further work in this area. However, Karpati and colleagues showed positive results following 4 and 10 months of having the sural

nerve connected to the denervated soleus muscle (Karpati, Carpenter, & Charron, 1981). However, they also reported that these positive results may be due to innervation from the small amount of motor fibers present in the sural nerve.

Instead of using a sensory nerve to protect a muscle, other groups have removed dorsal root ganglia (DRG) from donor animals and implanted them into the distal stump of a denervated muscle (Ochi, Kwong, Kimori, Chow, & Ikuta, 1992). The reported results showed a minimization of muscle atrophy, increases in tetanic force and weight in muscles that were protected with DRG for 72 to 286 days, suggesting a trophic influence of the implanted DRG. In a follow up study, the same group examined the effects of implanted DRG on two different muscles and found similar results in that atrophy was significantly minimized and muscle fiber area significantly increased (Ochi et al., 1996).

Hynes and colleagues re-examined the sensory to motor nerve crossover procedure that Karpati first published but used shorter time points of 1 and 2 months and showed a significant decrease in atrophy for muscles that had a sensory nerve connected to them compared to a purely denervated muscle (Hynes, Bain, Thoma, Veltri, & Maguire, 1997). They called this method sensory protection, as they hypothesized that clinically a sensory nerve may be used to babysit a denervated muscle until an appropriate motor supply is available.

Wang and colleagues extended the previous work by comparing several sensory protection treatments (DRG implantation, sensory nerve implantation, pre-ganglionic sensory nerve implantation, and sensory nerve crossover) in a single animal model over two different time points, 1 and 3 months (Wang, Gu, Xu, Shen, & Li, 2001). They found

that the effects of DRG implantation were not sustained over a 3 month period. Pre-ganglionic and distal sensory nerve implantation were no different from each other and showed significant benefits in reducing muscle atrophy and fiber area reduction in both 1 and 3 month periods. However, sensory nerve crossover had the most dramatic impact on denervation atrophy, confirming the results obtained by Hynes et al.

In a more clinically applicable study, Bain and colleagues extended the sensory protection model by investigating the effects on functional recovery by reconnecting a motor nerve following 2, 4, or 6 months of sensory protection (Bain, Veltri, Chamberlain, & Fahnestock, 2001). Results showed significant increases in twitch and tetanic forces along with muscle weight in 4 and 6 month sensory protected gastrocnemius muscle. Histological features were also improved. Sensory protected muscle had diminished levels of connective tissue and more regular shaped muscle fibers compared to denervated muscle. Papakonstantinou and colleagues supported these results by using sensory protection in a rodent upper limb model. Using a 6 month sensory protection period they reported superior results in terms of significant increases of muscle weight and improved behavioural tests for sensory protected compared to the denervated muscles (Papakonstantinou, Kamin, & Terzis, 2002).

Molecular aspects of sensory protection were examined and it was found that GDNF is maintained at levels closer to immediately repaired muscles than to denervated muscles (Zhao, Veltri, Li, Bain, & Fahnestock, 2004). Another study investigating the involvement of the distal stump concluded that sensory protection does not require the distal stump to impact functional recovery but that the stump may contribute to nerve

regeneration (Veltri, Kwiecien, Minet, Fahnestock, & Bain, 2005). More recently, a molecular investigation of the impact of sensory protection on the denervated stump showed that BDNF and GDNF levels are returned to control values and that these two molecules may play a role in communication between a nerve and its distal target (Michalski, Bain, & Fahnestock, 2008).

The early results of Weiss and Edds were extended by Elsohemy and colleagues in that sensory fibers do make their way to muscle and innervate spindles (Elsohemy, Butler, Bain, & Fahnestock, 2009). Following 3 or 6 months of sensory protection they showed that muscle spindle numbers were no different than in an immediately repaired muscle. Microscopic examination of the spindles showed they were much better preserved than those from denervated muscles. Electrophysiological responses to muscle stretch were also greater in the sensory protected muscles.

Lastly, the model of sensory protection has been demonstrated clinically with a favourable outcome. A patient who had a complete sciatic nerve palsy following hip arthoplasty reported early signs of proprioceptive responses and clinically significant functional outcomes 1 year following sensory protection (Bain, Hason, Veltri, Fahnestock, & Quartly, 2008).

There has been plenty of evidence to suggest that sensory protection has a positive impact on denervated muscle. However, muscle atrophy was only reduced and not ameliorated. Even with a favourable clinical outcome, recovery may have been enhanced if atrophy and other degenerative changes were eliminated. Nevertheless, sensory protection is a valid therapy for treating denervated muscle. A limited representation of

the outlined research for both treatments (soluble factors and sensory protection) is visualized in a timeline shown in Figure 1-11.

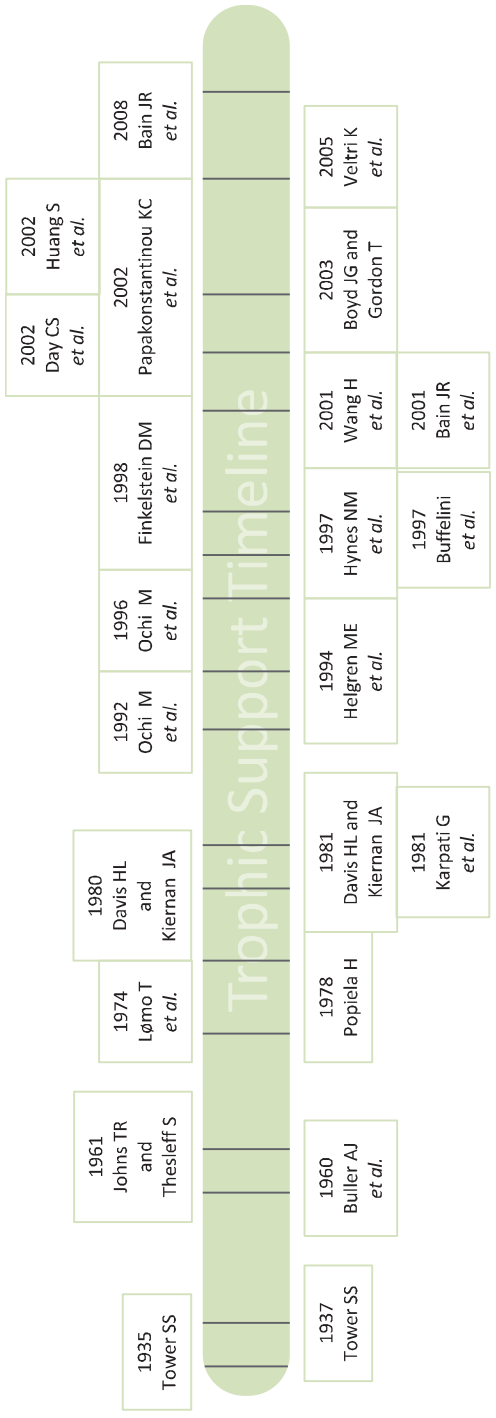


**Figure 1-11** A partial representation of published research where either soluble factors or sensory protection was used to treat denervated muscle.

## 1.10 Objective of This Thesis

The overarching goal of this work is to investigate the hypothesis that electrical stimulation of denervated muscle reduces muscle atrophy and improves functional outcome measures. This will be answered through the work of four separate but related studies that are presented as chapters in this thesis.

Although the background literature shows examples of how electrical stimulation minimizes atrophy, the protocols that were most effective used 24 hour stimulation or short term very low-frequency stimulation which can modify muscle contractile properties. Thus, one of the first questions to be answered is, can stimulation be effectively delivered in a one hour time frame and also decrease muscle atrophy while maintaining the muscle phenotype? This was answered through the development of a new one hour stimulus paradigm. This paradigm was then used to investigate the role of electrical stimulation in immediately repaired muscle and the impact on reinnervation of long term denervated muscle. Lastly, the combination of sensory protection and electrical stimulation following long term denervation was investigated.

### 1.10.1 Hypothesis

In summary, the overarching hypothesis for this thesis is that electrical stimulation of denervated muscle reduces muscle atrophy and improves functional outcome measures.

This will be tested through the following four specific hypotheses:

1) One hour of daily high-frequency electrical muscle stimulation reduces atrophy during short term muscle denervation.
2) Short term daily electrical muscle stimulation following immediate nerve repair improves functional outcome measures.
3) Chronic daily electrical muscle stimulation during long term denervation enhances reinnervation.
4) The combination of sensory protection and electrical stimulation enhances functional outcome measures more than either treatment alone.

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# Chapter 2: Instrumentation

## 2.1 Overview

This chapter describes the design and development of the necessary instrumentation that was built to test the hypothesis outlined in chapter 1. Three main pieces of hardware will be described: an electromyography (EMG) amplifier, a force transducer amplifier, and a multi-animal electrical muscle stimulator.

## 2.2 Electromyography (EMG) Amplifier Design Requirements

A multi-purpose amplifier was needed to amplify muscle action potentials with the ability to be adapted for nerve recordings. This amplifier was primarily used to obtain electromyography recordings during motor unit estimation. These are the high level design requirements for this amplifier to ensure flexibility of the device for use in multiple research objectives:

1. Low noise design to minimize instrumentation noise in low level signal acquisition recordings such as nerve action potentials;
2. High bandwidth to accommodate different biological signals;
3. Multiple gain selection;
4. Compatibility with existing data acquisition devices;
5. Device portability.

As EMGs are typically large signals (on the order of millivolts for surface EMG), commercial amplifiers sacrifice low noise design for lower cost. Nevertheless, commercial research amplifiers that are used in animal studies range in cost with most

being priced over $1000. Although low cost research amplifiers have been proposed (less than $10 in cost) they lack the robustness necessary for long term use in a laboratory setting (Bhaskar, Tharion, & Devasahayam, 2007). The proposed amplifier reduces cost by offloading all filtering to software, eliminating the need for multiple hardware amplifiers for filtering use. A simple Bayonet Neill–Concelman (BNC) connector was chosen to be used to interface with commercial data acquisition devices, as this type of connector is widely used. Portability of the device was achieved by using surface mount components to reduce the profile of the printed circuit board (PCB).

### 2.2.1 EMG Hardware Implementation

A block diagram of the amplifier is shown in Figure 2-1. The amplifier can be split into three main distinct stages: power conditioning, pre-amplifier, and gain.

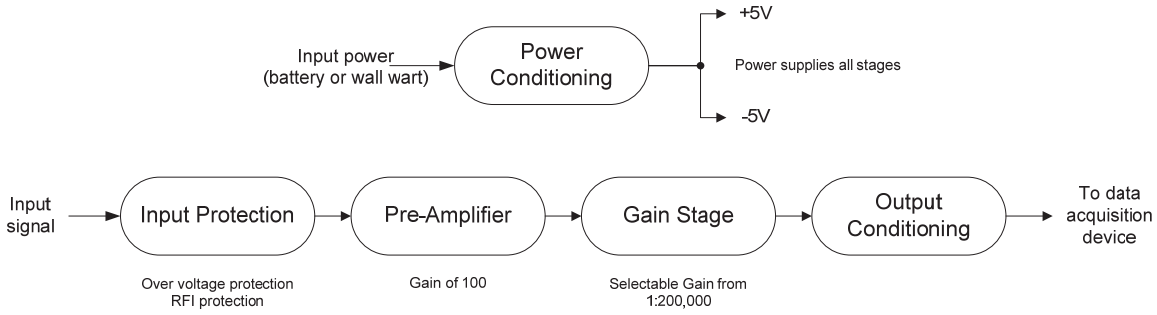


**Figure 2-1** A block diagram of the EMG amplifier design. The power conditioning block supplies power to all operational amplifiers in the pre-amplifier and gain stage blocks.

Input power is provided either via batteries that are regulated to a 5 V DC output voltage or by commercially available wall warts that are preset to convert mains power (120V AC) to a stable 5 V DC. The amplifier then conditions the input power by filtering it using a passive low pass LC design (cut off frequency at 27.3 KHz) and also passing it

through an integrated circuit (IC) (TPS60403 charge pump voltage inverter, Texas Instruments, USA) that converts the positive input to a negative voltage.

The pre-amplifier is the main differential amplifier used to remove common mode voltage and provides the first stage of amplification. A low noise (1 nV/√Hz) instrumentation amplifier IC was chosen to center the design on (AD8429, Analog Devices, USA). The trade-off with this IC is that the power requirements are much higher than typical instrumentation amplifiers (6.7mA quiescent current). The gain of the amplifier was chosen to maximize the common mode rejection ratio (CMRR) and minimize instrumentation noise. This gain was 100, resulting in a CMRR of 120 dB from DC to 60 Hz and 1 nV/√Hz noise from 100 Hz to 100 kHz. At frequencies above 60 Hz the CMRR dropped exponentially but was well over 100 dB at frequencies up to 1 kHz. To increase the robustness of the amplifier, the inputs to the IC were protected from high voltages using current limiting resistors and clamping diodes.

The gain stage consisted of four operational amplifiers (op-amps) connected in series, with two providing gain and two acting as buffers. The first op-amp was used to provide a range selection for amplifying the signal at a gain of 1, 10, or 100. This was followed by a high pass filter to remove any DC offsets and a buffer op-amp. The third op-amp acted as the primary gain amplifier with a selectable gain of 1, 25, 50, 100, or 200. This was followed once again by a high pass filter and a buffer op-amp. The resulting signal was fed to the BNC connector for output to the data acquisition device. Total gain of the amplifier can range from 1 to 2,000,000, which is useful for amplifying low level signals such as nerve action potentials or larger signals such as

electromyograms. The bandwidth of the amplifier was not limited, to preserve as much of the original signal as possible. A schematic of the amplifier is shown in Figure 2-2 along with renderings of the printed circuit board in Figure 2-3.

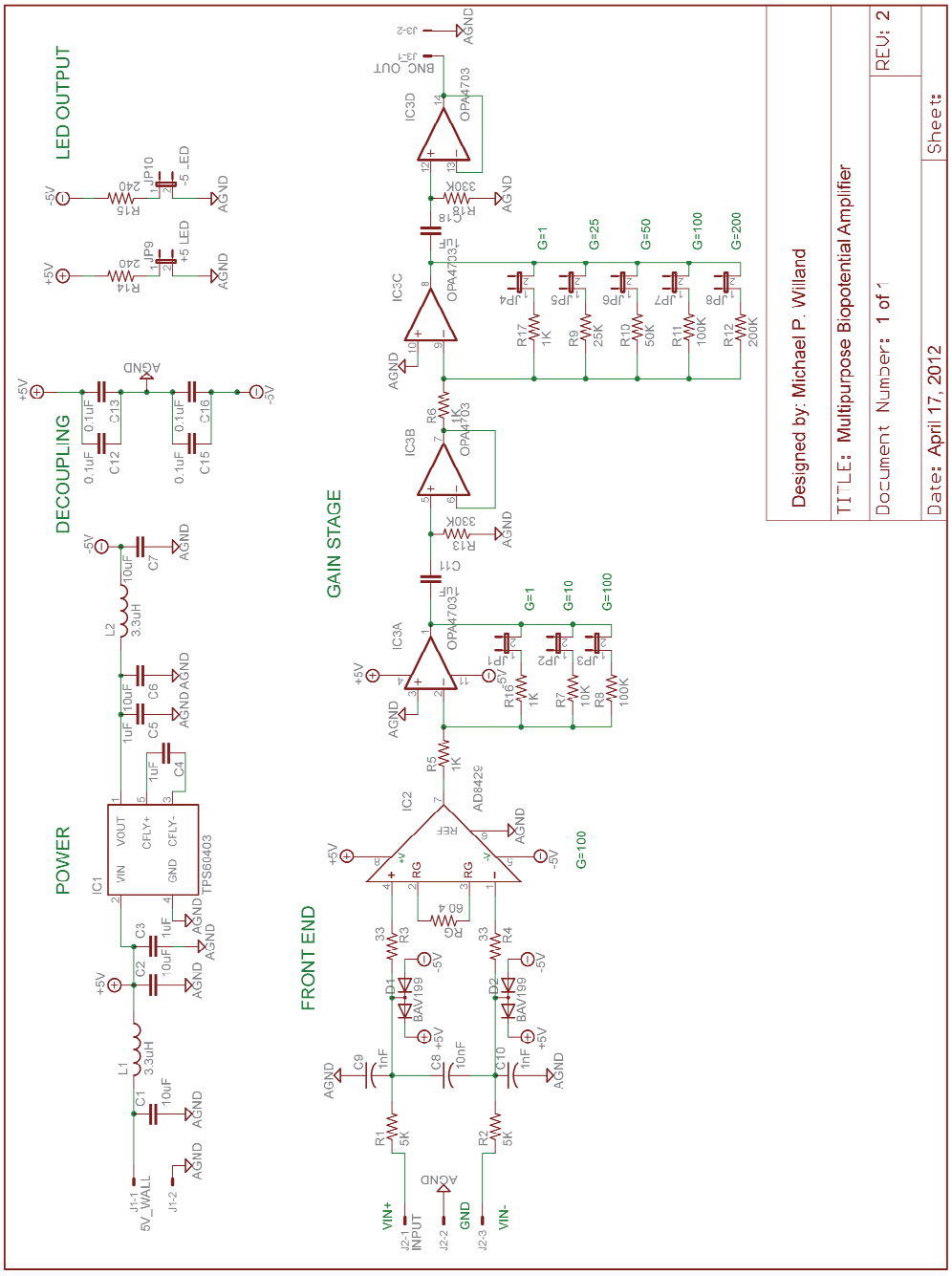


**Figure 2-2** Schematic of the electromyography amplifier.

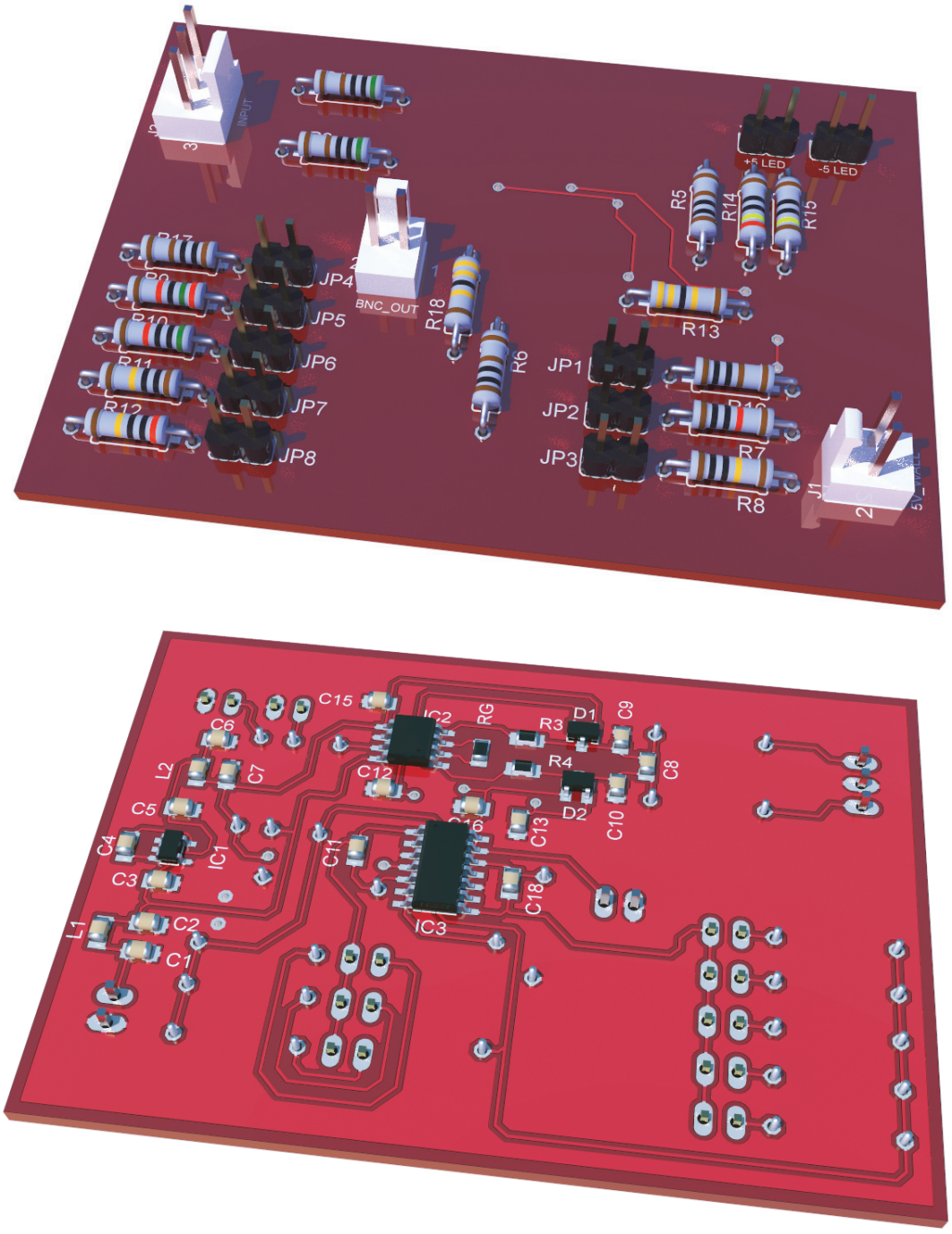


**Figure 2-3** Printed circuit board 3D renderings of the amplifier. The top of the board is shown above and the underside of the board is shown below.

## 2.3 Force Transducer Amplifier Design Requirements

All measurements of muscle tension or force were carried out using a Grass force displacement transducer (FT03, Grass Technologies, USA). This transducer is composed of four strain gage elements in a Wheatstone bridge configuration. As with the EMG amplifier, the commercial interface for this transducer costs well over $1000. Therefore, a low cost version was designed and built. The requirements for this amplifier were based on the commercial device:

1. Manual and Auto-zero feature to quickly remove offset voltages;
2. Multiple gain selection;
3. Device portability.

### 2.3.1 Force Transducer Amplifier Hardware Implementation

The amplifier design was split into two components, analog signal acquisition and digital baseline removal. A block diagram of the design is shown in Figure 2-4.

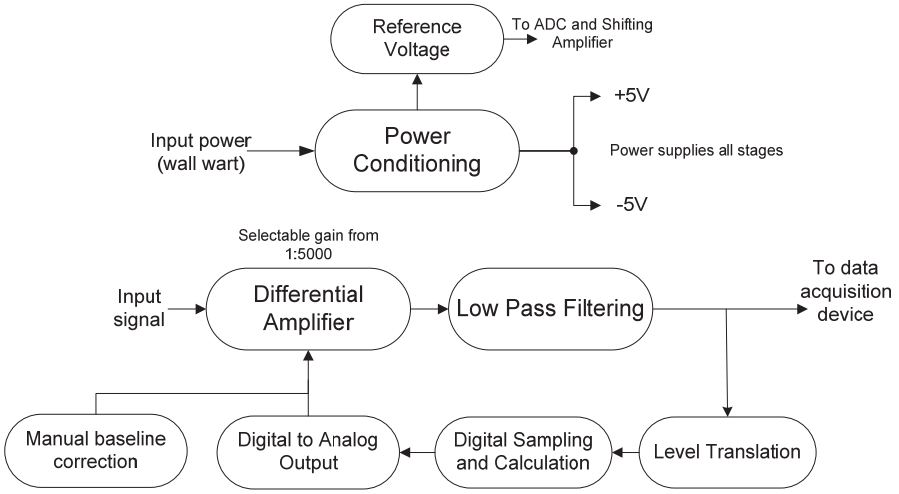


**Figure 2-4** Block diagram of the force amplifier design. The output from the low pass filter block is level translated and sampled for use in automatic baseline correction.

Input power to the device is provided from a regulated 5V wall wart plug. This is fed to a DC/DC converter (DCH010505D, Texas Instruments, USA) to create a negative voltage required for the Wheatstone bridge and also for the instrumentation amplifier. As the input signal to the amplifier is low level (on the order of millivolts) and contains common mode 60 Hz noise, it needs to be differentially amplified. A general purpose instrumentation amplifier (INA 128U, Texas Instruments, USA) was used to remove common mode signals and served as the primary gain stage. Variable gains from 100 to 5000 were designed into the device. Changing gains on an instrumentation amplifier also affects the common mode rejection ratio (CMRR). However, as the bandwidth of typical force profiles in muscle are much less than 1 kHz, at worst the CMRR is 125 dB at this frequency at a gain of 100. A selectable passive low pass filter follows the first stage and is used to remove any ringing artefact from the signal. The output signal is then fed to a BNC connector for connection with a data acquisition device. For manual adjustment of any offset voltages, a potentiometer was connected through an operational amplifier to the reference pin of the instrumentation amplifier. These offset voltages arise when the baseline force is non-zero. This is typical when a tendon is attached to the transducer and the muscle length is then optimized to produce maximal force. Although manual adjustment works well, it is tedious when baselines need to be corrected over many experiments. To address this issue a simple one button baseline removal technique was developed. This technique involved scaling and translating the output signal from the instrumentation amplifier in order for it to be digitized using a microcontroller. The scaling and translation was accomplished using an integrated circuit built for this purpose

(INA159, Texas Instruments, USA). A stable reference voltage (REF5050, Texas Instruments, USA) was used for accurate level translation and analog to digital conversion in the microcontroller. Scaled signals were fed into a microcontroller (PIC18F2620, Microchip, USA) and digitized. The baseline value of the digitized signal was calculated and then the digital value of appropriate negative voltage was sent to a digital to analog converter (MCP4821, Microchip, USA) that was connected to the instrumentation amplifier's reference input through a switch. The user can select whether to use a manual or automatic baseline adjustment through this switch. Schematics of this amplifier are shown in Figure 2-5 and printed circuit board renderings are shown in Figure 2-6.

