

## **SENSORY PROTECTION OF DENERVATED MUSCLE**

**TEMPORARY SENSORY PROTECTION OF DENERVATED SKELETAL MUSCLE:  
A QUANTITATIVE ULTRASTRUCTURAL AND FUNCTIONAL ASSESSMENT  
OF NERVE AND MUSCLE**

by

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## MAIN ABSTRACT

Peripheral nerve injuries have a devastating impact on muscle function due to the distance the axons must regenerate to reinnervate the target muscle. By the time the axons reinnervate the muscle, it has atrophied and lost its receptiveness resulting in impaired function. The objective of this study was to answer the following questions: 1. Do sensory axons delay muscle atrophy following denervation? 2. Does the protective effect observed by sensory axons translate into restored muscle function? 3. Do sensory axons preserve the architecture of the distal nerve sheath and provide a favourable regeneration substrate? 4. Do sensory axons influence the trophic effect on denervated muscle to maintain its “receptiveness” to reinnervation?

Following tibial nerve transection, rats were assigned to one of the following groups: (1) saphenous to distal tibial nerve neurotization (Nerve-to-Nerve sensory protected (SP)); (2) saphenous to gastrocnemius neurotization (Nerve-to-Muscle sensory protected (SP) in the absence of the distal nerve sheath); (3) Unprotected controls (tibial nerve transection) or, (4) immediate common peroneal to tibial nerve neurotization (Immediate Repair with a motor nerve). The unoperated contralateral leg of treated animals served as a control. After a 6-month denervation period followed by motor reinnervation, ultrastructure, histology, morphometrics of nerve and muscle were assessed and muscle function was measured.

Specimens of distal tibial nerve in the Nerve-to-Nerve (SP) group were superior to Unprotected controls shown by a significant increase in axon density, a significant decrease in collagen area, and improved axon-to-Schwann cell coupling. These features are characteristic of the original neural environment and reflect sustained neural integrity. Although axon number in the Nerve-to-Nerve (SP) group was similar to the Nerve-to-Muscle (SP) group, improved regeneration was evident in the Nerve-to-Nerve (SP) group shown by several axons at various stages of myelination, in a “normal” one-to-one



association with a Schwann cell. The Nerve-to-Nerve (SP) group also displayed a significant increase in mean axon area than the Nerve-to-Muscle (SP) group.

Gastrocnemius muscle specimens from both sensory protected groups displayed less collagenization and fat deposition than Unprotected control muscle, similarity in mean total muscle fibre size, and evidence of reinnervation (fibre type grouping) among regions of “mosaicism” suggesting a preservation of normal muscle features. Fast twitch fibres predominated in both sensory-protected groups (60% to 40%) as in normal muscle. Unprotected controls contained no fast twitch fibres and the total muscle fibre area of this group was significantly smaller than all other experimental groups. The mean area of fast twitch fibres in the Nerve-to-Muscle (SP) group was significantly larger than the Nerve-to-Nerve (SP) group suggesting a possible trophic influence on fast twitch muscle fibre area.

Mean compound muscle action potential amplitude in the Nerve-to-Nerve (SP) group was significantly higher than the Nerve-to-Muscle (SP) and Unprotected control groups. Although the Nerve-to-Muscle (SP) group demonstrated a significantly higher isometric contractile twitch force than Unprotected controls, this was only slightly increased possibly due to the unusually high values for the Unprotected controls.

These findings suggest that a feasible method to optimally diminish the denervation changes in muscle is to preserve the architecture of the distal nerve and concomitantly maintain the trophic influence on the muscle fibres.

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## PREFACE

The data contained herein are presented in 5 chapters, 2 of which are published articles in peer-reviewed scientific journals. Each of the 2 published articles contained in this thesis have been printed with permission from the publishers (letters of permission are enclosed). The thesis candidate is a coauthor on each of these published articles, as follows :

**Chapter 2 :** Hynes N, Bain JR, Thoma A, Veltri K and Maguire J. Preservation of denervated muscle by sensory protection in rats. *J Reconstr Microsurg* 13(5):337-343, 1997. (published with permission of Thieme, 2003)

**Chapter 3 :** Bain JR, Veltri KL, Chamberlain D and Fahnstock M. Improved functional recovery of denervated skeletal muscle after temporary sensory nerve innervation. *Neuroscience* 103(2): 503-510, 2001. (published with permission of Elsevier, 2003)

Each of the above published papers is followed by a brief Summary describing how the results relate to subsequent work described in Chapters 4 and 5.

Three chapters are contained herein as manuscripts prepared for submission to a scientific journal. Chapter 1 is a review paper which describes the anatomy and physiology of the normal peripheral motor nerve and skeletal muscle, denervation changes and current literature on denervation atrophy and modalities to diminish muscle atrophy. Chapter 4 and Chapter 5 present the findings of the current investigations comprising the thesis candidate's doctoral research to elucidate the mechanisms of "sensory protection".

The prepared manuscripts are :

**Chapter 1:** Karen Veltri, James R. Bain. Ultrastructure and physiology of the normal peripheral motor nerve, normal and denervated skeletal muscle, and current literature review of modalities to reduce muscle atrophy (submitted to Neuropathology and Applied Neurobiology)

**Chapter 4 :** K. Veltri, J.M Kwieciën, W. Minet, M. Fahnestock, J.R. Bain. Contribution of the distal nerve sheath to nerve and muscle preservation following denervation and sensory protection. (submitted to Neuroscience)

**Chapter 5:** Karen Veltri, James R. Bain, Margaret Fahnestock. A functional assessment of sensory protected muscle following denervation (submitted to J Applied Physiology)

The thesis candidate is the first author on each of the above submitted manuscripts.

#### Thesis Candidate's Contribution To Published Studies

The thesis candidate (KV) prepared the animals for surgery, calculated the dosage of anaesthetic and analgesics, administered anaesthesia and oxygen during surgery, administered analgesics after surgery and monitored the animals. The candidate also performed microsurgical procedures (denervation, sensory/motor nerve repair), performed isometric contractile function and electrophysiologic assessments, sacrificed the animals and procured the nerve and muscle specimens for histological / ultrastructural analysis. The candidate also examined the nerve and muscle morphology under the light microscope. The candidate set up all muscle recording apparatus, recorded the data and performed measurements of the data in addition to statistical analysis, interpretation and providing written drafts of the results, and conducted literature searches.

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## A. CHAPTER 1

Ultrastructure and Physiology of Normal Peripheral Motor Nerve, Normal and Denervated Skeletal Muscle,  
and Literature Review of Modalities to Reduce Muscle Atrophy

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**Abstract**

This paper describes the normal anatomy of the peripheral motor nerve and its innervation target (skeletal muscle) and the series of physiological events whereby a nerve action potential is translated into a muscle action potential via the neuromuscular junction. The integrity of the axonal impulse and ultimate muscular contraction can be identified using nerve conduction studies of compound muscle action potential amplitude, latency and conduction velocity and measures of the magnitude of the force generated by the muscle. Injury sustained by the peripheral motor nerve results in a loss of axonal communication to the target muscle leading to denervation, including fibrosis and fat deposition and fibre atrophy which substantially impairs the reinnervation potential of the muscle. Significant molecular, physiologic and morphological alterations occur in the nerve cell body, and the proximal and distal axon segments. These alterations, however, are crucial to the optimization of the reinnervation potential. This paper describes the denervation events at the molecular and physiologic level and explains the corresponding objective findings (ie. electrophysiologic and contractile force). These measures offer a means to objectively monitor how specific factors may enhance or confound the optimal recovery of denervated muscle. The literature is inundated with studies which have investigated a variety of modalities to optimize muscle recovery following denervation. By describing the events leading to muscle denervation and the functional correlates, it is hoped that a broader understanding of the mechanisms whereby these various modalities act will facilitate future research in the field of skeletal muscle denervation atrophy.

## Normal Peripheral Motor Nerve

### Ultrastructure of the Motor Axon

Mammalian peripheral motor nerves are comprised of axons whose cell bodies originate from the ventral horn of the gray matter in the spinal cord (Wyrick and Stern 1992). The “axoplasm” which contains cellular metabolites, organelles, and components of the cytoskeleton, is encapsulated by the axonal surface membrane (axolemma), a lipid bilayer containing channel proteins which is soluble to various ions, including sodium ( $\text{Na}^+$ ), potassium ( $\text{K}^+$ ), chloride ( $\text{Cl}^-$ ) and calcium ( $\text{Ca}^{2+}$ ). The “axon terminal” consists of synaptic vesicles containing “quanta” of acetylcholine (Ach), calcium channels and mitochondria (Figure 1).

Communication within the motor neuron must be maintained over a vast distance (up to 1 meter) in the peripheral nerve. This is accomplished by molecules transported anterogradely in the axoplasm from the cell body to the axon. Components of the plasma membrane and neurotransmitter storage vesicles proceed along the axon via “fast transport” whereas the “slow transport” system facilitates the flow of cytoskeletal elements such as microtubules, neurofilaments and microfilaments (Wyrick and Stern, 1992). Neurotrophic factors provide information from the peripheral nerve and target tissue to the cell body via retrograde transport.

The characteristic feature of a large-diameter myelinated axon is its interaction with a Schwann cell. The myelin sheath is formed by Schwann cell cytoplasm wrapping around the axon forming many layers of membrane containing a lipid substance (sphingomyelin) functioning as an insulator of electrical current flow. The myelinated regions of the axon are interrupted at regular distances up to 1 mm. These regions, devoid of myelin, are the “nodes of Ranvier” (Cormack, 1987). Schwann cell cytoplasm engulfs multiple small-diameter unmyelinated axons interspersed between the myelinated axons (Figure 2) (Weiss and Greep, 1977).

The axon is surrounded by an “endoneurial tube” consisting of loose connective tissue matrix (extracellular matrix) containing fibroblasts which secrete collagen, mast cells, and Schwann cells (Cormack, 1987). A group of axons (fascicle) is ensheathed by the “perineurium” (Figure 2, Panel A)



consisting of collagenous tissue with several layers of tightly-arranged flattened protective cells with their basement membrane (Wyrick and Stern, 1992). Axons are surrounded by 3 layers of collagen (Thomas 1964; Ushiki and Ide 1986). The innermost layer is comprised of the region where Schwann cell basal lamina interacts with axonal membrane. External to this layer is a region of collagen comprising the inner endoneurial sheath, beyond which is a thicker outermost layer of collagen.

A group of fascicles is contained by a superficial and internal layer of epineurium consisting of connective tissue with fibroblasts, macrophages, and mast cells. The superficial layer facilitates smooth nerve movement against surrounding structures, while the internal layer engulfs separate groups of fascicles (Figure 2) (Wyrick and Stern, 1992). The microvascular supply to the nerve arises from the vasa nervorum traversing the nerve through the endoneurium, perineurium and epineurium.

### Schwann Cells

Schwann cells show remarkable plasticity and possess multiple functions in peripheral nerve. An important function of Schwann cells is axon myelination which involves progesterone (Robert et al, 2001). Progesterone, synthesized and secreted by Schwann cells, upregulates genes encoding myelin proteins such as P<sub>0</sub>, myelin basic protein (MBP) and peripheral myelin protein (PMP22). Cell-surface progesterone receptors are expressed on the surface of Schwann cells (Robert et al, 2001).

Recent evidence indicates that signals derived from Schwann cells are critical to the development of neurons and connective-tissue cells. These signals also contribute to the survival of Schwann cells in the absence of axonal contact via autocrine loops (Jessen and Mirsky, 1999). The autocrine cycle involves growth factors which synergistically prevent Schwann cell death (Meier et al, 1999).

The regulation of axonal survival by Schwann cells stems from Schwann cell-derived signals (ie. myelin-associated glycoprotein (MAG), involved in the maintenance of axonal architecture) which regulate a "kinase-phosphatase cycle" (Jessen and Mirsky, 1999). This cycle is implicated in the maintenance of the neurofilament phosphorylation state which in turn affects the packing arrangement of neurofilaments and hence the axon diameter (de Waegh et al, 1992). Schwann cell-released factors are also involved in

stimulating the axonal membrane at the nodes of Ranvier to maintain Na<sup>+</sup> channel clustering (Black, et al, 1990).

Neurotrophins, a subfamily of neurotrophic factors, are critical in the development of the nervous system but in the adult of maintain neuronal differentiation, survival and plasticity. The neurotrophin family consists of nerve growth factor (NGF) and its receptor, tyrosine kinase (TrkA) (Thoenen and Barde, 1980; Bandtlow et al, 1987 ), brain-derived neurotrophic factor (BDNF) and its receptor, TrkB (Barde et al, 1982 ), neurotrophin-3 (NT-3) and its receptor, TrkC (Hohn et al, 1990) and neurotrophin (NT)-4 and its receptor, TrkB (Ip et al, 1992). These factors are secreted in low levels by Schwann cells under normal conditions (Varon and Adler, 1981). Schwann cells are also a source of ciliary neurotrophic factor (CNTF), a polypeptide neurotrophic factor which is non-secreted and differs in structure from the neurotrophins. Other factors secreted by Schwann cells at low levels include the cell-adhesion molecules (CAM), glial-derived neurotrophic factor (GDNF), and insulin-like growth factor (IGF) (Jessen and Mirsky, 1999).

### **Normal Motor Axon Physiology**

Peripheral motor nerves provide communication between the central nervous system and the target organ (muscle) and information is relayed via electrical impulses conducted along the length of the nerve (action potential). Depolarization of the axolemma causes changes in ion permeability in adjacent regions and subsequent depolarization. At rest, the axon membrane conductance for K<sup>+</sup> is between 50-100 times greater than for Na<sup>+</sup> (Guyton, 1987). The initiation of a nerve action potential occurs by excitation and discharge of motor neurons in the anterior horn cell of the spinal cord (Sternberg, 1997). This alters the permeability of the cell to Na<sup>+</sup> and K<sup>+</sup> whereby, when depolarization reaches threshold, voltage-gated Na<sup>+</sup> channels open to allow an influx of Na<sup>+</sup> ions giving rise to an “all-or-none” action potential. Propagation of the action potential occurs distally toward the axon terminal.

The myelinated regions of the axon prevent ion leakage as a result of high resistance to current flow (Dumitru, 1995). Conversely, at the nodes of Ranvier, a lack of myelin allows ion flow between the extracellular fluid and the axon. It is only at these nodes that nerve action potentials can arise by “saltatory

conduction", where electrical current stimulates each node consecutively along the nerve fibre and therefore increasing the efficiency and speed of nerve conduction.

When depolarization reaches the presynaptic terminal,  $\text{Ca}^{2+}$  ions subsequently enter the axoplasm in the presynaptic region thus increasing the intracellular level of  $\text{Ca}^{2+}$  (Sternberg, 1997). Synaptic vesicles then fuse with active zones of the axon membrane (Keeseey, 1989) and acetylcholine (Ach) contained within these vesicles (quanta) is simultaneously released into the synaptic cleft at the neuromuscular junction (Figure 1). This terminates the "message" to the target.

## **Normal Skeletal Muscle**

### **Normal Muscle Fibre Ultrastructure**

Mammalian skeletal muscle fibres are designed to convert mechanical force into chemical energy and developed along their long axis. Fibres in the long muscles (ie. strap muscles) usually run from one end of the muscle to the other end. Each cylindrical muscle fibre is comprised of several hundred muscle precursors (myoblasts) with nuclei typically located just below the sarcolemma along with mitochondria and glycogen granules (McComas, 1996). Each muscle fibre is comprised of a bundle of myofibrils, mitochondria and the sarcoplasmic reticulum which regulates  $\text{Ca}^{2+}$  levels within the fibre (Guyton, 1987). Each myofibril is distributed within the sarcotubular system comprised of transverse tubules (T-tubules) and sarcoplasmic reticulum (Dumitru, 1995). Transverse tubules form the transversely-arranged connecting complex within the muscle fibre and contiguous with the plasmalemma. The terminal cisternae of the sarcoplasmic reticulum lie on either side of the T-tubule. The "triad" of the reticulum is comprised of two terminal cisternae and the centralized T-tubule (Dumitru, 1995). Longitudinal elements of the sarcoplasmic reticulum connect consecutive terminal cisternae.

Surrounding each muscle fibre is a layer of connective tissue comprised of collagen. The epimysium is the layer which covers the entire muscle surface. The perimysium separates the muscle fibres into bundles (fascicles) and provides a vascular and neural pathway. Beneath the perimysium lie more delicate collagen fibrils, some of which are joined to the endomysium. Arterioles and venules are located in these areas along

with intramuscular nerve branches. The endomysium surrounds each muscle fibre and consists of small diameter collagen fibrils which join to the basement membrane (BM). This membrane comprises a glycoprotein layer (containing extracellular, transmembrane and intracellular membrane proteins) located external to the plasmalemma where Ach receptors are located (McComas, 1996). The basement membrane is crucial to neuromuscular integrity. Through its association with extracellular matrix proteins, a glycoprotein complex and dystrophin which is attached to the contractile components (i.e. actin), the BM is a crucial component in muscle fibre stability during contraction since it is linked via dystrophin to the cytoskeleton. Thus, changes in the composition of the muscle fibre basement membrane affect this stability (Sunada and Campbell 1995). The BM also functions to direct the formation of components located pre- and post-synaptically including agrin receptors (Carbonetto and Lindenbaum, 1995), acetylcholine receptors (Brenner et al, 1992) and acetylcholinesterase (AChE) (Anglister and McMahan 1985; Anglister et al 1994). The BM also acts as a diffusion barrier to macromolecules which may include toxins and antibodies that disrupt neuromuscular transmission (Oldfors and Fardeau 1983) storing factors which influence the growth and differentiation of regenerating axons at the nerve terminal (Sanes et al 1978). The BM acts as a scaffold for regenerating muscle cells after injury (Marshall et al 1977).

In the normal or "steady state", during which the axon is in contact with the muscle, neurotrophic factors produced by the muscle are transported in a retrograde fashion by the axon to maintain the neuron (Varon and Adler, 1980). Muscle-derived neurotrophins include BDNF, NT-3, and NT-4 (Chao MV 1992). Muscle fibres can also produce other polypeptides such as fibroblast growth factor-5 (FGF-5), leukaemia-inhibiting factor, insulin-like growth factor (IGF), and growth-promoting factors (McComas, 1996).

### Contraction of the Muscle Fibre

Each myofibril is comprised of a repeating structural unit of contraction, the "sarcomere". The light and dark banding appearance of the muscle fibres is due to the interdigitation of thick myosin and thin actin filaments (Dumitru, 1995). The sarcomere is comprised of thick filaments of the two dark "A bands" of myosin, and a small portion of actin comprising the region between successive Z lines (Buller and Buller,

1980), and thin actin filaments of the light "I band" which arise from a Z line at one end and extend between myosin filaments. The light H zone represents an area where actin filaments do not approximate when the muscle is under slight tension (Dumitru, 1995).

The actin filament consists mainly of actin. Actin contains a molecule of adenosine diphosphate (ADP) which is wrapped by tropomyosin which in the resting state, covers active sites on actin inhibiting its interaction with myosin. Actin binds the troponin complex consisting of 3 proteins: troponin I (Tn I) binds to actin, troponin T (Tn T) is attached to tropomyosin, and troponin C (Tn C) binds  $\text{Ca}^{2+}$  (Dumitru, 1995).

Acetylcholine released from the motor axon terminal binds to postsynaptic acetylcholine receptors (AChR) on the plasmalemma. The increase in  $\text{Na}^+$  and  $\text{K}^+$  conductance of the plasmalemma initiates an influx of  $\text{Na}^+$  ions accompanied by a depolarizing potential. This depolarizing potential represents the motor endplate potential (MEPP) (Sternberg, 1997).

The MEPP is propagated along the plasmalemma in both directions away from the endplate. The action potential propagates down the T-tubules and opens two types of  $\text{Ca}^{2+}$  channels to release  $\text{Ca}^{2+}$  from the sarcoplasmic reticulum. The dihydropyridine (DHP) channel, is a long-lasting  $\text{Ca}^{2+}$  channel, within the membrane of the T-tubules. The DHP channel is a voltage sensitive channel that transmits a signal to the second type of  $\text{Ca}^{2+}$  channel within the membrane of the sarcoplasmic reticulum. This other  $\text{Ca}^{2+}$  channel is the ryanodine receptor (RyR) protein. The top of the RyR channel attaches to the DHP channel and the change in conformation of the DHP channel due to a voltage signal in the T-tubule, triggers a conformation change on the top of the RyR channel allowing  $\text{Ca}^{2+}$  to be released from the sarcoplasmic reticulum (McComas, 1996). Calsequestrin contained within the terminal cisternae, has a high capacity but low affinity for  $\text{Ca}^{2+}$  ions and is tethered to the inner part of this structure (Dumitru, 1995).

Initiation of the actin-myosin "crossbridging" and muscle contraction results from the increased concentration of  $\text{Ca}^{2+}$  bathing the myofibrils. Calcium ions released from the sarcoplasmic reticulum activates the "tropomyosin-troponin complex". Two  $\text{Ca}^{2+}$  ions bind to each troponin C molecule laterally displacing tropomyosin and changing its conformation. This uncovers the ADP-active site on actin allowing the myosin heads bind the actin ADP-binding site forming a crossbridge (Dumitru, 1995). Upon binding,

the myosin head tilts towards its “neck” and facilitates the “sliding” action of the actin along the myosin in a “power stroke” (Guyton, 1987).

The source of energy for the power stroke arises from the prior linking of ATP, which is cleaved by the ATPase of the myosin head. Adenosine diphosphate (ADP) and phosphate ( $P_i$ ) are released thus cocking the myosin head and allowing ADP to bind to myosin. When the power stroke is complete, the ADP and  $P_i$  are released permitting another ATP molecule to bind to another newly uncovered site on the myosin head. For each ATP hydrolyzed into ADP and  $P_i$ , two  $Ca^{2+}$  ions are pumped into the sarcoplasmic reticulum. If  $Ca^{2+}$  remains and contraction continues, the myosin filaments reach the Z line, shortening the sarcomere resulting in muscle contraction. The muscle shortens with each power stroke, so a contraction involves several power strokes per second.

To cease muscle contraction,  $Ca^{2+}$  must be actively transported from the cytosol back into the sarcoplasmic reticulum. ATP is required for this process. Two  $Ca^{2+}$  ions are transported back into the sarcoplasmic reticulum in exchange for two  $K^+$  ions. As a result, the cytosol adjacent to the sarcoplasmic reticulum becomes negative. Once inside the sarcoplasmic reticulum, the  $Ca^{2+}$  ions are detached from the ATPase pump and uptaken by  $Ca^{2+}$ -binding proteins, i.e. high-affinity ATPase enzyme and the low-affinity calsequestrin. Tropomyosin is restored to its original conformation thereby blocking active sites and inhibiting the interaction between actin and myosin, and muscle relaxation occurs.

### **Motor Unit**

The peripheral nerve communicates with muscles the discrete quanta defined by motor units. The motor unit is comprised of an alpha motoneuron (derived from anterior horn cell in spinal cord) and the group of muscle fibres it innervates. Each motoneuron branches and multiple axon terminals contact the muscle plasmalemma at the neuromuscular junction (motor endplate), the site where the nerve action potential is converted into a muscle action potential (Figure 1) (Dumitru, 1995). Following excitation and discharge of motor neurons in the spinal cord cell permeability to  $Na^+$  and  $K^+$  is altered such that depolarization occurs (Sternberg, 1997). The  $Na^+$  influx caused by the opening of voltage-gated  $Na^+$

channels resulting in the “all-or-none” action potential stimulates the distal propagation of the action potential toward the axon terminal. The ensuing  $\text{Ca}^{2+}$  release promotes the release of acetylcholine into the synaptic cleft. The Ach then binds to its post-synaptical receptors on the muscle fibre, triggering a muscle fibre action potential which ultimately results in fibre contraction.

### Properties of Motor Units (Fast Twitch, Slow Twitch)

Skeletal muscle fibres are classified into various types. “Fast glycolytic”(type IIB) are white, contract rapidly and fatigue easily whereas “fast oxidative” (type IIA) are red, contract slowly and fatigue less easily. “Slow oxidative” (type I) are red, resist fatigue and function to maintain posture (Guyton, 1987). In the rat, the type II fibres are subdivided into a type IIX class.

Slow fibres receive multiple innervation, where more than one axon or axon branch terminates along the fibre length (Buller and Buller, 1980) localizing the contractile response to the active endplate rendering a slow contraction. Conversely, fast fibres are innervated by a single axon. A nerve action potential arriving at the endplate causes contraction of the entire fibre from the generation of a “muscle action potential” along the plasmalemma.

Within a muscle, motor unit size and muscle fibre diameters differ (Buller and Buller, 1980). The size of the smallest motor unit dictates the minimum force generated. Muscle fibres may be distributed throughout the muscle rather than confined to a discrete region. There are also differences in size of axons innervating different muscle fibres. Typically, large diameter axons supply “fast contracting” (fast twitch) muscle fibres which exert a large force. An increase in muscle force requires either repetitive stimulation of the same motor unit, recruitment of other motor units, or both. Slow twitch motor units are comprised of smaller axon and muscle fibre diameter and fewer fibres relative to fast-twitch motor units (Buller and Buller, 1980).

Biochemical/physiologic (enzyme profile) and histochemical (i.e. myosin ATPase stain) properties of fast and slow-twitch fibres are shown in Table 1 . In slow twitch fibres, glycolytic (anaerobic) enzymes (i.e. pyruvate kinase and aldolase) do not provide the primary energy production, and therefore stain weakly in

these muscle fibres. By contrast, oxidative enzymes (i.e. malic dehydrogenase, isocitrate dehydrogenase) provide most of the energy and thus stain strongly (Romanul 1964). Due to a reliance on energy sources which require oxidation, slow twitch fibres also contain a high levels of myoglobin delivered by a high density of capillaries and lipid components (Dux et al 1981; McPherson and Tokunaga, 1967). Thus, high triglyceride levels have been reported (Wittenberg et al, 1970). On the other hand, since fast twitch fibres employ glycogen as the primary energy source, resting glycogen levels in these fibres have been reported to be 16% higher than in slow twitch fibres (Vollestad et al, 1984).