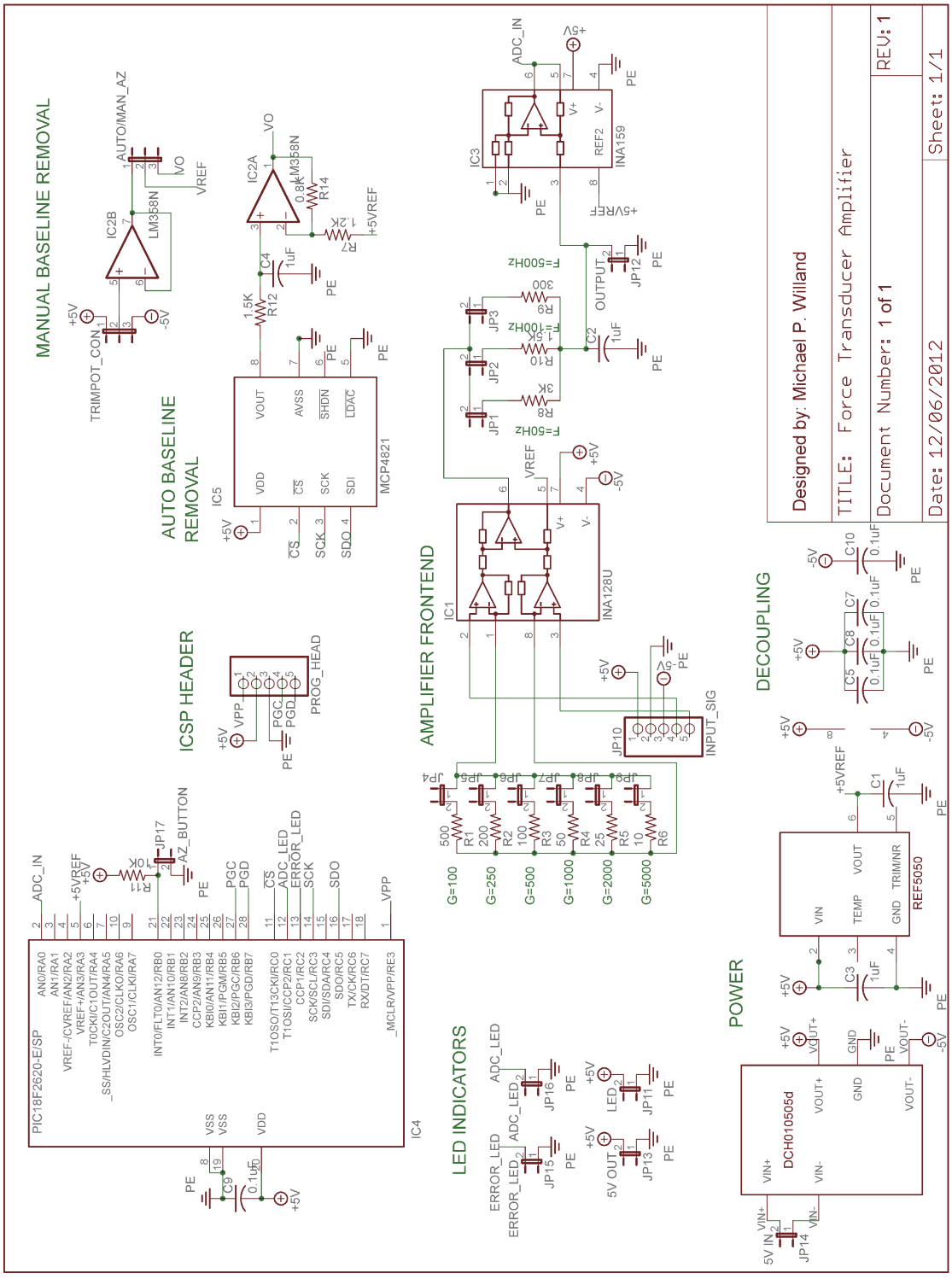


**Figure 2-5** Schematic of the force transducer amplifier.

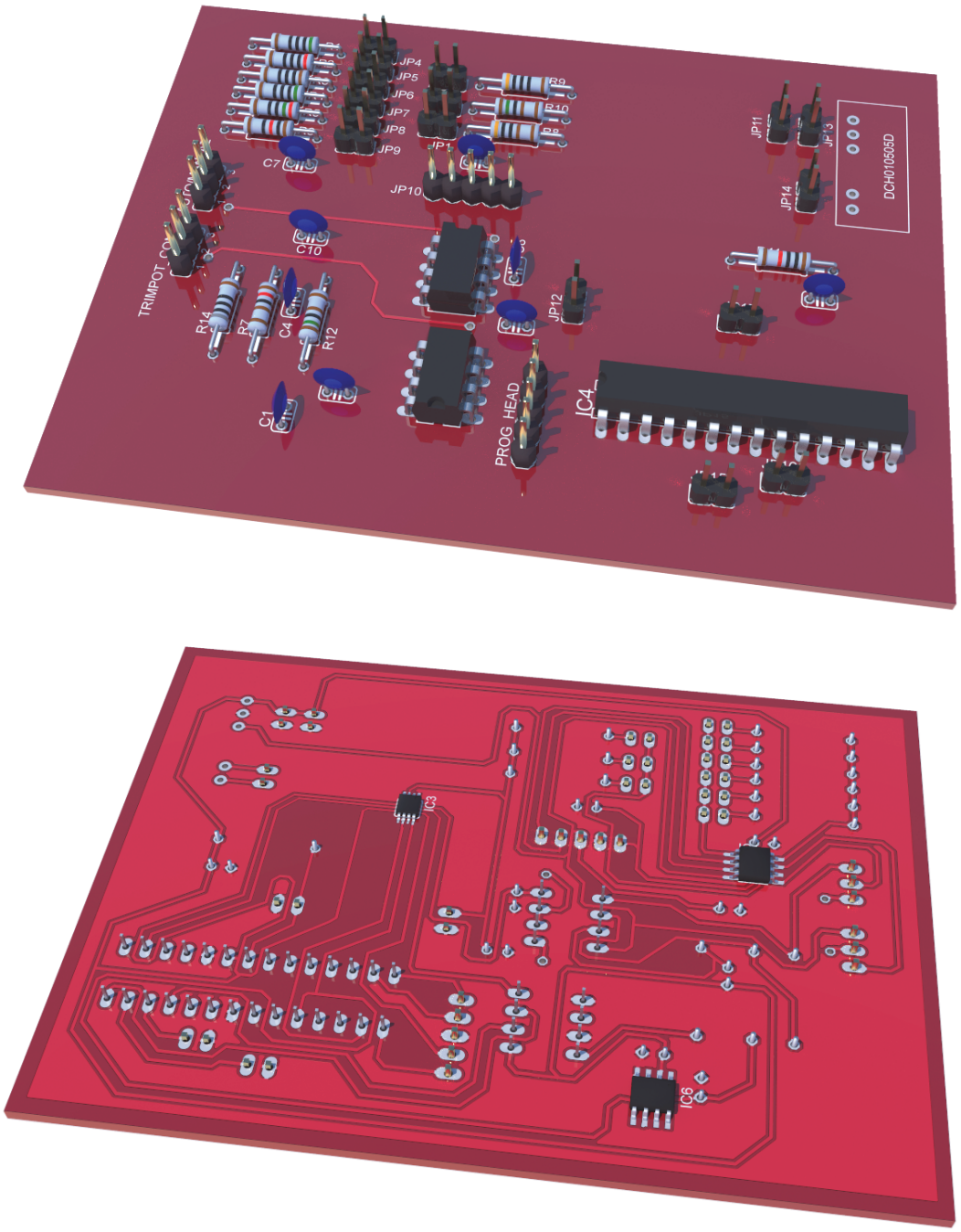


**Figure 2-6** Printed circuit board 3D renderings of the amplifier. The top of the board is shown above and the underside of the board is shown below.

## 2.4 Electrical Muscle Stimulator Design Requirements

An electrical muscle stimulator was needed to carry out all stimulation protocols throughout the experiments. Details of the design requirements and hardware implementation are discussed in Chapter 3 as they were presented in a published paper. The schematics and 3D renderings of the printed circuit boards, which were not presented in the paper, are shown in Figures 2-7 to 2-10. The extensive software used to control the system was also not described and will be outlined here.

### 2.4.1 Stimulator Software

All software used to control the stimulator was written in LabVIEW (National Instruments, USA). This programming language was chosen as it contains a number of functions and graphical user interface objects that make creating a fully functioning product easier and quicker. This way, the turnaround time to experimental use is minimized.

A number of input parameters are needed for control of the stimulus. These include: pulse width, frequency, train or single stimulus selection, number of pulses in a train, and stimulus amplitude. These parameters can be adjusted individually for each animal that is connected to the stimulator. There are also two global parameters that govern the entire stimulation session and these are total number of contractions in a session and the amount of time between successive contractions. These two parameters are used to ensure consistent delivery of the stimulus to all animals. As the hardware device is emulated as a serial port, all LabVIEW communication with the device is based on RS232 commands. When the user presses the “Stimulate” button, a handshaking

process is initiated. The LabVIEW program transmits an “s” character to the hardware and awaits the “go” command. Once this command is received, a parameter string is created featuring all the parameters for stimulation and is sent to the device. A triggering command, a “t” character, is then sent to the device to initiate delivery of the stimulus. If the user has enabled protocol stimulation, then the “Stimulate” button needs to be pressed only once and the software automatically delivers regular stimuli spaced by the global parameters that the user set. Indicators on the graphical user interface show when each channel is active, and the number of contractions remaining is also shown during protocol stimulation. A software flow chart is shown in Figure 2-11. As the stimulator contains five channels, the flow chart only shows the process for a single channel as the remaining channels are just duplicated versions.

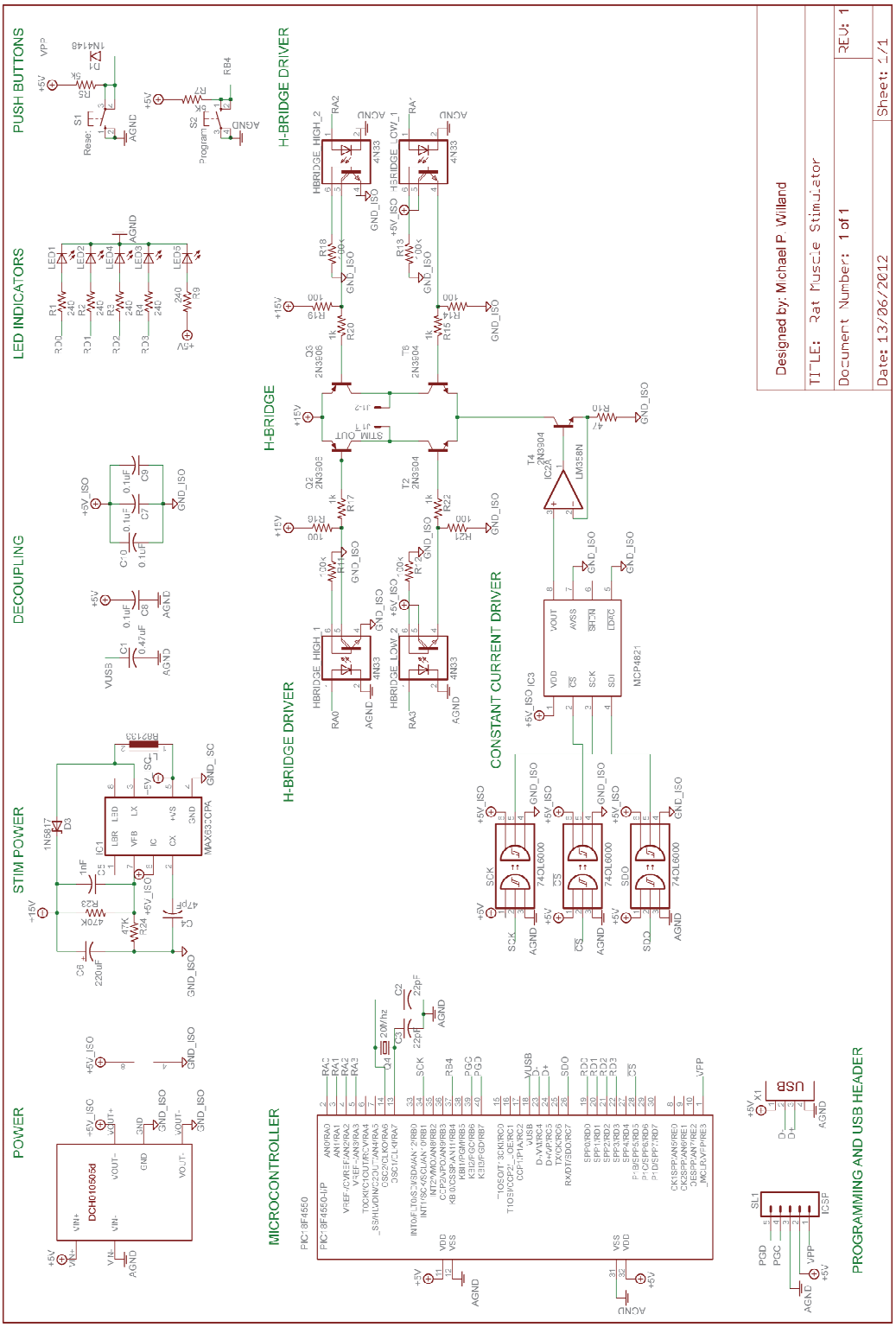


**Figure 2-7** Schematic of a single channel of the muscle stimulator.

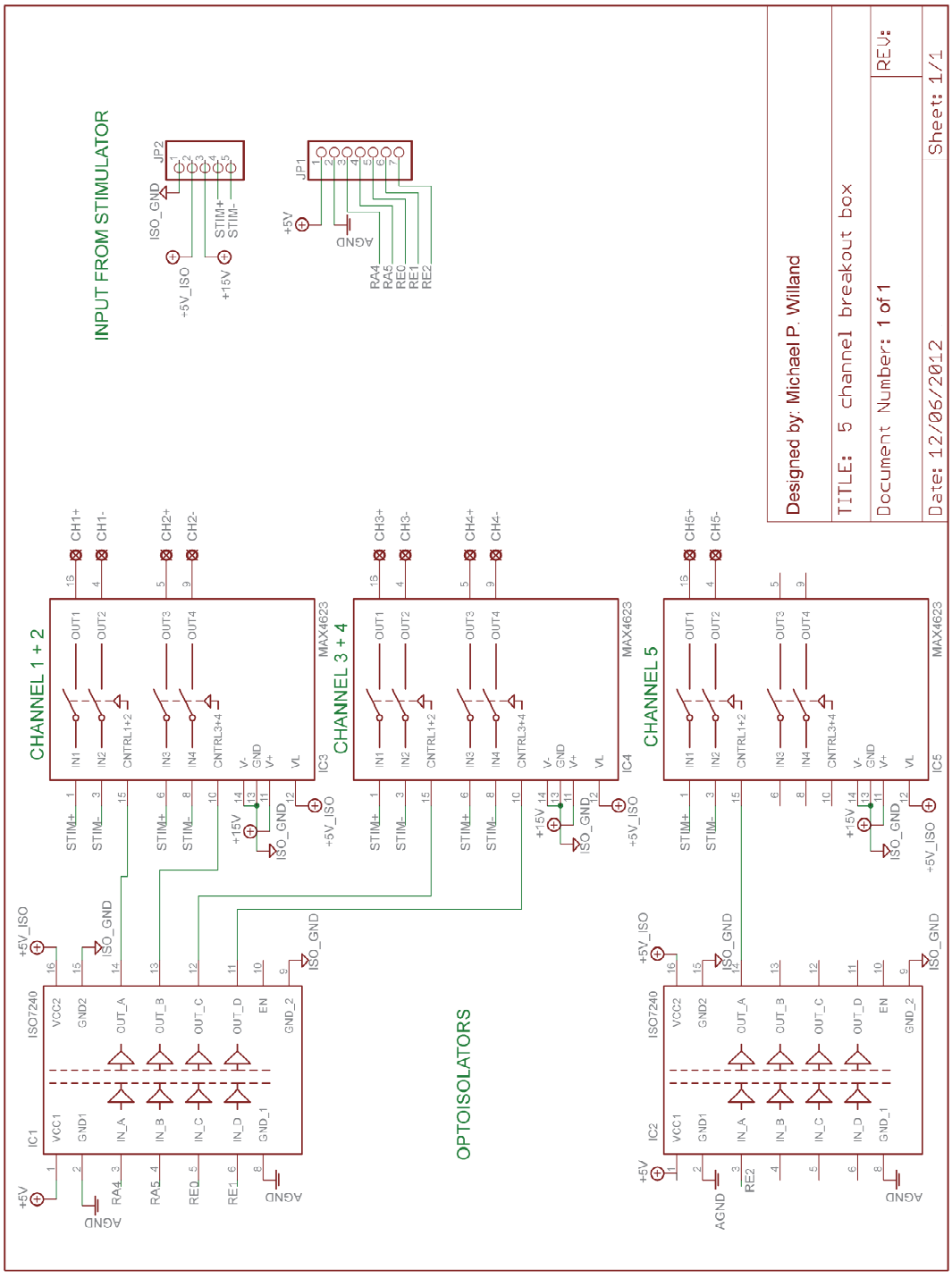


**Figure 2-8** Schematic of the 5 channel breakout box. This device is connected to the main stimulator and is used to provide a switching mechanism for stimulus delivery to five different animals.

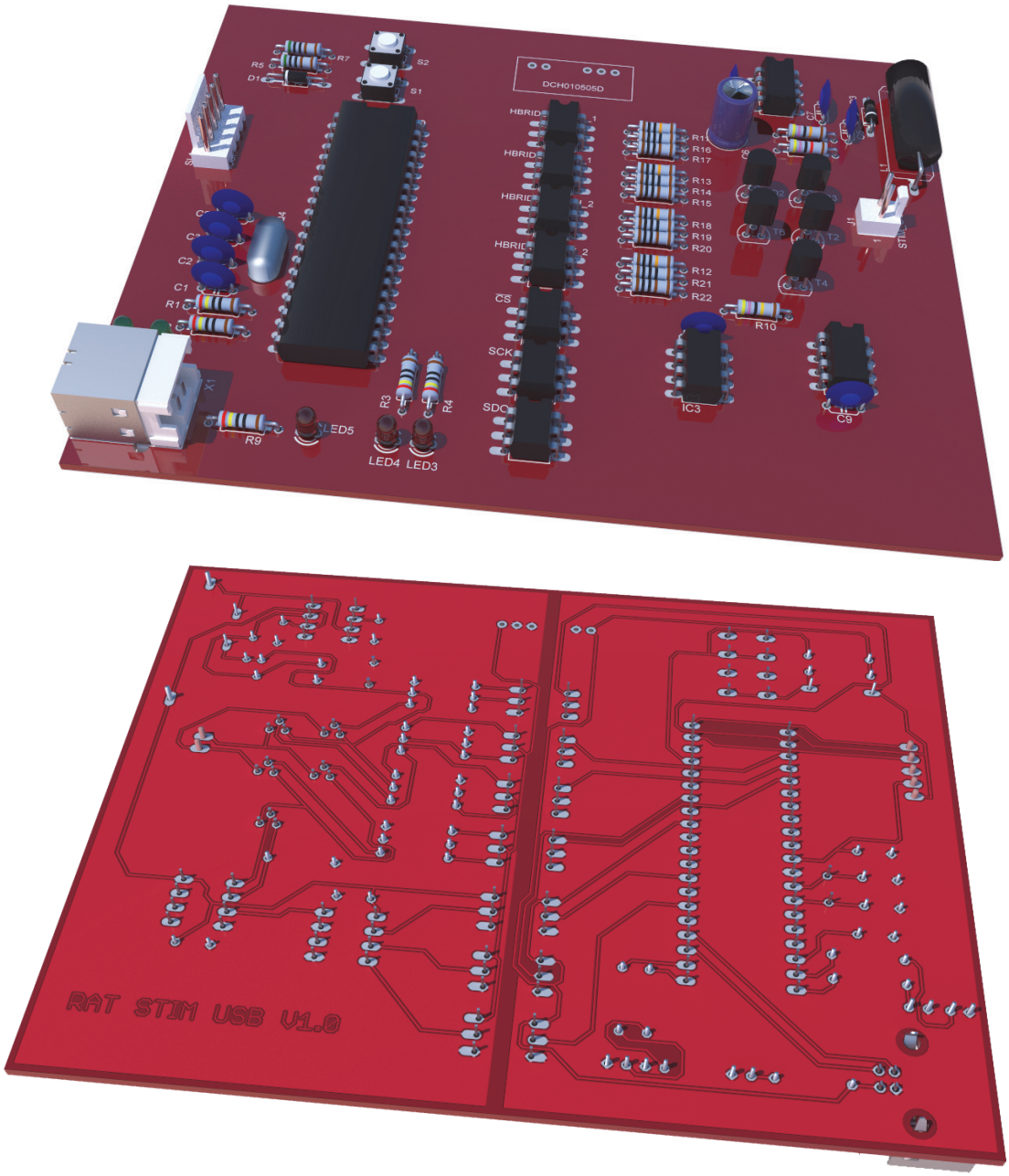


**Figure 2-9** Printed circuit board 3D renderings of the muscle stimulator. The top of the board is shown above and the bottom of the board is shown below.

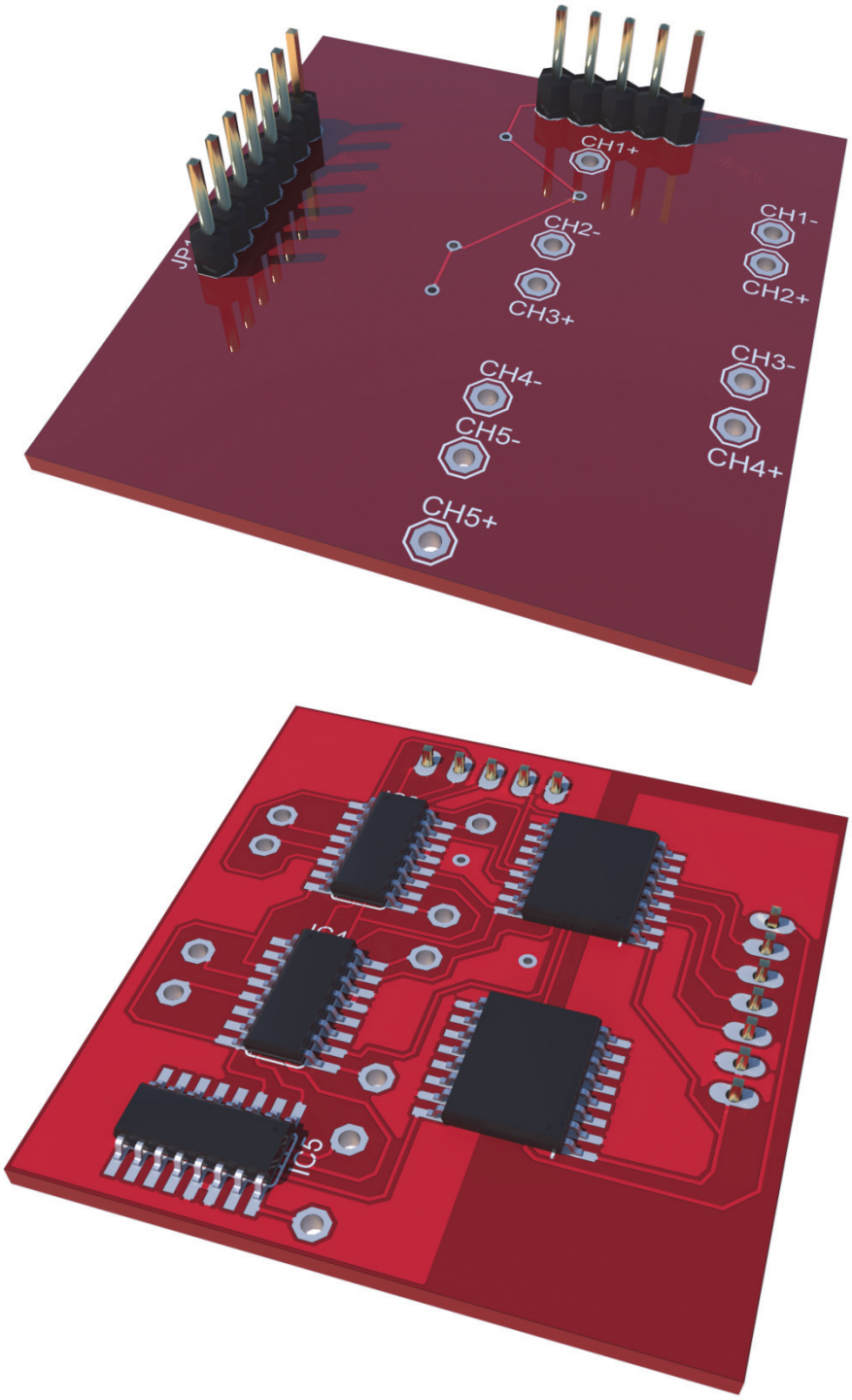


**Figure 2-10** Printed circuit board 3D renderings of the 5 channel breakout box.

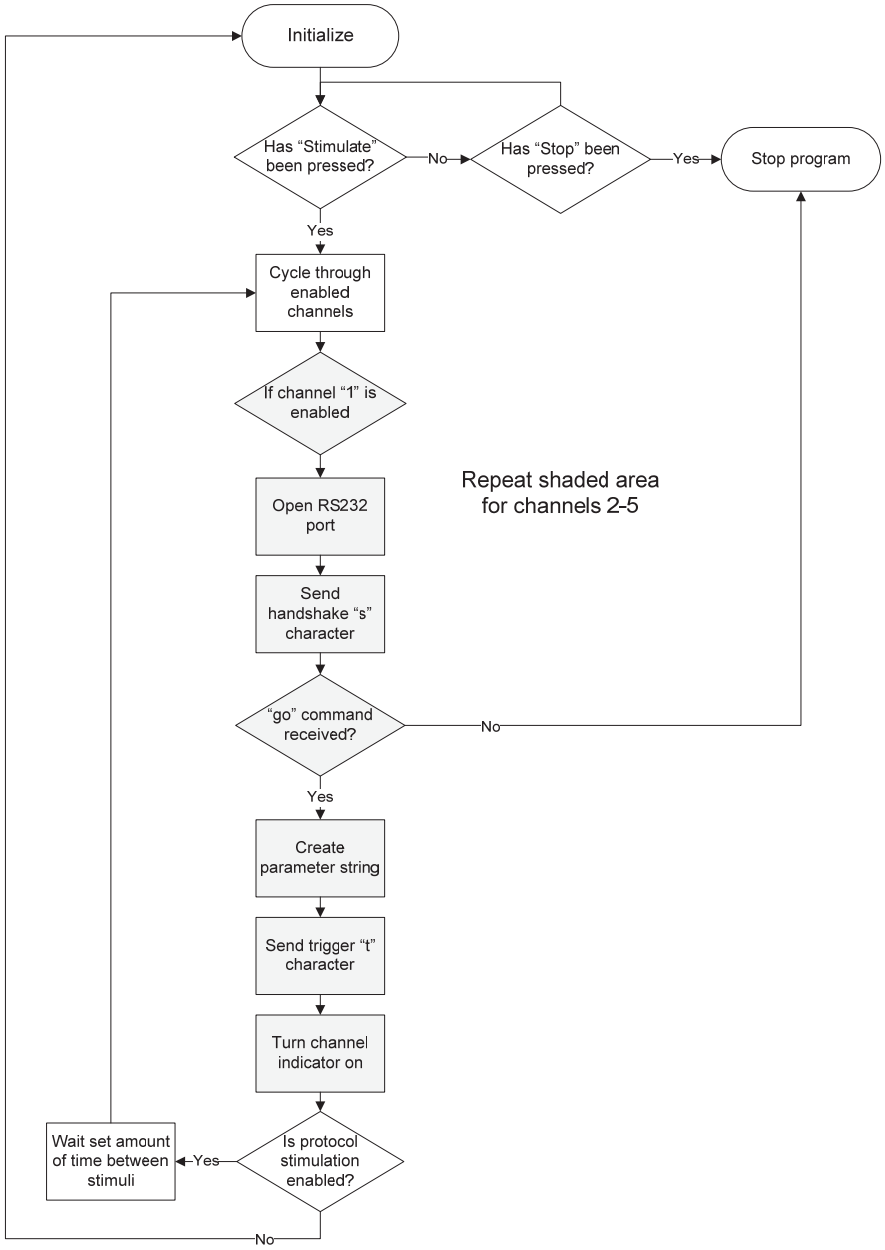


**Figure 2-11** Software flow chart for the LabVIEW program that controls the hardware stimulator.

## 2.5 References

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# Chapter 3: Paper One - A New System and Paradigm for Chronic Stimulation of Denervated Rat Muscle

**Authors:** M.P. Willand, J.P. Lopez, H. de Bruin, M. Fahnestock, M. Holmes, J.R. Bain

**Publication Information:** Journal of Medical and Biological Engineering. **2011**, 31(2): 87-92.

**Objectives:**

A new multi-animal muscle stimulator was designed to deliver a novel, short-duration stimulation protocol. This protocol involved daily stimulation sessions of one hour featuring a high frequency similar to the natural firing frequency of the muscle undergoing stimulation (gastrocnemius). This is in contrast to previous work that used slow frequency stimulation for a similar type of muscle. The new protocol presented was tested during a one month period of denervation.

**Main Contributions:**

1. A low cost system was designed and effectively delivered the proposed stimulation paradigm to five animals at one time

2. One hour per day stimulation using a fast frequency significantly increases muscle weight and twitch force

**A New System and Paradigm for Chronic Stimulation of Denervated Rat Muscle**

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**Abstract**

Traditionally, animal studies employing electrical stimulation for conditioning denervated muscle rely on 24 hour based stimulation paradigms, most employing implantable stimulators. While these stimulators provide the necessary current to cause muscular contraction, they have problems with battery life, programmability, and long term robustness. Continuous 24 hour stimulation, while shown to be effective in animals, is not easily translatable to a clinical setting. It is also difficult to evaluate animal comfort and muscular contraction throughout a 24 hour period. We have developed a system and stimulation paradigm that can stimulate up to five animals at one time for one hour per day. The constant current stimulator is a USB powered device that can, under computer control, output trains of pulses with selectable shapes, widths, durations and repetition rates. It is an external device with no implantable parts in the animal except for the stimulating electrodes. We tested the system on two groups of rats with denervated gastrocnemius muscles. One group was stimulated using a one hour per day, 5 days per week stimulation paradigm for one month, while the other group had electrodes implanted but received no stimulation. Muscle weight and twitch force were significantly larger in the stimulated group than the non-stimulated group. Presently, we are using the stimulator to investigate electrical stimulation coupled with other therapeutic interventions that can minimize functional deficits after peripheral nerve injuries.

**Introduction**

Peripheral nerves are the anatomical structures that connect our central nervous system to the structures that move our joints - muscles. When peripheral nerves are cut or crushed during injury, the muscles lose their connection with the central nervous system and become denervated. In order for full functional recovery to occur, peripheral nerves must grow from the site of injury until they reach the muscle and form functional connections with the muscle. Clinically, functional recovery after peripheral nerve injuries is poor, particularly if there is a prolonged delay before nerve-muscle contact is re-established [1]. Because peripheral nerves regenerate at a rate of 1 mm per day, injuries that are more proximal produce much longer periods of muscle denervation. Progressive muscle atrophy usually follows long-term denervation with a loss of muscle mass, muscle spindles, force, motor function and an increase in collagenization and fibrosis of the tissue [2,3,4].

Electrical stimulation of denervated muscle to prevent atrophy has been studied as early as the 1930s [5]. However, the use of stimulation as a clinical therapy is not widely accepted due to a lack of standards and questions of efficacy [6]. Indeed, efficacy is the main concern, since different studies show both positive and negative effects of stimulation [7,8]. These negative effects may be due to a lack of stimulation intensity needed to reach deep muscle fibers when using surface electrodes, incorrect frequency selection for stimulation, or stimulation protocols with long periods of rest between stimuli [9,10]. Nevertheless, intense stimulation of denervated muscle has been shown to have beneficial effects in human subjects and thus is a worthwhile approach to

maintaining muscle mass and force [11]. Numerous animal studies have been conducted using implantable stimulators to provide electrical muscle stimulation to denervated muscle [12-15]. These stimulators are costly, especially when conducting large scale studies involving numerous animals. They also require special manufacturing facilities since their small size requires custom designed integrated circuits and exclusively surface mount technology. The power output is always limited, and batteries may need to be changed during long-duration studies. The bulk of these studies also used stimulation paradigms that consisted of 24 hours per day of intermittent stimulation, something not easily translatable to a clinical setting unless expensive FDA-approved implantable stimulators are used [16]. It is also difficult to assess the level of muscular contraction over a 24 hour period.

In this paper, we present an external system designed to stimulate up to five animals simultaneously using a 1 hour per day, 5 days per week stimulation paradigm. The choice of an external system over an internal one was largely that of cost, ease of assembly, robustness and eventual clinical applicability. Moreover, a 1 hour per day/5 days per week stimulation paradigm is easily translatable to a clinical setting.

## Materials and Methods

### *System Design*

A block diagram of our system is shown in Figure 1. The stimulator utilizes a universal serial bus (USB) to provide power and communication with the host computer. Stimulation parameters (pulse width, frequency, amplitude, polarity) are sent as text

commands via custom LabVIEW (National Instruments) software running on the host computer and interpreted by the stimulator's microcontroller acting as a serial port emulator (PIC18F4550, Microchip Technology Inc.). This microcontroller was selected due to its easy USB interfacing. It uses the timing parameters to switch an isolated H-bridge. The H-bridge is coupled to a voltage controlled constant current source used to provide biphasic stimulus pulses. This current source consists of a transistor driven by an operational amplifier with a stimulation amplitude reference voltage provided by a digital to analog converter (MCP4821, Microchip Technology Inc.).

For chronic electrical muscle stimulation, biphasic pulses are necessary to prevent net ion flow that could result in tissue damage or electrode corrosion [17]. The purpose of having an isolated stimulator output is to ensure that no common ground loops are present and that the stimulus cannot be shunted through the animal's body to a common ground sink. As well, isolation is required to allow simultaneous recording of electrophysiological signals. Isolation is implemented in two ways: control signals arriving from the microcontroller are digitally isolated using optocouplers (4N33, Fairchild Semiconductor); stimulus voltage is provided by a 5-15 V step up converter (MAX 630, Maxim Integrated Products Inc.) that is isolated on the input side using a 5 V isolated DC/DC converter (DCH010505S, Texas Instruments). The designed 15 V maximum stimulator output has been shown in other studies to provide sufficient stimulus amplitude to contract denervated muscle [12]. The output of the H-bridge can be directly connected to an animal as a single channel device, or connected to a five-channel breakout box (not shown in Figure 1) for stimulation of multiple animals. The breakout

box is a set of analog switches (MAX 4623, Maxim Integrated Products Inc.) controlled by the microcontroller. This allows each of the five channels to have individual stimulus settings. System testing, using a resistance of 620 Ω placed across the stimulator outputs, showed it met design specifications and produced mono or biphasic pulses at selected amplitudes, durations, and repetition frequencies. This resistance is similar to that of denervated and stimulated muscle [12].

*Animals*

Ten male Lewis rats (Charles River, Quebec, Canada) weighing 250-350g were used for this study. This strain was chosen as it shows the least self-mutilation following surgery [18]. All housing, surgical procedures, analgesia and assessments were performed according to the Canadian Council on Animal Care Guidelines, using protocols approved by the Animal Care Committee at McMaster University.

*Surgical Procedure*

The animals were randomly assigned to either a stimulated or non stimulated group. Each group had the right gastrocnemius muscle denervated, as described previously, by cutting the tibial nerve approximately 13 mm from its entry point into the gastrocnemius muscle. The free distal stump was sutured to minimize extraneous innervation from other axons, and the proximal stump of the nerve was sutured onto the biceps femoris muscle to avoid reinnervation from proximal tibial nerve axons [4]. Teflon coated, stainless steel (Cooner Wire, AS 631) stimulating electrodes with ends bared of insulation were implanted into the belly of the denervated muscles of both groups using

an electrode suture complex [19] to minimize electrode migration. Slack wire was then coiled near the biceps femoris to allow for limb movement and animal growth. The electrode wires were threaded subcutaneously beneath the dorsal trunk skin, sutured in place, and externalized at the nape. The ends of the wire were bared for connection to the stimulator. This approach worked very well in chronic stimulation and caused no animal discomfort or infections at the wire exit sites.

*Stimulation Paradigm*

In order for the stimulation paradigm to be easily translatable to a clinical setting, we chose to use a 1 hour/day, 5 days/week muscle stimulation protocol. Previous studies using 1 hour durations for stimulation have shown some benefit. However, those studies employed a relatively low pulse repetition frequency (20 Hz) to stimulate a primarily fast twitch muscle (rabbit tibialis anterior) and elicited brief, unfused, tetanic contractions [15]. We opted to use a frequency of 100 Hz, more suited to a fast twitch muscle, which has also been used in previous studies [8,12] to elicit fused, tetanic contractions of the rat gastrocnemius muscle. A biphasic train of 400 ms duration (40 pulses at 100 Hz) was used with a pulse width of 200 μs per phase. In a preliminary study, we attempted to effectively match previous protocols and elicited 1200 contractions per stimulus session (one contraction every 3 seconds, similar to [15]). However, this fatigued the muscle significantly, and we consequently moved to our current protocol of 600 contractions per hour (one contraction every 6 seconds). The stimulus amplitude was adjusted for each animal until a visually strong contraction was produced.

*Muscle Assessment*

After the one-month experimental period, the animals were deeply anesthetized using halothane (5% induction, 2% maintenance) and the gastrocnemius muscle in both hind limbs was exposed, dissected free of the soleus and plantaris muscles, and connected to a force transducer (Grass FD03). Two fine-needle electrodes were placed in the belly of the muscle and served as stimulating electrodes. Muscle length was adjusted to the optimum length for force generation, and maximum twitch forces, time to peak ($T_{peak}$) and half-relaxation times ($T_{1/2R}$) were measured in both limbs, with the left hind limb serving as the fully innervated control for each denervated muscle. A fatigue test was then initiated which consisted of 13 pulses at 40 Hz delivered once every second [20], which is different from our stimulus protocol but used by others for endpoint fatigue testing [15,21,22]. The fatigue index (FI) was calculated by measuring the maximum tension after 2 minutes and dividing it by the maximum tension generated by the first stimulus. Once the force and fatigue tests were completed, the animal was sacrificed using an overdose of halothane and cervical dislocation. The muscle was excised, weighed, and immediately immersed in liquid nitrogen-cooled isopentane. Using a cryostat, 8-µm transverse sections were then taken from the frozen muscle and stained using hematoxylin and eosin for viewing under a light microscope. Ten images of each muscle were taken spanning the entire cross-section using a Nikon D300 camera adapted to a Carl Zeiss light microscope, and muscle fiber cross-sectional area was measured using ImageJ (NIH).

*Statistical Analysis*

To remove any variability due to animal weights, muscle weight and twitch force were expressed as percentages of the control (fully innervated, contralateral) limb. An unpaired T test was used to compare the stimulated group to the unstimulated group. In cases where the stimulated, unstimulated, and unoperated groups were compared, a one way ANOVA was used followed by a Bonferonni *post hoc* test. Significance was defined as $p < 0.05$.

**Results**

Stimulations began two days post-operatively, and each of the animals in the stimulated group underwent daily stimulation for 4 weeks (weekdays only). A biphasic train of 400 ms duration (40 pulses at 100 Hz) with a pulse width of 200 μs per phase was given every six seconds for one hour. Though the animals were fully awake during the stimulation period, they were restrained in custom designed restrainers that minimized movement. The restrainer with a rat inside of it is shown in Figure 2. The animals showed minimal discomfort throughout the stimulation period. One of the five animals in the stimulated group was not assessed physiologically, as the muscle was damaged during the dissection and could not be stimulated.

Maximum muscle twitches were elicited to provide a measure of the loss in denervated muscle's force output. Figure 3B shows the relative losses in maximum twitch forces for both denervated groups while Figure 4 shows typical twitches obtained from

one animal per group. These figures show that there was significantly less force loss in the stimulated than in the unstimulated group.

The shape and duration of the twitch are indicative of the fiber type, whether fast or slow twitch. While the times to peak force and half relaxation force in Table 1 were the same for control and unstimulated groups, showing that there was no change in contractile properties of the muscle fibers during one month of denervation, they were significantly increased for the stimulated group. Thus, when the muscle is electrically stimulated, there is a change in some muscle fibers from fast to slow twitch. Fatigue tests were done to examine if there were changes in the muscle's resistance to fatigue during one month of denervation, which would also indicate a change in fiber structure and/or metabolism. Table 1 shows that the stimulated group had a significantly higher FI (fatigue index) than the fully innervated controls. Although the mean FI for the denervated group also increased to more than double the control value, the change did not reach statistical significance.

Denervation for one month resulted in considerable loss of muscle weight as shown in Figure 3A with both groups having less than 50% of their contralateral muscle weight. However, the stimulated muscle group lost significantly less weight than the untreated group. Figure 5 shows that this loss was due primarily to a decrease in muscle fiber cross-sectional area rather than a decrease in the number of fibers. This is also confirmed by data in Table 2 which shows mean cross-sectional areas for the three groups. The average denervated fiber cross-section was less than 25% of the control

values and significantly less than the stimulated group's (36%). The ratio of stimulated to unstimulated cross sections (1.5) was close to the ratio of the whole muscle weights (1.3).

**Discussion**

Traditionally, denervated muscle studies conducted on animals utilize implantable stimulators that are costly and require special technical expertise and manufacturing facilities. Our system was built using a majority of standard components that are easily obtainable at low cost, making the stimulator an economical solution for animal studies requiring stimulation. The stimulator could easily produce the necessary amplitudes to elicit strong contractions in denervated muscle. Although only biphasic 400 μs pulses were used in the tests described in this paper, the stimulus output is completely flexible, allowing the researcher to select any pulse shape, duration and repetition pattern. Further, designing the system to reliably stimulate up to five animals concurrently increased laboratory efficiency considerably.

These data demonstrate that our stimulus paradigm is effective at increasing muscle weight, twitch force, and muscle fiber cross-sectional area. However, our twitch force and muscle weight values did not approach those values reported by others. Ashley et al. [15] used a similar number of pulses in a one hour stimulation protocol with force and weight values much closer to control values. However, their studies were done on rabbit tibialis anterior muscle using very long stimulus pulses, and stimulation began after 10 weeks of denervation. As the period between denervation and stimulation lengthens, the opportunity for recovery decreases [6] as muscle atrophies [4]. Our studies began

stimulation 2 days post denervation, similar to several other studies [8,12,23]. Although Dow et al. [12] also used 100 Hz stimulation bursts but spread over 24 hours, their results showed force and weight values exceeding those of controls. It may be that the amount of rest between contractions is an important factor in maintaining muscle weight and force [23], and a high frequency contraction every six seconds may too fast to maintain these properties. However, although our results did not approach control values, they did show a significant increase in muscle mass and twitch force, contrary to what Nix showed using a similar stimulation frequency in a fast twitch muscle [24]. Interestingly, the relative increase in force was much larger in our study than the relative increase in muscle mass. Other work has shown the opposite with stimulation resulting in a higher relative increase of mass than force. This may be because we elicited relatively strong contractions during stimulation. Other studies used very weak contractions throughout their stimulation protocol [12,15].

Some previous studies, using lower limb muscles of the rat other than the gastrocnemius, showed that both contraction and half relaxation times increased in denervated muscle [8,12,15,24]. In contrast, our results show that both of these twitch characteristics in denervated rat gastrocnemius muscle were no different from fully innervated control muscles (Table 1). This result is supported by other studies that found that the gastrocnemius remains a fast twitch muscle following short and long term denervation [25,26]. As well, they found that denervated, primarily fast twitch muscle like the gastrocnemius normally undergoes fiber type conversion from fast glycolytic to fast oxidative types (IIb to IIa) [25]. This would explain the increase in fatigue resistance

in both denervated groups compared to their contralateral innervated control muscles. However, this does not explain the increase in contraction and half-relaxation times for the stimulated group. Although a stimulation frequency (100 Hz) similar to the firing frequency of fast twitch fibers was chosen to avoid fiber type conversion, the results show that the contraction and half-relaxation times are more akin to those of slow twitch fibers (Figure 4). One explanation could be that chronic denervation increases the membrane time constant or the refractive period [24], resulting in a lower effective stimulation rate than the applied 100 Hz. Using a slower frequency on a fast twitch muscle has been shown to convert fast muscle fiber types to slow types [27]. It may be that such frequent stimulation (once every six seconds in this study) at high frequencies can overwhelm the ion channel mechanisms in denervated muscle, resulting in stimulation that is similar to a slow frequency protocol.

Our one hour stimulation paradigm was chosen because it can be translated to the clinical setting without the very high cost and invasive procedure of implanted stimulators. Of course the pulse repetition rate would be lower to match the longer twitch durations of most human skeletal muscles (e.g. 30 Hz). Clinical electrostimulation would then be delivered by removable surface electrodes, as is already done in physiotherapy clinics for muscle retraining or strengthening. Although the one hour 20 Hz paradigm used by Ashley et al [15] resulted in better weight and force gains than our approach, it required 20 ms stimulus pulses. In earlier work [28], we investigated the use of long, low-amplitude pre-pulses in surface stimulation of intact median nerves in normal subjects, and found such long duration pulses quite painful. However, it would be worth

investigating the effects of a one hour paradigm with stimulus rates lower than the 100 Hz used in our study (e.g. 50 Hz) but with pulse durations less than 1 ms. As well, we hypothesize that contractile input alone is not adequate to fully maintain denervated muscle, and the results of our studies fully support this.

**Conclusions**

The novel stimulator described in this paper proved very successful in both system and animal tests. Stimulus pulse parameters and repetition patterns are entered and controlled through a combination of a standard laboratory computer running a LabVIEW program and a dedicated microcontroller. This allows easy implementation and testing of new stimulus paradigms. The ability to simultaneously stimulate up to 5 animals with individual selectable pulse amplitudes is efficient, especially when large cohorts of animals are stimulated chronically over several months. The stimulation paradigm described above was effective in partially preserving muscle weight and force, compared to the untreated denervated muscle, with some results requiring further investigation. Our new electrical stimulation system and paradigm will be useful for investigating the combination of electrical stimulation and other therapeutic interventions to improve functional recovery after nerve injury.

**Acknowledgements**

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## Tables

**Table 1** – *Fatigue index (FI), time to peak (Tpeak), and half-relaxation time (T1/2R).* Numbers represent mean values ± standard error. *Denotes significant difference compared to the control group, $p < 0.05$. n = the number of muscles analyzed in each group.

| | Control | Denervated | Denervated-Stimulated |
|---|---|---|---|
| FI | 0.11 ± 0.03<br>n = 10 | 0.23 ± 0.02<br>n = 5 | 0.28 ± 0.05*<br>n = 4 |
| $T_{peak}$ (ms) | 39.8 ± 1.46<br>n = 10 | 39.2 ± 2.59<br>n = 5 | 56.8 ± 6.24*<br>n = 4 |
| $T_{1/2R}$ (ms) | 25.9 ± 2.07<br>n = 10 | 27.88 ± 1.29<br>n = 5 | 48.95 ± 6.91*<br>n = 4 |

**Table 2** – *Muscle fiber area.* Numbers represent mean values ± SEM. *Denotes significant difference compared to the denervated group, $p < 0.05$. †Denotes significant differences compared to denervated and stimulated groups, $p < 0.05$. n = the number of fibers counted. Each group consisted of 3 animals.

| | Control | Denervated | Denervated-Stimulated |
|---|---|---|---|
| Area ($\mu m^2$) | 2605 ± 45.7†<br>n = 489 | 618.7 ± 7.39<br>n = 1297 | 949.6 ± 12.09*<br>n = 1508 |

## Figures

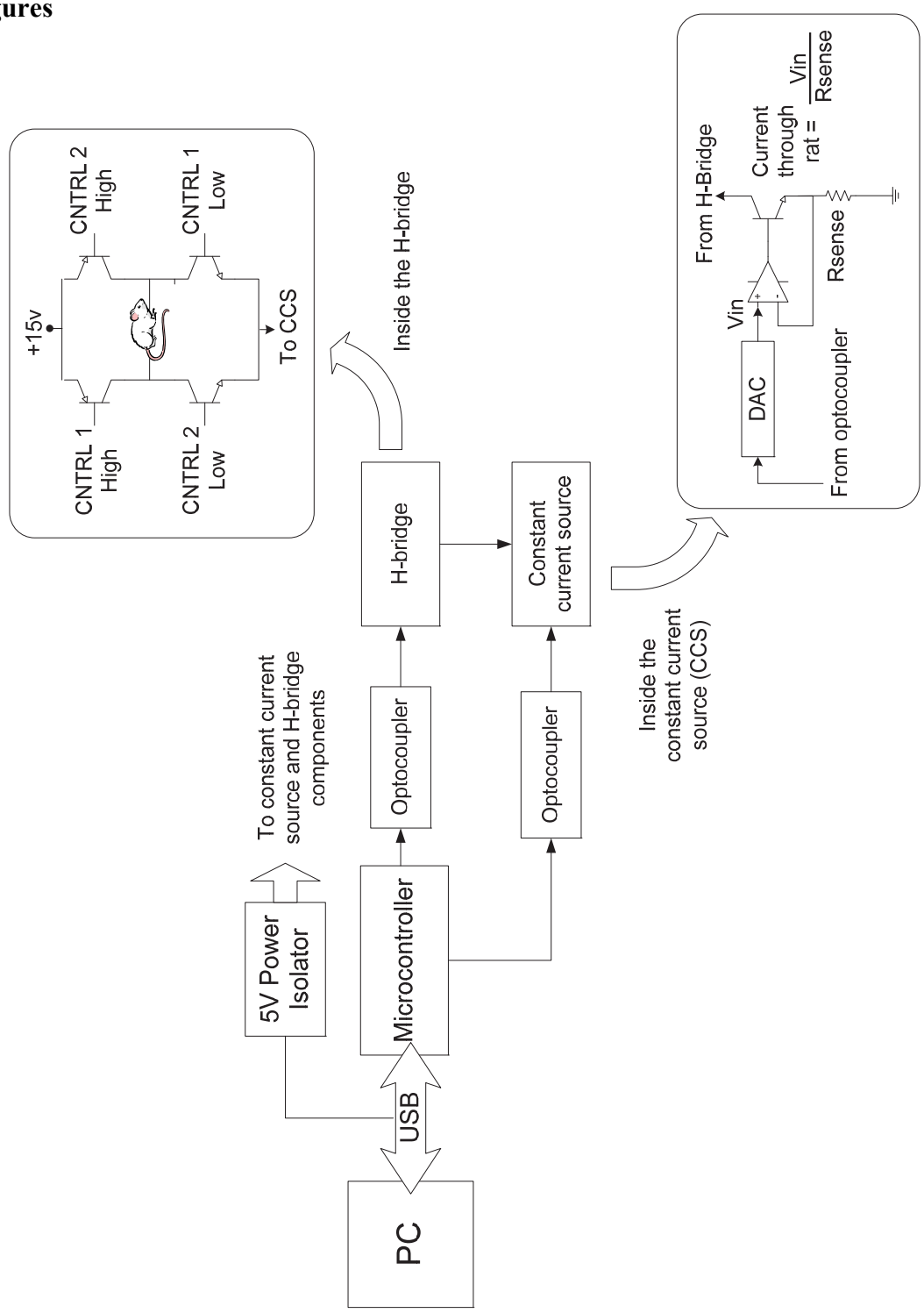


**Figure 1** – *Block diagram of the stimulator*. The expanded views detail the components of the H-bridge and constant current source.