**Table 1 . Skeletal Muscle Fibre Type Characteristics**

Property	Slow Twitch (Type I)	Fast Twitch (Type IIa)	Fast Twitch (Type IIx) *
Color	Red	Red	Light
Myoglobin	High	High	Low
Capillary Supply	High	High	Low
Mitochondria Density	High	High	Low
Glycogen	Low	High	High
Basic ATPase	Low	High	High
Acidic ATPase	High	Low	Intermediate
Oxidative Enzymes	High	Medium to High	Low
Banding	Wide Z lines		Narrow Z lines
Diameter	Small		Large

(adapted from McComas, 1996 and Cris dos Remedios, 2000) \* identified in rat muscle

Among the several histochemical staining methods used to distinguish muscle fibre types, the preferred method is the myosin (or myofibrillar) adenosine triphosphatase (M-ATPase) stain (Engel, 1962; Karpati and Engel, 1968). This method considers fibre type differences in myosin as the basis for fibre typing. Fibres which strongly stain for myosin ATPase following preincubation at pH 10.0 are classified as fast twitch fibres in contrast to weakly staining slow twitch fibres (Table 2).



**Table 2 . M-ATPase Histochemical Assay to Identify Muscle Fibre Types in Acidic (pH 4.3) and Basic (pH 10.0) Conditions (identified by light microscopy)**

	pH 4.3	pH 10.0
Type I	Dark colour	Light colour
Type IIA	Light colour	Dark colour
Type IIX*	Light colour	Dark colour
Type IIC	Intermediate colour	Dark colour

\* identified in rat muscle

[Note: In normal skeletal muscle, overlapping fast- and slow-twitch motor units result in “mosaic” pattern (i.e. dark and light areas) (Karpati and Engel, 1968) ]

#### **Electrophysiological Measurement of the Normal Nerve to Muscle Interaction**

When the nerve action potential reaches the motor endplate and stimulates all the fibres in the motor unit in an “all-or-none” fashion, the result is a motor unit action potential (MUAP). The synchronous summated contraction of all of the affected MUAPs results in a compound muscle action potential (CMAP) (Dumitru, 1995). Muscle contraction results from the consecutive recruitment of multiple motor units according to Henneman’s size principle (Henneman, 1957). The smallest sized motor units are recruited initially, while the largest motor units are recruited when maximum tension is required.

The configuration of a motor unit potential is dependent upon the anatomic characteristics of the motor unit including spatial and temporal relationships of individual muscle fibre potentials (Buchthal, 1960; Buchthal et al, 1954; Buchthal et al, 1957a ; Buchthal et al, 1957b ) and the number of muscle fibres innervated by the neuron (innervation ratio), the quantity of muscle fibres per cross-sectional area (fibre density), and the integrity of neuromuscular transmission (Kimura, 1983).

A motor unit potential, measured electromyographically, is described by 4 main components: amplitude, time to peak (rise time), duration, and number of phases (Daube, 1978). Amplitude is dependent on the number of axons stimulated. Time to peak is an indicator of the distance between the recording tip of

the electrode and the discharging muscle fibres, with a shorter time to peak indicating a closer proximity of the motor unit. The duration of a motor unit potential is a measure from the initial take-off of the negative peak until its return to baseline and is an indicator of the synchrony of the muscle fibre action potentials (Kimura, 1983).

Synchrony is influenced by the variation in distance between axon branch point and the endplate and thus the variable conduction velocity. The degree of synchrony also influences the number of phases comprising a motor unit potential. Normal motor units can be polyphasic being comprised of a maximum of 4 phases which indicates that the positive and negative peaks cross the baseline 3 times (i.e. number of baseline crossings plus 1) but most are monophasic.

An important functional difference between fast- and slow-twitch motor units is the duration and rate of onset of muscle contraction (Table 3).

**Table 3. Electrophysiologic Characteristics of Normal Skeletal Muscle**

Property	Slow Twitch (Type I)	Fast Twitch (Type IIa)	Fast Twitch (Type IIx) *
Twitch Time to Peak	Slow	Fast	Fast
Twitch Force	Small	Intermediate	Large
Fatigability	Low	Low	High

(adapted from McComas, 1996)

\* identified in rat muscle

### **Isometric Contractile Muscle Force**

Measurement of muscle contraction in whole muscle involves examining the contractile unit (sarcomere). Series elastic components (SES) such as tendons are arranged in series with the contractile component (Buller and Buller, 1980). Isometric muscle recording apparatus maintains the muscle at a constant resting length ( $L_0$ ) during contraction. Contraction force can be measured by a “force transducer” which converts the force to a proportional electrical signal for display.

The magnitude of the twitch response is influenced by several factors. The stimulus intensity, if weak, activates a subsample of the excitable motor axons, resulting in a small twitch contraction reflecting the stimulation of muscle fibres innervated by this subsample. Increasing the stimulus intensity activates a larger population of axons, until at supramaximal stimulation, all axons are recruited and no further increase in twitch contraction is achieved. The repetition rate of the single stimuli also influences the magnitude of the twitch response. To maintain the twitch amplitude up to a point, the nerve must be stimulated at regular and long intervals. Another factor is muscle temperature which, for an optimum twitch response, should be maintained between 36.7 ° C and 37.4° C (Buller and Buller, 1980). Finally, the magnitude of the twitch contraction linearly increases as a function of the resting muscle length ( $L_0$ ) (Buller and Buller, 1980).

### Properties of Muscle Contraction

The rate of isometric twitch contraction is characterized by its time to peak (TTP), defined as the time from contraction onset until the peak of maximum tension (Alway, 1985). Half relaxation time ( $\frac{1}{2}$  RT) is the time from the peak of maximum tension until the time at which force has decreased by one half. The TTP and  $\frac{1}{2}$  RT are shorter for fast-twitch muscle relative to slow-twitch muscle.

Tetanic contraction results from supramaximal stimulation of motor axons in rapid succession leading to a summation of the mechanical responses of the muscle before it resumes its resting state (Buller and Buller, 1980). Individual summing contractions can be distinguished when the stimulus frequency is low and tetani are unfused at a frequency of 10 Hz. By increasing the frequency to 80 Hz, individual contractions are less distinct but still unfused. In humans, however, fusion would occur at this frequency. At a stimulus frequency of 125 Hz, a smooth rise of tension is obtained and “fused tetanus” or “tetanic” contractions ensue. By increasing the stimulus frequency to fusion frequency, there is a progressive increase in the maximum force (force-frequency). If frequencies above fusion frequency are applied, no further increase in force is generated, but the rate of force development increases until a “maximum tetanic force” ( $P_0$ ) is achieved. In slow-twitch muscle, the rate of  $P_0$  development is reduced relative to fast-twitch muscle.

### **Motor Nerve Injury, Muscle Denervation and Regeneration (Histological Features)**

Peripheral nerve injury stimulates a symphony of changes from the central nervous system distally toward target organs.

#### **Nerve (Proximal Stump)**

Peripheral nerve injury results in morphologic and biochemical alterations in the nerve-cell body and its axon (Fu and Gordon, 1997). The neuron itself switches from “transmitting mode” to “growth mode” by increasing the synthesis of growth-associated proteins such as (GAP-43) (Miller et al 1989) and cytoskeletal proteins such as tubulin and actin (Tetzlaff et al 1988). Neurofilament proteins are downregulated (Tetzlaff et al 1988). Although several neurotransmitters are downregulated in neurons, several neuropeptides such as ciliary growth-related protein (CGRP) are upregulated (Haas et al 1990). These factors may also act as mitogens on glial cells (Brenneman et al, 1987). Injured axons, macrophages, platelets, and Schwann cells release mitogens such as fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and glial growth factor (GGF) (Reynolds and Woolf 1993). Cytokines, including the interleukins (IL-1, IL-2, IL-6), transforming growth factor (TGF- $\beta$ ) (Murphy et al 1995), and interferon- $\gamma$  (IFN- $\gamma$ ) (Olsson et al 1994), are also secreted by axotomized neurons and are mitogenic for Schwann cells.

Axons sprout from the proximal nerve stump and several neurotrophic factors are upregulated in Schwann cells and other glial cells, as well as in axotomized neurons (Thoenen et al, 1988). The motor neurons upregulate nerve growth factor (NGF) (Ernfors et al 1989), brain-derived neurotrophic factor (BDNF) and neurotrophin (NT)-3 (Kobayashi et al 1996), acidic fibroblast growth factor (FGFa) (Elde et al 1991), PDGF (Yeh et al 1991), GGF (Marchionni et al 1993), a cytokine, CNTF and the TrkB (Friedman et al 1995) and p75 (Verge 1992) receptors. The neurotrophic factors (and their receptors) include neurite-promoting factors to promote growth cone formation (Baron Van Evercooren et al, 1982) and neurite-outgrowth promoting factors which provide a substrate to which axons can adhere (including laminin and fibronectin in the extracellular matrix and basal laminae of Schwann cells). Schwann cell-derived factors which are upregulated include “recognition molecules” such as the cell-adhesion molecules (CAMs),

notably, neural cell adhesion molecule (N-CAM) and L1 (Cifuentes-Diaz et al 1994) which mediate the axon-to-glia cell interactions, myelin-associated glycoprotein (MAG), and lipid carrier proteins (Schachner, 1992).

The proximal nerve stump axons degenerate due to an influx of  $\text{Ca}^{2+}$ -associated proteases and swelling occurs as a result of the impaired transport of cellular components. A “growth cone” forms and axons regenerate from the first node of Ranvier proximal to the injury site and branch distally (Snyder et al 1988).

### **Nerve (Distal Stump)**

In the distal nerve stump, a sequence of events described as “Wallerian degeneration” occurs (Waller, 1850). This process involves the neuronal reactions, Schwann cell responses and macrophage recruitment and interactions with cellular components of the distal nerve stump (Griffin and Hoffman, 1993). Axon disruption stimulates an increase in vascular permeability (Olsson, 1966), Schwann cell dedifferentiation and proliferation (due to mitogenic TGF- $\beta$  released by macrophages) and upregulation of type IV collagen by Schwann cells and protrusion of cytoplasmic processes into the synaptic cleft (Thomas, 1966), macrophage infiltration of the region and phagocytosis of myelin debris by these cells and Schwann cells (Olsson and Sjostrand, 1969). Macrophages also synthesize interleukin-1 (IL-1) which upregulates neurotrophic factor synthesis by other cells (Lindholm et al, 1987). The neurotrophins, NGF, BDNF, NT-4, insulin-like growth factor (IGF)-1 and IGF-II are upregulated in the distal nerve stump after axon disruption (Heumann et al, 1987). NT-3 levels are unaffected by axonal injury.

Schwann cell differentiation is influenced by the relationship with the axon (Chiu, 1991). In one study, nerve injury resulted in apoptosis of the terminal (ie. neuromuscular junction) Schwann cells as well as those located in advance of the motor endplate (Trachtenberg and Thompson, 1996). These findings were attributed to the loss of interaction between Schwann cells and their axons following injury. Furthermore, when Schwann cells switch from “myelinating” to “growth” mode during axon degeneration, they downregulate genes encoding myelin-associated glycoproteins (MAG, PNP22) (DeLeon et al 1991) concurrent with the upregulation of L1 and N-CAM (Martini and Schachner 1988).

Schwann cells express the NGF receptor, TrkA, on their surface (Raivich and Kreutberg, 1987) to stimulate axon sprouting (Son and Thompson, 1995a). In contrast, the upregulation of the p75 receptor, which binds brain-derived neurotrophic factor (BDNF), was found to inhibit axonal regeneration (Funakoshi et al, 1993). Cell adhesion molecules, N-CAM, L1, and N-cadherin are upregulated in Schwann cells of the distal stump after injury of the mouse sciatic nerve (Lagenaur and Lemmon, 1987), providing a pathway for regenerating axons to reach their target. L1 and N-CAM have a role in facilitating interactions between the growth cone of regenerating axons and the columns of nonmyelinating Schwann cells (bands of Bungner) in the endoneurial tube of the distal nerve stump (Lundborg et al, 1994). Although GDNF is upregulated after axonal injury, Hoke et al (2002) found that chronically denervated Schwann cells failed to upregulate glial cell-derived neurotrophic factor (GDNF), an important motor neuron neurotrophic factor, which led to impaired ability of the Schwann cells to supply trophins to motor and sensory axons, impairing axon regeneration. Upregulation of components of the basement membrane (laminin, fibronectin, proteoglycans, and collagens) also occurs in Schwann cells upon axotomy (Bunge and Bunge 1983).

The axons swell and proteolysis occurs in which axoplasm and axolemma is degraded, and axonal segmentation precedes myelin retraction at the nodes of Ranvier with myelin lamellae disorganization and myelin sheath degradation. Ovoid bodies emanate from myelin striations. Further disruption of other organelles such as mitochondria, lysosomes, and axon microtubules, endoplasmic reticulum and neurofilaments occurs with further constriction of axons.

A member of the "cytokine" subfamily of neurotrophic factors, CNTF, is downregulated in the distal nerve stump within 1 day following injury (Seniuk et al 1992). By contrast, LIF and IL-6 are upregulated (Reichert et al 1996). The expression of FGF decreases in the distal nerve stump (Ishikawa et al 1992). CNTF, along with NGF, BDNF and their associated receptors are thought to influence the distal motor axon and reinnervation of denervated muscle (Funakoshi et al, 1993). CNTF has been shown to reduce muscle atrophy (Helgren et al 1994). *In vivo* studies demonstrated that CNTF sustains motor neurons in addition to inducing motor axon sprouting (Houenou et al, 1991). NGF is expressed in nerve and muscle, with increased expression following denervation (Amano et al, 1991).

These morphological and molecular processes which occur in the proximal and distal nerve segments following denervation are crucial to the removal of cellular debris and preparation of a more favourable substrate for axonal regeneration.

### Skeletal Muscle

Features of skeletal muscle denervation include decreased fibre cross-sectional area (atrophy), a decrease in fibre number and a resulting decrease in the size of the existing motor units, fibre type grouping and an increase in fibrosis in the endomysium with larger interfibrillar spaces in which fat and inflammatory cells collect (Sunderland 1991). Diminution of motor endplates and an alteration in the innervation ratio occurs due to an increase in the number of immature endplates. An inflammatory response to degenerating nerve tissue results in fibrosis also occurs in the perimysium (Tower, 1935; Fu and Gordon, 1995 a, b; Irintchev et al, 1990; Terzis and Smith, 1990; Schmalbrach et al, 1991; Jones ). Mitochondrial number decreases and myonuclei enlarge, change to a rounded shape and align centrally forming a chain-like appearance. Eventually, fibre necrosis occurs and the normal polygonal shape is replaced by small angular fibres, a hallmark of denervation (Bain et al, 2001).

Davis (1983) found that rat muscle that was denervated for 14 days and injected with sciatic nerve extract exhibited significantly less atrophy determined by muscle fibre cross-sectional area compared with uninjected control muscle. This finding was attributed to proteins (neurotrophins) within the peripheral nerve extract. Expression of neurotrophic factor mRNAs is differentially regulated in muscle following denervation injury (Funakoshi et al, 1993). Funakoshi et al demonstrated levels of BDNF mRNA were elevated, NT-4 mRNAs decreased and NT-3 mRNA was unchanged in denervated rat gastrocnemius muscle. The source of neurotrophin however, is controversial since Griesbeck et al (1995) later reported that the increase in BDNF mRNA was due to the expression of this factor in Schwann cells rather than muscle. Following muscle denervation, CNTF receptor  $\alpha$  expression in skeletal muscle was reported to increase (Helgren et al 1994).

Prolonged skeletal muscle denervation is linked to loss of viable muscle fibres and reduction in the satellite cell population, impairing muscle fibre regeneration. Helgren et al (1994) reported that the motor axon supplies trophic factors which sustain viability of muscle cells and their receptiveness to reinnervation. Muscle fibres after prolonged denervation are less receptive to regenerating motor axons due to loss of muscle fibres and the satellite cell pool exhaustion, with subsequent fibrosis (Irintchev et al, 1990).

A negative relationship exists between the time to reinnervation and the muscle recovery potential, which varies according to the quantity and quality of motor reinnervation (Karpati and Engel, 1968; Fu and Gordon, 1995a, b). Short-term denervation (i.e. less than 2 months) does not affect myofibre quantity or cross-sectional fibre area (Finkelstein et al, 1993), however myofibre type grouping, motor unit size and innervation ratio (Close, 1969) are affected. This short time delay of nerve regeneration results in a greater likelihood of successful muscle reinnervation Sunderland (1947).

More serious irreversible muscle characteristics are observed when reinnervation is delayed for more than 2 months (Irintchev et al, 1990; Terzis and Smith, 1990; Schmalbrach et al 1991; Fu and Gordon, 1995a, b). Fu and Gordon (1995b) reported that a time delay of up to 6 months resulted in the highest loss of muscle fibre content and reduction of fibre cross-sectional area. When reinnervated, the muscle exhibited a pronounced reduction in force capability. A delay exceeding 6 months is associated with greater muscle atrophy and a gradual decline in function (Fu and Gordon, 1995b; Gutmann and Young, 1944; Gordon and Fu, 1997). Despite showing evidence of regeneration at the microscopic level, these chronically denervated muscles showed serious degenerative characteristics of fibre necrosis and connective tissue hyperplasia (Fu and Gordon, 1995a, b; Irintchev et al, 1990; Schmalbrach et al, 1991; Terzis and Smith, 1990; Tower, 1935).

Effective nerve regeneration and muscle reinnervation rely on various factors. These include the survival of a sufficient number of neurons, appropriate target innervation, maintenance of the distal nerve stump and the ability of target fibres to accept reinnervation. Of equal importance is the maintenance of the satellite cell pool population leading to muscle regeneration, however, this is beyond the scope of this paper and can be reviewed in more detail in (Moss and Leblond, 1971). The impairment of muscle functional recovery following denervation may be due to any one or combination of these factors.



Irreversible changes in denervated muscle upon peripheral nerve injury are thought to arise from collagenization of endoneurial sheaths in the distal nerve stump (Morris et al, 1972) and fragmentation of the interface between the Schwann cells and their associated basal lamina, due to a lack of axons within the tubes (Fu and Gordon, 1995 a,b). Deterioration of the endoneurial sheaths provides poor growth substrate for regenerating axons and they fail to elongate toward appropriate target muscle fibres. Due to the collapse of the endoneurial sheaths, axon regeneration may proceed outside the endoneurial sheath and this has been purported to be less effective at maintaining trophic support, with fewer cell adhesion molecules and poorer regeneration in comparison to regeneration within endoneurial sheaths (Fu and Gordon, 1995 a,b; Holmes and Young, 1942).

Recent studies have reported the molecular changes which occur upon muscle denervation. Suarez et al reported that neuregulins (which modify the expression of Ach receptor genes and stimulate endplate development) regulate the uptake of glucose. These factors are also involved in alterations in glucose transport which occur following muscle denervation. Fischer et al (1999) reported that, following chronic denervation, an increase in the expression of the alpha 7 nicotinic acetylcholine receptor may indicate a possible role as a target for regenerating axons and in endplate integrity. Chronic denervation was also reported to result in a release of acetylcholinesterase from the muscle (Cater and Brimijoin 1981).

### **Peripheral Nerve Regeneration**

Axonal regeneration is defined by nodal collateral sprouting originating from nodes of Ranvier proximal to the nerve injury site (Hopkins et al, 1981; Hopkins and Slack, 1981; Cajal, 1968; Jacobsen and Guth, 1965), pre-synaptic collateral sprouts arising from more distal regions approaching the endplate, and terminal collateral sprouting occurring at the terminal endplate facilitating renewal or enlargement of existing neuromuscular connections.

Schwann cells play an important role in the success of regeneration. Hall (1986) report that proximal stump-derived axons with accompanying Schwann cells regenerate into an acellular nerve graft. Schwann cells provide neurotrophins which guide regenerating axon sprouts (Son and Thompson, 1995b; Nieke and

Schachner, 1985). Schwann cells hypertrophy and upregulate myelin proteins for remyelination to occur (Nitz and Matulionis, 1982). Regenerating axons from the proximal nerve stump must traverse scar tissue across the gap between the proximal and distal stumps under the guidance of Schwann cell bands of Bungner and ultimately elongate along the endoneurial tubes of the distal stump (Wyrick and Stern, 1992). Only those axons that make the correct connection to their target muscle survive, whereby others making inappropriate connections are pruned (Brushart and Seiler, 1987).

Regenerated axons from the proximal stump which reach the muscle fibre become established at the original endplate. In adult rats, nerve regeneration occurs at a rate of 0.3 mm/day immediately after nerve injury, and by 18 days, the rate reaches 3.0 mm/day (Dolene and Janko, 1976). Regenerating axons undergo collateral sprouting of surviving parent axons in which 5 or more daughter axons sprout and reinnervate denervated muscle fibres to expand the motor unit size and compensate for loss of fibres in existing motor units. Collateral sprouting is induced by the denervated muscle fibres which stimulate nearby intact motor axons to sprout. The denervated muscle fibre is able to receive an axon sprout and form a new endplate. In the absence of the original endplate, a new endplate will form in another area of the muscle fibre (McComas, 1996).

Upon reinnervation of partially denervated muscle, adjacent muscle fibres reinnervated by the same axon from which collateral sprouts have emerged, form a motor unit comprised of fibres of similar biochemical properties (i.e. M-ATPase activity). This "fibre type grouping" is illustrated at the light microscopic level by the clusters of dark and light fibres in enzyme-stained muscle cross-sections in contrast to the "mosaic" appearance of normal muscle (Cormack, 1987). Collateral sprouting serves to reinnervate muscle fibres thereby increasing the motor unit size (Dumitru, 1995).

The role of target-derived trophic factors has been implicated in regeneration and maintenance of nerve regeneration. Several studies report that skeletal muscle denervation stimulates neurite outgrowth and neuronal survival-promoting responses in extracts of denervated muscle (Henderson et al, 1983; Houenou et al, 1991).

## **Peripheral Motor Nerve Injury, Skeletal Muscle Denervation and Regeneration**

### **Electrophysiologic Correlates**

The cessation of neuronal-derived neurotrophin supply to the muscle fibres as well as electrical inactivity results in muscle “denervation” changes. Both factors are known to contribute since noticeable abnormality of the muscle fibres persists despite direct stimulation of the muscle (McComas, 1996).

Despite the increase in muscle fibre innervation resulting from collateral sprouting, immature motor endplates are not sufficient to generate an action potential, thus there is a decrease in compound muscle action potential (CMAP) amplitude. A decrease in CMAP amplitude is also attributed to a decrease in mean axon diameter and fewer axons.

Thin myelin sheaths, and shorter myelinated segments with more numerous and tight and irregularly-arranged nodes of Ranvier in regenerating axons render the axon inefficient at nerve conduction (Dumitru, 1995).

Clustering of synaptic vesicles at the endplate results in spontaneous discharge of motor end-plate potentials (MEPPs) with 8 hours of denervation, and electrical stimulation of the axon results in normal neuromuscular transmission. Synaptic vesicles maintain normal quanta of Ach and its release proceeds unimpeded. After 8 hours however, MEPPs cease and, upon axon stimulation, there is no muscle response due to a disruption of the endplate.

Following reinnervation, newly-formed immature motor endplates inefficiently respond to neural stimulation, release insufficient amounts of acetylcholine, and/or postsynaptic acetylcholine receptors respond inappropriately. The result is increased “jitter” and blocking (Hakelius and Stalberg, 1974). This is due to variability in subsequent discharges of the time interval between firing of action potentials between two muscle fibres within the same motor unit. The site of branching of collateral sprouts may not conduct an impulse upon motor unit stimulation at high frequencies (Dumitru, 1995), thus, if conduction fails at a neural branch point, action potentials in distal muscle fibres are blocked. During single-fibre EMG in which the axon is stimulated, if a group of muscle fibres is blocked simultaneously (neurogenic blocking), block of

neural transmission at a proximal axon branch point (node of Ranvier) is confirmed in contrast to more distal endplate block failure (neuromuscular junction block).

Upon denervation, the muscle fibre plasmalemma becomes highly sensitive to acetylcholine (Ach), and membrane instability results from spontaneous activity of partially denervated fibres. This is due to the topographic irregularity of the plasmalemma. Denervation is associated with the impairment of the  $\text{Na}^+ - \text{K}^+$  pump resulting in an altered permeability of the muscle fibre membrane to  $\text{Na}^+$ ,  $\text{K}^+$ , and  $\text{Cl}^-$  (Bray et al, 1976) which affects the muscle fibre action potential. Fibrillation potentials result from the subthreshold stimulation by the axon and an increase in Ach content associated with a decrease in acetylcholinesterase concentration.

Polyphasic (serrated) potentials occur as a result of random dropout of muscle fibres as well as altered nerve conduction velocities of multiple fibres which have been reinnervated by collateral axon sprouts, leading to increased temporal dispersion and asynchronous firing of the fibres. A decrease in CMAP amplitude and duration is also a reflection of the loss of muscle fibres from motor units and asynchrony of firing of fibres, leaving gaps in the summated voltage appearance of the waveform (Dumitru, 1995).

Positive sharp waves represent spontaneous irregularly-occurring depolarizations of single fibre potentials from an injured area of the muscle fibre. Action potentials are propagated but their magnitude is insufficient to generate a full action potential. Insertional activity increases due to increase sensitivity of injured muscle fibres to further disturbance of needle insertion. Fasciculations arise from spontaneous twitches of motor units which have lost a proportion of the myofibrils.

Hyperinnervation is a feature of reinnervated muscle fibres. Regenerated axons eventually return to the original endplate. If this occurs in the presence of an already established axon junction from a collateral sprout, the muscle fibre is innervated by two axons (Frank et al, 1974), resulting in an increase in CMAP amplitude due to the summation of action potentials arising from each axon. This polysynaptic reinnervation, however, is temporary.