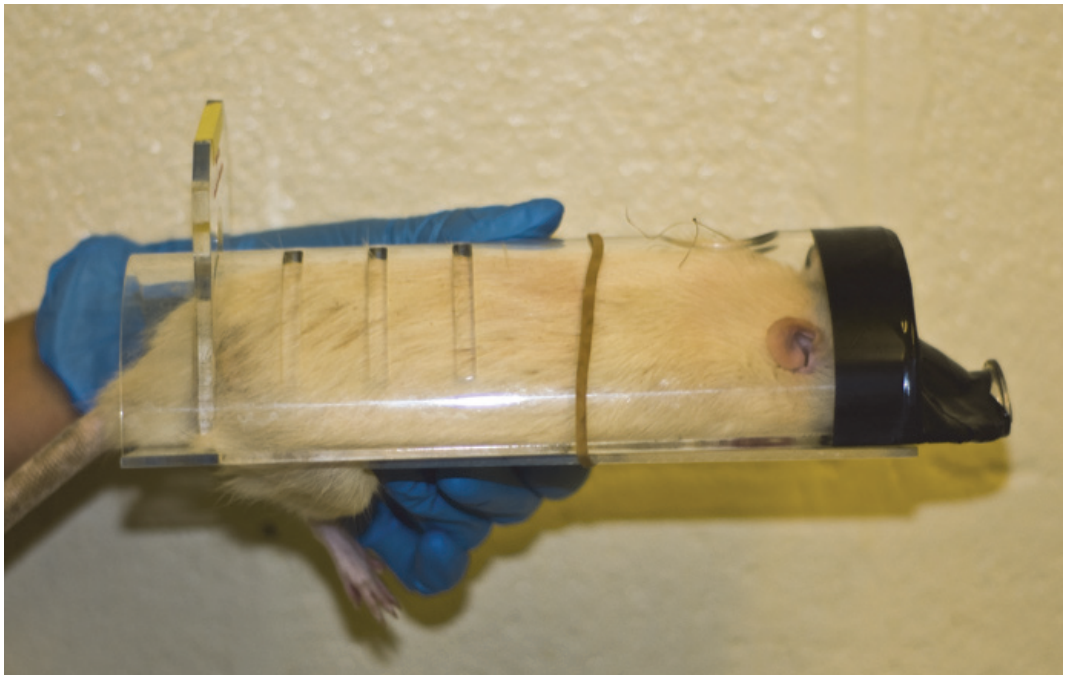

**Figure 2** – *Rat in custom designed restraining device.* Right leg is exposed for easy visualization of contractions.

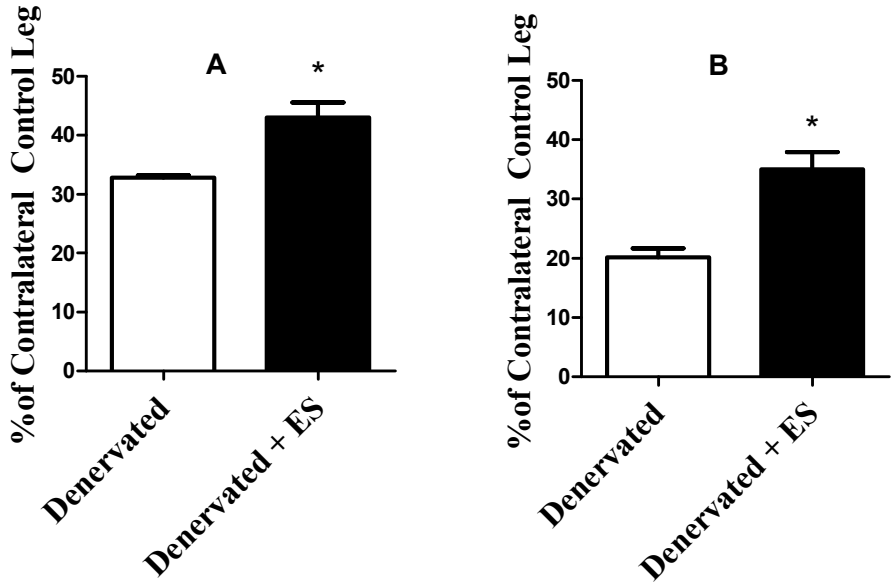


**Figure 3** – *Muscle weight (A) and twitch force (B) ratios* in rat gastrocnemius muscle 1 month after denervation or denervation and stimulation. Values represent the ratio of the denervated muscle to its contralateral unoperated control leg and are expressed as mean ± standard error. The means of the stimulated group in both the weight and twitch force were significantly higher than the unstimulated group (* $p<0.05$). n = 5 in the denervated group and n = 4 in the stimulated group.

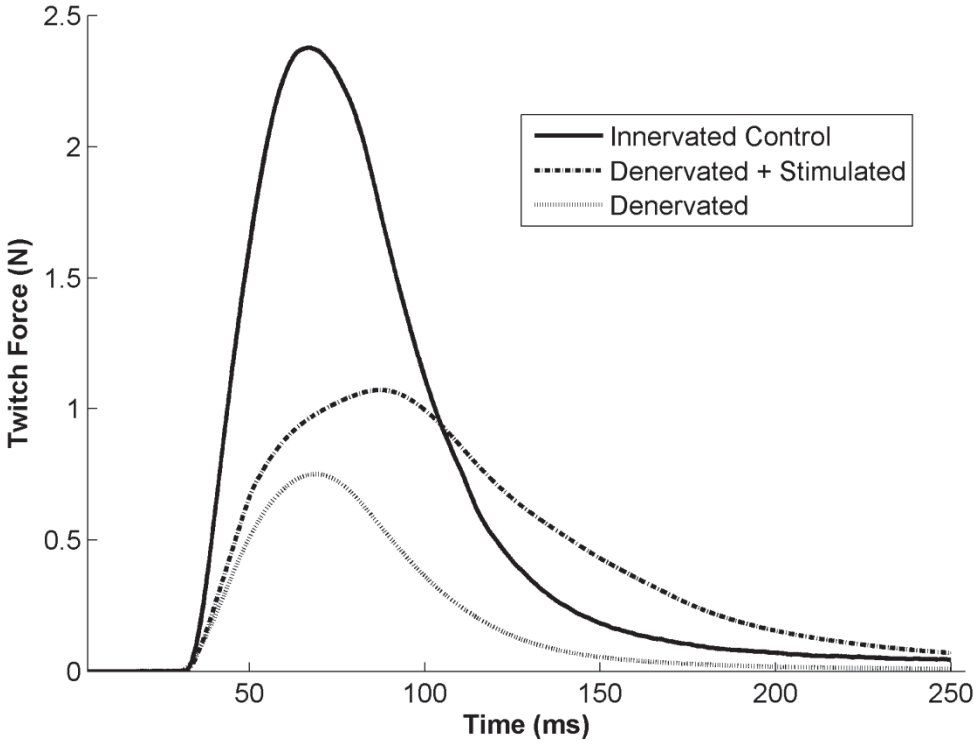


**Figure 4** – *Force profiles of the twitch response* for fully innervated muscle, denervated muscle, and denervated stimulated muscle. The profile of the stimulated muscle shows a lengthening of the contraction and half-relaxation times, indicating a possible conversion from fast to slow twitch muscle fibers. The innervated muscle profile shows no change in shape compared to the denervated muscle profile; only a change in amplitude is evident.

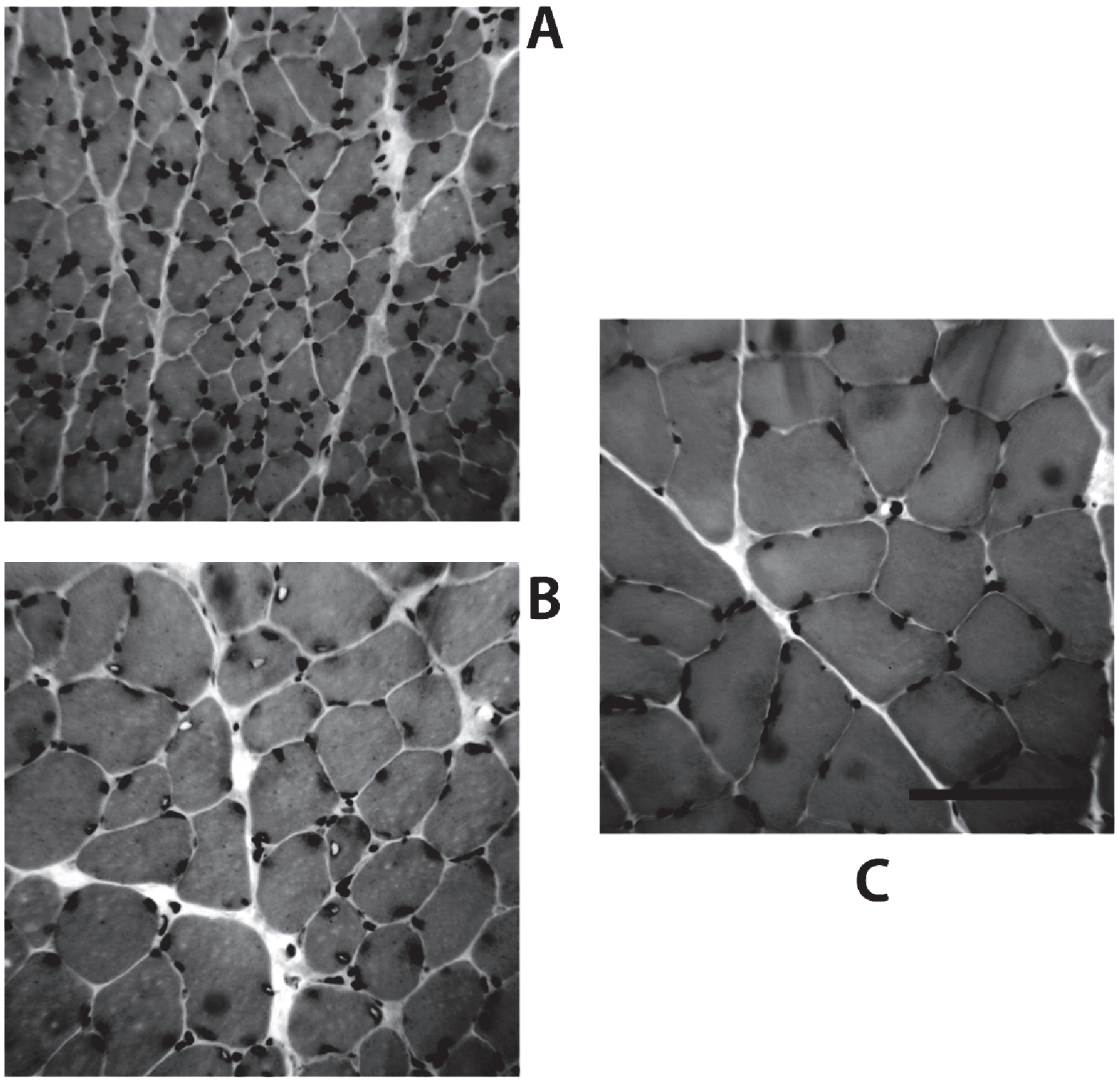


**Figure 5** – *Transverse sections of the medial gastrocnemius muscle* stained with hematoxylin and eosin (H&E). Sections were taken from the belly of the muscle. (A) denervated muscle; (B) denervated and stimulated muscle; (C) fully innervated muscle. Bar represents 100 μm and is applicable to all three panels.

# Chapter 4: Paper Two - Electrical muscle stimulation following immediate nerve repair reduces muscle atrophy without affecting reinnervation

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**Publication Information:** Submitted to Muscle & Nerve

**Objectives:**

Electrical muscle stimulation following immediate nerve transection and repair was investigated. Stimulation was delivered for one month following surgery and then animals were allowed to recover for an additional two months. The aim of this paper was to determine whether stimulation affects reinnervation and other functional measures during the period of nerve regrowth.

**Main Contributions:**

1. Motor unit counts were not significantly different in animals that were stimulated compared to unstimulated, demonstrating that one month of electrical muscle stimulation does not negatively impact reinnervation.

2. Type I muscle fibers were significantly larger in animals that were stimulated compared to unstimulated, showing that one month of electrical muscle stimulation preferentially increases the size of type I fibers.

3. Muscle weight and twitch force were significantly greater in stimulated animals confirming the findings of the previous paper.

**Electrical muscle stimulation following immediate nerve repair reduces muscle atrophy without affecting reinnervation**

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**Abbreviations:** IR, immediately repaired; IR+ES, immediately repaired with electrical stimulation; $T_{peak}$, time to peak; $T_{1/2R}$, half-relaxation time

**Abstract**

*Introduction:* Electrical stimulation of denervated muscle has been shown to minimize atrophy, fibrosis, and increase force in animal and human models. However, electrical stimulation following nerve repair is a controversial treatment due to questions of efficacy.

*Methods:* Using a rat model, we investigated the efficacy of short term electrical muscle stimulation for increasing reinnervation and preventing muscle atrophy. Following tibial nerve transection and immediate repair with the peroneal nerve, 1 month of electrical stimulation was applied 5 days/week for 1 hour to the gastrocnemius muscle via implanted electrodes.

*Results:* Following 2 months of further recovery without stimulation, muscle weights, twitch forces, and type I fiber areas were significantly greater in the stimulated animals than in repaired controls without stimulation. Motor unit size and numbers were not different between the two groups.

*Discussion:* Short term electrical muscle stimulation following nerve repair significantly reduces muscle atrophy and does not affect motor nerve reinnervation.

**Introduction**

Delaying the treatment of peripheral nerve injuries results in muscle atrophy and overall poor functional recovery[1–5]. Numerous studies have confirmed that immediate nerve repair, when possible, can lead to better functional outcomes[1,6–8]. Although new treatments have been proposed for immediate nerve repair[9], suturing remains the gold standard that is used clinically today[10]. The problem that arises with any type of nerve repair is that the muscle is without functional connections during the period of nerve regrowth. Even in proximal immediate repair cases the target muscle is denervated for weeks, allowing denervation-associated atrophy to take place.

One method used to minimize atrophy is to accelerate nerve regeneration rates. It was observed by Gordon et al[11] and others[12] that axon regeneration across a suture site is delayed due to staggered axonal growth. Electrically stimulating the proximal nerve for one hour following surgical reconnection significantly increases the rate of regeneration across the suture site, allowing the muscle to receive functional connections much sooner[13]. However, the acceleration of outgrowth is only present across the suture site and once axons cross this site, the regeneration rate is not further enhanced. For proximal injuries this treatment may reduce the amount of time a muscle remains denervated, but the long distance required for nerve regrowth still allows atrophy related changes to take place.

A more direct method of minimizing atrophy is to electrically stimulate the muscle. This treatment has been used clinically with positive results on long term denervated muscle[14,15]. A number of animal studies have investigated the use of electrical

stimulation following immediate nerve repair, but the length and parameters of stimulation all vary, leading to both positive and negative effects of stimulation[16–20]. Most of these studies continued to stimulate muscle once reinnervation took place, potentially negatively influencing functional outcomes[21].

It has been suggested in the literature that short term electrical muscle stimulation during the period of nerve regrowth following nerve repair may be beneficial[22]. Based on observations by others and in our pilot studies, we estimated that with a suture site approximately 12-15 mm away from the muscle all motoneurons would cross the repair site and form neuromuscular connections in 5-6 weeks. In keeping with this time course, in our previous work we showed that one month of electrical stimulation of denervated gastrocnemius muscle significantly increased muscle force, weight, and fiber area[23]. We used a high-frequency stimulation paradigm that was suited to the fiber type of the treated muscle, with treatment taking place five days per week for one hour per day, a clinically translatable protocol. The goal of the present study was to investigate one month of electrical stimulation using this paradigm during the nerve regrowth phase in immediately repaired rat gastrocnemius muscle.

## Materials and methods

*Animals*

Experiments were performed on 22 Lewis rats (Charles River, Quebec, Canada) weighing 200-250 g. All animal housing, anesthesia, analgesia, surgical procedures, and terminal assessments were performed according to the Canadian Council on Animal Care

Guidelines. Protocols were approved by the Animal Care Committee at McMaster University.

*Surgical procedure*

Rats were randomly divided into two equal experiment groups: immediately repaired (IR) and immediately repaired with electrical stimulation (IR+ES). Each experimental group had the right gastrocnemius muscle denervated as described previously[1]. Briefly, the tibial nerve was severed approximately 10-13 mm away from the gastrocnemius muscle. The proximal tibial nerve was then sutured into the biceps femoris to prevent reinnervation of the gastrocnemius muscle. The peroneal nerve was then transected and immediately sutured in an end-to-end method to the distal tibial stump using 10-0 Ethilon nylon suture (Ethicon Inc.). The use of this cross-innervation model allows for manipulation in the timing of the repair while eliminating variability due to proximal nerve degeneration during long term axotomy. This is particularly important for investigating the effects of electrical stimulation in long term denervated muscle where chronic axotomy is a major factor limiting functional recovery.

Two Teflon coated stainless steel wires (Cooner Wire, AS 631) with ends stripped of insulation were implanted into the belly of the muscle using an electrode suture complex[24]. Electrode leads were then threaded subcutaneously along the dorsal wall and exposed at the nape. A portion of the externalized electrode lead was then stripped of its insulation in order to connect to the stimulation unit.

The animals were allowed to recover for three months before terminal muscle assessments (force, electrophysiological, and histological measurements) were made.

*Electrical stimulation*

The stimulation protocol was delivered using custom designed hardware as previously described[23,25]. All animals were awake throughout the treatment and placed in modified plastic rodent restrainers (RSTR544, Kent Scientific Corp., Torrington, CT) with their right leg exposed for easy visualization of the contraction as previously described[23]. Stimulated legs were freed from the restrainer to reduce any effects of loading on muscle recovery. Animals in the stimulated group (IR+ES) received one hour per day of electrical muscle stimulation five days per week for one month starting one day post-operatively. This duration was chosen based on our estimates from our pilot study that it would take at least one month for functional connections to be made and was similarly suggested by Dow et al.[22].

Each hour-long treatment featured 600 contractions (one contraction every 6 seconds) with each contraction consisting of 40 biphasic pulses at 100 Hz (pulse width of 200 μs per phase). The frequency of stimulation was chosen to suit a primarily fast twitch muscle such as the gastrocnemius and has been shown by others to be effective at maintaining muscle weight and force in fast twitch muscle following denervation[26–28]. Stimulus amplitudes were adjusted daily to ensure that strong, but not painful, visible contractions were being elicited. Other studies have previously also used a visual method to assess the strength of contraction prior to starting treatment[26,28–31].

*Motor unit number estimation (MUNE)*

Following the three month period of nerve repair, animals were anesthetized using isoflurane and estimates of the number of functional motor units were made using an

automated version of the McComas incremental stimulation method[32]. This method allows for measurements of functionally connected muscle fibers to be made which provides an estimate of the number of reinnervating axons in the muscle. The peroneal nerve was exposed and two hook stimulating electrodes were placed around the proximal end. Two needle electrodes were then inserted into the muscle's proximal and distal ends and served as recording electrodes. Monopolar stimulus pulses, 50 μs duration, were delivered to the nerve and M-wave responses recorded using a custom designed amplifier. Data were sampled at 8 kHz, and a custom written LabVIEW program was used to trigger, record and analyze the results[33]. Stimulus amplitude was gradually increased until 18-20 unique responses were recorded. The average motor unit size was calculated by finding the peak to peak amplitude of the largest unique response and dividing this by the number of responses. Motor unit counts were then calculated by dividing the maximum compound muscle action potential by the average motor unit size as previously described[25,34].

*Force recordings*

Isometric twitch and tetanic measurements were taken from both limbs of all animals with the left limb serving as a within-animal control. The gastrocnemius muscle was exposed, with the soleus and plantaris muscles removed, and calcaneal tendon severed. The proximal end of the calcaneal tendon was attached to a force transducer (Grass FD03) and two needle electrodes were placed in the muscle and connected to a stimulator. The animal's knee was fixated and muscle length was adjusted to produce maximum twitch force. Single maximal stimuli (1 ms duration) were used to elicit twitch

contractions. Contraction and half-relaxation times were measured from these recordings. Tetanic contractions were elicited using 40 pulses at 100 Hz, similar to our stimulation protocol. Once tetanic measurements were made, the muscle was allowed to rest for 2 minutes prior to fatigue testing. This involved a 325 ms pulse train at 40 Hz for 2 minutes as described by Burke et al.[35] The fatigue index was then calculated by taking the maximum tension at 2 minutes and dividing it by the maximum tension produced during the first contraction.

*Muscle weights*

Once all functional measurements were made, the muscle was excised, blotted to remove excess moisture, and weighed. A 3x3x8 mm long section was then cut from the belly of the medial head of the gastrocnemius, immersed in optical cutting temperature embedding medium (Tissue-Tek OCT Compound, Sakura Finetek U.S.A, Inc.), and flash frozen in isopentane pre-cooled in liquid nitrogen.

*Histology*

Muscle samples from 4 animals in each group were cut into 12 μm transverse sections using a cryostat and mounted on glass slides. Sections were then stained for myofibrillar ATPase and incubated at pH 10 in order to identify type I and type II muscle fibers as previously described[1,36]. Slides were imaged using a Nikon D300 camera adapted to a Zeiss light microscope. Approximately 2000 fibers per animal were measured for muscle cross sectional area. This analysis examined both type I and type II fibers and was carried out using Image J software (NIH). Fiber type ratio was determined

by counting the number of type I fibers and expressing it as a percent of total fibers counted. Fiber areas were determined and smoothed histograms were calculated using a kernel density estimator[37]. Further analysis was completed using MATLAB software.

*Statistical analysis*

Our previous work[1] showed no compensatory changes in weight or force in the contralateral limb in treated animals compared to unoperated animals. Therefore, to minimize variability between animals, the left limb was used as a fully innervated control, and values for weight and force were expressed as a ratio of the treated right limb to the contralateral control limb. Results from muscle weight, force, contractile properties, and motor unit estimates were analyzed using a two-tailed t-test. Histological data was compared using a non-parametric one-way ANOVA (Kruskal-Wallis test) and when significant a *post-hoc* Dunns test was used to compare groups. Significance was defined as $p<0.05$. All data is presented as means ± standard error of the mean (SEM).

**Results**

One rat from the IR+ES group was removed from analysis due to the presence of ectopic innervation from the tibial nerve discovered during endpoint measurements. Efferent connections from the tibial nerve may influence final motor unit counts or possibly provide the muscle with early innervation and minimize atrophy. A second rat in the IR+ES group had the tendon of the left control limb damaged during dissection and consequently twitch and tetanic forces were not able to be measured. The remaining 20 animals exhibited no signs of ectopic innervation.

To confirm the validity of using the contralateral limb as a control, we implanted 5 rats with electrodes in the right limb (without denervation) and assessed these animals 3 months following implantation. Histological examination of the muscle showed that implantation produced no major effects. Results for muscle weight, twitch and tetanic forces for the contralateral limb of these animals were no different than the contralateral limbs of immediately repaired animals (with or without stimulation). Results are shown in Table 1.