Unlike normal muscle in which small motor units are recruited first (Henneman, 1957) followed by larger ones, in reinnervated muscle, motor unit recruitment pattern may abnormally start with large motor

units (Milner-Brown et al, 1974). The motor unit action potential (MUAP) may therefore initially appear large as well as an initially large twitch force and increased rate of twitch. Needle electromyography would reflect this abnormal recruitment of motor units (Dumitru, 1995).

In denervated muscle, loss of myofibrils and atrophy impairs adequate isometric contractile force generation. The maximum rate of rise of tetanic tension increases, and twitch rate is decreased due to the reduced NCV attributed to a prolonged duration of activity following a single stimulus (Winter, 1979). Atrophied muscle fibres exhibit an increased duration of muscle fibre action potentials due to the large ratio of sarcoplasmic reticulum to myofibril mass thus  $Ca^{2+}$  remains for a longer period of time.

#### **Literature Review of Modalities to Reduce Denervation Atrophy**

Various techniques have been employed to prevent denervation atrophy. These include, exercise as a means of directly stimulating the muscle (Hie et al, 1982), chronic electrical stimulation of muscle (Schmalbrach et al, 1991) or short-term muscle stimulation (Al-Majed et al, 2000), as well as growth factor administration (Helgren et al, 1994). Others (Lohof et al, 1993; Yan and Miller, 1993) report that survival of motor neurons, axon sprouting, and formation of neuromuscular junctions are improved with exogenous application of neurotrophic factors such as pan-neurotrophin-1 (PNT-1) which incorporates the active sites of NGF, BDNF and NT-3 and binds and activates all neurotrophin receptors (Funakoshi et al, 1998).

Microsurgical reconstructions of the nerve with nerve grafts, foreign nerve transfers (motor and sensory donors) also offer viable treatments for denervation atrophy (Mackinnon et al, 1988). A major drawback is that the donor nerve(s) must be sacrificed. Although these efforts at early reinnervation have resulted in preservation of muscle fibre size and number, they have been less effective at preserving motor unit number (Dautel et al, 1992; Fu and Gordon, 1995a, b). This may be attributed to axon deterioration during Wallerian degeneration (Waller, 1850) and a distance-from-injury-related time delay in axon regeneration into the motor endplates (Dautel et al, 1992; Karpati et al, 1981; Weiss and Edds, 1945).

Terzis (1990) pioneered the surgical method of “motor nerve babysitting” on patients with facial muscle paralysis by combining cross-facial nerve grafting and partial hypoglossal nerve transfer. In addition

to the aesthetically pleasing outcome of facial symmetry, its most notable feature is that the ipsilateral hypoglossal nerve preserved the bulk of the facial muscles without sacrificing tongue innervation.

In 1994, a rat model for facial paralysis and blink reflex test was established (Terrell and Terzis, 1994). Using a saphenous nerve graft to bridge a section of the hypoglossal nerve to the main zygomatic branch of the facial nerve supplying the orbicularis oculi muscle (OOM), Mersa et al (2000) established that 40% partial neurectomy of the hypoglossal nerve resulted in sufficient restoration of OOM function even after prolonged denervation, while still maintaining some degree of tongue bulk.

Using a rat model (Frank et al, 1974), the superficial fibular nerve was the foreign nerve which was cut and neurotized to the soleus muscle eliciting hyperinnervation. This foreign nerve was found to have formed synaptic junctions on soleus muscle fibres upon denervation of the muscle by cutting the tibial nerve. Soleus twitch tension produced by the foreign nerve in all 8 rats studied was increased or equal to earlier measurements. Histological findings showed that 92% of muscle fibres were innervated by the foreign nerve.

Direct implantation of a motor nerve into denervated muscle has been investigated by Brunelli in the early 1970's and reported in *The Journal of Hand Surgery* in 1982 (Brunelli, 1982) in 21 rabbits where tibial nerve was excised and peroneal nerve directly neurotized to the denervated gastrocnemius muscle. In subsequent studies, motor neurotization was performed in 40 rats in which the distal end of the peroneal nerve was unravelled into several fasciculi and neurotized to increase contact area (Brunelli, 1982). In both series of experiments, motor endplates were formed near the nerve implant sites and muscle contraction at 1 month followup was elicited after electrical stimulation of the peroneal nerve.

Based on earlier animal studies, Brunelli and Monini (1985) performed a clinical study in which 28 patients with tibial nerve transection injury underwent muscle neurotization in which one end of the motor nerve graft was sutured to the proximal tibial nerve stump and the distal end of the graft was split into several fasciculi and implanted into denervated muscle. In 15 of 28 patients followed up to a maximum of 9 years, the motor function score was M4 or greater (based on the Medical Research Council scale of 0 to 5 where a score of 0=no motor function and a score of 5=optimum motor function).

Chiu et al (1991) demonstrated that neurotization of common peroneal nerve (CPN) to muscle resulted in minimal difference in electrophysiological findings and histology compared to CPN neurotization to the transected tibial nerve stump, suggesting that the type of motor nerve innervation was equally effective at preservation of muscle electrophysiology.

Three types of muscle neurotization techniques were also performed in 40 rabbits which underwent lateral gastrocnemius denervation (Askar et al, 2001). One group of animals underwent neurotization with peroneal nerve upon tibial nerve transection. In another group, dual motor innervation was established by neurotizing peroneal with tibial nerve re-anastomosis. In the third group, hyperneurotization was performed where fascicles of peroneal nerve were neurotized while maintaining an intact tibial nerve. Function was recovered in all neurotization groups, however, in groups with tibial nerve transection, muscle weight was reduced compared to groups with intact tibial nerve.

In rat sternomastoid muscle denervated between 1 and 5 months, foreign reinnervation by motor, sensory or autonomic neurons was examined (Zalewski, 1970). This author reported that only axons from the original motor sternomastoid and hypoglossal (motor) nerves resulted in functional reinnervation. Conversely, muscle reinnervated by sensory or sympathetic neurons histologically resembled chronically denervated muscle. It was concluded that neither sensory nor autonomic axons bestowed a neurotrophic effect on muscle. However, a significant flaw in this study is that muscle contractile function was assessed using subjective measures rather than standardized nerve conduction studies of CMAP amplitude, latency, nerve conduction velocity. At the short-term followup (5 months), axon quantity may not have sufficiently reached threshold to elicit a subjective visually recordable response. However a sufficient number of axons may have elicited a more sensitive objective electromyographic response. They did not provide motor reinnervation after a period of foreign innervation which may have restored motor function.

Nomoto (1989) identified that newly-formed endplates at sites of previously denervated endplates in cat intrinsic laryngeal muscles, arising from axon sprouting from intact autonomic nerves surrounding laryngeal muscle vasculature. These intact axons possibly entered the Schwann tubes to elongate and establish new motor endplates. This coincided with spontaneous electrical discharge which occurred in 6

of 8 cases. In a later study, they demonstrated fibrillation-like activities within 5 weeks of nerve transection using a similar model (Nomoto et al, 1991).

#### **Literature Review of Modalities to Reduce Denervation Atrophy (Sensory Protection)**

Since the 1940's, investigators have performed sensory-motor nerve repair to provide muscle reinnervation (Close, 1969; Dautel et al, 1992; Karpati et al, 1981; Weiss and Edds, 1945). We and others have reported the benefits of this type of repair ("sensory protection") specifically when the denervation period exceed 3 months (Weiss and Edds, 1945 ; Gutmann, 1945; Karpati et al, 1981; Dautel et al, 1992 ; Ochi et al, 1992; Ochi et al, 1996; Zhang et al, 1997; Hynes et al, 1997; Ebert and Terzis, 1999; Wang et al, 2001; Papakonstantinou et al, 2002) for preserving both nerve and muscle integrity and function. Our recent work (Bain et al, 2001) corroborated these findings however, the main difference from these previous studies was that the intention was to allow the sensory nerve to temporarily maintain the muscle until appropriate motor reinnervation was established. Therefore, our study design differed from previous studies in that we provided subsequent motor reinnervation following the period of sensory protection.

In one study (Dautel et al, 1992), the nerve supply of a pedicled gracilis flap in the rat was either left intact (positive control), transected (unprotected), or immediately repaired with a motor or sensory donor nerve. Muscle weights in the sensory protected group were found to be intermediate (39.8% of control) between the unprotected group (37.2% of control) and the immediate motor repair group (41.3% of control). Histological features of the sensory protected muscle were also intermediate. Although it appears that a sensory protection effect is evident, since the positive control muscle weight was 50% less than the normal muscle, these results remain controversial.

In another study (Karpati et al, 1981), the soleus muscle in guinea pigs was examined histologically and electrophysiologically 4 to 10 months following transection and sensory repair (sural nerve) to the motor nerve supply. Although it appeared that sensory protection maintained function (3/38 animals exhibited motor contraction following electrical stimulation of the sural) and facilitated muscle reinnervation (histological features), they could not exclude the possibility that the effect was elicited by the ectopic



innervation provided by motor fibres in the sural nerve. An interesting finding in this study was the presence of “amphibian-like” motor endplates comprised of the connection between the sensory fibres and the muscle surface.

Classical experiments reported by Weiss and Edds (1945) used the saphenous nerve to the femoral nerve in the rat to test the effect of sensory protection on the quadriceps musculature. Although they reported no benefit of sensory protection in terms of histological appearance and electrophysiology, the flaw in this study is that, due to a dehiscence of the surgical repair in one rat, they relied on data from only 2 of 3 animals. It is possible that with a larger sample size, the results may have supported previous studies.

Ochi et al (1992) and Ochi et al (1996) transplanted dorsal root ganglia into denervated muscle in the rat and found improved muscle weight and an increase in tetanic contractions by direct muscle stimulation in DRG-transplanted muscle.

Nishimura et al (1991) reported that axons originating from spinal motoneurons which had elongated into a sensory cutaneous nerve (caudal cutaneous sural) exhibited restored electrical properties when regenerated into skin.

Deckwerth and Johnson (1993) found that sensory neurons are sustained by an increase in nerve growth factor (NGF) binding to its receptor (trkA) on sensory nerves. Motor nerves do not express this receptor. NGF is reported to influence the release of BDNF (Michael et al, 1997) and, upon distal nerve transection, sensory neurons release and deliver BDNF to the denervated stump (James et al, 1998). Under these conditions, the rate of functional recovery increased (Lewin et al, 1997).

The variability in results among these studies may be due to different animal models, different donor nerves, failure to provide motor reinnervation, duration of the sensory protection interval, variability in outcome measures, and follow up assessment times.

Among these *in vivo* models, several *in vitro* studies have reported the benefit of sensory influence on denervated muscle (Peterson and Crain, 1972; Hooisma et al, 1978). Lentz (1971) reported this effect on muscle cholinesterase activity.

**Figure Legend**

Figure 1 Skeletal muscle neuromuscular junction. The inset (shown by thick arrow) shows the pathway for acetylcholine synthesis in the axon terminal.

ScN=Schwann cell nucleus; ScC=Schwann cell cytoplasm; Mt=mitochondria;

Ach=acetylcholine; AchE=acetylcholine esterase; MF=muscle fibre;

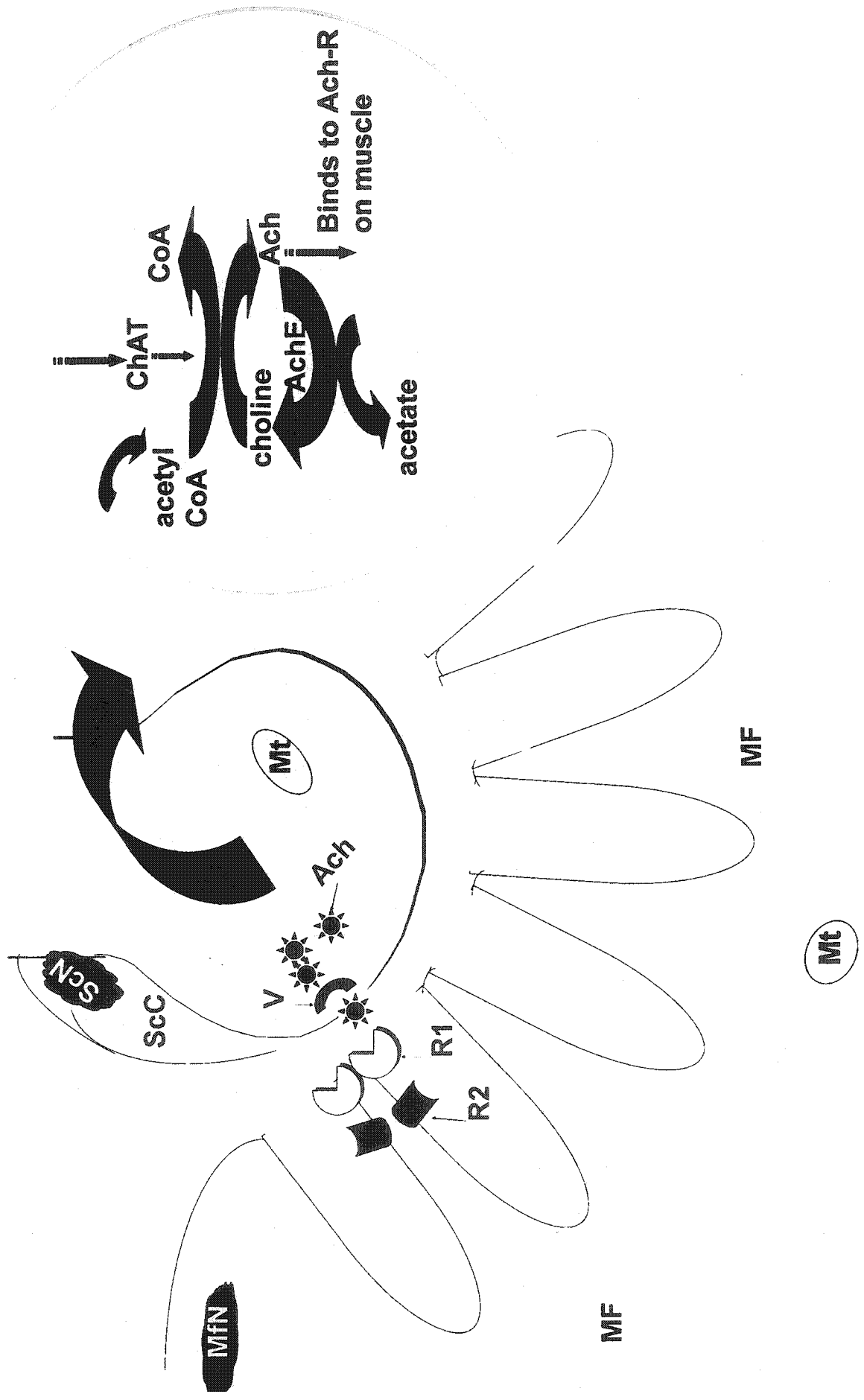
MfN=muscle fibre nucleus; CoA=coenzyme A; ChAT=choline acetyl transferase;

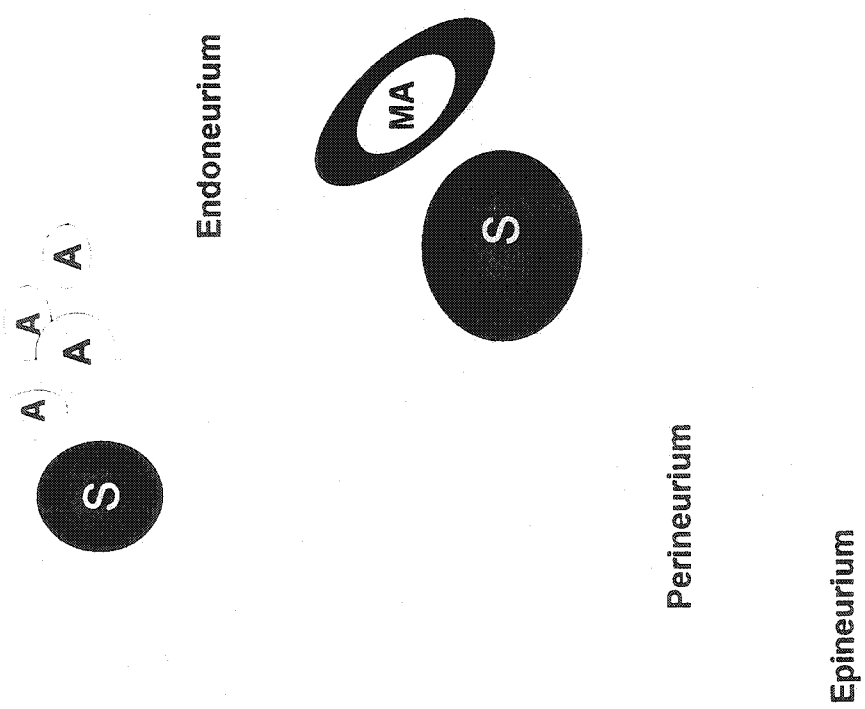
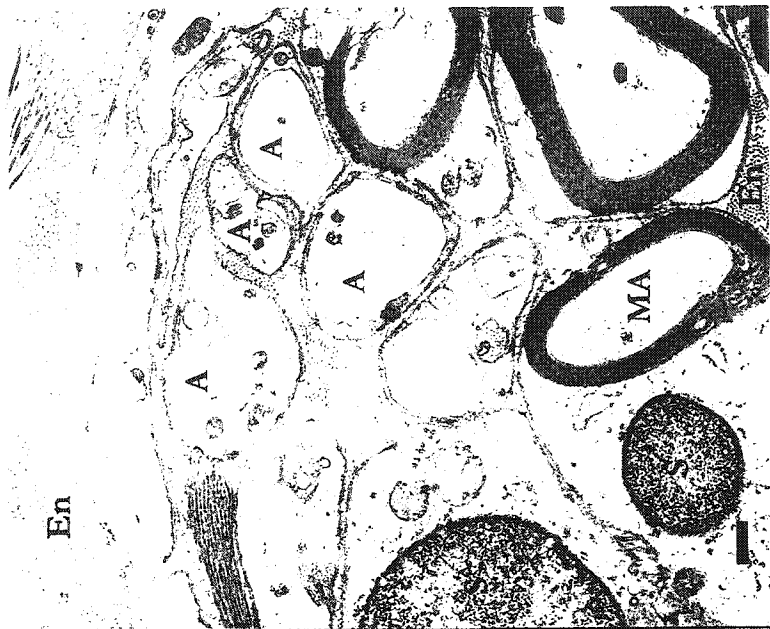
R1=Ach receptor 1; R2=Ach receptor 2

Figure 2 (Panel A) Diagrammatic representation of peripheral nerve showing myelinated axons (MA), unmyelinated axons (A), Schwann cells (S) and 3 layers including endoneurium, perineurium and epineurium.

(Panel B) Electron micrograph of normal peripheral nerve showing features outlined in Panel A. Scale Bar=500 nm (nanometres)

Fig. 1





B

A

Epineurium

Perineurium

Endoneurium

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## **B. PURPOSE OF PRIMARY STUDIES (Chapter 2, 3, 4, 5)**

To date, no evidence exists regarding the mechanism of “sensory protection”. The pilot study (Chapter 2, Hynes et al, 1997) was designed to investigate the role of sensory axons in accomplishing this delay in muscle atrophy. The subsequent study (Chapter 3, Bain et al, 2001) was designed to investigate whether the protective effect observed by sensory axons translated into a restoration of muscle function. The studies in Chapter 4 and Chapter 5 were designed to elucidate the mechanisms of sensory protection. This information would hopefully lead to the development of a clinically feasible surgical technique which would diminish irreversible muscle atrophy and impairment in function in patients with peripheral nerve injury. The objective is to improve the quality of life of the patient.

## **C. CLINICAL RELEVANCE OF PRIMARY STUDIES**

There is no question as to the significant impact that major peripheral nerve injuries such as brachial plexus palsy, Bell’s palsy or proximal nerve transections have on a patient’s quality of life. Often, these patients represent a younger population of employed individuals whose injuries result in a loss of motor function which, in some, may hinder employment and/or activities of daily living.

Treatment of distal nerve transection injuries by immediate or early microsurgical nerve repair often results in the best outcome (sensory and motor recovery). However, proximal nerve transection injuries present a more devastating problem given the distance over which the nerve must regenerate back to the target muscle. Given that the rate of regeneration of peripheral nerve is about 1 mm/day, time is a significant factor in determining functional recovery of these proximal injuries. Thus, injury to the ulnar nerve at the level of the elbow would constitute a profound loss of function given 10 months of regeneration is required to reinnervate the motor endplates of the hypothenar muscles. By this time, the muscles would atrophy to an extent that muscle recovery would be almost negligible, since a delay of more than 6 months results in a substantial decline in muscle recovery. Thus, in the absence of interim treatment measures, permanent muscle atrophy occurs and this necessitates other secondary surgical procedures such as tendon transfers, tenodesis or joint fusion, free functioning muscle transfer in cases of facial nerve palsy and others,

to restore function. The drawback to these procedures is the need for additional donor tissue. Where no secondary reconstructions are possible, prostheses or orthotics are the remaining options.

Conversely, several treatment options have been proposed which would preserve the muscle fibres, intending to delay atrophy in the interim until appropriate motor reinnervation is restored. Addition of exogenous neurotrophins has been attempted experimentally but is not clinically relevant to date. Foreign motor and autonomic nerve reinnervation has also been attempted. However, the drawbacks to these procedures is that an essential nerve (ie motor, autonomic) is sacrificed. In the majority of nerve injuries, a sensory nerve resides in the vicinity of the denervated muscle. Ideally, this sensory nerve could be detached and transposed to the distal nerve stump or sutured to the denervated muscle. Depending on the location, the sensory nerve may not be as essential as a motor nerve, and sacrifice of the sensory nerve would be less of a disruption to the patient's function. In the interim, if the sensory axons preserve the distal nerve sheath and also protect the denervated muscle, the delay to reinnervation would be less important. A temporary sensory nerve repair would be required until the target muscle is reinnervated. A second surgery would be performed to restore motor innervation.

#### **D. HYPOTHESES**

The present thesis tests the following hypotheses:

- Hypothesis 1. Sensory nerve repair following muscle denervation diminishes the changes associated with denervation including atrophy, fibrosis/fat and preserves normal features of muscle fibre arrangement.
- Hypothesis 2. Sensory nerve repair following muscle denervation preserves muscle twitch and tetanic contractile function.
- Hypothesis 3. Sensory nerve repair following muscle denervation preserves the architecture of the distal endoneurial sheath and sustains a favorable regeneration substrate.
- Hypothesis 4. Sensory nerve repair following muscle denervation influences a trophic effect on muscle fibres and maintains their receptiveness to reinnervation.



## E. DEFINITIONS OF TERMS

For the purposes of this study, the following definition of terms shall apply:

### Muscle Physiological Characteristics

*Isometric twitch tension* refers to the amount of force exerted by a muscle fibre, motor unit or entire muscle during a muscle contraction in which the muscle length does not change.

*Twitch* refers to a phasic contraction of short duration elicited by a muscle fibre or the brief sudden contractile response of a motor unit or all motor units within a muscle in response to a supramaximal stimulus.

*Twitch time to peak (TTP)* refers to the time (milliseconds) from the onset of the muscle contraction to the maximum peak value.

*Half relaxation time ( $\frac{1}{2} RT$ )* refers to the time (milliseconds) from the peak of the muscle contraction to half the peak value.

*Twitch total duration (TTD)* refers to time (milliseconds) from the onset of the muscle contraction to its completion .

*Tetanus* refers to the continuous contraction of muscle caused by repetitive stimulation or discharge of the nerve or muscle.

### Muscle Fibre Type Determination

*Myosin ATPase (adenosine triphosphatase)* is the myosin catalyst of the hydrolysis of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) and phosphate ( $P_i$ ) used as a source of energy for muscle contraction. Myosin ATPase staining is a commonly used method to identify muscle fibre types.

*Fast twitch fibres* are muscle fibres which have short contraction duration usually less than 60 ms and short relaxation duration.

*Slow twitch fibres* are muscle fibres which have long contraction duration usually greater than 60 ms and long relaxation duration.

*Type I fibres* feature myosin ATPase which is acid stable and alkaline labile (i.e. the enzyme is active at acidic pH 4.3, but inactive at alkaline pH 10.0 )

*Type II fibres* feature myosin ATPase which is alkaline stable (i.e. at alkaline pH 10.0) and acid labile (at acidic pH of 4.3).

### Nerve Electrophysiological Characteristics

*Compound muscle action potential* refers to the summation of the contractile responses of all motor units in response to a supramaximal stimulus.

*Compound muscle action potential baseline to peak amplitude* is the distance between the maximum peak of the negative spike and the imaginary baseline directly beneath it.

*Nerve conduction velocity (NCV)* is determined by measuring the distance between the site of nerve stimulation and recording and the time it takes the leading portion of the action potential to cover this distance (i.e.  $100 \text{ mm} / 10 \text{ ms} = 10 \text{ m/s}$  ).

**F. Prelude to Chapter 2 (Hynes et al, 1997)**

Investigators in previous studies on sensory to motor crossover were cautious to attribute the positive outcomes (histology, electrophysiology) to sensory axons since the confounder of ectopic innervation was not controlled for. Our pilot study (Hynes et al, 1997) was designed to eliminate this confounder by introducing a novel rat model where the hindlimb musculature was denervated by transecting the tibial nerve and burying the proximal portion on the adjacent adductor muscle, outside of the tibial nerve compartment, thereby removing any likelihood of reinnervation of the denervated muscle by axons arising from the proximal tibial nerve stump. Muscle histology was compared in 3 groups of rats: denervated, motor-motor crossover and sensory-to-motor crossover.

The following Chapter is a published paper on which the doctoral candidate was a coauthor:

Nicolas M. Hynes, James R. Bain, Achilleas Thoma, Karen Veltri and John A. Maguire. Preservation of denervated muscle by sensory protection in rats. *Journal of Reconstructive Microsurgery* 13(5):337-343, 1997.

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## G. CHAPTER 2

### Preservation of Denervated Muscle By Sensory Protection in Rats

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Materials in this paper were presented at the annual meetings of the Canadian Society of Plastic Surgeons, Halifax, Nova Scotia, May 1996, and the American Society for Peripheral Nerve, St. Louis, Missouri, June 1996 (received an award of merit).

### Abstract

The goal of this study was to determine whether sensory motor nerve crossover could alter post-denervation atrophy of skeletal muscle. Sixty adult Lewis rats were divided into three groups: 1) unilateral transection of the tibial nerve alone; 2) unilateral transection of the tibial nerve with immediate repair; and 3) unilateral tibial and sural nerve transections with repair of the proximal sural nerve (sensory) to the distal tibial nerve (motor). The unoperated hind legs acted as positive controls. At 1 and 2 months postoperatively, posterior compartment musculature was harvested, weighed, then fixed and stained for histologic analysis.

One month postoperatively, mean muscle weight in Group 1 animals (transection alone) was  $23.0 \pm 2.6$  percent of the control side; for Group 2 animals (motor-motor repair) was  $40.9 \pm 42$  percent; and for the sensory-protected Group 3 animals (sensory-motor repair) was  $26.7 \pm 2.8$  percent of controls ( $n=15$  per group). Two months postoperatively, the mean weights were  $14.5 \pm 0.9$  percent,  $58.8 \pm 7.3$  percent, and  $21.1 \pm 3.1$  percent of controls for Groups 1, 2 and 3, respectively ( $n=5$  per group). Differences between groups were statistically significant.

Histologic analysis of Group 1 specimens revealed generalized atrophy of all muscle fibers. In Group 2, specimens showed evidence of reinnervation and less atrophy. Group 3 specimens demonstrated an atrophic pattern with islands of non-atrophic fibers scattered throughout.

Sensory protection was thus shown to have a significant effect on post-denervated atrophy in rat skeletal muscle.

## Introduction

The management of proximal peripheral-nerve injuries continues to challenge reconstructive surgeons. Despite advances in microsurgical technique, the regeneration of repaired nerves is often too slow to reach the end-organ before irreversible muscle atrophy and eventually fibrosis have taken place (1). This atrophy translates into suboptimal motor function and the frequent need for secondary procedures. Complete understanding of the mechanism(s) of post-denervation muscle atrophy continues to elude investigators. However, several factors affecting the motor neuron and the muscle are known to contribute to poor motor recovery.

Previously identified neurogenic factors include: 1) death of motor neuron cell bodies, particularly following proximal axotomy (2) ; 2) inappropriate reinnervation (i.e. motor/sensory /autonomic mismatch)(3) ; and 3) deterioration of intramuscular nerve sheaths (4-6). Myogenic factors include: 1) inability of muscle fibers to accept reinnervation due to cell death (7); 2) replacement of muscle by fibrous tissue (8); and 3) depletion of the satellite cell pool (9, 10).

The importance of neurotrophins not only to the regenerating axons but also to the target tissue (muscle) has been suggested. Helgren et al (11) showed that rat skeletal muscle expresses functional receptors for ciliary neurotrophic factor ( CNTF) and that *in vivo* administration of CNTF has the ability to reduce the magnitude of atrophy and functional changes of denervated muscle. Peripheral nerves, particularly Schwann cells which are actively remyelinating, are an abundant source of CNTF and other trophic factors. Schwann cells are present in both motor and sensory peripheral nerves, and therefore the potential exists for sensory nerves to be a delivery mechanism for neurotrophic factors to denervated muscle.

Various techniques to minimize muscle wasting have been utilized both experimentally and clinically. Isometric and isotonic exercise (12), direct electrical stimulation of target muscle (9, 13) and administration of various exogenous growth factors (11) have been attempted. Previous sensory-to-motor nerve crossover investigations (14-16) have been reported, but only limited conclusions can be drawn due to small numbers and variable techniques.

The purpose of the present study was to determine whether sensory/motor crossover can prevent or reduce atrophy in rat skeletal muscle in the early denervation period, and to characterize the histologic pattern in muscle with and without sensory protection.

## Materials and Methods

### Animal Model

Adult male Lewis rats (Charles River) weighing 200 to 250 g were used. Animals were housed in a central animal care facility according to Canadian Council on Animal Care guidelines and were given rat chow and water *ad libitum*.

### Experimental Design

Sixty animals were randomized into three groups: 1) tibial nerve division without repair; 2) tibial nerve division with immediate repair; or 3) tibial nerve division and sensory/motor nerve crossover (Fig. 1). The contralateral hind limb of each animal remained unoperated and served as a within-subject control. End-point evaluations were performed at 1 month (15 animals per group) or 2 months (5 animals per group) and included gross muscle weights, qualitative histologic examination, and morphometric analysis.

### Surgical Procedure

Animals were induced and maintained using inhalational halothane anesthesia. Using sterile technique, a gluteal muscle-splitting incision was made in the left hind limb of each animal to expose the sciatic nerve and its branches. Using microsurgical technique, the tibial nerve was sharply transected and buried in the adjacent adductor muscle in Group 1 animals. In Group 2, the tibial nerve was transected and immediate epineurial repair was performed using 10-0 nylon. In Group 3, both the tibial and sural nerves were divided, and the proximal stump of the tibial nerve was buried in the adjacent muscle, while the proximal sural nerve was repaired to the distal stump of the tibial nerve. Postoperative fluids (5 cc subcutaneous normal saline) and analgesia (buprenorphine 0.03 mg/kg subcutaneously) were administered.



### Gross Muscle Weights

Animals were sacrificed with intraperitoneal injection of pentobarbital sodium. The operative site of each subject was examined under X3.5 loupe magnification to confirm the integrity of the previous repair. Posterior compartment musculature was then harvested from the Achilles tendon insertion to muscle origin bilaterally. Wet weights were obtained and the weight on the experimental side was compared to the contralateral, unoperated side and expressed as a percentage.

### Histologic Evaluation

Fresh muscle specimens were snap frozen in liquid nitrogen and cut on a cryostat at  $-20^{\circ}$  C. Full mount sections were procured from the mid-portion of the muscle specimens to represent maximal diameter. Hematoxylin and eosin (H&E) and ATPase stains at pH 4.3 and 10.0 were used. Specimens were examined under the light microscope and a qualitative assessment was done. Specifically, muscle fiber atrophy, fibrosis, reinnervation pattern, and fiber type distribution and size were evaluated by a blinded observer.

### Morphometric Analysis

A Kontron computerized image analysis system was used to determine total cross-sectional area (CSA), mean fiber cross-sectional area, and fiber type percentages. Briefly, five specimens representative of each group were selected and multiple fields evaluated. A minimum of 200 fibers per field were measured. Fibers not cut in cross-section and fibrous tissue were eliminated from the analysis.

### Statistical Analysis

Parametric data were compared by one-way analysis of variance (ANOVA) to determine whether significant differences were present between groups. Paired Student's *t*-tests were used for multiple comparisons, if justified by the analysis of variance. A *p* value of less than 0.05 was defined as significant.

## Results

### Muscle Weights

Statistically significant differences in wet muscle weights were identified between all groups at both 1 and 2 months postoperatively (Fig. 2). Of particular note, muscle weight in the sensory-protected Group 3 animals ( $X = 21.08$  percent  $\pm 3.06$  percent) was significantly greater than muscle weight in the purely denervated Group 1 animals ( $X = 14.46$  percent  $\pm 0.91$  percent) at 2 months ( $p=0.0097$ ).

### Histologic Evaluation

Histologic examination of control muscle specimens revealed normal architecture and minimal fiber-size variability (Fig 3A). No evidence of inflammation or fiber injury was identified. Group 1 (denervated control) muscle specimens showed generalized atrophy and early fibrosis. As expected, atrophy was worse at 2 months (Fig. 3B). In contrast, Group 2 muscle showed evidence of motor reinnervation, including fiber-type grouping and much less atrophy (Fig. 3C). There was reinnervation at the periphery being noted by 2 months.

Group 3 sensory-protected muscle displayed a marked difference. There was a patchy atrophic pattern, with islands of non-atrophic fibers of both fiber types scattered throughout (Fig. 3D). In addition, much less fibrosis was seen in the sensory-protected group than in the denervated controls at 2 months.

### Morphometric Analysis

Morphometric analysis of muscle total cross-sectional area paralleled muscle weight findings (Fig. 4). Differences between groups were statistically significant using ANOVA at both 1 and 2 months. In addition, a post hoc Student's t-test demonstrated a significant difference between the mean total cross-sectional area of Group 1 and Group 3 muscles at 2 months ( $p=0.022$ ).

Muscle-fiber cross-sectional area in each group was also analyzed morphometrically. ANOVA revealed a significant difference between the mean fiber cross-sectional areas among groups at both 1 and 2

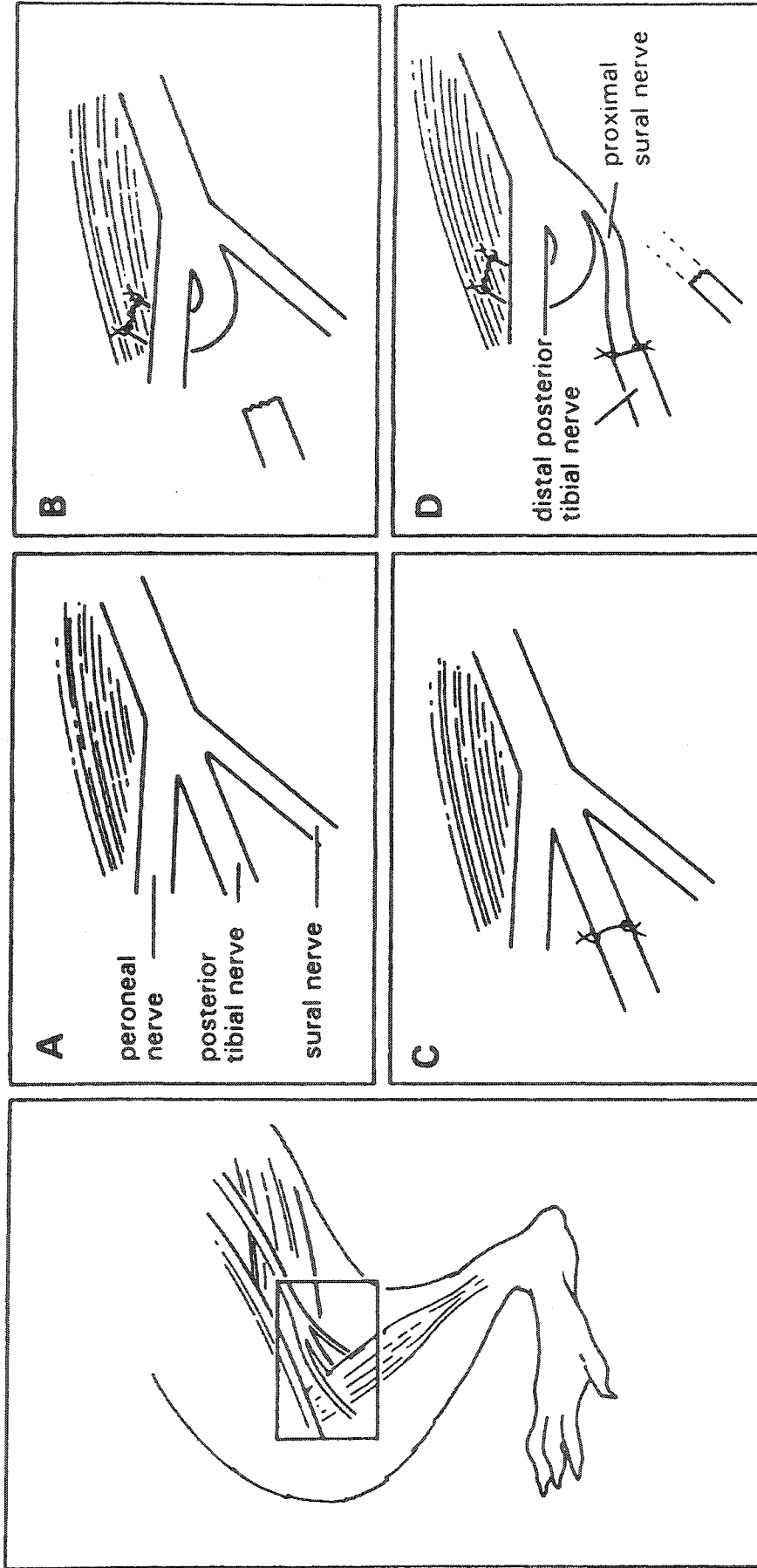


Figure 1. A, Branches of the sciatic nerve in the rat hindlimb. B, Group 1 animal (control denervated). Note that the proximal end of the divided tibial nerve is buried in the adjacent adductor muscle. C, Group 2 animal (motor reinnervated). Immediate microsurgical repair of the tibial nerve is performed. D, Group 3 animal (sensory-protected). Proximal end of the divided sural nerve (sensory) repaired to the distal end of the divided tibial nerve (motor).

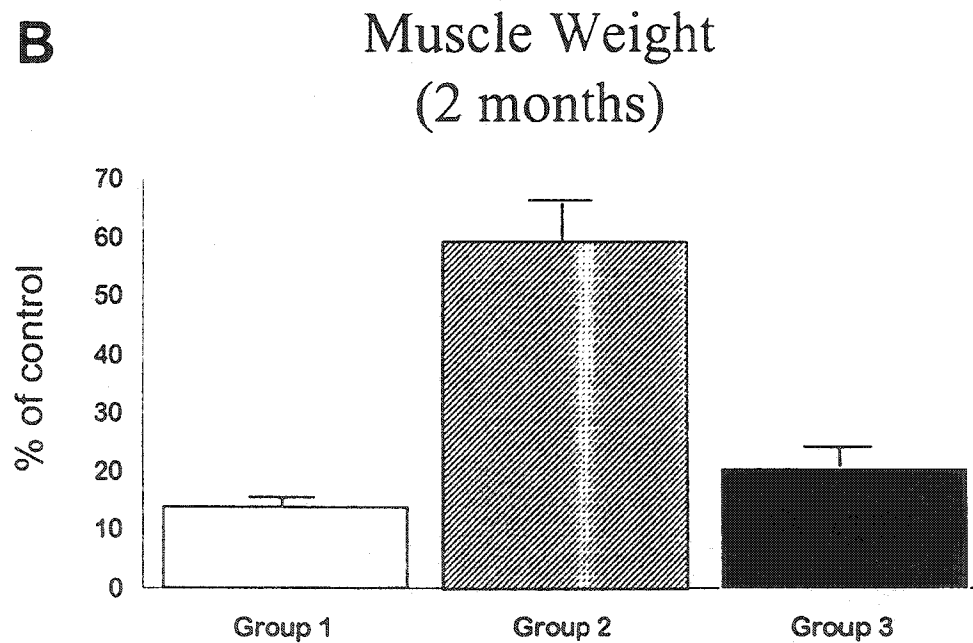
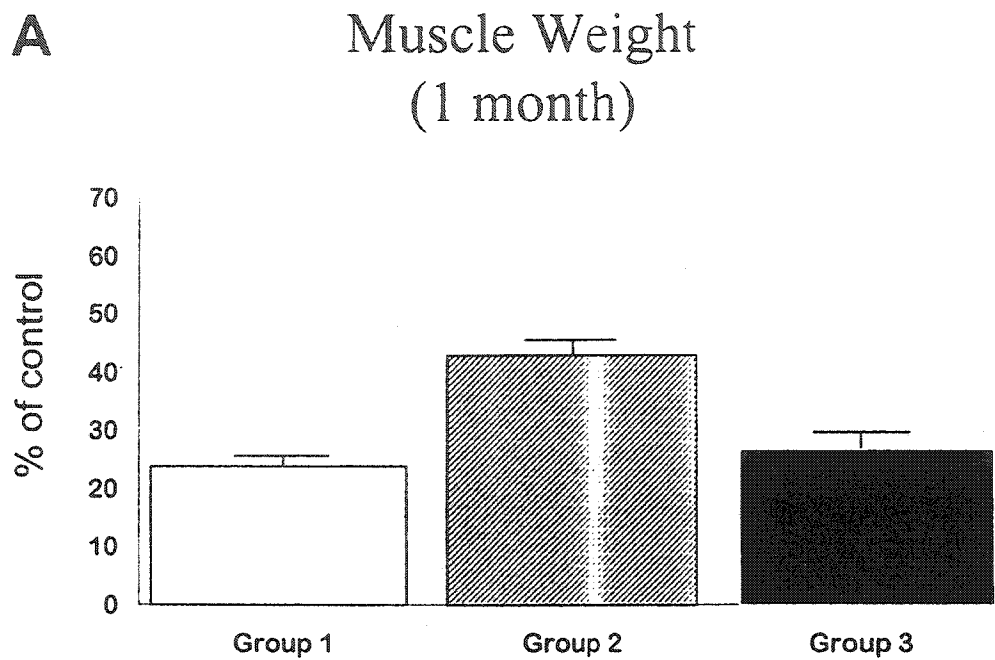


Figure 2. Wet muscle weights at 1 and 2 months postoperatively (expressed as percent of control side).

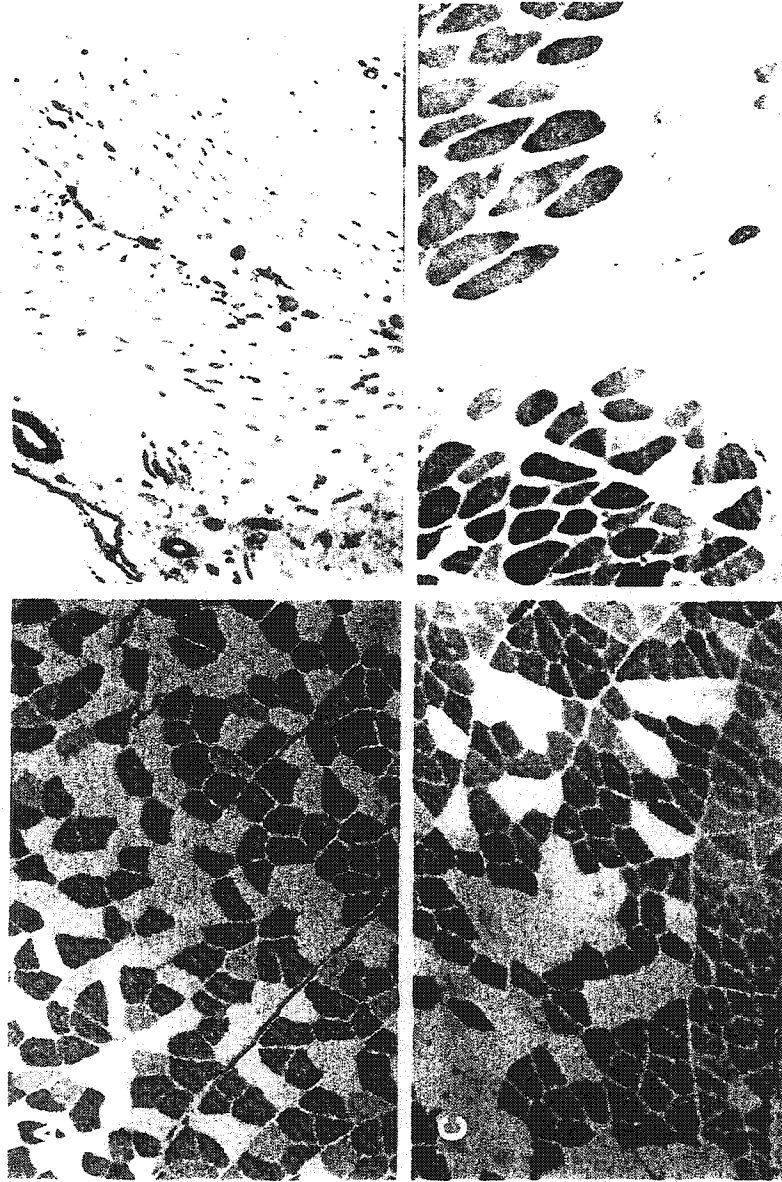


Figure 3. Representative photomicrographs of muscle specimens at 2 months postoperatively (ATPase stain at pH 4.3; magnification =  $\times 10$ ). A, Control specimen. B, Group 1 animal (control denervated). Note generalized atrophy of muscle fibers. C, Group 2 animal (motor reinnervated). Note fiber type grouping and normal fiber diameters. D, Group 3 animal (sensory-protected). Note islands of non-atrophic fibers within the atrophied muscle specimen.

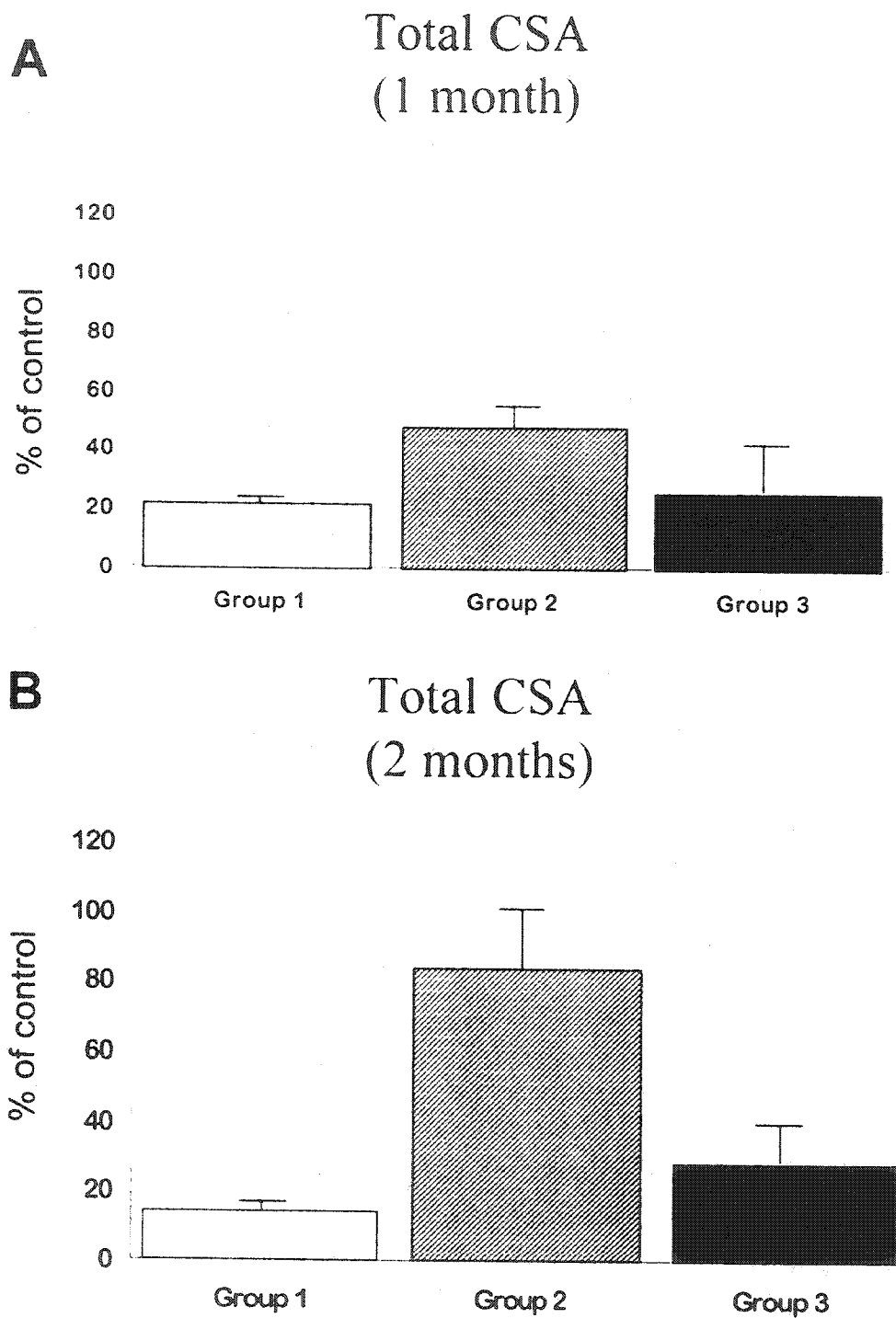


Figure 4. Muscle total cross-sectional area (total CSA) at 1 and 2 months postoperatively (expressed as percent of control side).