*Motor unit number estimation*

Animals that were stimulated had motor unit counts no different ($p>0.05$) than the unstimulated group (70 ± 6.3 and 82 ± 6.2, respectively) as shown in Figure 1A. Average motor unit sizes were also calculated and together with motor unit counts provide an estimate of the innervation ratio. Our previous work[25] suggested that long term denervated muscle has lower motor unit counts and smaller mean motor unit sizes leading to a low innervation ratio. In this study, both stimulated and unstimulated repaired groups had large mean motor unit sizes (0.212 ± 0.01 mV and 0.219 ± 0.018 mV, respectively) that were no different from each other ($p>0.05$) as shown in Figure 1B.

*Muscle weight, force, and contractile properties*

The muscle weight was expressed as a ratio of the treated limb to the fully innervated contralateral limb. This minimized any variability due to animal weights. Figure 2A shows that 1 month of stimulation significantly ($p<0.01$) increased muscle weight ratio (74 ± 3.1 % and 63 ± 1.7%). Twitch force (Figure 2B), also expressed as a

ratio, was significantly greater ($p<0.05$) in the stimulated than unstimulated group (48 ± 2.9 % and 39 ± 1.6 %, respectively). Although tetanic force ratios were higher in the stimulated group (62 ± 5.4 % vs. 56 ± 2.3 %, $p=0.31$) there was no significant difference between the groups ($p>0.05$, Figure 2C). Interestingly, the increase in the muscle weight ratio was no different from our previous work where we stimulated denervated muscle for one month[23]. In the latter case, the muscle weight ratio increased by approximately 10 % when compared to unstimulated muscle. This suggests that our protocol effectively increased muscle size regardless of whether a nerve was connected to the distal stump or not.

Twitch force profiles as measured by contraction time and half-relaxation time give an indication of the overall fiber type composition of the muscle. Contraction and half-relaxation times were no different between the two groups (Table 2), suggesting that 1 month of electrical stimulation did not alter the overall fiber type composition. Twitch/tetanus ratios serve as a measure of the activation properties of the muscle[38]. No differences were found in the twitch/tetanus ratios ($p>0.05$) between the stimulated and unstimulated groups (0.27 ± 0.007 and 0.27 ± 0.012, respectively, Table 2) which indicates that short term stimulation does not interfere with activation properties. Fatigue index measurements were significantly greater in the stimulated group ($p<0.05$) compared to the unstimulated group (0.28 ± 0.04 and 0.19 ± 0.03, respectively, Table 2). Denervation alone increases the percentage of type IIA fibers which increases fatigue resistance compared to untreated muscle[39,40]. The stimulated group showed greater fatigue resistance compared to unstimulated limbs, suggesting that our stimulation

protocol may further alter the metabolic properties (increased type IIB to IIA conversion) of the muscle, increase the number of slow twitch fibers, or produce a combination of both. Results for all electrophysiological measurements are shown in Table 2.

*Histology*

In order to further investigate the mechanism for the stimulation-induced increase in fatigue resistance, we stained muscle sections for type I and type II muscle fibers. Mean fiber areas for type II fibers were significantly larger ($p<0.01$) following one month of stimulation than for unstimulated repaired muscle ($2434 \pm 12\ \mu m^2$ vs. $2259 \pm 12\ \mu m^2$, Table 3). Both groups had significantly smaller type II fibers compared to the contralateral control limbs ($p<0.01$). However, the increase in mean fiber area for stimulated animals was even more pronounced in type I fibers, which were significantly greater ($p<0.01$) than unstimulated ($2275 \pm 74\ \mu m^2$ vs. $1825 \pm 22\ \mu m^2$, respectively, Table 3) and no different from contralateral control limbs ($p>0.05$). Smoothed histograms representing the averaged fiber area statistical distributions are shown in Figure 3A for the type I and Figure 3B for type II muscle fibers. It is evident from these distributions that there is an increase in the size of type I fibers in the stimulated compared to the unstimulated group, suggesting that our stimulation protocol targets these fibers. The fiber composition ratio is also shown in Table 3 and is no different between the groups. These values are consistent with fully innervated control muscles shown by others[41].

## Discussion

*Stimulation does not affect reinnervation*

Numerous studies have concluded that electrical stimulation adversely affects muscle innervation[18,42,43]. It has been suggested that stimulation prior to innervation may alter trophic factor levels and decrease muscle receptivity to forming new functional connections[43]. In our present study we demonstrated that 1 month of stimulation had no adverse effect on muscle innervation and significantly benefited overall muscle weight and force even 2 months after cessation of stimulation. Although muscle weight was approximately 70% of contralateral control legs in stimulated animals, further enhancement may be possible by using the tibial nerve for nerve repair which contains twice as many motor axons as the peroneal nerve[1]. Average motor unit sizes along with motor unit counts were no different between the two groups, demonstrating similar innervation ratios. An argument can be made that our present study may not have had a long enough denervation period to produce significant atrophy and affect receptivity. However, a previous study we conducted demonstrated that stimulation during 3 months of denervation did not affect muscle reinnervation[25]. It is widely known that nerves form functional connections with a muscle in an asynchronous manner. It may be that stimulation of a partially innervated muscle can have adverse effects for the remaining nerves that have not reached the muscle and this can compromise functional reinnervation[21]. In our study the gastrocnemius muscle may have been only sparingly innervated at the 1 month time point when electrical stimulation was stopped, reducing any negative impact that stimulation may have on functional reinnervation.

*Histological examination*

Interestingly, our high-frequency stimulation protocol targets type I muscle fibers preferentially, as shown by the histological results. Our fiber type area analyses clearly show an increase in the size of type I fibers in stimulated compared to unstimulated animals, restoring type I fiber area to control levels. The scale of increase of the size of type I fibers is similar to the overall wet muscle weight increase. Similarly, these fiber type changes were reflected in the twitch characteristics of our previous 1 month study of stimulated denervated and unrepaired muscle[23]. In that study, and based on the literature[40,44,45], we hypothesized that a type IIB to IIA fiber conversion was taking place and increasing the fatigue index of the muscle. At that time we could not explain the concurrent increase in half relaxation times for the stimulated group. In our present study, it is clear that our stimulation paradigm targets type I fibers, and this can contribute to increases in the fatigue index and half-relaxation times. However, in the present study the half-relaxation times were no different between the stimulated and unstimulated groups, in contrast to our previous work. This is likely due to the difference between our previous work with denervated muscle and the present results using repaired muscle. It has been shown by others that motor neurons can affect fiber type distributions[41]. Because the peroneal nerve normally innervates a fast twitch muscle, when functional connections are made with the gastrocnemius muscle the fast fibers may be preferentially maintained[46,47]. While the fiber type ratios were not statistically significantly different from one another, the stimulated group had a mean value approximately half of the control group. The hypertrophy of type I fibers in this group may maintain the contribution to half-relaxation

times while the lower type I percentage and increased fatigue resistance suggest a greater conversion of type IIB to IIA fibers. Further investigation using immunohistochemical staining techniques may provide more information about these fiber type changes.

*Stimulation paradigm targets type I fibers*

Our stimulation protocol elicited fiber hypertrophy 2 months post stimulation. Surprisingly, given the high frequency of our stimulation paradigm, type I fibers were preferentially stimulated. We chose this frequency as it was shown by many different groups that stimulation of muscle at its natural firing frequency, whether denervated or not, can maintain normal contractile characteristics and fiber type distributions[26,28,48,49]. Low frequency stimulation of fast twitch muscles was shown to convert the muscle to a slow variety[50,51]. The number of pulses per day, 24 000, was similar to what Ashley et al. showed to be effective in maintaining denervated rabbit tibialis anterior muscle[29]. Although we did not notice muscle fatigue throughout the 1 hour stimulation period, a likely explanation for our results may be that the separation between successive contractions is too short[27]. This could result in type IIA fibers fatiguing and not contracting while type I fibers continue to be stimulated.

More research is needed to compare various stimulation protocols in a single animal model. To date, there are numerous studies using a variety of survival periods, muscles, and stimulation frequencies, making it difficult to compare results. There is growing evidence that daily short bursts of stimulation can be effective at reducing muscle atrophy[29]. Our work supports this model and provides evidence that stimulation immediately following nerve repair reduces muscle atrophy and does not cause

detrimental effects to motor re-innervation. Electrical muscle stimulation, in combination with immediate surgical intervention, may aid functional recovery following peripheral nerve injury.

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## Tables

**Table 1 –** Measurements for contralateral limbs of immediately repaired animals and animals that had only electrodes implanted into their right limb (control) with no denervation.

| Measurement | Immediately repaired | Immediately repaired with electrical stimulation | Control |
|---|---|---|---|
| Twitch Force (N) | 6.58 ± 0.433<br>n=11 | 5.79 ± 0.512<br>n=9 | 5.77 ± 0.289<br>n=5 |
| Tetanic Force (N) | 17.74 ± 0.551<br>n=11 | 16.45 ± 0.792<br>n=9 | 18.5 ± 0.825<br>n=5 |
| Muscle Weight (g) | 2.29 ± 0.05<br>n=11 | 2.23 ± 0.032<br>n=10 | 2.21 ± 0.075<br>n=5 |

Number of animals per group is denoted by *n*.

**Table 2 –** Electrophysiological measurements for gastrocnemius muscle following immediate repair with or without electrical stimulation

| Measurement | Immediately repaired | Immediately repaired with electrical stimulation |
|---|---|---|
| Twitch Force Ratio | 39.73 ± 1.695<br>n=11 | 48.11 ± 2.941*<br>n=9 |
| Tetanic Force Ratio | 56.36 ± 2.340<br>n=11 | 62.11 ± 5.443<br>n=9 |
| Twitch/Tetanus Ratio | 0.2726 ± 0.007<br>n=11 | 0.2771 ± 0.012<br>n=10 |
| $T_{peak}$ (ms) | 30.05 ± 0.83<br>n=11 | 30.60 ± 1.25<br>n=10 |
| $T_{1/2R}$ (ms) | 30.09 ± 1.23<br>n=11 | 28.32 ± 1.24<br>n=10 |
| Fatigue Index | 0.1962 ± 0.03<br>n=11 | 0.2855 ± 0.04 *<br>n=10 |

* represents significant difference between groups ($p<0.05$). All values are expressed as a mean ± standard error of the mean. Number of animals per group is denoted by *n*.

**Table 3 –** Gastrocnemius muscle fiber area and fiber type ratios following immediate nerve repair with or without electrical stimulation

| Measurement | N | Immediately repaired | Immediately repaired with electrical stimulation | Contralateral control |
|---|---|---|---|---|
| Type I Area ($\mu m^2$) | 4 | 1819 ± 21.81† | 2275 ± 34.88* | 2295 ± 26.90 |
| Type II Area ($\mu m^2$) | 4 | 2246 ± 12.05† | 2434 ± 12.16*† | 2872 ± 14.61 |
| Fiber Type Ratio (%) | 4 | 12.52 ± 4.874 | 9.294 ± 0.5842 | 18.94 ± 2.422 |

Fiber Type Ratio = number of type I fibers expressed as a % of total fibers counted. *represents significant difference from immediate repair group ($p<0.05$). †represents significant difference from contralateral control group ($p<0.05$). All values are expressed as a mean ± standard error of the mean. N = number of animals per group.

## Figures

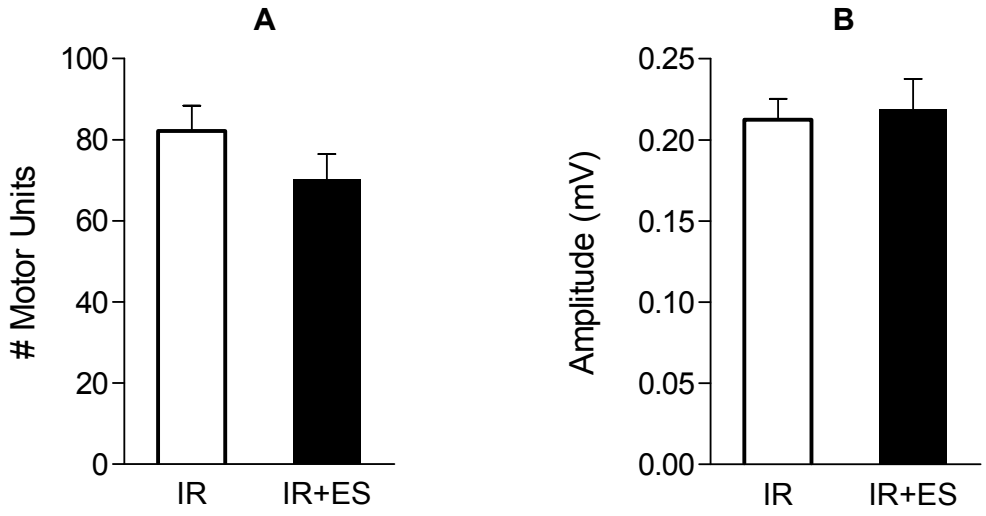


**Figure 1** – (A) Mean motor unit counts for 1 month stimulated and unstimulated animals. No significant difference (p=0.19) was found between groups, indicating that electrical stimulation did not have a negative impact on reinnervation. (B) Mean motor unit size, represented as the mean peak to peak amplitude value from recorded motor units, was also not different between groups (p=0.76). All values are represented as a mean ± standard error of the mean.

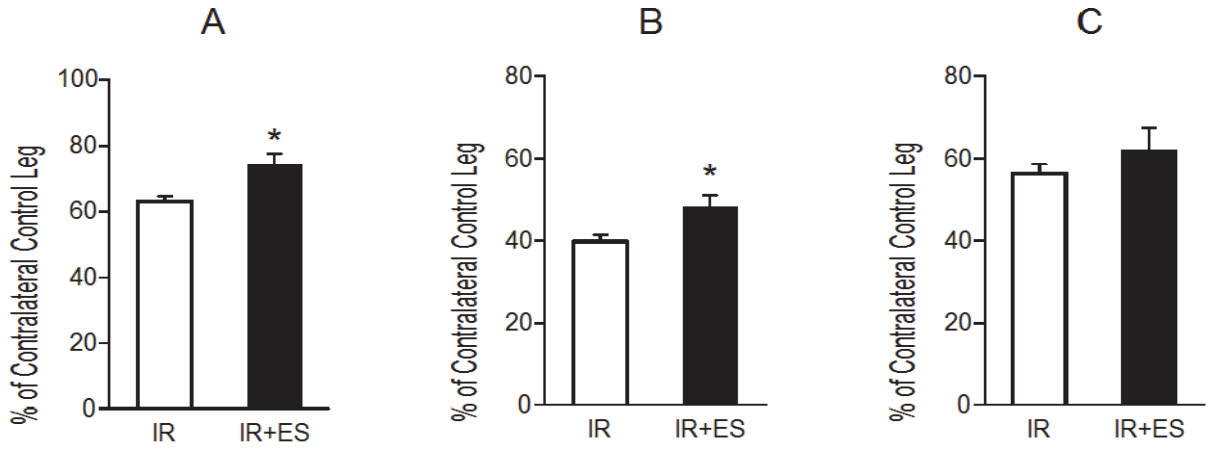


**Figure 2** – (A) Muscle weight represented as a ratio of repaired to contralateral limb. One month of stimulation significantly improved muscle weight ($p<0.05$). (B)Twitch forces were also significantly greater in stimulated limbs ($p<0.05$). However, mean values for tetanic forces (C) were trending higher but were not significantly different ($p>0.05$). All values are represented as a mean ± standard error of the mean.

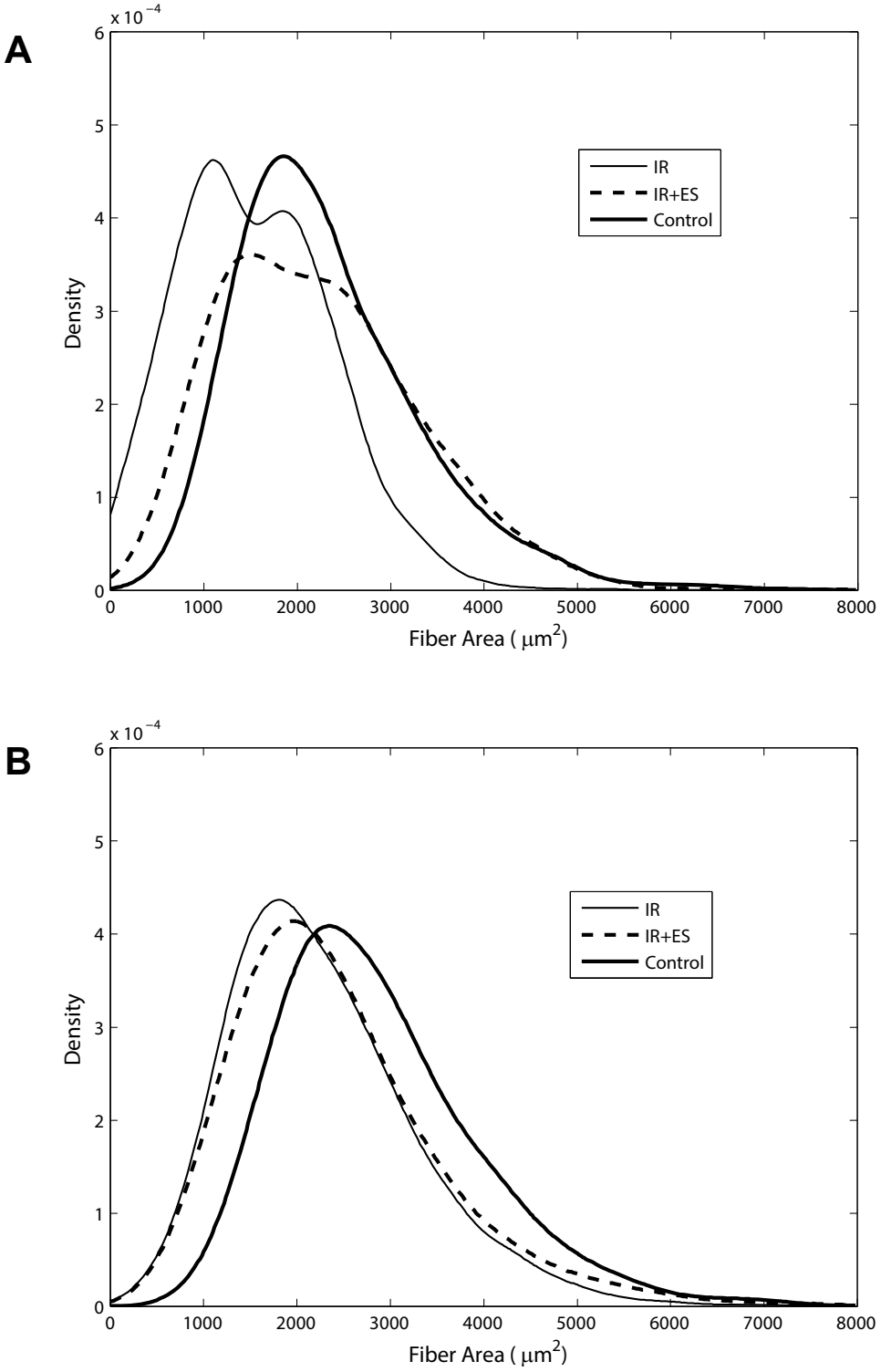


**Figure 3** – Averaged muscle fiber area statistical distributions for (A) type I muscle fibers and (B) type II muscle fibers. The distribution of type I fibers clearly shows a shift to larger fiber areas.

# Chapter 5: Paper Three - Determining the effects of electrical stimulation on functional recovery of denervated rat gastrocnemius muscle using motor unit number estimation

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**Objectives:**

The effect of chronic electrical muscle stimulation on reinnervation was investigated. Stimulation was delivered for three months following denervation surgery and then one month following nerve repair. Animals were allowed to recover for an additional two months. The aim of this paper was to determine whether long term muscle stimulation throughout denervation affects reinnervation (specifically motor unit numbers).

**Main Contributions:**

1. Motor unit counts were significantly higher in animals that were denervated and stimulated. These numbers were no different than immediately repaired animals.
2. Chronic electrical stimulation of denervated muscle enhances reinnervation.

**Determining the Effects of Electrical Stimulation on Functional Recovery of Denervated Rat Gastrocnemius Muscle using Motor Unit Number Estimation**

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**Abstract**

The use of electrical muscle stimulation to treat denervated muscle prior to delayed reinnervation has been widely debated. There is evidence showing both positive and negative results following different protocols of electrical stimulation. In this study we investigated the role electrical stimulation has on muscle reinnervation following immediate and delayed nerve repair using motor unit estimation techniques. Rat gastrocnemius muscle was denervated and repaired using the peroneal nerve either immediately or following three-months with and without electrical stimulation. Motor unit counts, average motor unit sizes, and maximum compound action potentials were measured three-months following peroneal nerve repair. Motor unit counts in animals that were denervated and stimulated were significantly higher than those that were denervated and not stimulated. Both average motor unit sizes and maximum compound action potentials showed no significant differences between denervated and denervated-stimulated animals. These results provide evidence that electrical stimulation prior to delayed nerve repair increases muscle receptivity to regenerating axons and may be a worthwhile treatment for peripheral nerve injuries.

**Introduction**

Despite the ability of peripheral nerves to regenerate following injury, complete functional recovery is rare [1]–[3]. The best case scenario for treating nerve injuries is immediate surgical repair; however, this is not always possible [1]–[4]. As soon as a nerve is injured or transected, the muscle has lost its functional connection to the nervous system and is termed denervated. As the length of time of muscle denervation increases, the chance for complete functional recovery significantly diminishes. Progressive muscle atrophy usually follows long-term denervation with a loss of muscle mass, muscle spindles, force, motor function and an increase in collagenization and fibrosis of the tissue [2], [3], [5]. Other important factors contributing to poor recovery are incomplete or inappropriate muscle reinnervation and decreased receptivity of muscle to reinnervation [5], [6]. One of the potential maintenance treatments for denervated muscle is electrical stimulation (ES) following injury. The issue with this treatment is that it is not widely accepted due to a lack of standards and questions of efficacy [7]. Many studies have shown both positive and negative results. Some beneficial effects include an increase in muscle fiber area, maintenance of fatiguability properties, and increases in muscle force [8]–[10]. Some negative effects include reduced intramuscular axonal sprouting [11], [12] and compromised reinnervation [7]. Others have shown no benefits for the use of ES prior to delayed nerve repair [13]. Because of the wide range of positive and negative effects there is still no consensus regarding the optimal electrical muscle stimulation protocol. Some studies have utilized a 24-hour paradigm employing implanted stimulators [14], [15], while others have shown that short term (20 minutes to 5

hours daily) stimulation provides just as much benefit [8]. Recently, our group has shown that a 1 hour per day stimulation protocol was effective at significantly increasing muscle weight, force, and fiber area in one month denervated rat gastrocnemius muscle [16]. In a second more comprehensive study we have looked at the longer term effects of electrical stimulation in both three month denervated and immediately repaired muscle. This paper specifically addresses the issue of ES and reinnervation using motor nerve stimulation and motor unit number estimation.

## Materials and methods

*Animals and surgical procedure*

The experiments were performed on male Lewis rats (Charles River, Quebec, Canada) weighing between 200-250g. This strain was chosen as it shows the least self mutilation following surgery [17]. All housing, surgical procedures, analgesia and assessments were performed according to the Canadian Council on Animal Care Guidelines, using protocols approved by the Animal Care Committee at McMaster University. Thirty-two animals were randomly assigned to one of four groups: immediate repair (IR), immediate repair with electrical stimulation (IR+ES), denervated (DEN), or denervated with electrical stimulation (DEN+ES). Each group had the right gastrocnemius muscle denervated, as described previously [2], by cutting the tibial nerve approximately 13 mm from its entry point into the gastrocnemius muscle (Fig. 1). The free distal stump was ligated to minimize extraneous innervation from other axons, and the proximal stump of the nerve was inserted and sutured into the biceps femoris muscle

to avoid reinnervation from proximal tibial nerve axons [2]. Teflon coated, stainless steel (Cooner Wire, AS 631) stimulating electrodes with ends bared of insulation were implanted into the belly of the right gastrocnemius muscle in all groups using an electrode suture complex to minimize electrode migration [18]. For both the IR and IR+ES groups the peroneal nerve was transected and connected directly to the distal tibial stump. For DEN and DEN+ES groups, the peroneal to tibial union was delayed by three months after the initial denervation. Following the peroneal to tibial repair, the muscles were allowed to recover for three months before final motor unit assessments were made.

*Electrical muscle stimulation*

The stimulation paradigm featured a 1 hour session per day using 400 ms of 100 Hz frequency, 200 μs per phase biphasic pulses followed by 6 seconds of rest. Stimulus amplitudes were adjusted to maintain a strong visual contraction. The paradigm was delivered using a custom designed stimulator capable of stimulating five rats simultaneously using the protocol mentioned above. Details of the stimulator design and stimulation paradigm are discussed elsewhere [16]. Animals in the IR+ES group received one month of electrical stimulation following the initial surgery. The rationale for this is that following peroneal to tibial union the muscle is without any functional connections for approximately one month. This is the estimated time it takes for regenerating motor axons from the peroneal nerve to reach the muscle and form functional connections. Animals in the DEN+ES group received three months of stimulation following the initial denervation surgery and one month following the peroneal to tibial repair surgery.

*Motor unit number estimation (MUNE)*

Motor unit estimation was performed on all animals following the three-month recovery period after peroneal to tibial repair. Two needle recording electrodes were placed in the gastrocnemius muscle and two stimulating hook electrodes on the proximal peroneal nerve. Stimulation pulses of 50 μs duration were delivered using an isolated stimulator and a custom designed EMG amplifier was used to record M-wave responses at a sampling rate of 8 kHz. A custom written LabVIEW program was used to trigger, record, and analyze the results. M-wave pattern recognition for template formation in this technique was accomplished using a 3-level wavelet decomposition classification scheme [20]. Maximum compound muscle action potentials (CMAPs) were first recorded and then twenty templates were recorded for each leg in the animal by gradually increasing the stimulus amplitude from threshold. Motor unit numbers were then estimated using the incremental method [19]. This involved dividing the area of the maximum CMAP by the average motor unit action potential contribution. The stimulating electrode was then moved a few millimetres proximally and the procedure repeated. Motor unit counts were averaged over these two trials. Fig. 2 shows a typical result of the properly characterized templates using wavelet pattern recognition.

Average motor unit action potential (MUAP) size was found by taking the largest template peak to peak value and dividing it by the number of steps required to reach that value. To ensure that there were no contributions to the motor unit count from the previously cut tibial nerve, stimulating electrodes were placed proximal to the level of the initial denervation and 50 μs stimulus pulses were delivered. The acquisition of an M-

wave by the recording electrodes would suggest ectopic innervation and hence that animal's results would be discarded.

*Statistical analysis*

To determine any significant differences between the groups a one-way ANOVA was performed followed by a Tukey post hoc test. Significance was defined as $p<0.05$.

**Results**

Two animals died during the initial surgery and three animals had noticeable ectopic innervation upon proximal tibial nerve stimulation. The latter three animals were excluded from any analysis.

Fig. 3 shows the motor unit estimates from all the groups. One month of stimulation in the IR+ES group provided no additional increases in motor unit numbers when compared to the unstimulated group (IR). Interestingly, three months of electrical stimulation in the DEN+ES group resulted in motor unit numbers not significantly different from either IR group. Most importantly, all three groups had values significantly greater than the DEN group, $p<0.05$. The fully innervated control group (CNTRL), which is the untreated contralateral limb in each animal, is shown for comparison.

Fig. 4 shows the average MUAP size. The post hoc test found no significant differences between the groups because of the large variability in each group's results. However, there is clearly a trend in that both the DEN and DEN+ES groups have smaller average values than both IR groups.

The last measure to be evaluated was the maximum peak-to-peak amplitude of the compound muscle action potential (Fig 5). There was no significant difference between both IR groups; however, both of these were significantly greater than both the DEN and DEN+ES groups ($p<0.05$). There was also no statistical difference between the DEN and DEN+ES groups although the mean values for the stimulated group were approximately 30% greater than for the unstimulated.

**Discussion**

This study provides clear evidence that electrical stimulation of denervated muscle plays a positive role in functional recovery. Motor unit numbers were not significantly different than the best-case scenario: immediate repair. Interestingly, one month of electrical muscle stimulation provided no added benefit in the immediate repair case. This may be due to the fact that the majority of motor axons found in the peroneal nerve create functional connections with the muscle and there is little room for improvement. However, these values were much less than the typical motor unit count in the gastrocnemius. Fully innervated and untreated gastrocnemius muscles in our animals have motor unit counts approximately 2 times that of our IR and IR+ES groups (155±32 vs. 82±20 and 80±20, respectively). This is consistent with a study that showed the tibial nerve has about twice as many motor axons as the peroneal nerve [21]. Our motor unit numbers in the control leg are also similar to those published by other authors [22].

We expected that the average MUAP size for the DEN group would be smaller than that of the DEN+ES group. Our rationale was that because of the severe fiber

atrophy and fibrosis taking place over three months of denervation, the muscle would not be as receptive to reinnervation. Consequently, motor axons that do make their way towards the muscle are forming connections with a small number of extremely atrophied fibers. Our results from a previous study [16] showed that after only one month the untreated denervated muscle had significantly smaller average muscle fiber sizes compared to a denervated-stimulated and fully innervated muscle. MUAPs from these motor units should then be smaller than MUAPs from a motor unit containing the same number of muscle fibers but with a much larger fiber area. Our results are not consistent with this hypothesis as both the DEN and DEN+ES MUAPs were the same size.

Our results do, however, show that significantly fewer axons were able to form functional connections with the denervated muscle fibers. One explanation for the larger than expected average MUAP size for this group may be that, during the three month recovery period following peroneal repair, those fewer motor axons that have made early functional connections with the denervated muscle will have had additional time to sprout collateral branches to the remaining denervated fibers and to further increase the size of the motor unit. Indeed, preliminary histological observations of muscle fiber area from this study also indicate that the DEN+ES group has a much larger fiber area than the DEN group. That is, the denervated untreated MUAPs are larger than expected because these motor units have a greater number of small muscle fibers. CMAP values provide an indication of the size of muscle fibers and consequently the force output. The results were as expected with the DEN+ES group being higher (although not significantly) than the DEN group. Control values for CMAP were not statistically different than either of the

immediate repair groups. However, histological examination shows larger fiber areas in control muscles than in both IR and IR+ES groups indicating larger force output. Indeed, preliminary measurements of tetanic force confirm this result.

## Conclusion

Contrary to what other studies have shown [13], electrical stimulation of denervated muscle prior to nerve repair provides beneficial effects over a longer period of time with both increased motor unit numbers and higher CMAP values. The data from this study may provide clinicians with the information necessary to start investigating electrical stimulation as supplementary treatment for peripheral nerve injuries.

## Acknowledgements

This work was supported in part by grants #IMH-87057 and #CPG-99371 from the Canadian Institutes of Health Research. The authors wish to thank Matthew Macdonald and Mary Susan Thompson for their contributions to the histological aspect of this study. We would also like to thank Larissa Schudlo and Christine Gabardo for their help with the animal muscle stimulation.

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**Figures**

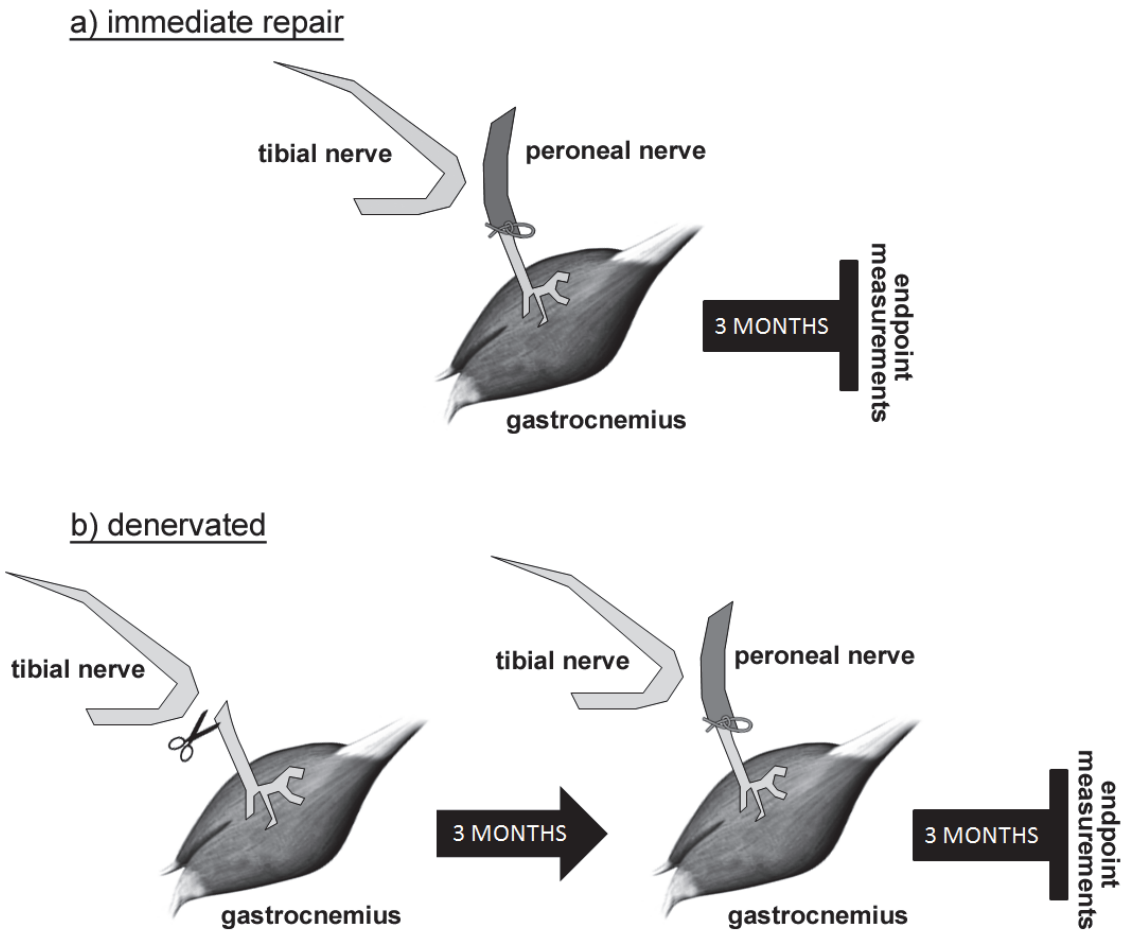


**Figure 1** – Surgical procedure and time points for experimental muscles. (a) immediate peroneal to tibial repair followed by 3 months of recovery. (b) complete denervation followed by a delayed (3 month) peroneal to tibial repair and a 3 month recovery period. At the end of the recovery period functional measurements are taken. Both of these groups were duplicated and included 1 month of electrical stimulation in the immediate repair case and 3 months of electrical stimulation in the denervated case. Animals in all groups had electrodes implanted into the belly of the muscle.

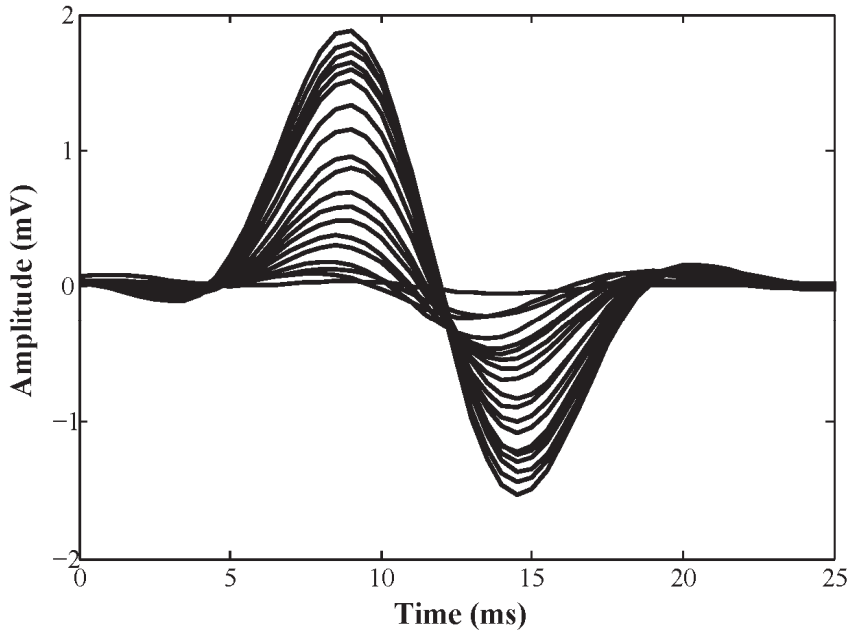


**Figure 2** – A typical template set for a fully innervated rat gastrocnemius muscle.

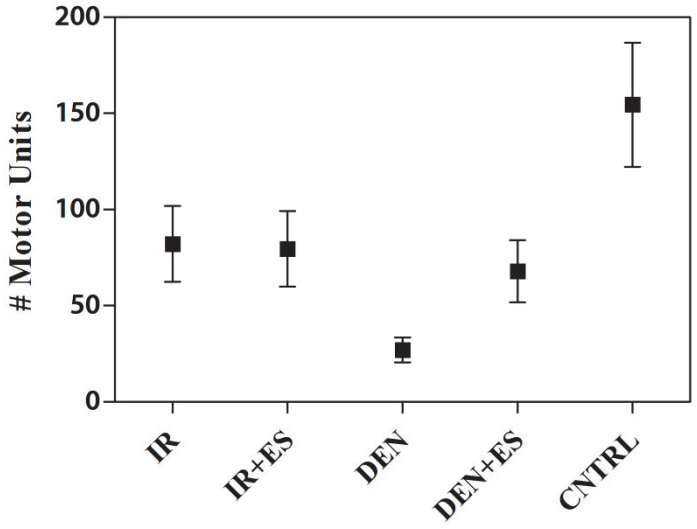


**Figure 3** – An estimation of the number of motor units shown for the immediately repaired (IR), immediately repaired with electrical stimulation (IR+ES), denervated (DEN), denervated with electrical stimulation (DEN+ES), and unoperated gastrocnemius muscle from the contralateral limb (CNTRL). Error bars represent standard deviation.

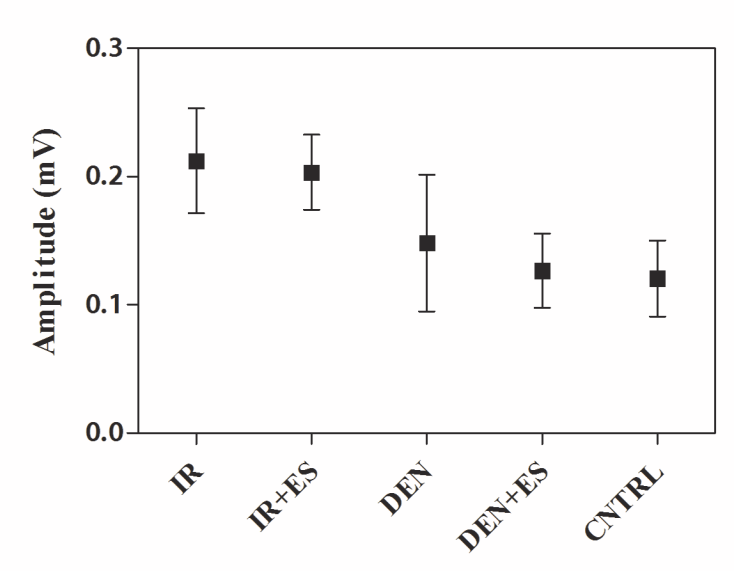


**Figure 4** – Average peak-to-peak MUAP amplitude values. Error bars represent standard deviation.

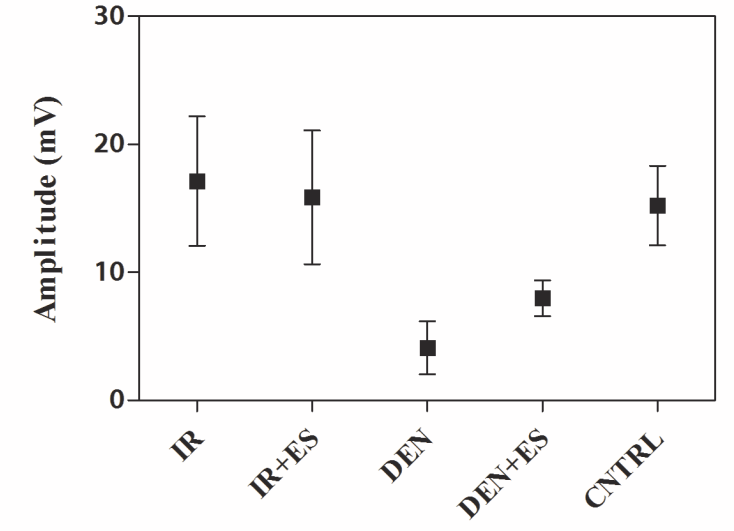


**Figure 5** – Maximum peak-to-peak amplitude values for the compound muscle action potentials. Error bars represent standard deviation.

# Chapter 6: Paper Four - Combined sensory protection and electrical stimulation of denervated rat skeletal muscle improves functional recovery

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**Publication Information:** Submitted to the Journal of Physiology

**Objectives:**

Muscle stimulation alone is not adequate to maintain muscle force and weight near control levels following denervation. Therefore, the effects of chronic electrical muscle stimulation combined with sensory protection were investigated. Stimulation, sensory protection, or both were administered for three months following denervation surgery and then a nerve repair was conducted. Animals were allowed to recover for an additional three months. The aim of this paper was to determine whether long term muscle stimulation throughout denervation combined with sensory protection can provide superior functional outcomes compared to each treatment alone.

**Main Contributions:**

1. Motor unit numbers were significantly greater in all treatment groups compared to denervation alone with the combination treatment having the greatest mean value
2. Muscle weight and twitch force were significantly greater in animals receiving the combination treatment compared to all other groups.
3. The combination treatment did not produce an additive effect, suggesting the individual treatments work through both common and different mechanisms.

**Combined sensory protection and electrical stimulation of denervated rat skeletal muscle improves functional recovery**

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**Abstract**

Long term muscle denervation leads to severe and irreversible atrophy, coupled with a loss of muscle spindles, fiber type distribution, force, motor function and fibrosis. These factors contribute to poor functional recovery following delayed reinnervation. Our previous work demonstrated that suturing a sensory nerve to the distal motor stump (sensory protection) significantly improves functional and histological outcome measures prior to reinnervation. More recently, we have shown that one month of electrical stimulation of denervated muscle also significantly improves outcome measures. In this study, we tested whether a combination of sensory protection and electrical stimulation would enhance functional recovery more than either treatment alone. Rat gastrocnemius muscles were denervated by cutting the tibial nerve. The peroneal nerve was then sutured to the distal tibial stump following three months of treatment (electrical stimulation, sensory protection, or both). Three months following peroneal repair, functional measurements were taken and muscles excised. The results showed that all treatment groups had significantly higher muscle weight ($p<0.05$) and twitch force ($p<0.001$) when compared to the untreated group (denervated), but fiber type compositions did not differ between groups. Importantly, muscle weight and force were significantly greater in the combined treatment group ($p<0.05$) compared to stimulation or sensory protection alone. The combined treatment also produced motor unit counts significantly greater than sensory protection alone ($p<0.05$). Thus, electrical stimulation combined with sensory protection significantly improves functional measures following delayed nerve repair,

suggesting these approaches work through different mechanisms. The use of the combined therapy may provide new options for clinical treatment of denervated muscle.

**Introduction**

Peripheral nerve injuries ranging from crushing to severing of the nerve can have a serious impact on distal muscle structure and function. Without an intact nerve connection, progressive muscle atrophy takes place. Long term denervation is accompanied by irreversible atrophy along with proliferation of connective tissue (Gutmann & Young, 1944; Savolainen *et al.*, 1988; Veltri *et al.*, 2005), disintegration of muscle spindles (Swash & Fox, 1974; Elsohemy *et al.*, 2009), and muscle fiber necrosis (Schmalbruch *et al.*, 1991). These structural changes result in decreases in muscle force (Finol *et al.*, 1981) and motor function and contribute to incomplete or inappropriate muscle reinnervation (Fu & Gordon, 1995*a*). Taken together, these factors contribute to poor functional recovery following peripheral nerve injury. The gold standard for treatment of these injuries is immediate nerve repair within two months of sustaining an injury (Isaacs, 2010). However, nerve transfers often have a high failure rate due to nerve tension or use of grafts, and if the site of injury is very far from the muscle then regardless of the time the initial surgery was performed, functional recovery and outcomes will be poor as the target musculature will be denervated for long periods of time (Fu & Gordon, 1995*a*).

One method that has been traditionally used to minimize muscle atrophy is electrical stimulation. This technique has been investigated as early as the 1840s as a

potential treatment for denervated muscle (Reid, 1841). However, the efficacy of this approach is controversial, with various studies demonstrating positive and negative effects (Cole & Gardiner, 1984; Dow *et al.*, 2004, 2007; Marqueste *et al.*, 2006; Sinis *et al.*, 2009; Gigo-Benato *et al.*, 2010). This is partly due to uncertainty regarding optimal stimulation paradigms, different animal models used, and a lack of standardization in animal studies. Thus, electrical stimulation has not been widely adopted as a clinical therapy (Eberstein & Eberstein, 1996).

Many studies have used a 24-hour stimulation paradigm in animals that was shown to be effective at maintaining muscle mass and force at control levels (Williams, 1996; Dennis *et al.*, 2003; Dow *et al.*, 2004, 2005). However, when translated to a clinical setting it proved to be a costly endeavour (Nicolaidis & Williams, 2001). We and others have shown that a simple 1-hour per day regimen of stimulation, easily translated to a clinical setting, can effectively increase mass and force (Ashley *et al.*, 2008; Willand *et al.*, 2011*b*). However, in these reports outcome measures following long term stimulation of denervated muscle were not near those of immediately repaired or fully innervated animals, providing evidence that electrical stimulation alone may not fully recover function.

Another method used to prevent muscle atrophy and enhance recovery during nerve regeneration is sensory protection. We demonstrated in our previous studies that cross-anastomosis of a sensory nerve to the distal motor stump can protect the muscle from denervation-associated atrophy and improve functional recovery (Hynes *et al.*, 1997; Bain *et al.*, 2001; Veltri *et al.*, 2005; Elsohemy *et al.*, 2009). This method has been

successfully used clinically (Bain *et al.*, 2008). Although sensory protection provides significant benefits, it does not on its own completely prevent denervation-associated changes in muscle.

To the best of our knowledge, there have been no studies investigating the combination of sensory protection and chronic electrical muscle stimulation. Sensory protection was shown to maintain structure (Hynes *et al.*, 1997; Zhang *et al.*, 1997; Wang *et al.*, 2001; Papakonstantinou *et al.*, 2002) and neurotrophic factor levels (Michalski *et al.*, 2008) in the distal tibial stump closer to that of a normal muscle while innervating and protecting muscle spindles which are essential for proprioceptive function (Elsohemy *et al.*, 2009). While sensory protection does not produce muscle contractions, previous work showed that muscle morphology was improved compared to denervation alone (Bain *et al.*, 2001). On the other hand, electrical muscle stimulation involves contraction of muscle fibers which has been shown to bulk muscle fibers leading to concomitant increases in force (Dow *et al.*, 2004; Kern *et al.*, 2005; Ashley *et al.*, 2008; Willand *et al.*, 2011*b*). Others have also shown that the muscle excitability is increased (Kern *et al.*, 2002) and that stimulation promotes selective reinnervation (Zealear *et al.*, 2002).

Given these facts the hypothesis of this present study is that the combination of the two therapeutic modalities will improve functional recovery better than each of these alone. To assess functional recovery a number of assays were used similar to our previous studies. Gross changes in the muscle were assessed through muscle weight and force measurements along with contractile properties. Analysis of fiber types and structural

changes were assessed through histological means. Lastly, motor unit number estimation provided evidence of muscle reinnervation.

## Materials and Methods

### *Ethical approval*

All experiments were performed on male Lewis rats (175g-200g) and approved by the Animal Care Committee at McMaster according to the Canadian Council for Animal Care Guidelines. This strain of rat was chosen because it demonstrates the least amount of self-mutilation following surgical procedures (Carr *et al.*, 1992).

### *Surgical Procedure*

Forty-two rats were randomly divided into four experimental groups: denervated (DEN, n=10), denervated with electrical stimulation (ES, n=10), sensory protected (SP, n=11), and sensory protected with electrical stimulation (SP + ES, n=11).

Figure 1 summarizes the experimental conditions and surgical procedures. Details of the surgical procedures have been previously described (Bain *et al.*, 2001). Animals were anesthetised using 5% isoflurane follow by 2% isoflurane to maintain anaesthesia. Each animal then had their right gastrocnemius denervated by transecting the tibial nerve approximately 13 mm from the point of entry into the muscle. The proximal tibial nerve stump was then sutured into the biceps femoris muscle in order to minimize ectopic innervation (Bain *et al.*, 2001). Animals in the denervated groups (DEN, ES) had the distal tibial stumps ligated and no further surgical procedures were performed until the delayed nerve repair (see below). In sensory protected animals (SP, SP+ES) the

saphenous nerve was transected and sutured to the distal tibial stump (Bain *et al.*, 2001). Teflon coated stainless steel stimulating electrodes (Cooner Wire, AS 631) were inserted into the right gastrocnemius muscle of all animals, immobilized with an electrode-suture complex, and externalized at the nape to allow for connection to the external stimulator (Willand *et al.*, 2011*b*). Following three months of denervation, stimulation, or sensory protection with stimulation, all animals were repaired by transecting the common peroneal nerve and suturing it to the distal tibial stump (Bain *et al.*, 2001). A further three month recovery period followed the common peroneal nerve repair, for a total of six months following initial denervation.

*Electrical Stimulation*

Animals that were assigned to the groups with electrical stimulation received a daily one-hour session of stimulation five days per week. This paradigm was used throughout the treatment portion of the study (3 months) and for one month following common peroneal repair. Each stimulation session featured delivery of 600 contractions using a frequency of 100 Hz. Each contraction consisted of a biphasic pulse train of 400 ms duration with the width of each pulse phase being 200 μs in duration. Subsequent contractions were delivered following a 6-second rest. To ensure a strong contraction was elicited, the amplitude was adjusted at each daily session until a visibly strong contraction was established. Others have also used a visual method to assess the strength of the contraction (Dennis *et al.*, 2003; Marqueste *et al.*, 2006; Dow *et al.*, 2007; Ashley *et al.*, 2008). The stimulus was delivered using a custom designed stimulator capable of stimulating five rats simultaneously using our protocol (Willand *et al.*, 2011*b*).