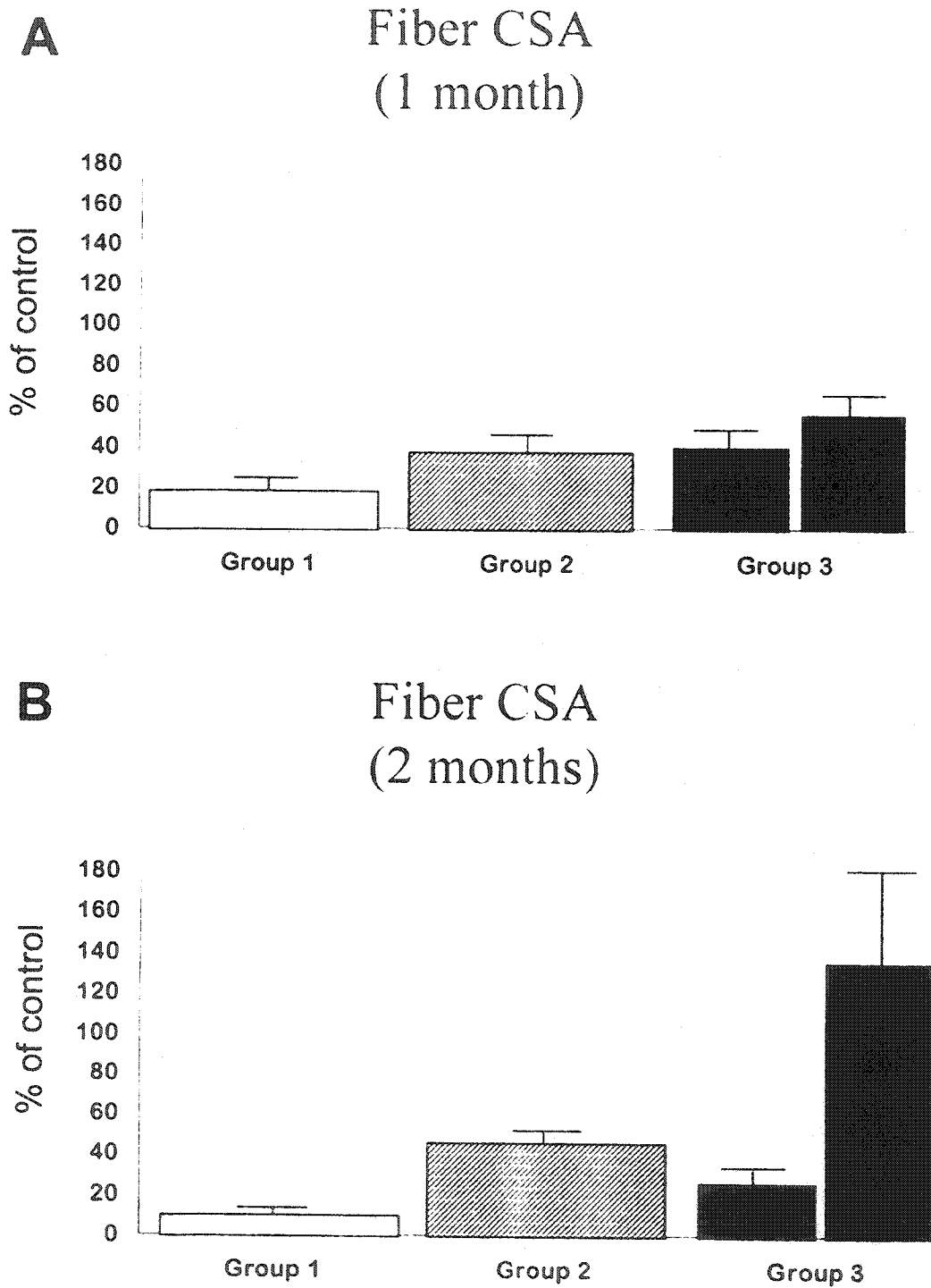


Figure 5. Muscle-fiber cross-sectional area (fiber CSA) at 1 and 2 months postoperatively (expressed as percent of control side).

months. *Post hoc* t-test analysis revealed a significant difference between Groups 1 and 3 at 1 and 2 months ( $p=0.011$  and  $p=0.0068$ ), respectively).

Of interest, the distribution of diameters was different between groups. There was a unimodal distribution of fiber cross-sectional area in Group 1 (denervated control) and Group 2 (motor reinnervated) muscles and these differed significantly at both 1 and 2 months ( $p=0.0013$ ) and  $p<0.0001$ , respectively). In contrast, because of the distinct histologic appearance of the Group 3 muscle specimens, there was a bimodal distribution with respect to muscle-fiber cross-sectional area (Fig. 5). It is important to note that the smaller diameter fiber population of Group 3 muscle was significantly different from Group 1 (denervated control) muscle at 2 months ( $p=0.024$ ). The large fiber population of the sensory-protected group was also statistically greater than even the motor-motor (Group 2) control at 2 months ( $p=0.020$ ).

Fiber-type analysis revealed a significant increase in the proportion of type I fibers in the Group 3 specimens at 2 months, when compared to controls. This was not observed in the other experimental groups.

## Discussion

Several investigators have attempted sensory-to-motor nerve crossover studies (14-16). Weiss and Edds (14) performed a series of nerve crossovers in rats utilizing the saphenous nerve and motor nerve to quadriceps. However, their methodology predated modern microsurgical techniques and experimental design. They repaired sensory-to-motor nerves in only three animals, with one gross dehiscence. They were unable to demonstrate transmissive connections with the muscle fibers or changes in muscle with sensory-motor crossover.

Karpati et al (15) repaired the sural nerve to the motor nerve to soleus in guinea pigs. They evaluated animals at 4 to 10 months following nerve crossover. Half the nerve repairs dehisced. In the animals with intact nerve repairs, about half showed histologic evidence of partial reinnervation. However, the authors attributed this finding to aberrant innervation from the proximal motor nerve, rather than to any protective effect of the sensory fibers.



Dautel and colleagues (16) raised a pedicled gracilis flap in a rat model and subjected its neural pedicle to three conditions: intact, nerve transection and repair, or transection and repair with a sensory donor nerve. It is difficult to interpret their results, since the control muscle (i.e. nerve intact but muscle flap raised) had a postoperative weight of only 50 percent that of the unoperated control. The weight of the sensory neurotization group (39.81 percent) was intermediate between the transection alone group (37.21 percent) and the motor-motor repair repair group (41.27 percent). They concluded that there was no significant difference (although some effect was seen). The histologic appearance was also intermediate in the sensory-motor group.

Indirect support for the hypothesis of sensory protection of denervated skeletal muscle comes from the clinical microsurgical literature. Chang et al (17) published a report of ten patients who underwent free muscle transfer for coverage of heel soft-tissue defects. A variety of sensory reinnervation procedures were performed. Five patients had sensory-to-motor nerve repair. They had good weight-bearing tolerance and good muscle bulk (some requiring debulking at a second stage). Of interest, the histologic appearance of the muscle presented by the authors appeared superior to that expected for muscle denervated for 4 years. However, the authors were not looking specifically for any trophic effect of sensory nerve input and therefore drew no conclusions regarding this finding.

The present study shows that sensory protection has a significant effect on denervated rat skeletal muscle, as measured by muscle weights and histologic evaluations. Several important questions are raised by this finding. First, does sensory protection have any functional significance? Second, what is the mechanism for sensory protection? Third, is there a clinical application for this technique?

Previous investigators have hypothesized that sensory input alone may effect a functional benefit (i.e. muscle contraction) with the initial crossover being the only intervention. We hypothesize that the sensory nerve would act only as a "baby-sitter" protecting muscle until appropriate reinnervation from a motor nerve occurs. The presence of sensory axons in the distal nerve sheath of motor denervated skeletal muscle could protect against atrophy, by preventing deterioration of the intramuscular sheath and/or by secreting

neurotrophic factors that prevent irreversible muscle changes. Previous authors have never restored the proximal motor nerve to the muscle.

In the next phase of our investigation, we will address this issue by performing secondary motor repairs to reinnervate muscle in animals with and without sensory protection. Functional assessments will include walking track analysis (18), electrophysiologic measurements, and isometric contractile function studies. In addition, quantification of neurotrophic factors and their receptors will be carried out in both sensory-protected and control groups.

Clinically, application of this technique could prevent or ameliorate muscle atrophy, leading to excellent restoration of motor function in patients with proximal nerve injuries. In most nerve injuries, there is a donor sensory nerve in the region of the denervated muscle that can be readily transferred to the distal motor nerve. By protecting the muscle, the time delay of motor axon regeneration from the site of injury to the distal muscle would not have a significant detrimental effect, and function would be comparable to that of distal injuries. Once the motor nerve regenerated to the distal muscle, the donor sensory nerve could be returned to its original site.

The present study lays the groundwork for many exciting possible applications in surgery of the peripheral nerve.

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#### H. Summary of Chapter 2 (Hynes et al, 1997)

Microsurgical nerve repair following injury often results in muscle atrophy and eventual fibrosis since axonal regeneration rate is inadequate to allow axons to reach the motor endplates to prevent atrophy. Sensory-to-motor studies have been undertaken but insufficient sample size and variable techniques led to limited conclusions. The objective of this study was to establish a clinically feasible microsurgical technique which would delay or prevent irreversible muscle atrophy and subsequent impairment in muscle function. The hypothesis stated that sensory protected muscle fibres exhibit less atrophy than unprotected fibres.

A sensory (sural) nerve was used as a "babysitter" to protect denervated rat hindlimb calf musculature. Histological assessments of muscle fibre samples were performed after 1- and 2-month intervals. One month post-denervation, in the sensory protected group, mean muscle weights had a significantly higher percentage of control weight than unprotected muscle (26.7 vs 23 g), with a more pronounced difference at 2 months (ie. sensory protected muscle weights (percent of control) was 21.1 g. compared to 14.5 g for unprotected muscle. Regions of non-atrophic muscle fibres were also identified in the sensory-protected specimens.

This pilot investigation demonstrated less muscle fibre atrophy and improved muscle fibre architecture supporting the role of sensory axons in delaying muscle atrophy.

### I. Prelude to Chapter 3 (Bain et al, 2001)

Hynes et al (1997) did not rule out the possible contribution by motor axons in the “mixed” sural nerve to the improved muscle histology. Furthermore, Hynes et al (1997) and earlier studies on sensory-to-motor crossover failed to provide motor reinnervation to sensory protected muscle, and muscle function was not evaluated.

The purpose of the subsequent study (Bain et al, 2001) was to provide motor reinnervation after sensory protection to establish the functional outcome. The hypothesis stated that the presence of sensory axons within endoneurial sheaths of the distal nerve stump release trophic factors which preserve the distal nerve environment and prevent the irreversible denervation changes in muscle. A purely sensory nerve (saphenous) was used to perform an end-to-end sensory-motor repair for a maximum duration of 6 months after which motor reinnervation was provided by cross-repair to the common peroneal nerve. End-point assessment included electrophysiological measures and isometric contractile muscle force.

This study was designed to investigate whether the protective effect observed by sensory axons in our initial study translated into a restoration of muscle function. Previous studies indicated that sensory protection (with no motor reinnervation) did maintain muscle histology, but no studies looked at muscle function and histology following a period of motor reinnervation.

The following Chapter is a published paper on which the doctoral candidate was a coauthor:

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## J. CHAPTER 3

### Improved Functional Recovery of Denervated Skeletal Muscle After Temporary Sensory Nerve Innervation

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## Abstract

Prolonged muscle denervation results in poor functional recovery after nerve repair. The possible protective effect of temporary sensory innervation of denervated muscle, prior to motor nerve repair, has been examined in the rat. Soleus and gastrocnemius muscles were denervated by cutting the tibial nerve, and the peroneal nerve was then sutured to the transected distal tibial nerve stump either immediately or after two, four or six months. In half of the animals with delayed repair, the saphenous (sensory) nerve was temporarily attached to the distal nerve stump. Muscles were evaluated three months after the peroneal-to-tibial union, and were compared with each other, with unoperated control muscles and with untreated denervated muscles. After four to six months of sensory "protection", gastrocnemius muscle weighed significantly more than unprotected muscle, and both gastrocnemius and soleus muscles exhibited better preservation of their structure, with less fiber atrophy and connective tissue hyperplasia. The maximum compound action potentials were significantly larger in gastrocnemius and soleus muscles following sensory protection, irrespective of the delay in motor nerve union. Isometric force, although less than in control animals and in those with immediate nerve repair, remained reasonably constant after sensory protection, while in unprotected muscles there was a progressive and significant decline as the period of denervation lengthened.

We interpret these results as showing that, although incapable of forming excitable neuromuscular junctions, sensory nerves can nevertheless exert powerful trophic effects on denervated muscle fibers. We propose that these findings indicate a useful strategy for improving the outcome of peripheral nerve surgery.

**Key Words:** muscle denervation, muscle reinnervation, nerve regeneration, nerve repair, neurotrophism



## Introduction

Major peripheral nerve injuries are best treated by early microsurgical repair (18). Thus, when surgery follows within two months of injury, both the numbers and the sizes of the reinnervated muscle fibers are well maintained (5), even though the muscle may contain fewer motor units than normal (3, 7). In contrast, if surgery is delayed for six months or more, recovery is usually incomplete, and weakness and wasting are both present (7,11). Although affected muscles may have microscopic evidence of muscle fiber regeneration from satellite cells, there are degenerative features, including fiber necrosis and connective tissue hyperplasia, which are irreversible (7,14,23,25,26). Furthermore, even when nerve surgery is performed early, there will still be a long period of muscle denervation if the distance from the site of injury is substantial, and the operative results are likely to be correspondingly poor.

Two major factors have been proposed to account for the impaired motor recovery after denervation. First, the regenerating nerve sprouts may not grow well along the distal nerve stump because of collagenization of the endoneurial tubes and fragmentation of the Schwann cell basal lamina (11, 24). Second, the denervated muscles may become less receptive to those motor axons which reach the muscle, because of loss of muscle fibers, exhaustion of satellite cell regeneration and fibrosis (14).

One possible solution to the latter problem would be to provide the denervated muscle with temporary trophic support from a sensory nerve. Sensory-to-motor innervation experiments have been attempted before (4,10,15,28-30) usually to determine to what extent a sensory nerve can substitute for a motor nerve. Such investigations have shown the sensory nerve to be incapable of forming excitable neuromuscular junctions, but there have been indications of trophic effects exerted on the muscle. On the basis of these early observations, our working hypothesis is that temporary sensory innervation ("sensory protection") of denervated muscle will allow improved recovery of muscle structure and function when motor reinnervation subsequently takes place. The results of a pilot study have been published elsewhere (13).

## Animals

The experiments were performed on female Lewis rats (Charles River, Quebec, Canada) weighing 200-250 g; this strain was chosen as it shows the least self-mutilation following surgery (2). 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. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

## Surgical procedures

Fifty-four female rats were randomly assigned to one control group and four treatment groups. The unoperated sides of the treated animals also served as controls. All the treated animals underwent unilateral denervation of the calf muscles under halothane anesthesia. This procedure was performed by exposing the tibial nerve in the posterior thigh, freeing the nerve from the popliteal vessels and then cutting it. The distances from the transection to the nerve entry points in two muscles under study, the medial gastrocnemius and the soleus, were approximately 13 and 27 mm, respectively. The central stump of the tibial nerve, after further mobilization from the sciatic nerve, was then sutured on to the superficial surface of the biceps femoris muscle. In group 1 rats (denervation without repair) no further surgery was performed until the assessment (Fig. 1a). In group 2 animals (immediate repair), the ipsilateral peroneal nerve was also transected and the epineurial sheath of the proximal stump was attached to that of the distal stump of the tibial nerve with two to three sutures of 10-0 nylon (Fig. 1b). In group 3 and 4 animals (delayed repair, sensory protected and unprotected) the peroneal-to-tibial union was delayed from two, four or six months after the initial denervation. Group 3 rats (Fig. 1c) differed from those in group 4 (Fig. 1d) in that, during the interval between the two operations, the saphenous nerve, a purely sensory nerve, was used to provide “sensory protection” of the denervated calf muscles. For this, the saphenous nerve was freed from the saphenous vein in the medial aspect of the thigh and was then guided through an opening in the medial thigh muscles until it lay in the popliteal fossa adjacent to the distal tibial nerve stump, to which it was then

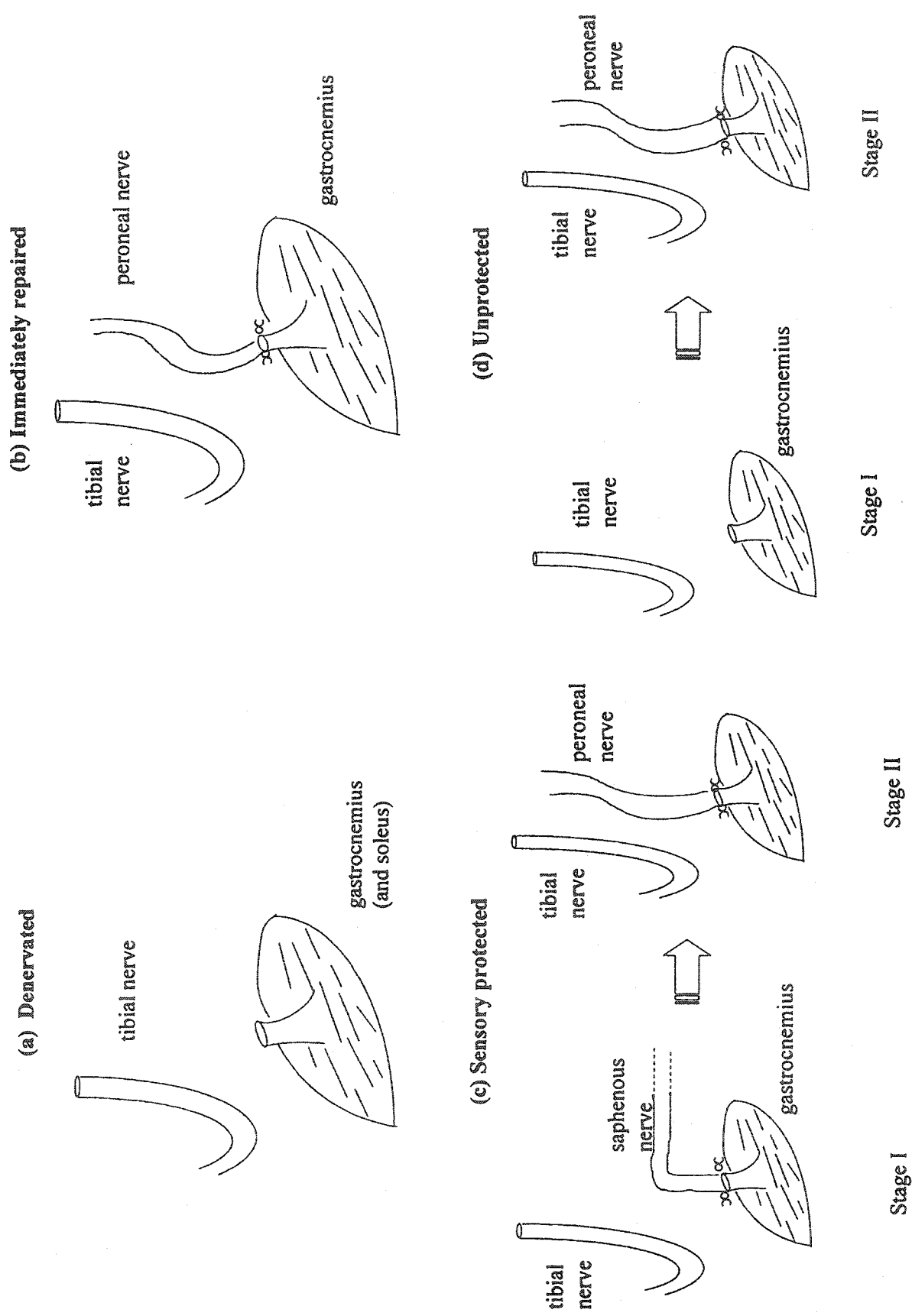


Fig. 1. Surgical procedure for each group of experimental muscles. (a) Group 1. Denervation without repair. (b) Group 2. Immediate peroneal-to-tibial nerve repair. (c) Group 3. Delayed peroneal-to-tibial repair with temporary sensory protection from the saphenous nerve. (d) Group 4. Delayed peroneal-to-tibial repair without sensory protection ("unprotected" muscles).

sutured (Fig. 1c). In the remainder of the text, the group 3 and 4 animals are referred to as “sensory protected” and “unprotected”, respectively. In both groups the tibial nerve was stimulated electrically at the time of the delayed peroneal-to-tibial union, and the calf muscles were carefully inspected under the dissecting microscope. Any contractions would have indicated the presence of ectopic motor innervation or excitable sensory neuromuscular junctions.

A three-month period of recovery was allowed before the animals were evaluated after the initial denervation (groups 1 and 2) or the subsequent peroneal-to-tibial repair (groups 3 and 4). Included in the evaluation was microscopic inspection of the site of the earlier nerve surgery for any disruption of the nerve repair. At the conclusion of the physiological assessment (see below), the animals were killed by an intracardiac injection of sodium pentobarbitone.

#### M-wave recordings

The maximal compound action potentials (M-waves) in the soleus and medial gastrocnemius muscles were recorded in the treated and control legs under halothane anesthesia, following sciatic nerve stimulation. The stimuli were 50  $\mu$ s current pulses, applied through bipolar stimulating electrodes from a portable electromyography machine (NeuroMax, Excel Tech, Oakville, Ontario, Canada). To diminish any cross-talk between muscles, the evoked M-waves were recorded with a coaxial needle electrode and were then amplified, displayed and automatically measured by the same electromyography machine. The muscles were kept moist with Ringer-lactate and their surface temperatures were maintained at 31-33° C with a heating lamp.

#### Force recordings

After completing the M-wave studies, the tibia was fixed firmly to the recording frame with a 22-gauge needle and the tendon of the gastrocnemius muscle was tied to a force transducer (model FT10, Grass Instruments, Quincy, MA, USA). Single maximal stimuli were then delivered to the peroneal nerve and the twitch tension ( $P_t$ ) of the entire (medial and lateral) gastrocnemius recorded at the optimal muscle length.

Tetanic tension ( $P_0$ ) was then determined following 80-Hz stimulation. The procedure was repeated for the soleus. After amplification, the muscle tensions were digitized and analysed automatically (WINDAQ software, DATAQ Instruments, Akron, OH, USA). The force recording system was calibrated with known weights and shown to be linear.

#### Muscle weights

At the conclusion of the force recordings, the soleus and the entire gastrocnemius muscles were dissected free of their tendons, blotted and weighed separately.

#### Histology and Histochemistry

Fresh muscle specimens were snap-frozen in liquid nitrogen, and complete transverse sections of the middle portions of the medial gastrocnemius and soleus muscle bellies were made with a cryostat. For examination of general morphology, sections were stained with hematoxylin and eosin, or with a modified Gomori trichrome stain (19). For fiber type assessment, the myosin ATPase reaction was used, after acid (pH 4.3) or alkaline (pH 10.0) preincubation. "Blind" evaluations were made of the muscle fiber sizes and the fiber type distributions, as well as the presence or absence of central myonuclei, increased connective tissue and inflammatory cell infiltrates.

#### Statistical analysis

In the text and table, mean values have been expressed with their standard deviations. To determine the significance of any differences between means, a one-way analysis of variance (ANOVA) was followed by Tukey's procedure. Significance was defined at  $P < 0.05$ .

## Results

### Muscle weights

Three animals died during the second operation and one from unknown causes. A further animal was excluded because nerve stump stimulation at the time of peroneal-to-tibial repair evoked weak contractions in the previously denervated calf muscles; this result was attributed to ectopic motor innervation.

In the operated legs, the weights of the gastrocnemius and soleus muscles were significantly lower than those in control legs, the values depending on the type and timing of the nerve repair. Immediate peroneal-to-tibial union (group 2) gave the best results, the mean gastrocnemius and soleus weights being 48% and 45% of control, respectively, while the untreated denervated muscles (group 1) showed the greatest loss of muscle mass, the values for gastrocnemius and soleus being only 14% and 9.5% of control, respectively (Table 1). The results for the sensory-protected (group 3) and unprotected (group 4) animals fell between these extremes. Regardless of the delays before the animals had undergone peroneal-to-tibial repair, the mean weights of the gastrocnemius and soleus muscles in the sensory-protected rats were always higher than those in the unprotected animals. As shown in Table 1, the differences reached statistical significance for gastrocnemius muscles when the nerve repair had been delayed for four to six months, and for the soleus muscles when the delay was two months.

### M-waves

The mean amplitudes of the M-waves evoked in treated muscles are also shown in Table 1. Although, in both gastrocnemius and soleus muscles, the amplitudes had declined after six months of sensory protection, the values for these animals remained significantly larger than those for unprotected animals, regardless of the delay in nerve repair. Indeed, among the unprotected animals with six months of denervation before repair, there were some with no detectable muscle responses.

### Twitch and tetanic tensions

As anticipated from the relative sizes of their muscle bellies, the mean twitch and tetanic tensions of the gastrocnemius muscles ( $2.13 \pm 0.26$  and  $16.6 \pm 1.1$  N, respectively) were considerably larger than those of soleus muscles in control legs ( $0.27 \pm 0.02$  and  $1.99 \pm 0.07$  N,  $P < 0.001$  in both cases). Moreover, the values were in agreement with those reported for these muscles in rats of similar weights (3,9) allowing for the fact that the combined medial and lateral gastrocnemius muscles were used in the present experiments. The mean tensions for animals with immediate nerve repair were approximately half the control means, although tetanic tension in the gastrocnemius muscles showed a better recovery (Fig. 2). In the rats with sensory protection, the tension values declined when nerve repair was delayed for two months, but then showed little further change (Fig. 2). In contrast, the contractile forces in the unprotected muscles became smaller the longer that nerve repair was postponed, and the differences between the sensory-protected and unprotected muscles became statistically significant.

### Histology and histochemistry

In gastrocnemius and soleus muscles subjected to immediate nerve repair, the general appearance of the fibers and surrounding connective tissue was close to normal, and a typical section is shown in Fig. 3A. In muscles with sensory protection, the structure was also preserved. For example, in Fig. 3B, taken from a gastrocnemius with six months of protection, the only abnormalities are a greater variation in fiber cross-sectional area and a slight increase in connective tissue. In contrast, sections from unprotected muscles displayed wide areas of atrophied fibers and considerable connective tissue hyperplasia; moreover, the larger, presumably reinnervated, fibers tended to have rounded outlines rather than the normal polygonal appearance (Fig. 3C). The untreated denervated muscles showed only atrophied muscle fibers and replacement of muscle by overgrowth of fibrous and fatty connective tissue (Fig. 3D). Histochemical staining, using the myosin ATPase reaction, confirmed the presence of reinnervation in the muscles that had undergone nerve repair; the evidence consisted of abnormal grouping of muscle fibers of the same histochemical type (data not shown).

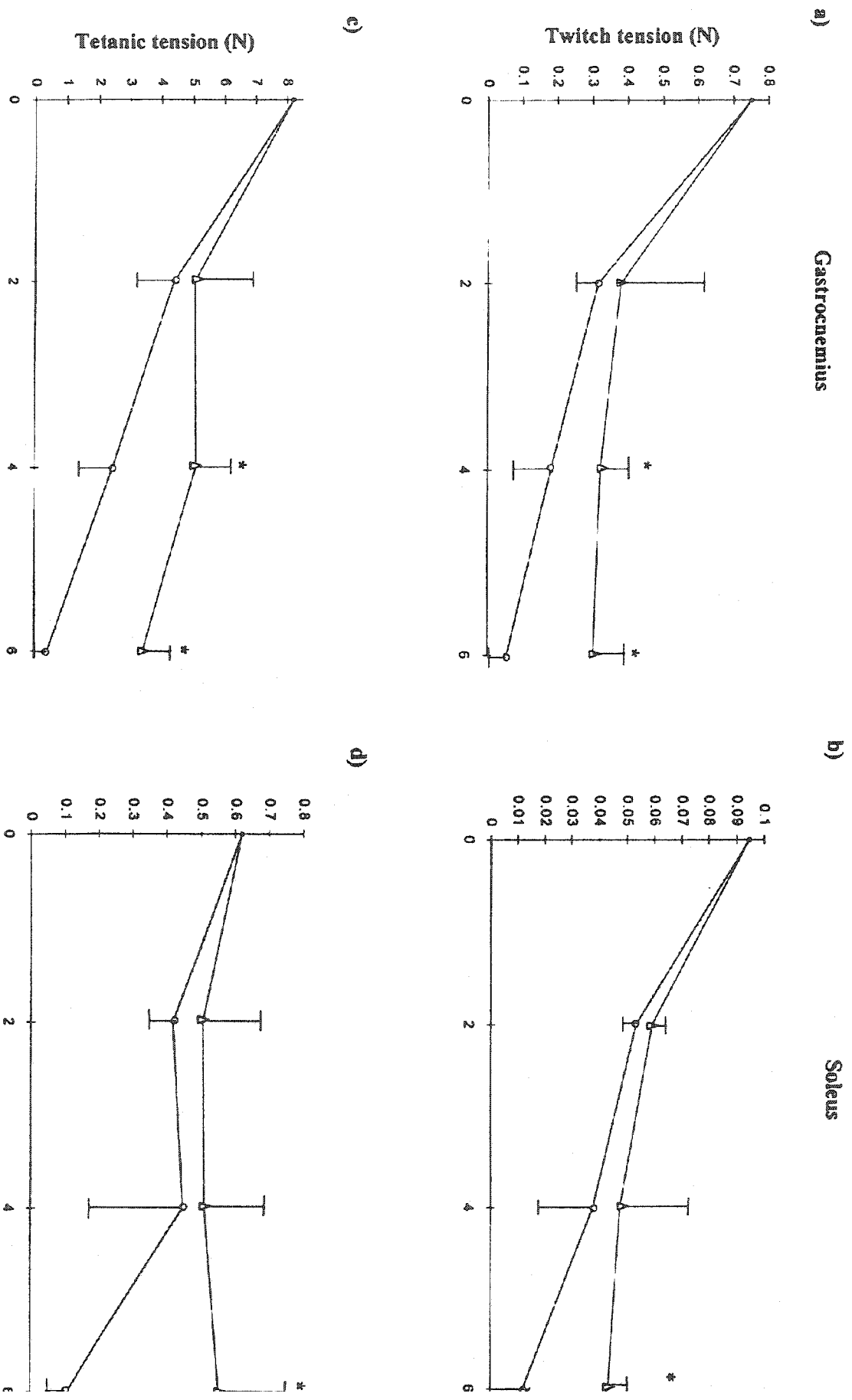


Fig. 2. Effects on twitch and tetanic tensions of delaying peroneal-to-tibial nerve repair on gastrocnemius and soleus muscles which were either unprotected (○) or had sensory protection (△) for varying periods. Values at zero time are for muscles with immediate peroneal-to-tibial nerve repair. Mean values are given with standard deviations; asterisks denote significant differences.



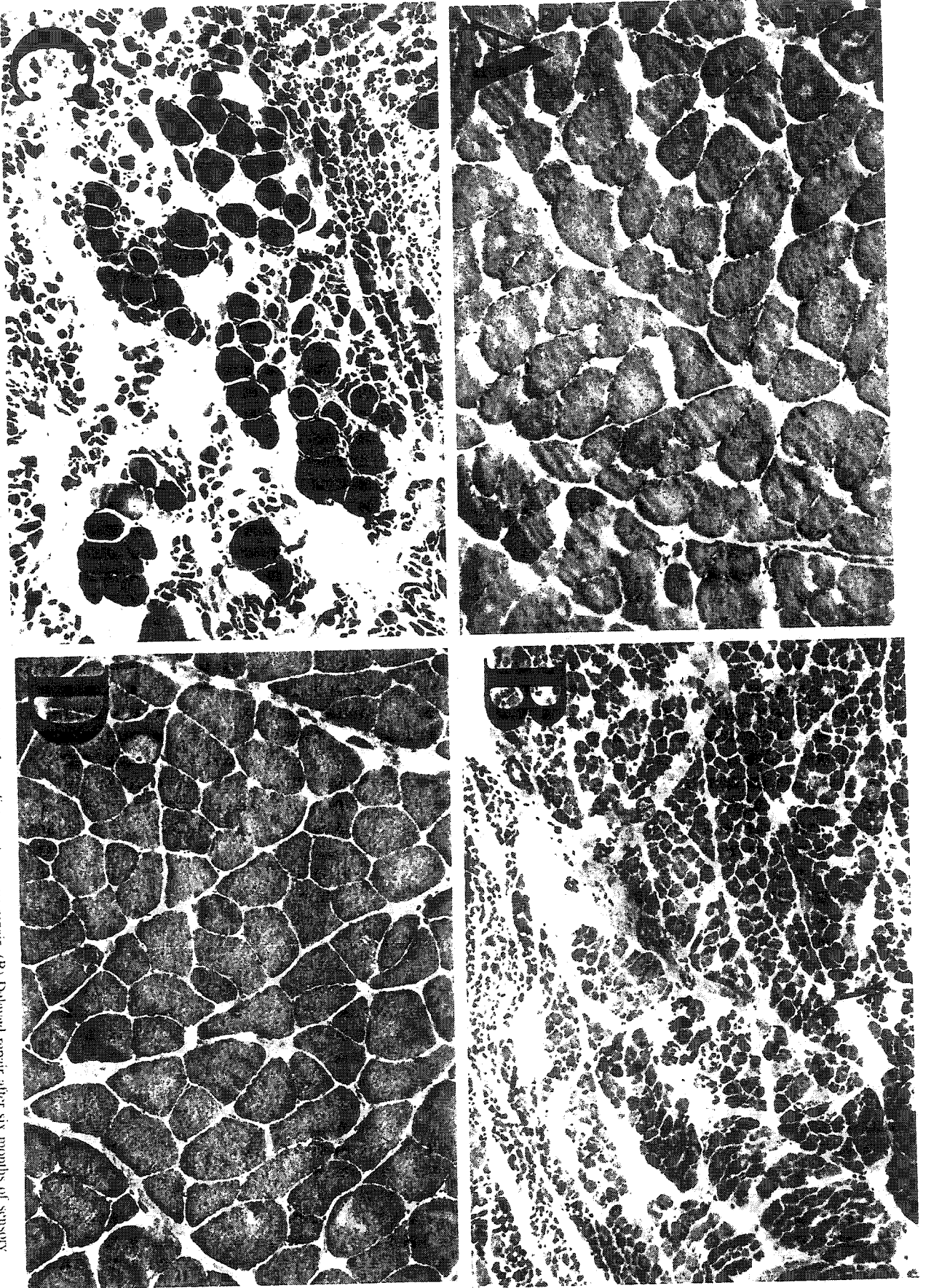


Fig. 3. Photomicrographs of medial gastrocnemius muscle sections treated with Gomori trichrome stain. (A) Immediate motor nerve repair. (B) Delayed repair after six months of sensory protection. (C) Delayed repair, muscle unprotected for six months. (D) Denervated muscle without nerve repair. See text for descriptions. Magnification: X 100.

## Discussion

As noted previously, the clinical outcome of peripheral nerve surgery is likely to be poor if reinnervation is delayed, either because of the timing of the operative intervention or because of the long distance required for nerve fiber regeneration. In this context, the quantitative study by Fu and Gordon (6) is especially relevant. In rats, they found that delaying nerve repair caused an exponential decline in the numbers of regenerating axons forming motor units. Although the numbers and sizes of the muscle fibers were well preserved in that study, others have found that degenerative features may not be fully reversible in muscles after prolonged denervation (14,23,25,26). The present experiments were undertaken to see whether a period of sensory innervation might improve structure and function when denervated muscles were subsequently innervated by motor axons.

Sensory innervation of denervated muscles was first attempted by Weiss (28,29), with the intention of determining whether excitable neuromuscular junctions could be established. Although the results were negative, there was less atrophy, and greater force on direct stimulation, than in untreated muscles. Our pilot investigations also demonstrated less atrophy and improved muscle fiber architecture (13). While this improvement could have resulted from a trophic influence exerted by the sensory nerves, it is important to exclude other explanations. Thus, Karpati et al (15), in their own experiments, reported abundant sensory nerve fibers in the denervated muscles, together with immature neuromuscular junctions, but they attributed the improvements in muscle mass and architecture to ectopic innervation by "foreign" motor nerve fibers.

In the present study, we have extended this line of investigation by subsequently replacing the sensory innervation with a motor nerve and comparing the results with those of animals without a period of sensory protection. In addition, we have looked for, and excluded, any effects due to ectopic innervation; the chances of ectopic innervation would, in any case, have been minimized by transecting both the tibial nerve, in the initial denervation, and the peroneal nerve, in the later cross-union procedure.

The results of the present study were convincing. In terms of muscle mass, M-wave amplitude, twitch and tetanic tensions, and histological appearance, the sensory-protected muscles were significantly superior to the unprotected ones. This enhancement of structure and function took place in the absence of excitable

sensory neuromuscular junctions, since electrical stimulation of sensory fibers at the time of the peroneal-to-tibial repair evoked contractions in only one animal, a result attributed to ectopic reinnervation. The present findings, of a benefit with sensory protection, are in keeping with those recently obtained by Ochi et al (20), who employed a different approach. After transplanting dorsal root ganglia into denervated muscles in rats, these authors observed enhanced muscle weights and greater tetanic forces, on direct stimulation, in the treated animals.

Although the improvements in structure and function of the sensory-protected muscles in the present experiments were considerable, even more impressive results might have been obtained if the original nerve had been used in the subsequent nerve repair. Thus, the peroneal nerve contains approximately half as many motor axons as the tibial nerve (600 vs more than 1000) (22). This prediction would be consistent with our finding, in pilot experiments, that, if the tibial nerve rather than the peroneal nerve was used for immediate repair, there was a greater preservation of muscle mass both for the gastrocnemius (73.1% vs 48.0%) and for the soleus (69.9% vs 45.1%).

Regarding the nature of the trophic effect of the sensory nerve, there is now abundant evidence that neurotrophins (NTs), produced by motor and possibly sensory nerves, may have powerful actions on skeletal muscle fibers. For example, denervated soleus muscles treated with exogenous ciliary neurotrophic factor have larger masses and develop greater twitch and tetanic tensions (12) while NT-3 and brain-derived neurotrophic factor have been shown to influence the development and functional maintenance of skeletal muscle and its innervation (16,17,27). In addition, injured and regenerating nerves alter NT profiles, the levels of NT-4 and brain-derived neurotrophic factor both increasing (8).

Apart from its putative neurotrophic effect, the temporary sensory innervation may have improved the outcome in the present experiments by helping to preserve the architecture of the distal nerve stump prior to the subsequent motor nerve union. Original studies by Sunderland and Bradley (24) describe collagenization, collapse and fragmentation of the intramuscular motor nerve fiber sheaths with prolonged denervation. Given that maintenance and regeneration of the basal lamina of the endoneurial tubes require axon-Schwann cell interaction (1), the sensory fibers may maintain this relationship. Similarly, the initial

stimulus for Schwann cell proliferation and NT production following nerve injury could conceivably be prolonged by the presence of sensory fibers within the distal motor nerve sheaths (c f. Ref. 21). In addition, the sensory fibers may prevent endoneurial tube collapse (7), and hence improve the later entry by regenerating motor axons.

While further experiments are needed to clarify the full nature of the protective mechanism(s) provided by the sensory nerve fibers, the present results point to a useful new strategy in dealing with peripheral nerve damage.

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Table 1. Muscle weights and M-wave amplitudes of muscles which remained denervated or were subjected to nerve repair, either immediately (0 months) or after defined intervals (two to six months)

	n	Operative Delay (months)						Denervated	
		0	2	4	6	10			
Muscle Weight (% of control)									
Gastrocnemius	10	50.0 ± 4.2	37.9 ± 3.1	30.0 ± 8.0	31.6 ± 5.4	19.7 ± 5.5*	32.7 ± 16.3	13.2 ± 1.5*	14.0 ± 1.8
Soleus	10	45.1 ± 3.1	34.9 ± 6.7	15.2 ± 5.0*	25.6 ± 10.8	17.4 ± 8.5	20.7 ± 4.8	15.3 ± 13.0	9.3 ± 3.3
M-wave (mV)									
Gastrocnemius	10	17.1 ± 3.8	15.3 ± 1.7	7.8 ± 4.2*	12.7 ± 2.6	7.2 ± 3.1*	9.5 ± 3.7	2.3 ± 2.1*	-----
Soleus	10	9.6 ± 6.0	10.7 ± 2.8	5.5 ± 1.5*	7.2 ± 3.0	2.8 ± 1.1*	4.0 ± 0.7	1.6 ± 1.8*	-----

Data are mean values ± S.D.; n refers to the number of animals analysed. \* Significant differences between sensory-protected (SP) and unprotected (UP) muscles.

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#### **K. Summary of Chapter 3 (Bain et al, 2001)**

End-point assessments revealed that, following 6 months of sensory protection, gastrocnemius muscle weight was significantly higher relative to unprotected muscle weight (32.7 % of control vs. 13.2 % of control), respectively. This study also showed that in comparison to unprotected controls, muscle histological features were maintained in sensory protected muscle in addition to contractile function and electrophysiological properties.

This study concluded that although sensory axons could not mimic motor axons by stimulating a muscle contractile response, sensory axons conferred a trophic effect on the denervated muscle fibres.

#### **L. Prelude to Chapter 4 (Veltri et al)**

Karen Veltri , Jacek Kwiecien, Wyatt Minet, M. Fahnstock and James R. Bain. Contribution of the distal nerve sheath to nerve and muscle preservation following denervation and sensory protection (submitted to Neuroscience)

The previous work supported the role of “sensory protection” in preserving muscle histology and function. However, a thorough understanding of the mechanism(s) of sensory protection continued to elude investigators. It was yet uncertain as to whether sensory axons within the endoneurial sheaths maintain the neural environment of the distal nerve stump to maintain a favorable regeneration substrate for regenerating axons or whether sensory axons provide trophic factors to the muscle and maintain its “receptiveness” to motor reinnervation.

Irreversible changes in denervated muscle upon peripheral nerve injury were purported to arise from collagenization of endoneurial tubes in the distal nerve stump and fragmentation of the interface between the Schwann cells and their associated basal lamina, due to vacant endoneurial tubes. Deterioration of the distal nerve endoneurial sheaths is insufficient growth substrate for regenerating axons and they fail to elongate to reach the target muscle fibres. This mechanism has been demonstrated experimentally by Fu and Gordon

(1995) using a motor nerve to sustain the denervated muscle. These authors have shown that motor axon regeneration outside of endoneurial sheaths directly into muscle fibres is less effective at maintaining trophic support, cell adhesion molecules and the overall neural environment than motor axon regeneration within the endoneurial sheath.

Using the Fu and Gordon (1995) study as a basis for the next investigation (Chapter 4), a similar model was used but sensory axons substituted the use of motor axons. This study used a similar model to that previously used (Bain et al, 2001) and another type of sensory repair whereby the sensory nerve was neurotized to the muscle to determine the contribution of the endoneurial sheath. End-point assessments included nerve and muscle ultrastructural morphometry and fibre type analysis.

The following work has been prepared for submission to Neuroscience and is included herein as a manuscript.

## M. CHAPTER 4

### Contribution of the Distal Nerve Sheath to Nerve and Muscle Preservation Following Denervation and Sensory Protection

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Running Title: Contribution of the distal nerve sheath to sensory protection

## Abstract

Foreign sensory nerve repair to a distal motor nerve target (“sensory protection”) preserves muscle weight, histologic features (Hynes et al 1997) and muscle function (Bain et al 2001) following denervation. The purpose of this study was to determine the contribution of the distal nerve sheath to sensory protection.

Following tibial nerve transection, rats were assigned to one of the following groups: (1) saphenous to tibial nerve neurorrhaphy (Nerve-to-Nerve repair (sensory protected)(SP) connecting a sensory nerve to the distal tibial nerve sheath); (2) saphenous to gastrocnemius neurotization (Nerve-to-Muscle repair (sensory protected)(SP) connecting the sensory nerve to the muscle in the absence of the distal nerve sheath); (3) Unprotected Controls (tibial nerve transection) or, (4) immediate common peroneal to tibial nerve neurorrhaphy (Immediate Repair with a motor nerve). The unoperated contralateral leg of treated animals served as a control. After a 6-month denervation period followed by motor reinnervation, ultrastructural, histological, and morphometric analyses were performed on distal tibial nerve and gastrocnemius muscle cross-sections.

Specimens of distal tibial nerve in the Nerve-to-Nerve (SP) group were superior to Unprotected controls shown by a significantly higher axon density, reduced collagen area and improved axon-to-Schwann cell coupling. These are features of the original neural environment and reflect sustained neural integrity. Although axon number in the Nerve-to-Nerve group was similar to the Nerve-to-Muscle (SP) group, the Nerve-to-Nerve (SP) group displayed improved regeneration shown by several axons at various stages of myelination, in a “normal” one-to-one relationship with an adjacent Schwann cell. This suggests superior neural architecture. The Nerve-to-Nerve (SP) group contained significantly less collagen and a significantly larger mean axon area than the Nerve-to-Muscle (SP) group. Specimens of gastrocnemius muscle from both sensory protected groups demonstrated less collagenization and fat deposition than Unprotected control muscle, similarity in mean total muscle fibre size, and evidence of reinnervation (fibre type grouping) among regions of overlapping fibre types (mosaicism) suggesting a preservation of normal muscle features. Fast twitch fibres predominated in both sensory-protected groups (60% to 40%) as in normal muscle. Unprotected control muscle lacked fast twitch fibres and the total muscle fibre area of this

group was significantly smaller than all other experimental groups. The mean area of fast twitch fibres in the Nerve-to-Muscle (SP) group was significantly larger than the Nerve-to-Nerve (SP) group in support of a trophic influence on fast twitch muscle area.

These data suggest that multiple mechanisms may contribute to long-term sensory protection. Sensory axons directly neurotized to muscle maintain existing muscle integrity in terms of less fibrosis, collagenization and fat deposition, and preserve the distribution pattern of fast twitch fibres in the absence of the distal nerve sheath. However, during prolonged denervation, neurorrhaphy of the sensory nerve to the distal tibial nerve (thus using the distal nerve sheath) improves the existing endoneurial sheath structure which enhances nerve regeneration.

**Key Words:** sensory protection, denervation, ultrastructure, morphometry, nerve

## Introduction

Chronic denervation results in changes in the distal neural environment and target muscle that may prevent successful reinnervation and functional recovery after injury. Denervation-induced changes can be identified by deteriorating distal axon endoneurial sheaths (Cormack, 1987). Deterioration of the distal endoneurial sheath is purported to arise from collagenization (Morris et al 1972) and fragmentation of the interface between the Schwann cells and their basal lamina, due to vacant endoneurial tubes (Fu and Gordon, 1995a, b). Wallerian degeneration of the distal axon is characterized by axonal retraction and axonal loss, Schwann cell proliferation and phagocytosis of cellular debris (Waller, 1850). Unsuccessful axonal regeneration and loss of contact with Schwann cells leads to shrinkage of the “bands of Bungner” guides for regenerating axons leading back to their original endplates (Reynolds and Woolf, 1992) with only basement membrane and scar tissue remaining, reducing the potential for successful nerve regeneration and subsequent muscle recovery.

Chronically denervated muscle fibres exhibit muscle fibre atrophy and shape alteration, necrosis and ultimately fibrosis (Helgren et al 1994; Irintchev et al 1990). Loss of motor innervation is associated with an increase in intermysial connective tissue with large deposits of fat and inflammatory cells (Sunderland 1991). Alterations in fibre type grouping are also a characteristic feature of chronically denervated muscle (Irintchev et al, 1990). The contribution of the nerve upon muscle atrophy following denervation is not clear. It may arise from deterioration of the distal endoneurial sheath leading to loss of muscle fibre receptiveness and denervation atrophy (Fu and Gordon, 1995a, b). Alternatively, the loss of neural input, including neurotransmitters, neurotrophic factors, and other signals, may lead to muscle fibre atrophy, and subsequent fibrosis.

We and others have previously reported the benefits of sensory-to-motor nerve repair, “sensory protection” (Weiss and Edds, 1945 ; Gutmann, 1945; Karpati et al, 1981; Dautel et al, 1992 ; Ochi et al, 1992; Ochi et al, 1996; Zhang et al, 1997; Hynes et al, 1997; Ebert and Terzis, 1999; Wang et al, 2001; Papakonstantinou et al, 2002) for preserving both nerve and muscle integrity and function. Bain et al (2001)

defined the concept of “sensory protection” after significant improvement in motor function was observed following temporary sensory-motor neurorrhaphy. To date, the mechanism of “sensory protection” is unclear. The present study tests the relative contribution of two possible mechanisms. One involves the interaction between sensory nerve axons and Schwann cells of the endoneurial sheath which prevents collagenization of the sheath, thereby maintaining endoneurial sheath architecture. Another involves a direct effect of sensory nerve-derived or sensory nerve-stimulated factors such as neurotransmitters or neurotrophins to preserve muscle integrity. In the first case, innervation of the muscle by a sensory nerve would require the distal nerve sheath, and direct neurotization to the muscle would not be beneficial. In the second case, direct sensory neurotization to the muscle would be more beneficial to the existing muscle fibre integrity independent of the distal nerve sheath.



## **Experimental Procedures**

### **Animals**

Male Lewis rats (Charles River, Quebec, Canada) weighing between 200-250 g were used for all experiments as this strain has demonstrated very little limb self-mutilation following sciatic nerve surgery (Carr et al, 1992). Housing, surgical procedures, administration of analgesics and assessments were performed according to the guidelines of the Canadian Council on Animal Care (CCAC), and animal utilization protocols (AUP) were approved by the Animal Care Committee (ACC) at McMaster University. A gaseous mixture of halothane (2.5%) in oxygen was used to anesthetize the animals during the initial surgery. All surgeries were performed under sterile conditions using a Zeiss operating microscope (Carl Zeiss, Oberkochen, Germany). Postoperatively, 5 cc of normal saline was administered subcutaneously to replenish fluid loss, analgesia (buprenorphine 0.03 mg/kg) was administered subcutaneously, and animals were kept warm with a heating pad. Endpoints were established to minimize animal suffering. The smallest sample size necessary to produce adequate statistical power was used.

### **Experimental Design**

The main effect, independent variable was surgical intervention (repair) classified into 4 groups: sensory-to-motor (Nerve-to-Nerve sensory protected, i.e. N-N), sensory-to-muscle (Nerve-to-Muscle sensory protected, i.e. N-M), denervated (Unprotected control, UP), and motor-to-motor (Immediate Repair, IR). Thirty-eight (n=38) rats were randomly assigned to one of these groups. The unoperated contralateral hind limb of the treated animals served as a control. This sample group of animals was also used to obtain data on muscle function. Dependent variables, axon integrity, size, density, collagen deposition, axon-to-Schwann cell interaction, muscle fibre organization, size and fibre type distribution were assessed by an independent observer. Dependent variables also included morphometric measures of axon area, myelin sheath thickness, collagen area, Schwann cell area and muscle fibre area and distribution.

### Surgical Procedures

In all groups, denervation was performed as described in Bain et al (2001) with some modifications. Briefly, the tibial nerve (TN) was exposed using sterile microsurgical technique and transected distally approximately 13 mm from the nerve entry point in the lateral head of the gastrocnemius (Figure 1A). The free distal stump was ligated with 9-0 nylon suture to minimize extraneous innervation from other axons. The proximal stump of the nerve was sutured with 9-0 nylon onto the superficial surface of the biceps femoris muscle to avoid reinnervation from proximal tibial nerve axons.

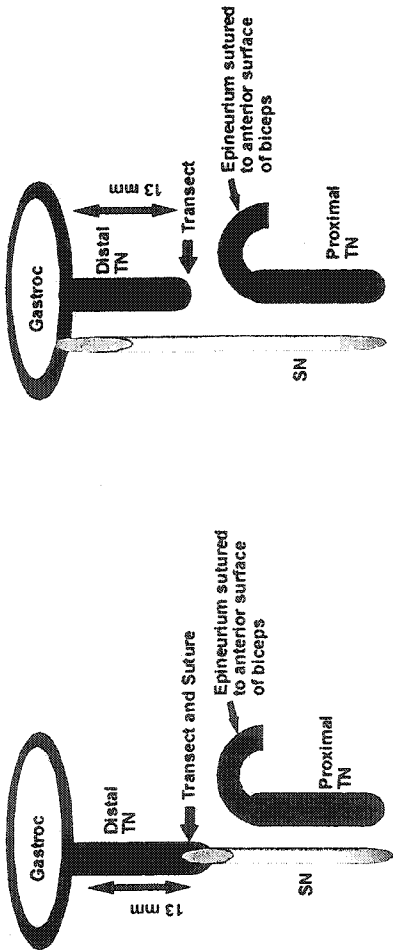
Sensory-to-motor repair (Nerve-to-Nerve sensory protected, designated N-N (SP)) following denervation was performed as described in Bain et al (2001) and shown here in Figure 1A. The saphenous nerve (SN) was chosen as a sensory nerve donor to avoid potential ectopic innervation by motor axons in a mixed nerve. The site at which the saphenous nerve was transected and transposed into the popliteal fossa, the segment of the saphenous nerve just distal to the bifurcation of the femoral nerve, is where the nerve divides into its purely sensory branch to the medial thigh (Harsh et al, 1991), avoiding motor axon influences.