*Motor Unit Number Estimation*

Following the six-month period of denervation and treatment, animals were anesthetised with isoflurane and functional end point measurements were taken. To ensure that no ectopic innervation took place, the proximal sciatic nerve was stimulated. Animals that showed twitch responses in the gastrocnemius muscle were removed from the study.

Estimation of the number of motor units was carried out on the experimental right limb in each animal six months after the initial denervation. The incremental method of estimating motor units (Galea *et al.*, 1991) was used in conjunction with custom written LabVIEW software. Briefly, a pair of stimulating hook electrodes was placed on the proximal peroneal nerve (right limbs only) and two recording needle electrodes placed in the gastrocnemius muscle. Stimulation pulses (50 μs duration) were then delivered using an isolated stimulator. Compound muscle action potentials were recording using a custom designed amplifier and sampled at 8 kHz. The LabVIEW software then characterized each motor unit template using a 3 wavelet decomposition pattern recognition scheme (Salvador & de Bruin, 2006). For each limb a maximum response was first obtained followed by 18-20 incremental templates. Each template response corresponded to the average of a minimum of three identical motor unit combination responses. The stimulating electrodes were then moved and the procedure repeated. This provided two trials for which the motor unit counts could be averaged. Motor unit counts were estimated by taking the area under maximum response, dividing it by the area of the

maximum template and multiplying by the total number of templates (Willand *et al.*, 2011*a*).

*Force Measurements*

Once the number of motor units was estimated, the calcaneal tendon was severed and attached to a force transducer (Grass FD03). The tension of the muscle was adjusted in order to maximize force production. Two needle electrodes were placed in the belly of the muscle and served as stimulating electrodes. These were then connected to an isolated stimulator and maximum responses were elicited using a 1 ms duration stimulus pulse. For tetanic force measurements a 100 Hz train was delivered for 400 ms. Both twitch and tetanic force measurements were amplified using a custom designed amplifier and recorded using custom written LabVIEW software. From the twitch force curves, contraction and half relaxation times were measured as these served as an approximation to the overall fiber type distribution of the muscle. The procedure was repeated for the contralateral limb in all animals.

*Muscle Weight*

When all functional measurements were completed, animals were killed by overdose of isoflurane gas and cervical dislocation. The gastrocnemius muscle was excised and weighed. A section from the mid-belly of the medial gastrocnemius muscle (approximately 3 mm thick x 3 mm wide and 8-10 mm in length), was then dissected, mounted in embedding medium (Tissue-Tek, OCT Compound, Sakura Finetek U.S.A.,

Inc.; Torrance, CA), immediately frozen in liquid nitrogen cooled isopentane, and stored at −80°C for future analysis.

*Analysis of fast (type II) and slow (type I) twitch muscle fibers*

Muscle samples were sectioned on a Leica CM3050 S cryostat cooled to −20°C (Lecia Microsystems Inc.; Richmond Hill, Ontario). 10-12 μm sections were mounted onto glass slides and stained for myofibrillar ATPase at a pH of 10 (Brooke & Kaiser, 1970). Slides were imaged using a Nikon D300 adapted to a Zeiss Universal light microscope using a 16x objective. Muscle fiber area and type ratios were analyzed by counting and measuring approximately 2000 fibers (2017 ± 158, mean ± SEM) per animal using ImageJ software (NIH). To visualize fiber area distributions, a smoothed histogram was calculated using a kernel density estimator (Lexell & Taylor, 1991) based in Matlab (The MathWorks, Inc.; Natick, MA).

*Statistics*

Statistical analysis was accomplished using GraphPad Prism 5 software (GraphPad Software, Inc.; La Jolla, CA). To reduce variability due to differing animal weights, the ratio of experimental to contralateral limb was used when expressing results from muscle weight and force (twitch and tetanic). A one-way ANOVA followed by a Tukey *post-hoc* test was used to compare groups. Histological data was analyzed using a non-parametric Kruskal-Wallis test with a Dunns *post-hoc* test to compare all groups. The results were significant when $p < 0.05$.

**Results**

In the denervated group one animal died during the initial surgery and one animal was found to have ectopic innervation during the terminal surgery. Of the remaining eight animals in this group, we had difficulty with obtaining motor unit estimates in one of them and data for that animal was not included in motor unit number analysis. One animal in the stimulated group was discarded from final analysis, as the common peroneal to tibial surgical procedure was not adhered to.

*Motor unit number estimation*

As expected, the denervated group showed the lowest number of motor units (36.4 ± 6.4, Figure 2). Stimulation during 3 months of denervation and for 1 month following common peroneal nerve repair significantly increased motor unit numbers compared to denervation alone ($p<0.05$, 73.8 ± 10.7). Although 3 months of sensory protection did not produce a statistically significantly greater number of motor units, the mean value was trending higher compared to the denervated group ($p=0.097$, 63.16 ± 4.3). The combination of sensory protection and electrical muscle stimulation produced motor unit counts (91.9 ± 7.4) no different than electrical stimulation alone but significantly higher than either denervation or sensory protection alone ($p<0.001$ and $p<0.05$, respectively, Figure 2). These results show that the use of sensory protection and electrical muscle stimulation is accompanied by an increase in the number of reinnervated motor units.

*Muscle weights*

The muscle weights of the treated limb of each animal were expressed as a percentage of the unoperated contralateral limb in order to minimize within-animal variability. Our previous work (Bain *et al.*, 2001) showed that the unoperated limb does not undergo compensatory increases in weight and force allowing the use of this limb for normalization purposes. Denervation without any treatment (29 ± 2.6 %) showed the lowest muscle weight, while the combination of sensory protection and stimulation had significantly higher weights than all other groups ($p<0.05$, Figure 3). Although the combined treatment was higher than either stimulation (41 ± 3.4 %) or sensory protection (41 ± 2.2 %) alone, the mean value (53 ± 2.2 %) was not the result of an individual additive effect of the treatments, suggesting the treatments may work through both common and different mechanisms. There was no difference in weight between stimulation alone and sensory protection alone. Regardless of the treatment type, mean weights were significantly greater than denervation alone ($p<0.05$, Figure 3).

*Muscle forces and contractile properties*

Tetanic tension measurements were elicited using 40 pulses at 100 Hz. Results showed the denervated group had significantly lower peak tension values (9 ± 2.6 %) compared to all other groups ($p<0.01$, Figure 5). Electrical stimulation significantly increased tetanic force compared to the denervated group ($p<0.001$, 29 ± 4.1 %). Similarly, sensory protection also significantly increased tetanic force ($p<0.0$ 1, 25 ± 1.7 %). The combination treatment (38 ± 2.9 %) exhibited increased tetanic force compared to denervation alone and was significantly greater than sensory protection alone ($p<0.01$).

There were no observed differences between the combined treatment and stimulation alone.

Similarly, muscle twitch forces were elicited using 1 ms pulses and contractile properties were calculated from the resulting tension profiles. Twitch forces as a percentage of the contralateral limb force followed a similar pattern as muscle weights with the denervated group (8 ± 1.2 %) having significantly lower values than all other groups ($p<0.001$, Figure 4A). Similarly, stimulation (26 ± 3.4 %) and sensory protection (28 ± 1.7 %) twitch forces alone were not different from each other. However, the combination of sensory protection and stimulation (37 ± 3.2 %) was significantly different than stimulation alone ($p<0.05$), and mean values were trending higher than sensory protection alone ($p=0.07$). Typical tension profiles for each group are shown in Figure 4B. It is evident from this graph that contractile properties in the muscle remain unaltered. Indeed, there were no statistically significant differences between groups observed in either contraction or half-relaxation times, suggesting that regardless of the treatment, overall fiber type composition is not altered (Table 1). Results from these measurements are summarized in Table 1.

*Muscle fiber type distribution and fiber area*

In order to determine whether fiber type was altered by these treatments, analyses of muscle fiber area and type distribution were assessed in 4 animals per group, with approximately 2000 fibers examined per animal. Gross histological examination showed that sensory protection and stimulation minimized the number of denervated fascicles (Figure 6). Muscle sections from animals treated with sensory protection alone or

stimulation alone exhibited areas of atrophy, suggesting that reinnervation did not take place in some areas. The general appearance of denervated muscle showed large areas of atrophy and fiber necrosis. Mean type I muscle fiber areas in stimulated animals were significantly larger than fiber areas in denervated animals ($p<0.05$, Figure 7, Table 2). Similarly, stimulated animals had significantly larger type II fiber areas when compared to denervated animals ($p<0.01$, Figure 7, Table 2). Type I and II fiber areas in sensory protection alone and the combined therapy were not significantly different from all other groups and were intermediate between denervated and stimulation alone. This suggests that stimulation alone has more impact on fiber area than sensory protection alone. However, an examination of the fiber area distributions showed changes that are not reflected in mean area values. A type I fiber density plot (Figure 8A) clearly showed that in the denervated group, type I muscle fibers with areas under 1000 $\mu m^2$ predominated. Stimulated animals had a bimodal distribution of type I fibers with many more fibers exhibiting larger areas compared to the other groups, suggesting that stimulation alone targets these fibers. Results from our previous studies also showed that our stimulation protocol may be targeting type I fibers (Willand *et al.*, 2011*b*; Willand *et al.*, 2012). Sensory protected and sensory protected stimulated type I muscle fibers had Gaussian shaped distributions.

A type II fiber density plot (Figure 8B) shows that all groups except the combined treatment group had bimodal distributions. This is consistent with gross histological examination showing atrophied fascicles in these groups. The combined treatment group had a more Gaussian distribution of type II fibers, consistent with the general appearance

of the muscle where fascicle atrophy is minimal. The fiber type composition of the muscle was no different between groups, with approximately 10 % of the counted fibers stained as type I in all groups. Mean fiber area and type composition are shown in Table 2.

**Discussion**

Recovery following peripheral nerve injury is poor if treatment is not provided within days in an animal model or within a few months in a clinical model (Mackinnon, 1988; Finkelstein *et al.*, 1993). We have previously demonstrated that sensory protection alone can increase functional recovery following long term denervation (Bain *et al.*, 2001, 2008). We also recently showed that our electrical stimulation protocol can minimize denervation-induced atrophy and increase innervation following long term denervation (Willand *et al.*, 2011*a*). However, neither treatment alone produces complete functional recovery. In this study, we demonstrated that the combination of sensory protection and electrical stimulation provides additional benefits beyond either treatment alone.

It was previously reported that electrical muscle stimulation alone has no effect on functional recovery following long term denervation (Dow *et al.*, 2007). Our work contradicts that study, with 3 months of electrical stimulation providing significantly greater values in all our endpoint tests compared to the denervated group. An important difference between the studies is that we used a freshly axotomized nerve for motor repair whereas Dow et al. did not. It is known that long term axotomy can lead to poor functional recovery (Fu & Gordon, 1995*b*). Another difference is in the stimulation

paradigms used. Our stimulus paradigm employs one hour of daily contractions whereas Dow et al. provide continuous stimulation over a 24 hour period. There is evidence that continuous stimulation may alter muscle reinnervation (Love *et al.*, 2003). Nevertheless, our results confirm that intramuscular stimulation alone can improve reinnervation and functional recovery.

When electrical stimulation was combined with sensory protection mean motor unit counts for the combined treatment were larger than for either treatment alone, but not significantly greater than for electrical stimulation alone. When compared to our previous results (Willand *et al.*, 2012), motor unit values from the combined treatment were statistically no different ($p>0.05$) from an immediately repaired animal ($91.9 \pm 7.4$ vs. $82.1 \pm 6.3$, respectively), immediate repair being the gold standard exhibiting the best functional recovery following peripheral nerve transection. The results from this functional assay suggest that both treatments improve reinnervation but that electrical muscle stimulation has a greater role.

Further investigation of enhanced reinnervation in sensory protected and stimulated muscle was carried out through histological examination of the muscle. This showed that the combined treatment group had a more uniform looking muscle with intact fascicles consisting of less atrophy compared to all other groups, confirming the improved reinnervation in this group. Fiber type compositions were no different between treated groups, suggesting that our treatments did not change muscle fiber types, with fast twitch (type II) fibers being the dominant type. As shown by the density plots, all treatments were effective at increasing type I muscle fiber size. However, a bimodal

distribution of both type I and II fibers was present in animals that received stimulation alone. Microscopic examination showed that the areas of atrophy were concentrated in the periphery of the muscle, suggesting that the stimulation field may not have spread to the entire muscle. Similarly, a bimodal distribution was found for type II fibers in animals with sensory protection alone. The combined treatment group did not exhibit these characteristics, but rather showed a decrease in the number of larger type II muscle fibers compared to the stimulated group alone. This suggests that sensory protection may limit the increase in fiber size provided by electrical muscle stimulation. On the other hand, further investigation into the stimulus amplitudes used showed that animals that were sensory protected and received stimulation had lower amplitudes delivered during each session compared to animals receiving stimulation alone. Contractions were visibly strong in both cases, however, discomfort was more evident at higher amplitude levels in those receiving the combined treatment. As the saphenous nerve is a sensory cutaneous nerve, electrical stimulation of the muscle during the time that it is sensory protected may depolarize the sensory axons themselves. This stimulus may be perceived as cutaneous pain. Other sensations such as proprioception, temperature changes or pressure may be present as well. Indeed, we have shown that clinical use of sensory protection returns the sense of proprioception (Bain *et al.*, 2008). This limited our use of higher amplitudes in these animal studies. The use of lower amplitudes results in lower drive to the muscle and may reduce muscle fiber size. Animals receiving stimulation alone had higher amplitudes delivered, resulting in greater maintenance of larger fibers. Indeed, the fiber area distribution of animals receiving stimulation alone was similar to that of an immediately

repaired animal (Willand *et al.*, 2012). These low amplitudes may have limited therapeutic gain in animals receiving the combined treatment. Stimulation paradigms that lower the amplitude requirements through the use of long sub-threshold prepulses (Willand & de Bruin, 2008) may warrant investigation in order further enhance functional outcome measures.

Other functional outcome measures such as muscle weight and twitch force were significantly higher in the combined treatment compared to either treatment alone. However, when outcome measure values from sensory protection and stimulation alone were combined the results were not additive. Each outcome measurement was influenced differently by the treatments. Electrical muscle stimulation had a greater role in producing larger tetanic forces in the combined treatment. This is not surprising as the contractile mechanism is not used in sensory protected animals. Both stimulation and sensory protection seemed to equally influence twitch forces in animals that received the combined treatment. It is known that twitch contractions can be elicited in the absence of external calcium ions (Stroffekova, 2008) while tetanic contractions require calcium release and reuptake from the sarcoplasmic reticulum. This suggests that daily muscular contraction elicited through electrical stimulation may specifically influence the sarcoplasmic reticulum. Muscle weight in the combined treatment group was significantly higher than either treatment alone suggesting that weight is influenced by both treatments in different ways. The partial additivity of these results may be due to the use of lower stimulus amplitudes in the combined treatment which would decrease the number of large fibers and result in lower twitch force and muscle weight. The treatments may also work

through both common and different mechanisms. Our previous work supports this theory as sensory protection was shown to maintain the structure of the distal nerve stump (Veltri *et al.*, 2005), the proprioceptive machinery (Elsohemy *et al.*, 2009), and morphological features without contraction of the muscle fiber. Electrical stimulation further enhances the morphology through muscle fiber contraction while improving muscle receptivity to reinnervation. As the distal stump has been shown to play an important role in functional recovery (Veltri *et al.*, 2005) and neurotrophin secretion following denervation (Michalski *et al.*, 2008), maintenance of this structure may be important to enhanced recovery (Gordon *et al.*, 2011). Muscle atrophy is also an important contributor to poor functional outcomes following nerve injury, and the use of electrical muscle stimulation has been widely shown to reduce atrophy (Gordon *et al.*, 2011; Willand *et al.*, 2012). Taken together, protection of the distal stump and maintenance of muscle receptivity may provide enhanced functional recovery. The molecular and cellular mechanisms of both sensory protection and electrical stimulation are unclear and warrant further investigation. Although twitch and tetanic force levels were not near values we reported for immediately repaired muscle (Willand *et al.*, 2012), this could be the product of a stimulation paradigm that is not optimal for chronic long term denervation studies. Further work to investigate different paradigms may be necessary to recover force at levels close to immediately repaired muscle.

*Conclusion*

Sensory protection and electrical stimulation of denervated muscle have both separately been demonstrated to enhance functional recovery in animal models and in a clinical setting (Bain *et al.*, 2001, 2008; Kern *et al.*, 2005; Willand *et al.*, 2012). In this study, we show in a rat model that both treatments together improve reinnervation, muscle weight and force. Our results provide evidence that sensory protection and electrical muscle stimulation enhance functional recovery at least partially through different mechanisms. The differing results in individual outcome measures may be attributed to the different mechanisms. The data presented in this paper support the use of both treatments together to enhance functional recovery following denervation, and this may provide a new strategy for clinical treatment of denervated muscle.

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## Tables

**Table 1 –** Twitch and tetanic force, contraction and half-relaxation time measurements of denervated rat gastrocnemius muscle subjected to various treatments.

| Measurement | DEN N=8 | ES N=9 | SP N=11 | SP+ES N=11 |
|---|---|---|---|---|
| Twitch Force Ratio | 8.1 ± 1.277 | 26.2 ± 3.44 | 28.4 ± 1.71 | 37.4 ± 3.25 |
| Tetanic Force Ratio | 9.4 ± 2.62 | 28.9 ± 4.14 | 25.36 ± 1.72 | 37.9 ± 2.94 |
| Twitch/Tetanus Ratio | 0.32 ± 0.0358 | 0.34 ± 0.0288 | 0.42 ± 0.0148 | 0.33 ± 0.0168 |
| $T_{peak}$ (ms) | 31.05 ± 0.55 | 32.49 ± 0.544 | 30.44 ± 0.536 | 30.73 ± 0.902 |
| $T_{1/2R}$ (ms) | 32.08 ± 1.863 | 34.62 ± 2.282 | 32.55 ± 1.033 | 36.22 ± 1.151 |

DEN = denervated; ES = electrical stimulation; SP = sensory protection; SP+ES = sensory protection and electrical stimulation. N=number of animals per group.

**Table 2 –** Rat gastrocnemius muscle fiber area and type distribution after various treatments

| Measurement | DEN N=4 | ES N=4 | SP N=4 | SP+ES N=4 |
|---|---|---|---|---|
| Type I Area ($\mu m^2$) | 301 ± 188 | 2055 ± 326 | 1054 ± 123 | 1109 ± 172 |
| Type II Area ($\mu m^2$) | 569 ± 259 | 2224 ± 328 | 1196 ± 63 | 1648 ± 217 |
| Percentage of Type I | 6.59 ± 1.379 | 12.51 ± 2.626 | 10.63 ± 1.552 | 11.67 ± 4.248 |
| Percentage of Type II | 93.41 ± 1.379 | 87.49 ± 2.626 | 89.37 ± 1.552 | 88.33 ± 4.248 |

DEN = denervated; ES = electrical stimulation; SP = sensory protection; SP+ES = sensory protection and electrical stimulation. N=number of animals per group.

## Figures

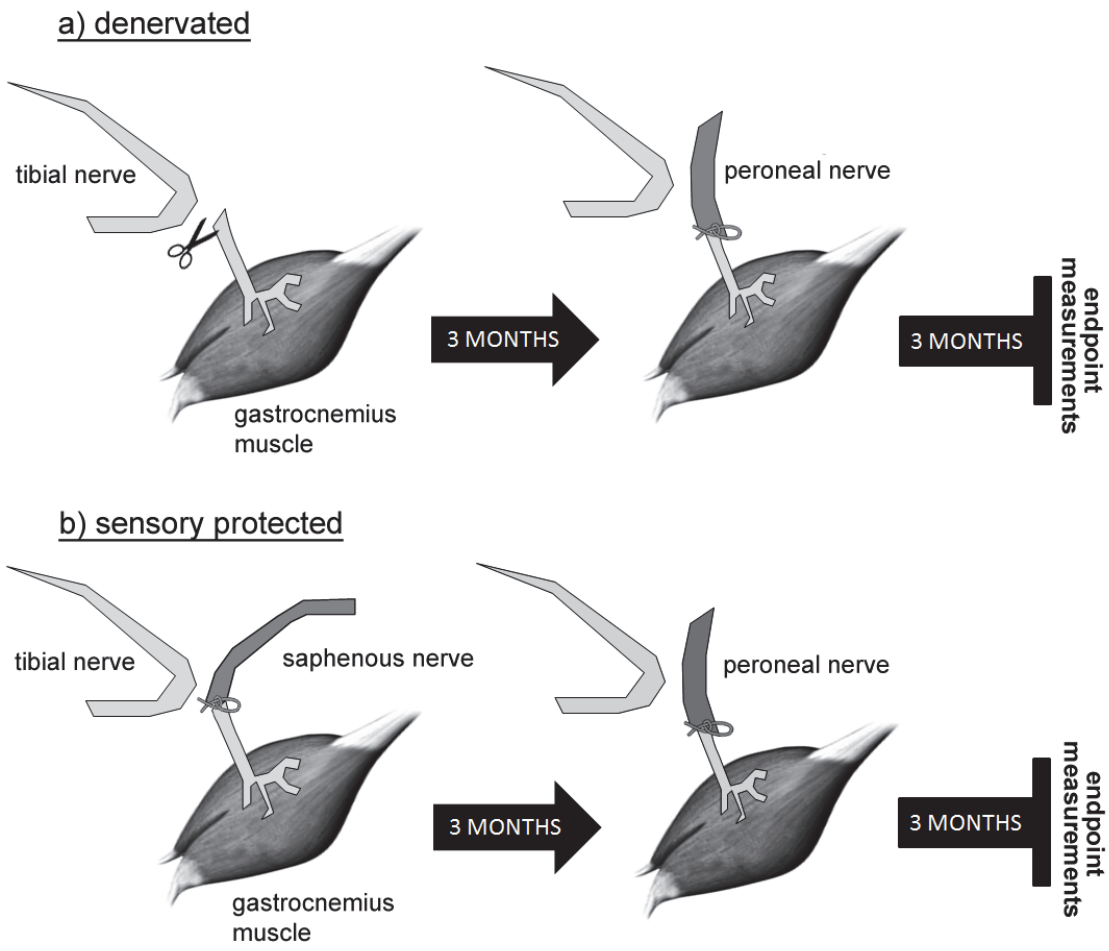


**Figure 1** – *Surgical procedures*. The right gastrocnemius muscle in each animal was either denervated (a) or sensory protected (b). Each group also had electrodes implanted into the muscle. Animals that were in the stimulated group underwent daily electrical stimulation during the initial 3 months of denervation or sensory protection followed by 1 month immediately after surgical nerve repair. The contralateral unoperated limb in each animal served as a control.

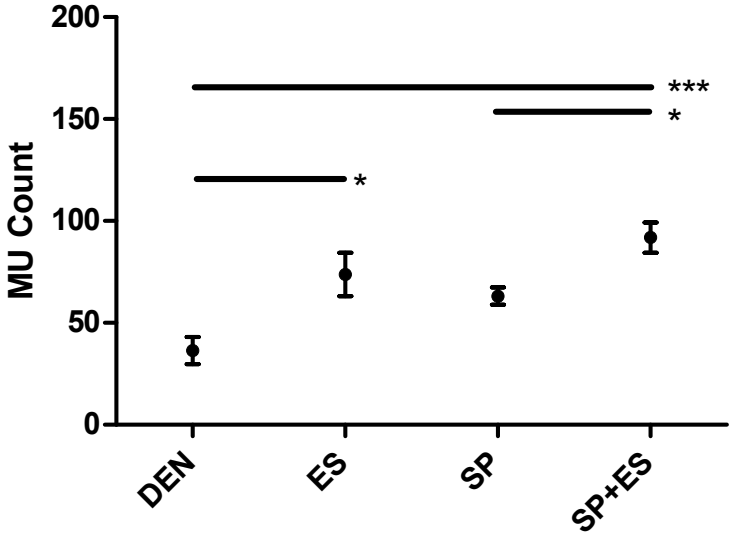


**Figure 2** – *Motor unit number estimation*. Motor unit (MU) numbers were estimated 3 months following nerve repair. In each animal 18-20 unique responses were used to estimate the total number of units. The combined treatment of sensory protection and stimulation (SP+ES) was significantly greater than sensory protection (SP) and denervation alone (DEN). Stimulation alone (ES) was significantly greater than DEN. Error bars represent SEM (one way analysis of variance followed by Tukey post hoc test, *p<0.05, ***p<0.001).