Sensory-to-muscle repair (Nerve-to-Muscle sensory protected, designated N-M (SP)) was performed by suturing the proximal segment of the transposed saphenous nerve to the aneural zone of the lateral gastrocnemius muscle (Brunelli 1982) using 9-0 nylon (Figure 1A).

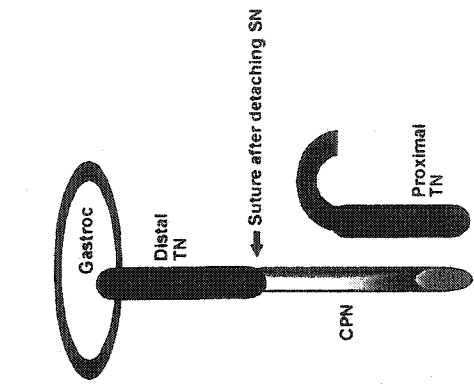
Immediate motor repair (IR) was performed as described in Bain et al (2001) and shown here in Figure 1B. The proximal stump of the common peroneal nerve (CPN) was sutured to the distal tibial nerve stump. Muscle reinnervation started after approximately 14 days and was mature 3 months following this repair. In the Unprotected control group, during the 6-month denervation period, no sensory repair was performed (Figure 1B).

In the sensory protected (N-N, N-M) and UP groups, motor innervation was re-established after 6 months by detaching the saphenous nerve (in sensory protected groups) and suturing the freshly transected proximal stump of the common peroneal nerve as described in Bain et al (2001) to the distal tibial nerve

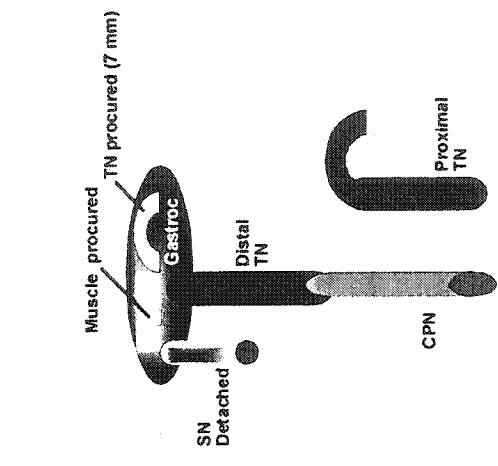
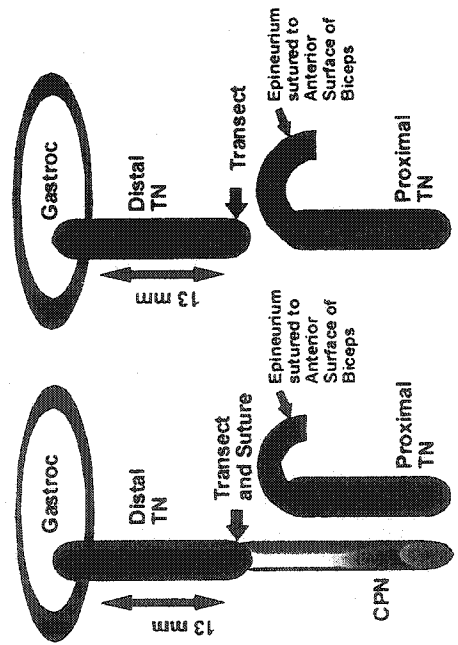
C.



A.



D.



Nerve-to-Muscle

Nerve-to-Nerve

Immediate Motor Repair

Unprotected Control

stump (Figure 1C). A 6 month denervation period maximized the effect of axon sprouting *in vivo* (Kobayashi et al, 1997).

In all groups, the tibial nerve was stimulated using a portable surgical nerve stimulator (Model 85-62010, C-Line, Vary-Stim III, Xomed-Treace) prior to the second surgery, and the gastrocnemius muscle was examined for evidence of muscle contractions which would have indicated ectopic motor innervation, resulting in the animal being excluded. Similarly, any sign of dehiscence of the nerve repair would have led to animal exclusion.

### **Morphometric Assessments**

Final assessments were performed on all animals 3 months following the second reinnervation surgery in sensory protected (N-N, N-M) and UP groups. The Immediate Repair group animals were also assessed 3 months after the initial surgery.

Ultrastructural examination of tissue was performed following electrophysiological and contractile assessments (Bain et al, 2001). Animals were killed by an intracardiac overdose of sodium pentobarbital (65 mg/ml).

### **Nerve Processing**

Three representative samples of distal tibial nerve from each group were used for analysis. In each group, a segment from the distal intramuscular portion of the tibial nerve (7 mm) was harvested at a standard distance (20 mm) distal to the neurotomy site (Figure 1D). The nerve segment was oriented to identify the distal and proximal ends, and immediately immersed in Bilbao's primary fixative (2.5% glutaraldehyde in 0.025M sodium cacodylate buffer, pH 7.4) at 4° C, for a minimum of 1 hour. Specimens were then post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.4 (for 1 hour at room temperature), then dehydrated in graded ethanol. Nerve segments were embedded in Epon-Araldite and polymerized at 65° C. Transverse semithin sections (0.5-1.0 µm) were cut using a Reichert Ultracut S microtome (Leica, Vienna, Austria) and stained with 1% toluidine blue in 1% sodium borate (David and Aguayo, 1985).

The present study combines automated image analysis with a sampling scheme used by Ghalib et al (2001). Briefly, 6 randomly selected cross-sections from each nerve specimen were digitized using a light microscope. Morphometric evaluation of axon area and number was performed using computer-assisted image analysis software (Metamorph morphometric software, Version 4.6.7., 1992-2001). Inner perimeters of myelinated axons were used to calculate axon area.

#### **Distal Tibial Nerve: Light Microscopy**

Semithin toluidine blue-stained (David and Aguayo, 1985) nerve sections were examined by light microscopy (Zeiss Axioplan Universal Microscope, Carl Zeiss) and representative regions were selected for morphometric analysis.

#### **Distal Tibial Nerve: Electron Microscopy**

Representative ultrathin sections (~90 nm) chosen from semithin sections of nerve were placed onto copper/palladium 200 mesh grids and counterstained with uranyl acetate (saturated in 50% ethanol) and Reynold's lead citrate (Loeb and Gans, 1986). Specimens were viewed in a transmission electron microscope (Jeol 1200EX Biosystem, Tokyo, Japan) at 80 kV.

#### **Axon Measurement Criteria**

Axon identification was based on the following criteria: the characteristic axolemma defining the axon boundary; axoplasm within the axon boundary was identified by an amorphous and electron-translucent appearance. Filamentous structures such as neurofilaments and microtubules longitudinally oriented along the axon and, on cross-section, identifiable as small round dark structures and donut-like structures on cross-section, within the axoplasm (Jenq and Coggeshall, 1985). Myelinated axons were identified by peripherally-situated darkly-stained myelin and, at higher magnification, periodicity of myelin lamellae (Weiss and Greep, 1977).

A high degree of myelination is represented by increased myelin thickness (Nitz and Matulionis, 1982). Regeneration is identifiable by a high variability in myelin thickness due to small immature unmyelinated, thinly myelinated or axons undergoing myelination by Schwann cells, interspersed by large diameter thickly myelinated axons occupying a single trough of Schwann cell cytoplasm (Jenq and Coggeshall, 1985).

#### **Schwann Cell Identification**

Schwann cells were identified by the presence of a large nucleolus and cytoplasm encircling myelinated or unmyelinated axons (Weiss and Greep, 1977) and a double layer of basement membrane. Apoptotic Schwann cells were defined by dark regions of coarse chromatin “clumping” and loss of cytoplasmic organelles (Grinspan et al 1996).

#### **Interaxon Collagen Identification**

Deposits of interaxonal collagen were noted after viewing nerve cross-sections with a light microscope. Collagen was identifiable by its characteristic features including fibril arrangements of irregular orientation and circular domains of collagen fibrils (sometimes associated with a fibroblast) interspersed between the axons, and periodicity (Bradley et al 1998).

#### **Morphometric Analysis of Nerve**

##### **Light Microscopy**

Stereologic digitized images of semithin cross-sections of nerve were captured using a camera (Photometrics Cool Snap HQ) mounted to a computer-assisted light microscope (Zeiss Axioplan Universal Microscope, Carl Zeiss). Measures of axon number and area were obtained using Metamorph morphometric software (Version 4.6.7., 1992-2001).

### Electron Microscopy

Morphometric analyses of collagen total area, Schwann cell total area and myelin sheath thickness were performed on glossy electron micrographs from ultrathin cross-sections of nerve, using a stereologic computerized image analysis system (Kontron MOP-Videoplan, Version 5.4).

### Morphometric Sampling of Nerve

Mean axon number and area were based on light microscopic digitized images from representative regions of distal nerve, photographed (63X magnification) and calibrated to a standard reference image area (Kroeber et al, 2001).

Roughly 20% of total tibial nerve axons (tibial nerve contains approximately 1000 axons) were sampled in experimental groups in which axon density was sufficient. In the UP group, fewer than 100 axons were available for examination. However, when equal sample sizes were considered for all experimental groups, statistical analyses of means remained unchanged.

Measurements of collagen area, Schwann cell area and mean myelin sheath thickness were obtained from enlargements of original electron micrographs taken from representative regions of distal tibial nerve. Only regions occupied by nerve were contained within the reference areas.

Mean myelin sheath thickness was measured on a minimum of 10 randomly selected axons from each group. The distance ( $\mu\text{m}$ ) between the inner and outer myelin sheath was recorded at three arbitrarily chosen sites at the periphery of the axon, and the mean of the 3 measurements was used (Ghalib et al, 2001).

## **Muscle**

### Muscle Ultrastructure: Processing

In all groups, the same region of the lateral gastrocnemius was harvested (Figure 1D) and transverse muscle sections were processed for ultrastructural analysis according to the protocol described above for processing of nerve.

### **Assessment of Muscle Fibre Morphology**

Cross-sections of representative samples of muscle were examined by light microscopy and electron microscopy by an independent blinded assessor.

### **Muscle Histochemical Processing**

The lateral gastrocnemius muscle was detached from its tendinous insertion, cut transversely, oriented, embedded in optimal cutting temperature (O.C.T.) embedding medium (Tissue Tek, Sakura Finetechnical Co. Ltd, Tokyo) and immediately immersed in liquid nitrogen. Samples were stored at  $-70^{\circ}\text{C}$  until use.  $10\ \mu\text{m}$  cross-sections were cut in a cryostat at  $(-20^{\circ}\text{C})$  and mounted on glass slides.

### **Myofibrillar Adenosine Triphosphatase (ATPase) Reaction**

The slides containing muscle samples were stained for myofibrillar ATPase (ATP, Sigma A2383) following separate incubations at pH 4.3 and pH 10.0, based on the protocol described by Dubovicz (1985). Slides were mounted using Permount (Fisher SP15-100) and stored for morphologic and morphometric analysis.

### **Muscle Fibre Morphometry**

#### **Fibre Type Classification**

Muscle fibre type classification and fibre measurements were based on the method described in Daemen et al (1998). Briefly, fibre measurements were determined from digitized images captured by a camera mounted to a light microscope (Olympus BX60, United Kingdom) attached to a desktop computer with morphometric software (Image Pro Plus, Version 4 for Windows, 1993-1998, Media Cybernetics L.P.).

#### **Muscle Fibre Area**

Muscle fibre cross-sectional areas and relative proportions were measured from non-overlapping light microscopic images selected from several representative muscle regions (Fu and Gordon, 1995a,b) and



based on a sample of approximately 200 fibres per group (Karpati and Engel, 1968). Only whole fibres contained within each field were included.

### Statistical Analysis

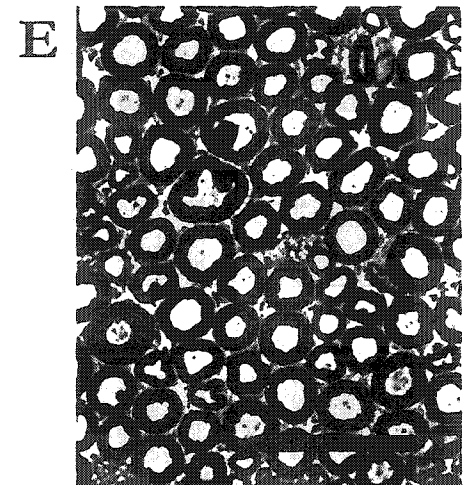
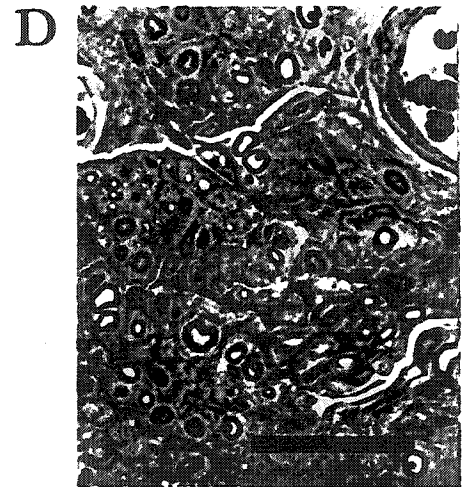
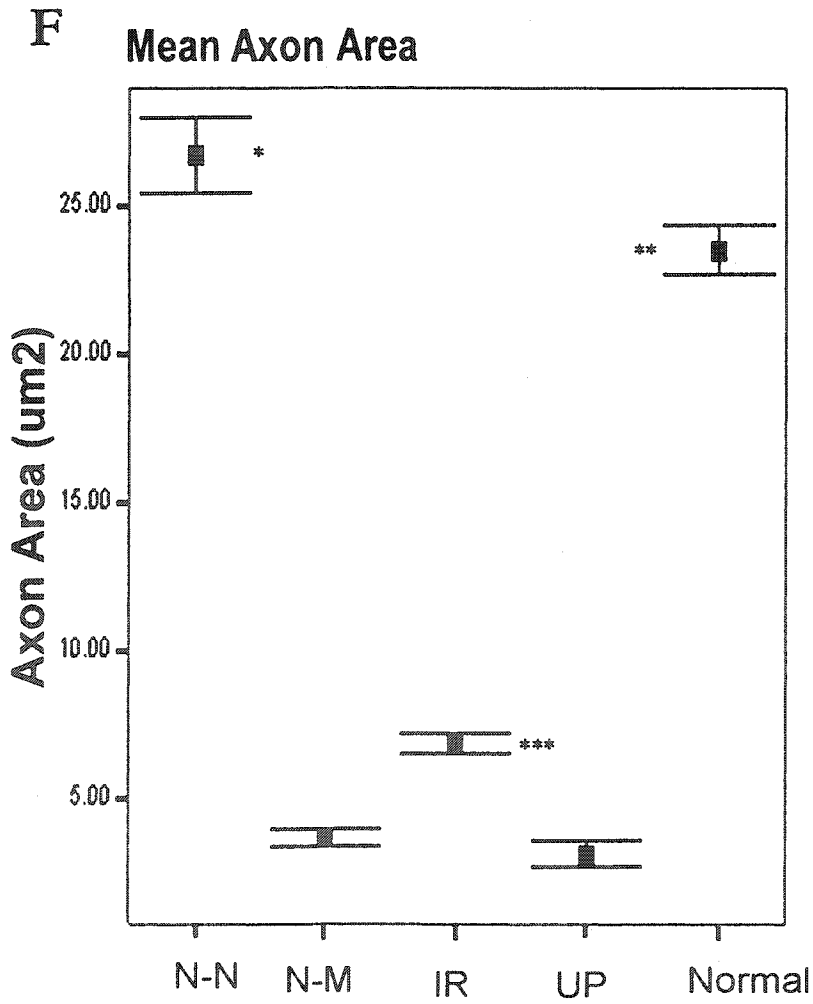
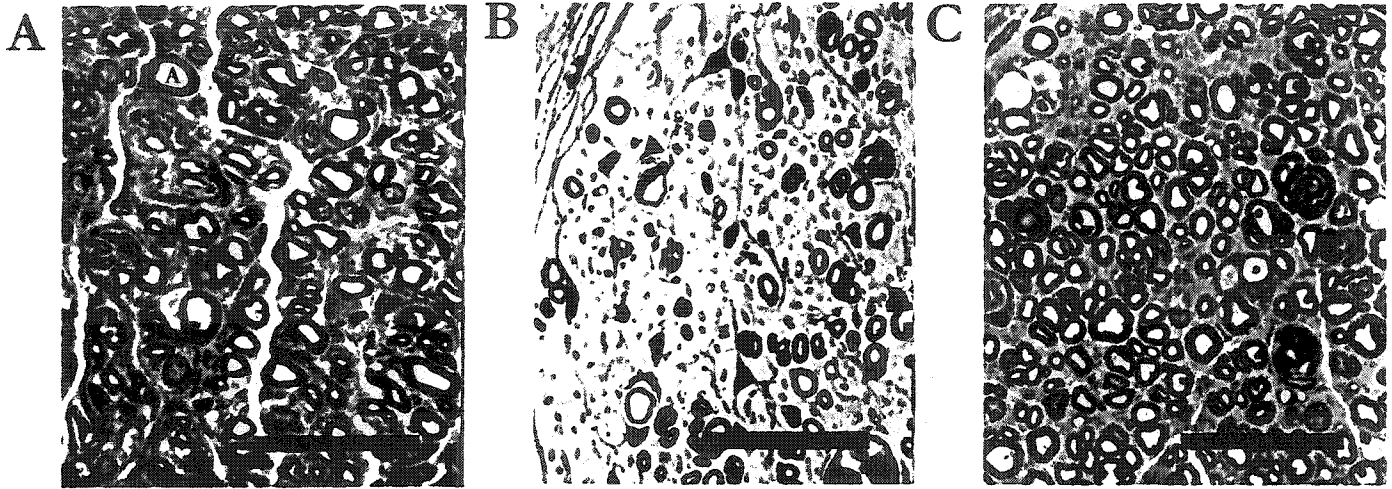
Morphometric data from dependent variables (axon density, axon area, collagen area, Schwann cell area, myelin sheath thickness, muscle fibre area) were subjected to one-way analysis of variance (ANOVA) using SPSS (Statistical Software Version 8) followed by a post hoc Tukey test for intergroup comparisons. A *p* value of less than 0.05 ( $p < 0.05$ ), generated from the analysis of variance, was deemed to be statistically significant. Data are presented as means with standard error.

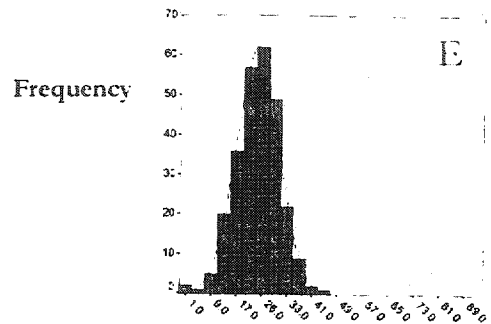
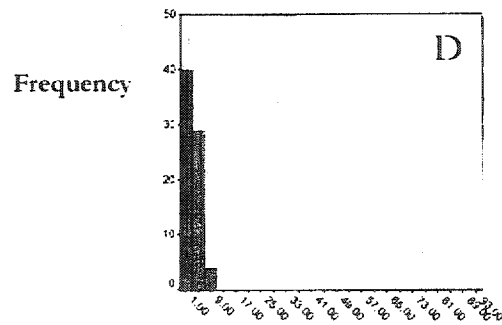
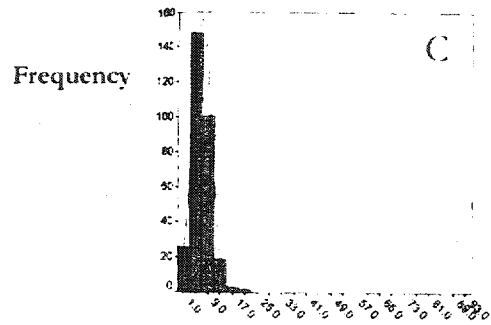
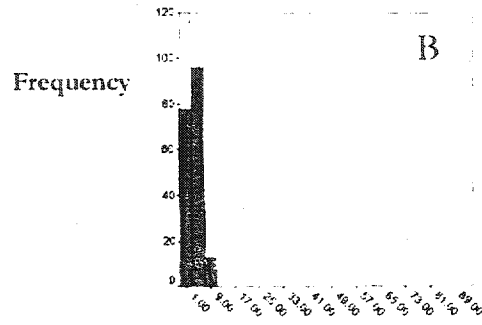
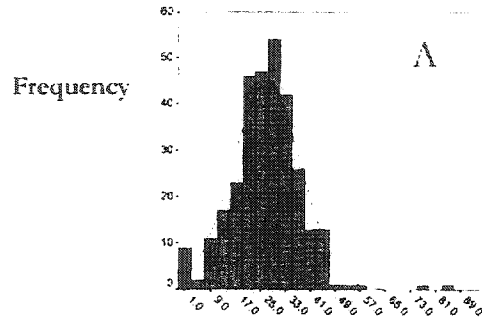
### Results

One animal in the N-N group died during the initial surgery and 4 animals in the UP group died during the second surgery. Thus, 33 of the initial 38 animals were used for final assessments. We saw no evidence of ectopic reinnervation or nerve dehiscence in any of the remaining animals.

### Nerve

Normal tibial nerve was similar to previous studies (Ghalib et al, 2001; Nitz and Matulionis, 1982), with homogeneity in myelination, predominance of tightly-spaced, large, thickly-myelinated axons, and a negligible amount of interaxonal collagen (Figure 2E). Axon areas in the normal group were significantly larger (Figure 2, Panel F) than in the remaining groups except for the N-N group and were normally distributed (Figure 3, Panel E). The N-N group (Figure 2A) exhibited a significantly larger axon area (Figure 2F) compared to the N-M group (Figure 2B) and an axon area distribution pattern similar to normal (Figure 3, Panels A,E). The N-M group (Figure 3, Panel B) featured an extreme left-skewed distribution of axon areas. However, the IR group (Figure 2C) contained significantly larger axons than the N-M and UP groups (Figure 2F) and significantly smaller axons than the N-N and Normal group (Figure 2F). The IR group featured an extreme left-skewed distribution of smaller axons (Figure 3C). By contrast, UP controls





Axon Area (um<sup>2</sup>) (excluding myelin)

(Figure 2D) exhibited a significantly smaller axon size relative to all other groups except the N-M (Figure 2F). Notably, the IR group (Figure 4C) contained significantly higher axon numbers than normal (Figure 4F). Axon number in the N-N, N-M and normal groups was similar (Figure 4A,B,E,F), was significantly reduced compared to the IR group (Figure 4C,F), and significantly increased relative to the UP group (Figure 4D,F). The axon number in the UP specimen (Figure 4D) was significantly smaller than all other groups (Figure 4F) with an extreme left-skewed distribution shown in Figure 3 D.

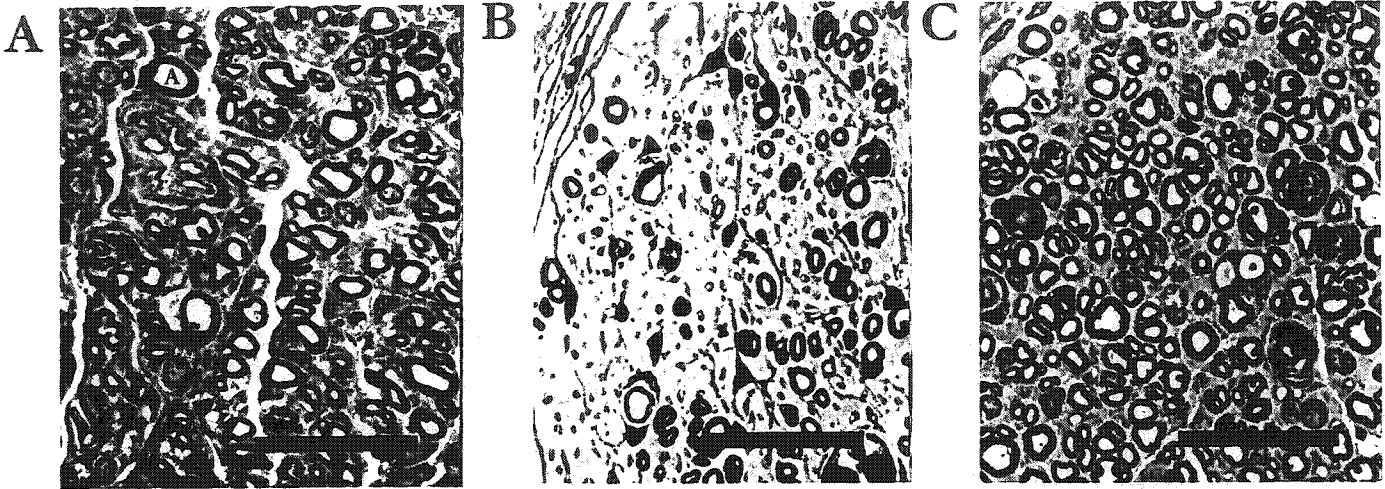
In agreement with previous reports (Nitz and Matulionis, 1982), collagen content of the Normal nerve specimen was minimal (Figure 5E). UP nerve (Figure 5D) and that of the N-M group contained a significantly higher mean area of collagen relative to all other groups (Figure 5F). Notably, the low collagen content of the N-N group was similar to normal (Figure 5F), and was significantly reduced 5-fold relative to the N-M group and UP group (Figure 5F). The IR group (Figure 5C) contained significantly less collagen than the N-M and UP groups (Figure 5F).

In the UP nerve specimen, several darkly stained Schwann cells with nuclear pycnosis and karyorrhexis lay adjacent to dying Schwann cells (data not shown), similar to changes observed in apoptotic Schwann cells by Grinspan et al (1996). The N-N specimen featured a predominance of one-to-one axon-to-Schwann cell coupling (Figure 5A), a feature of Normal nerve (Syroid et al, 1996).