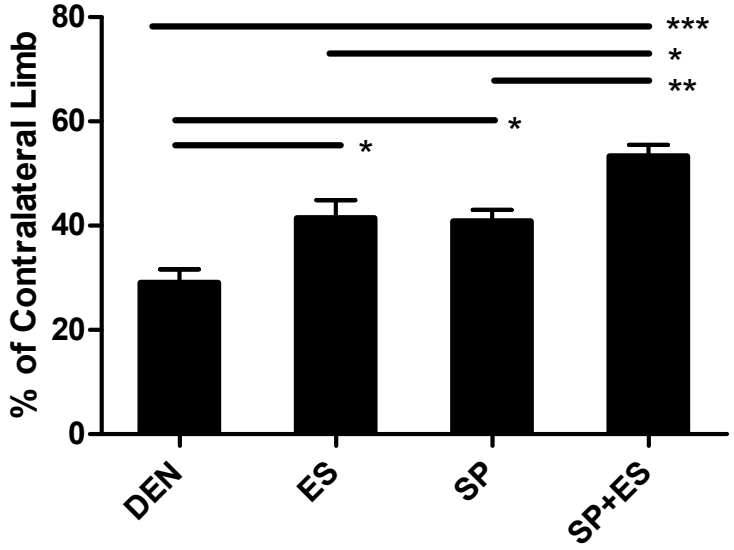


**Figure 3** – *Muscle weight ratio*. Muscles were excised and weighed 3 months following nerve repair and weights are expressed as a ratio of the treated to contralateral control limb. The combination treatment of sensory protection and stimulation (SP+ES) was significantly greater than all other groups. Stimulation alone (ES) was no different than sensory protection (SP) alone, and both were greater than denervated (DEN) muscle. Error bars represent SEM (one way analysis of variance followed by Tukey post hoc test, *p<0.05, **p<0.01, ***p<0.001).

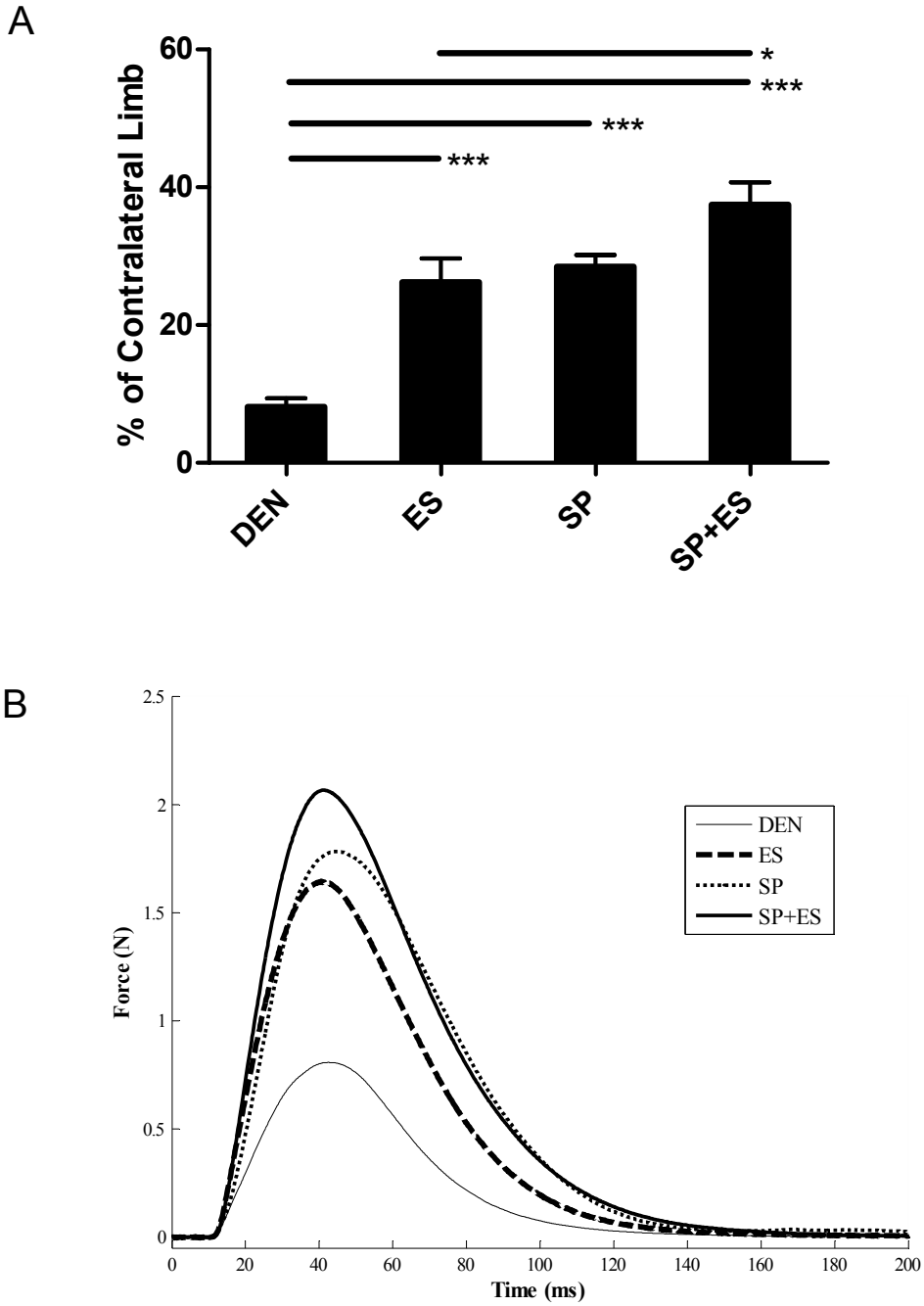


**Figure 4** – *Twitch force measurement.* A, mean twitch force values are represented as a ratio of the treated to contralateral control limbs. Force ratios were significantly greater in the combined therapy (SP+ES) compared to stimulation (ES) or denervation (DEN) alone. Sensory protected muscle (SP) was not significantly different from either ES or SP+ES. Error bars represent SEM (one way analysis of variance followed by Tukey post hoc test, *$p<0.05$, ***$p<0.001$). B, individual twitch profiles for each group. Twitch characteristics such as contraction time and half relaxation time were not different between groups (see Table 1).

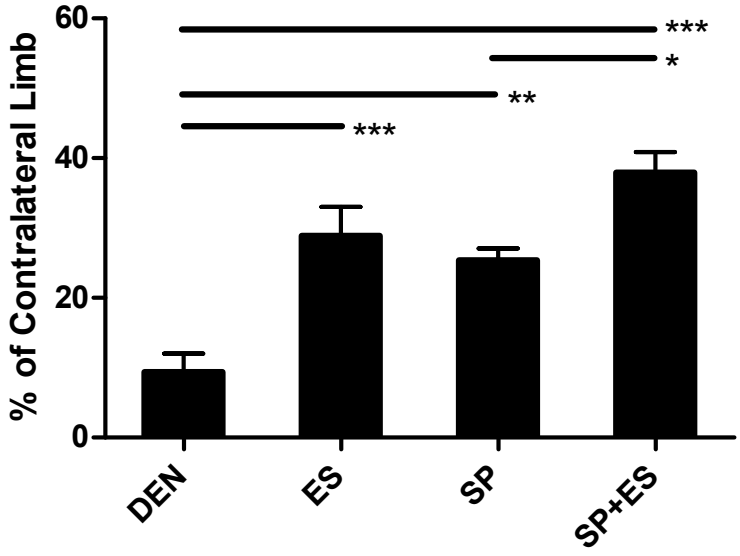


**Figure 5** – *Tetanic force measurement*. Mean tetanic force values are represented as a ratio of the treated to contralateral control limbs. For each animal, 40 pulses at 100 Hz were used to obtain tetanic contractions. Stimulation alone (ES) was not different from the combined treatment (SP+ES), but tetanic force of the SP + ES group was significantly greater than that of the sensory protected group (SP). All groups had significantly greater force ratios than the denervated group (DEN). Error bars represent SEM (one way analysis of variance followed by Tukey post hoc test, *$p<0.05$, **$p<0.01$, ***$p<0.001$).

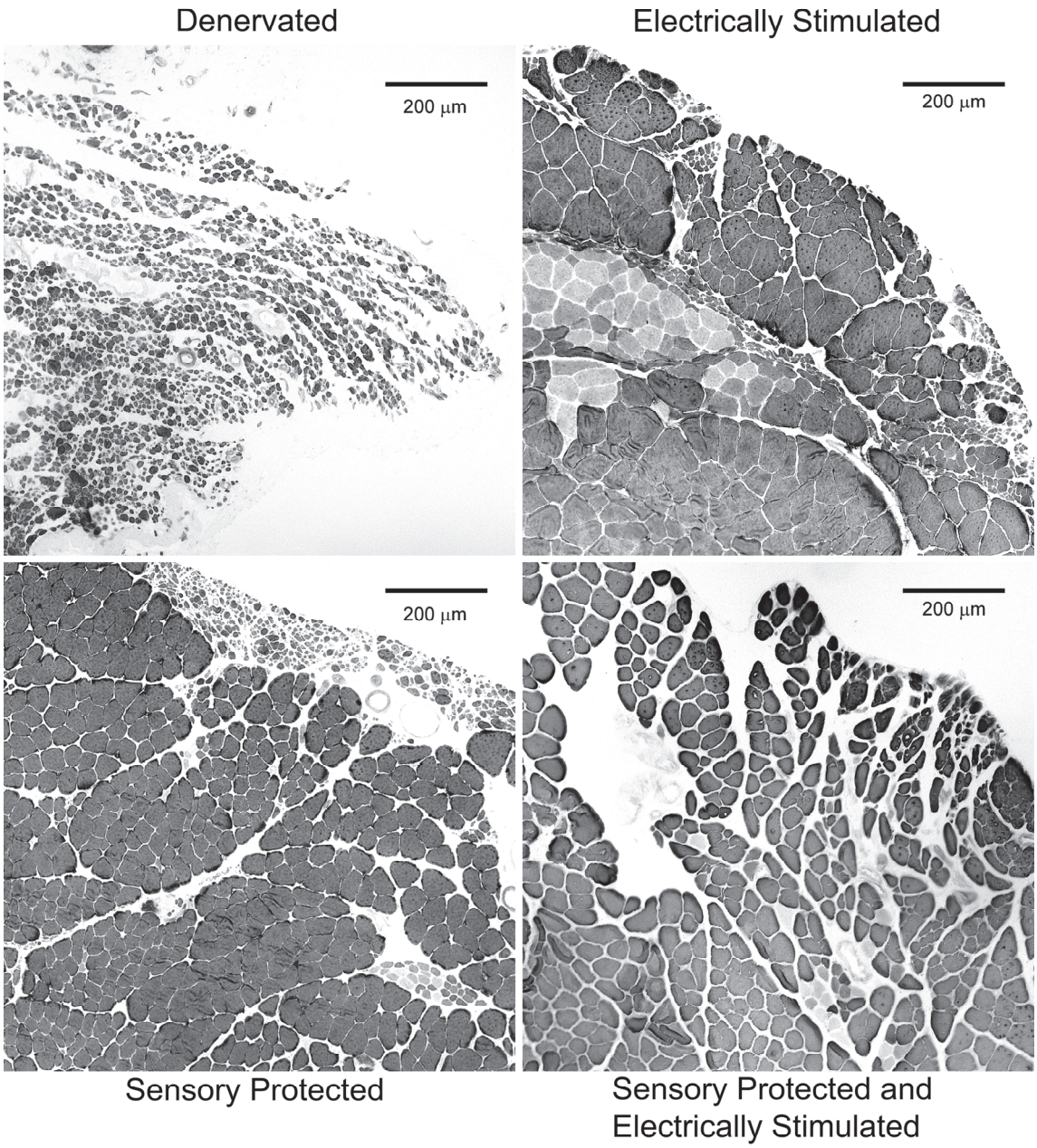


**Figure 6** – *Micrographs of ATPase stained gastrocnemius muscle*. Denervated (top, left), stimulated (top, right), sensory protected (bottom, left), and sensory protected and stimulated (bottom, right) sections. Darkly stained fibers represent type II fast twitch fibers and lightly stained fibers represent type I slow twitch muscle fibers. Electrical stimulation, sensory protection, or the combination of both, increase fiber area when compared to denervated muscle. Large areas of atrophy are noticeable in all groups except in the combined treatment. Scale bar represents 200 μm.

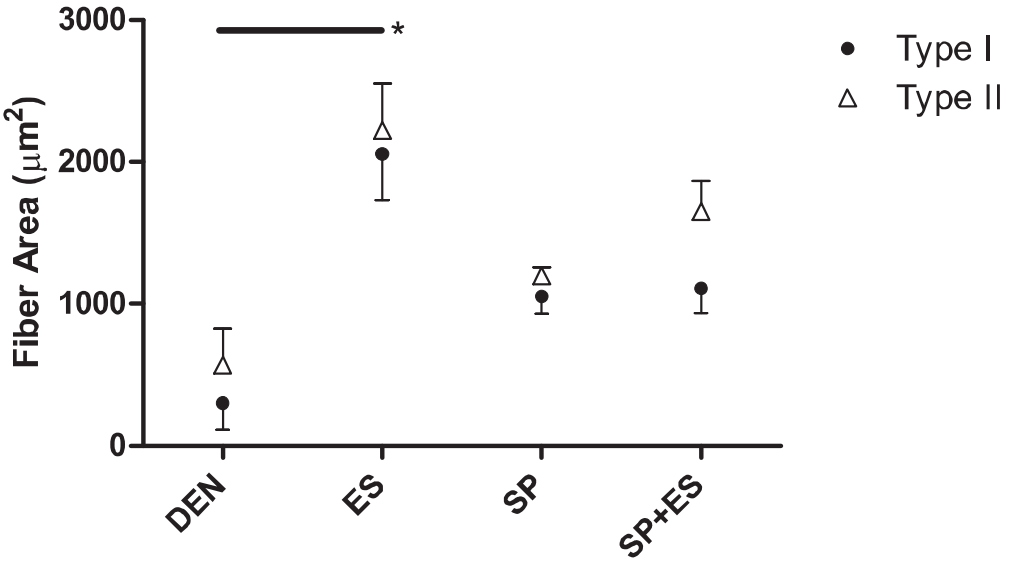


**Figure 7** – *Muscle fiber area*. Type I and II fiber areas are shown for each group. Stimulation alone (ES) was significantly greater than denervated (DEN) for both type I and II fibers. SP = sensory protected; SP + ES = sensory protected + electrical stimulation. Error bars represent SEM (one way analysis of variance followed by Tukey post hoc test, *$p<0.05$).

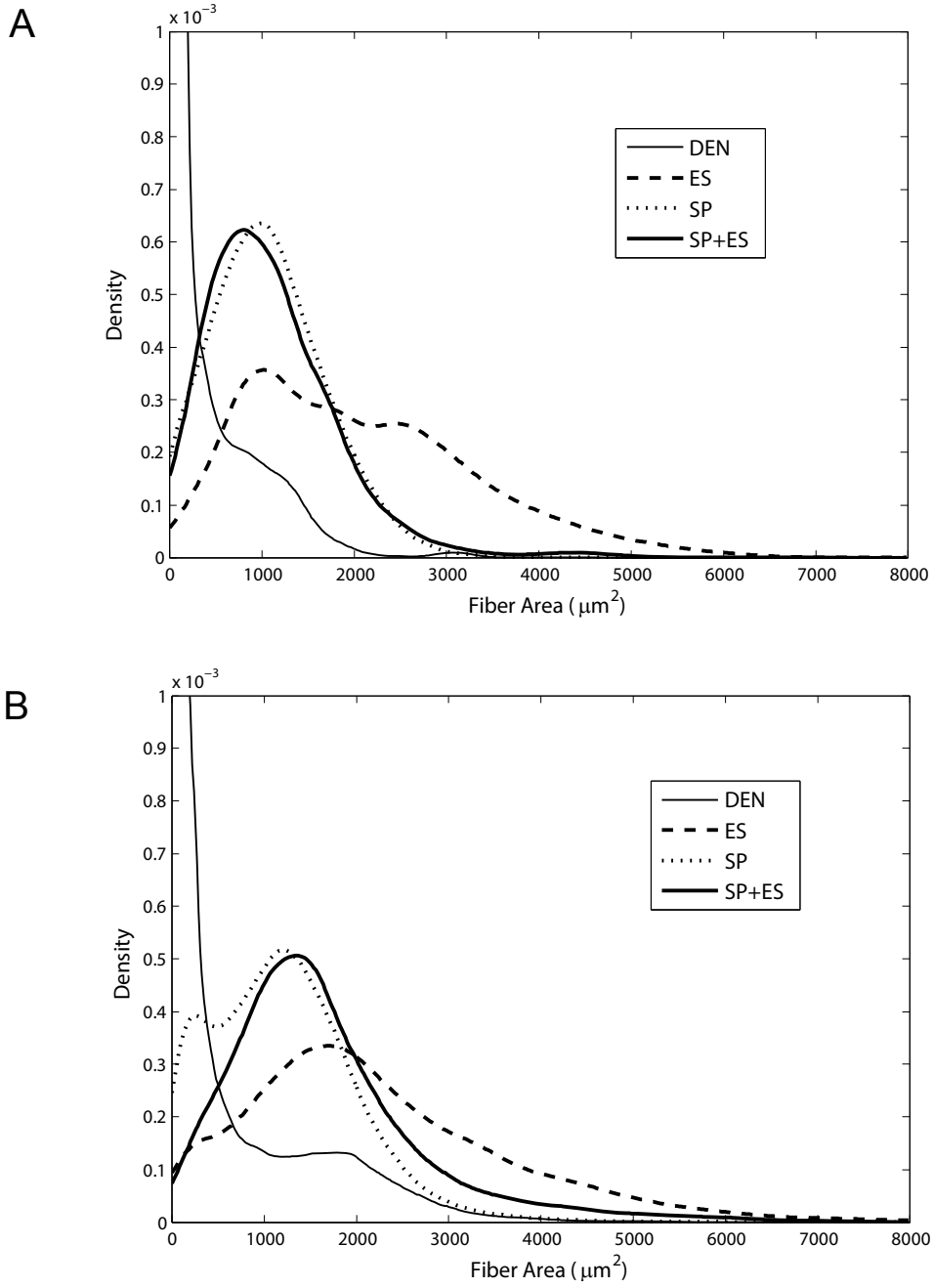


**Figure 8** – *Fiber area density*. Distributions of fiber types are represented as density graphs. A, type I fibers, B, type II fibers. The peak of the denervated (DEN) group is not shown for scaling purposes. For type I fibers, stimulation alone (ES) had a greater proportion of larger fibers compared to other groups. For type II fibers, both stimulation (ES) and sensory protection alone (SP) had bimodal distributions corresponding to fascicles that are atrophied. The distribution of sensory protection and stimulation (SP+ES) is uniform in appearance.

# Chapter 7: Conclusion and Future Work

## 7.1 Conclusion

Poor functional recovery following muscle denervation is a major clinical problem (Bain, Hason, Veltri, Fahnestock, & Quartly, 2008). The most effective treatments today involve surgical coaptation of the nerves. However, when injuries are sustained more proximally, there is a longer period of time that the muscle remains denervated and atrophy starts to take place which prolongs recovery. Electrical muscle stimulation has been used in the past to counteract changes due to atrophy. However, the proper stimulation paradigm to use has always been controversial (Eberstein & Eberstein, 1996). With advances in microelectronics, implantable stimulators have shown great promise but come with an increased price and potential requirements for multiple surgeries (Nicolaidis & Williams, 2001; Williams, 1996). More recently, short duration stimulation protocols have been shown to significantly reduce atrophy in animals (Ashley et al., 2008). These protocols represent a change from continuous 24 hour stimulation using implantable stimulators and they are more likely to be adhered to in a clinical setting. In this thesis, a new protocol was developed and tested in denervated and denervated-repaired muscle. More specifically, the first paper (Chapter 3) presented the design, development, and testing of a new stimulator that can efficiently deliver the stimulation paradigm which consisted of daily one-hour sessions featuring 600 contractions per session. This low cost stimulator reduced the need for expensive implantable stimulators and is similar to what would be used in a clinical setting. The implications of this first paper are that short term electrical stimulation, on the order of one hour per day, is

effective at significantly reducing muscle atrophy, increasing muscle fiber size, and muscle force.

Furthermore, the second and third papers (Chapters 4 and 5, respectively) demonstrated that electrical stimulation does not negatively impact muscle reinnervation, as suggested by others (Love, Son, & Thompson, 2003; Sinis et al., 2009). The use of electrical stimulation following immediate nerve repair (paper two, Chapter 4) did not significantly impact reinnervation and showed a benefit in terms of significantly larger slow twitch fiber areas and twitch force. Electrical stimulation during three months of denervation (paper three, Chapter 5) also showed significantly greater reinnervation numbers compared to denervation alone. The implications of these papers are that electrical muscle stimulation can increase functional reinnervation following long term denervation. Papers two and three (Chapter 4 and 5 respectively) provide significant evidence that electrical stimulation, even at intervals as low as one hour per day, can be used as a treatment for denervated muscle. However, functional outcome measures from these two papers were not near control values and it may be that electrical stimulation alone is not adequate enough to completely preserve muscle.

The last paper (paper four, Chapter 6) combined electrical stimulation with a method called sensory protection. This method involves the use of a sensory nerve coapted to the distal motor stump during the period of denervation. This method protects or babysits the muscle during this denervation period. Previous work has shown that sensory protection significantly reduces muscle atrophy, fibrosis, and preserves muscle spindles (Bain, Veltri, Chamberlain, & Fahnestock, 2001; Elsohemy, Butler, Bain, &

Fahnestock, 2009; Hynes, Bain, Thoma, Veltri, & Maguire, 1997; Veltri, Kwiecien, Minet, Fahnestock, & Bain, 2005). Force measures were also increased but again not near control values (Bain et al., 2001). This method has also been successfully demonstrated in a clinical setting (Bain et al., 2008). The results of paper four demonstrate that the combined treatments (electrical stimulation and sensory protection) do provide additional benefit that is not present in either of these treatments alone. Muscle atrophy was significantly reduced in the combined treatment compared to the individual treatments. Muscle force followed a similar trend. However, the effects were not additive suggesting that these two treatments work through both similar and different mechanisms. More specifically, muscle weight, force, and fiber area benefited from both therapies whereas motor unit numbers were primarily increased through electrical muscle stimulation. The maintenance of muscle spindles and the distal nerve stump were primarily contributed by sensory protection. The implications of this paper are that combining a treatment that focuses on supporting the contractile mechanism (electrical muscle stimulation) and one that regulates the trophic environment (Davis & Kiernan, 1980, 1981) and protects the distal nerve stump (sensory protection) can provide significant functional improvement during long periods of denervation. Since both of these treatments have separately been used in a clinical setting it may be beneficial to combine the two to enhance functional recovery.

The work presented in this thesis made significant contributions to areas of electrical muscle stimulation and treatment of denervated muscle. More importantly, the new stimulation protocol described has potential to be translated to a clinical setting and

benefit patients following a nerve injury. Furthermore, the combination of two distinct treatments shows promising results that may also be quickly adapted to a clinical setting but also can stimulate new research into the combination of exogenous trophic support along with electrical muscle stimulation.

## 7.2 Recommendations for Future Work

With short term stimulation paradigms showing positive results, more research needs to be done to quantify the impact or varying the number of stimulation sessions per day and number of contractions. It has been suggested by Dow that a long rest period between stimulation sessions may not retard muscle atrophy as much as more frequent sessions (Dow, Faulkner, & Dennis, 2005). Therefore, a future aspect for investigation would be to split the current one hour paradigm into two thirty minute sessions each featuring half the number of contractions (300) as the full one hour session (600). This would potentially reduce the rest period by half and potentially further reduce muscle atrophy while maintaining a clinically translatable protocol.

A further enhancement to the stimulation device can be made in order to miniaturize the apparatus. Currently, a lot of through-hole components are used that are being phased out by manufacturers and replaced by surface mount varieties. Using a surface mount design will further reduce costs along with physical size.

The use of electrical stimulation following immediate repair was shown to have positive effects (Chapter 4). However, an approach to quantify motor unit reinnervation throughout the recovery period can assess whether stimulation accelerates functional recovery. At present, all motor unit analysis takes place during terminal measurements.

Improvements to this technique could allow for continuous measurements of motor unit numbers throughout an experiment. To accomplish this, two approaches could be used. The first requires an implantable stimulator connected to a cuff electrode to stimulate the proximal nerve. As intramuscular electrodes are already present and used for muscle stimulation they can be also connected to a recording amplifier for observing motor unit action potentials. The second approach forgoes the use of implantable stimulation devices. A cuff electrode can be attached to the proximal nerve with the leads being externalized along with the intramuscular electrodes. The stimulation apparatus would be an external device. Both of these approaches provide a solution to obtaining motor unit counts throughout an experiment. With this method in place a further change to the stimulation protocol following immediate nerve repair can be investigated. This change would affect the duration of stimulation. Although one month of stimulation did not affect reinnervation perhaps a different length of time may be more beneficial.

Finally, sensory protection and electrical muscle stimulation have shown great promise as a future treatment. However, the mechanisms by which each treatment works need to be thoroughly investigated. Extensions of this work can be carried out by combining electrical muscle stimulation with exogenous trophic factors such as GDNF. Previous studies have shown that local delivery of GDNF following denervation can promote axonal regeneration (Boyd & Gordon, 2003).

The treatments outlined in this thesis are targeted to preserve muscle health. An approach that combines accelerating nerve growth through short term proximal nerve

stimulation (Gordon, Brushart, & Chan, 2008) with the treatments in this thesis may provide superior functional recovery following a nerve injury.

There is a bright future for patients with nerve injuries and it is the hope that the work in thesis may facilitate and strengthen current research into treatment of denervated muscle.

## 7.3 References

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