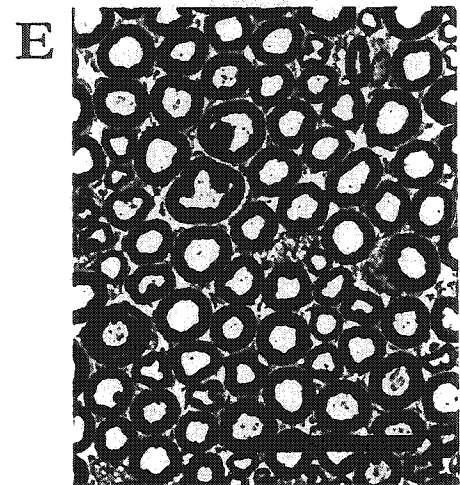
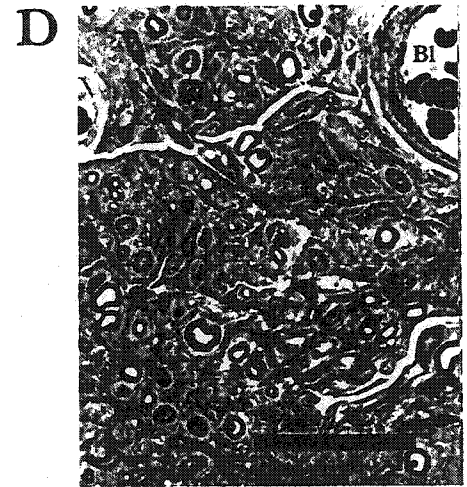
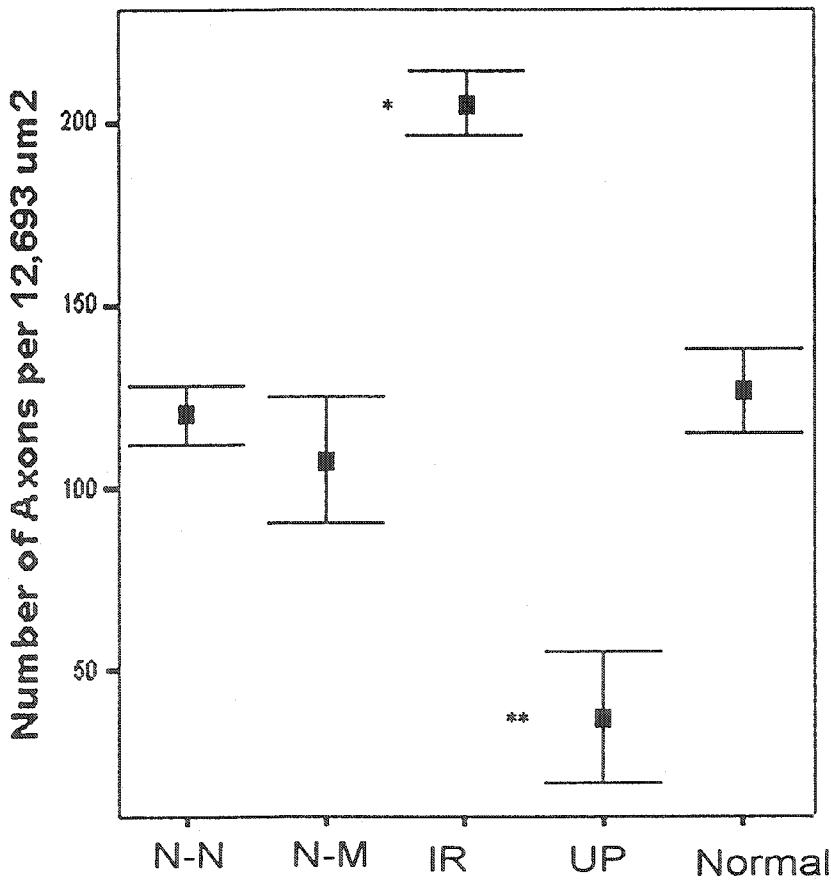
The myelin sheath surrounding axons in the normal nerve specimens was thicker in relation to the remaining groups (Figure 6E, F). Morphometric analysis revealed a significantly increased myelin sheath thickness in the normal group relative to the remaining groups with no significant difference among the remaining groups (Figure 6F)[from Kroeber et al 2001 Mean myelin thickness =  $2.1 \pm 0.4 \mu\text{m}$  (n=8)] .

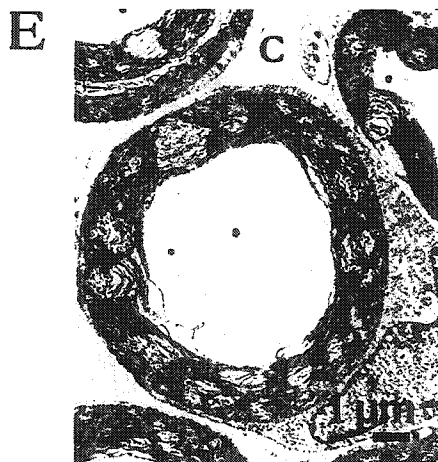
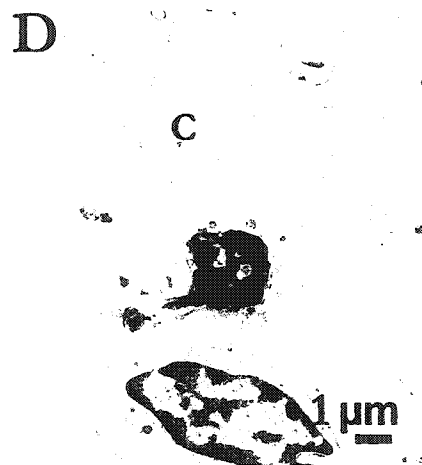
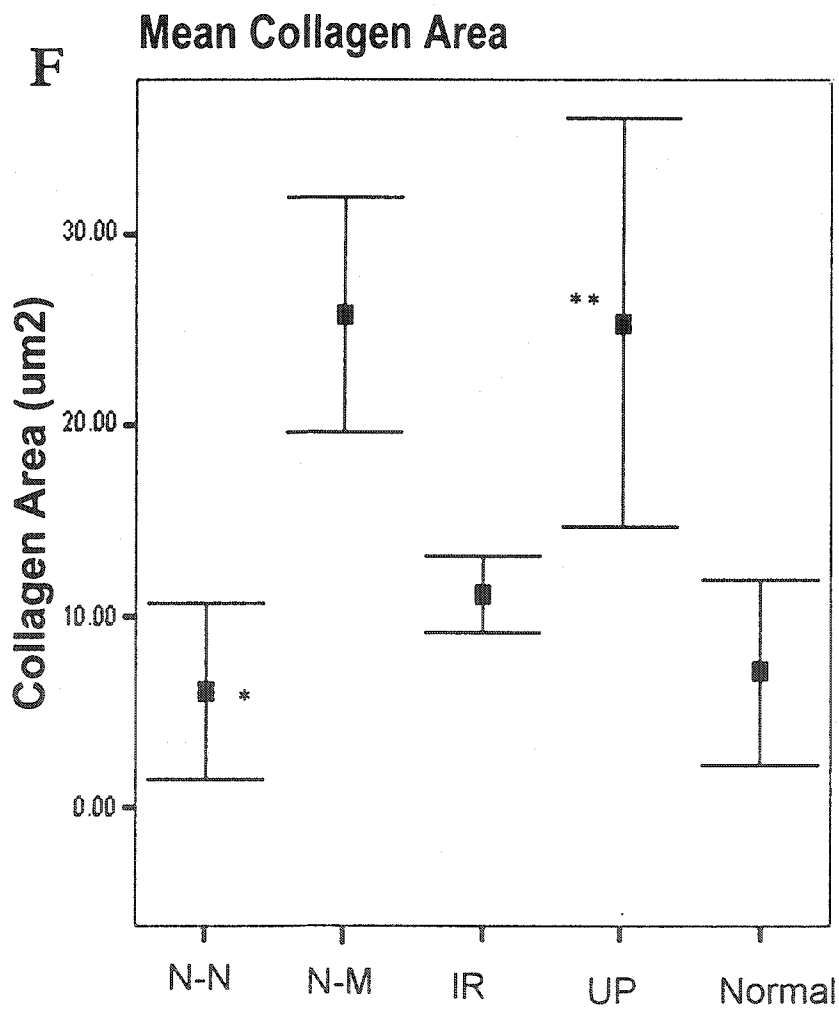
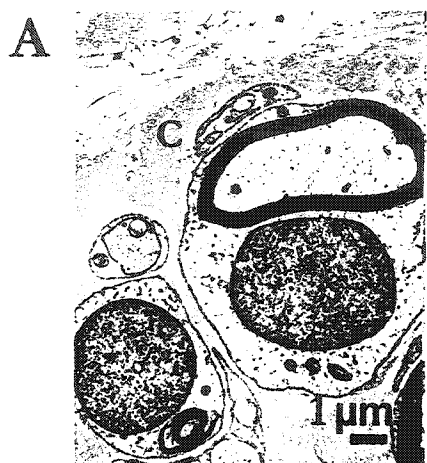
## Muscle

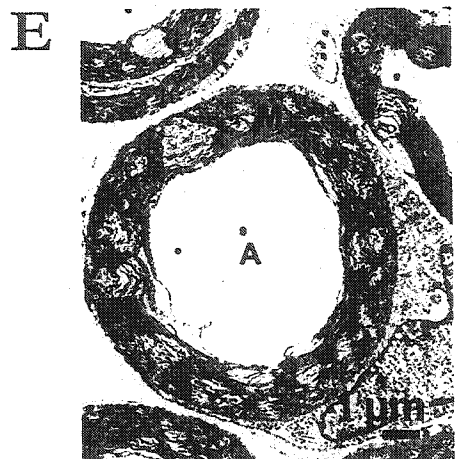
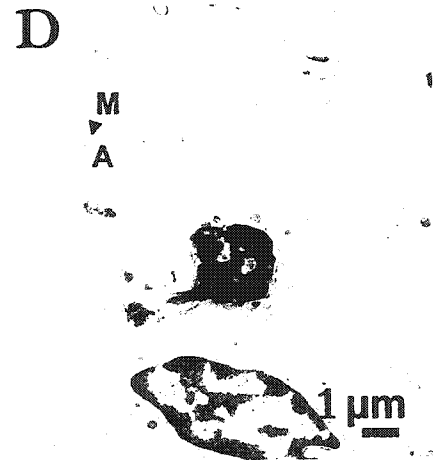
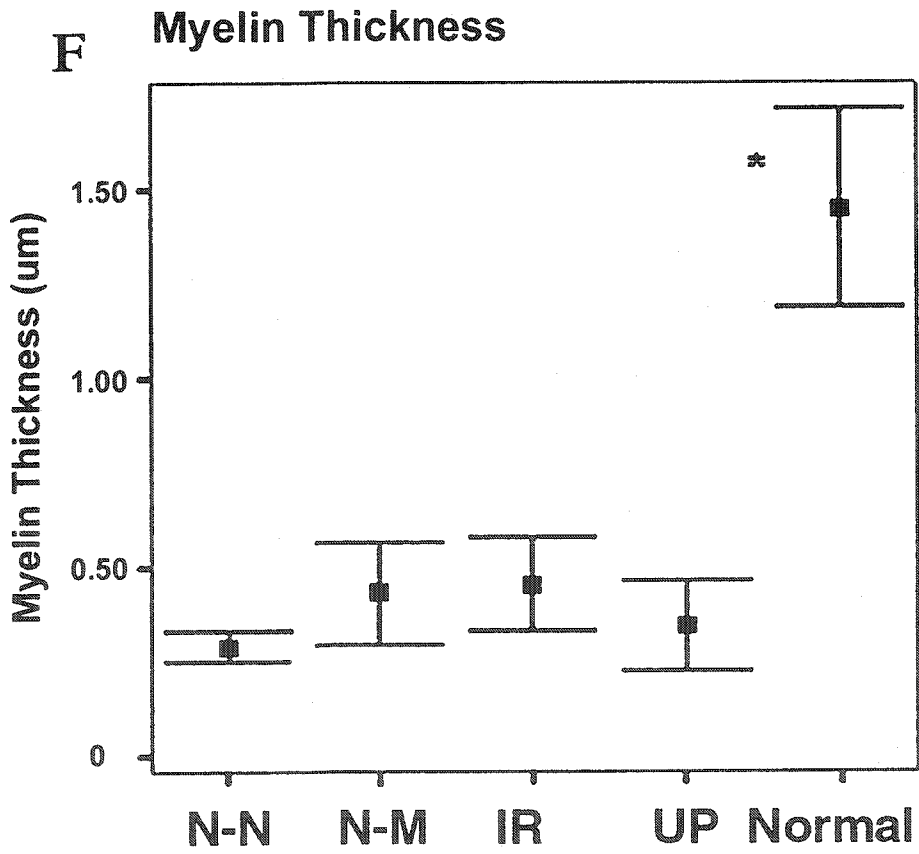
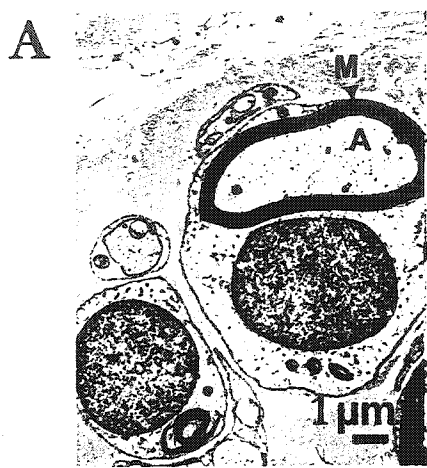
The appearance of normal muscle cross-sections under the transmission light microscope was similar to other published literature (Cormack, 1987). These included tightly-arranged, large, homogeneous, polygonal fibres interspersed with a minimal amount of interstitial collagen (Figure 7E). The N-M specimens (Figure 7B) demonstrated a morphology almost identical to normal muscle (Figure 7E) with



F Mean Number of Axons





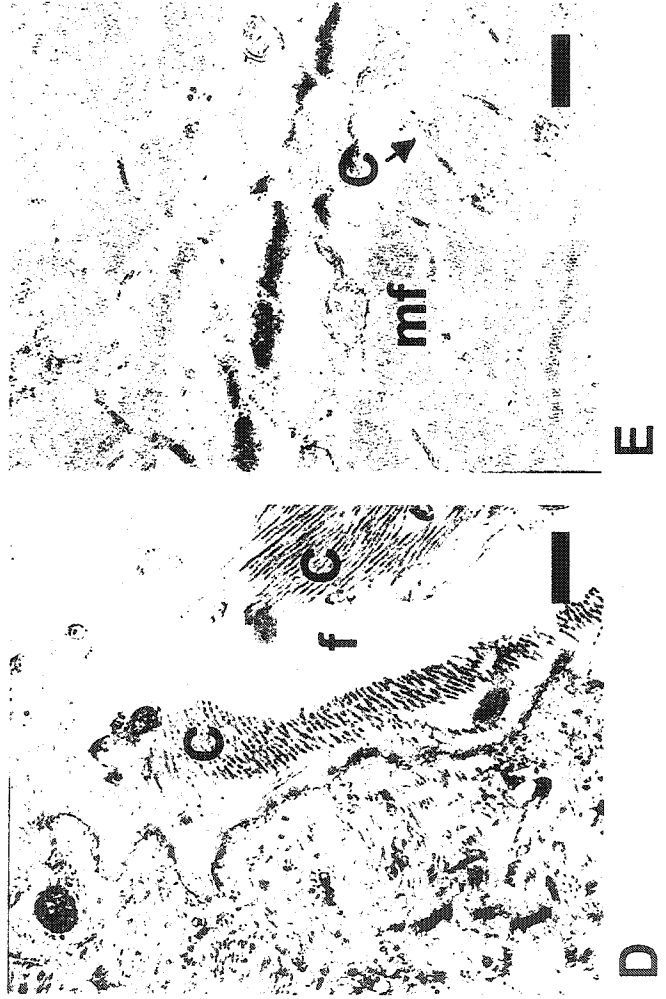
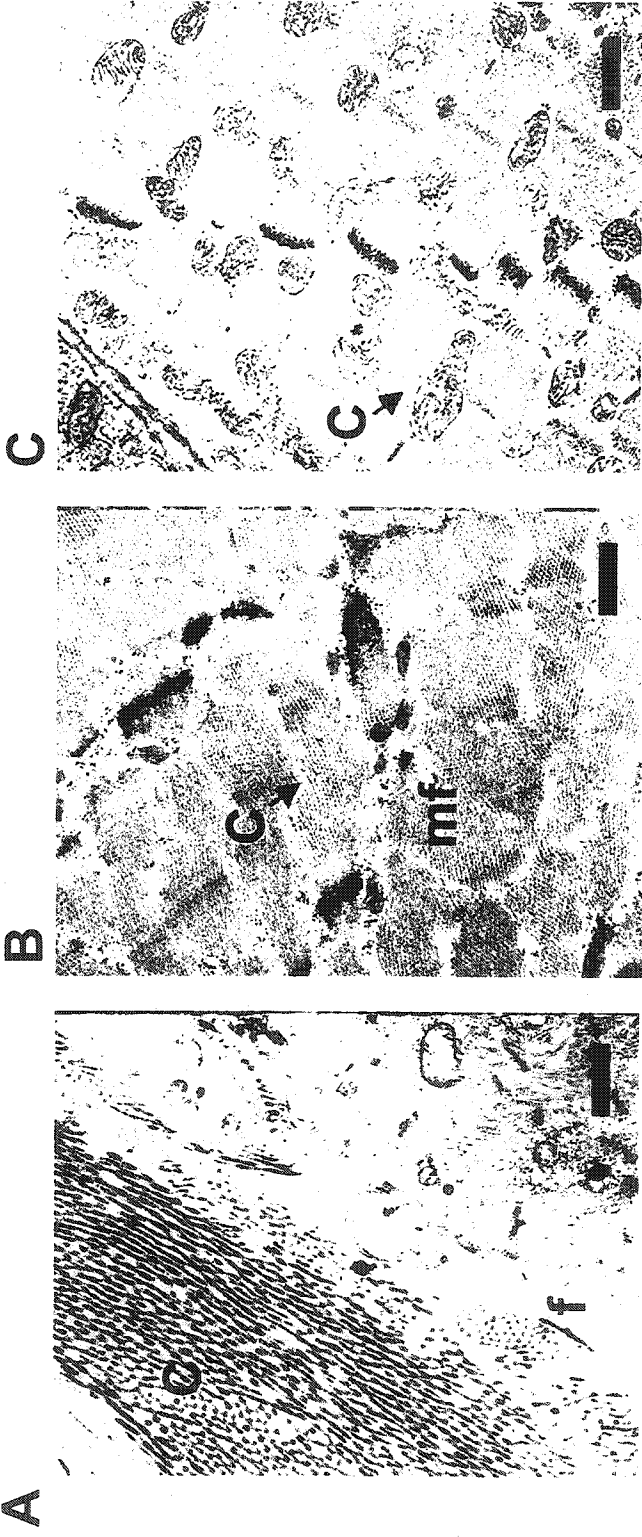


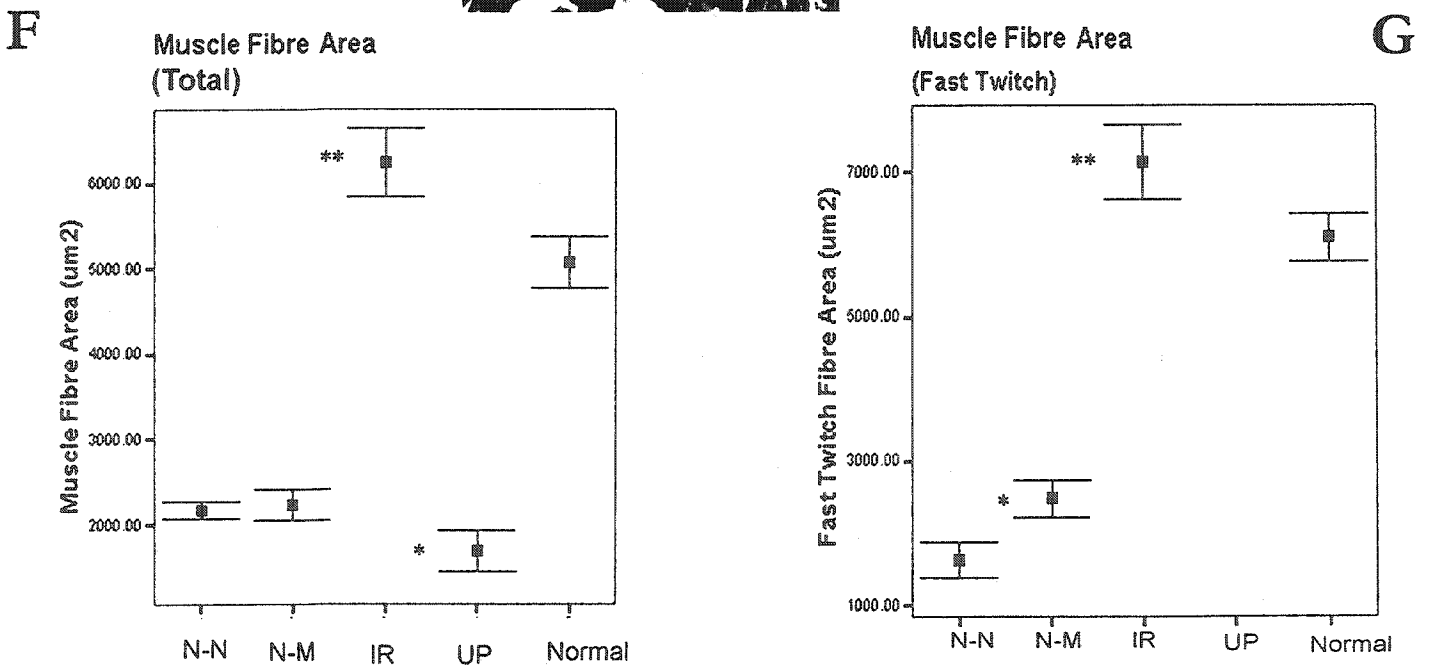
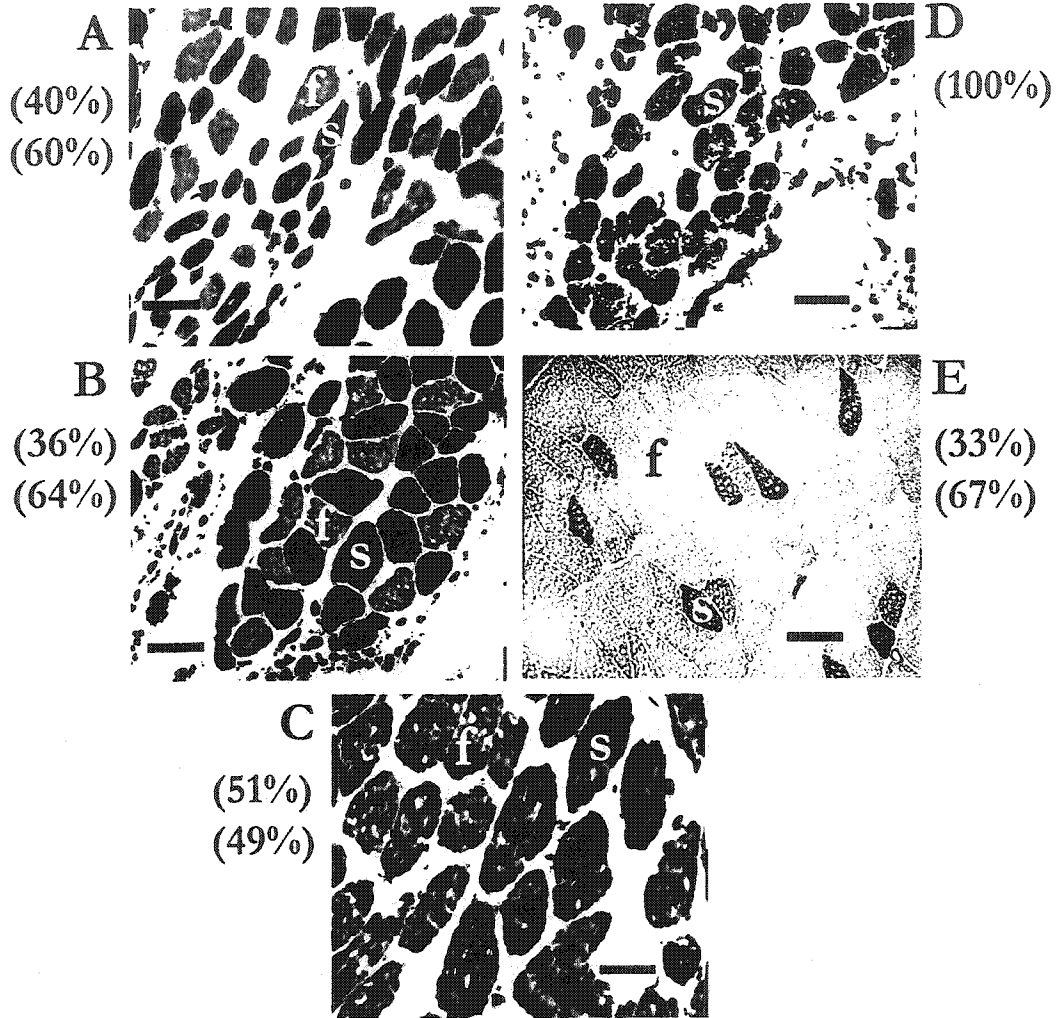
large, homogeneous, tightly-arranged fibres and minimal interstitial fat and collagen. The N-N group (Figure 7A) and UP group (Figure 7D) appeared similar to each other, but different from normal. The N-N muscle (Figure 7A) and UP muscle (Figure 7D) contained large areas of abnormally-arranged, rounded, atrophic fibres interspersed with a considerable amount of interstitial degenerative tissue observed to be fat and collagen as previously reported (Bain et al, 2001).

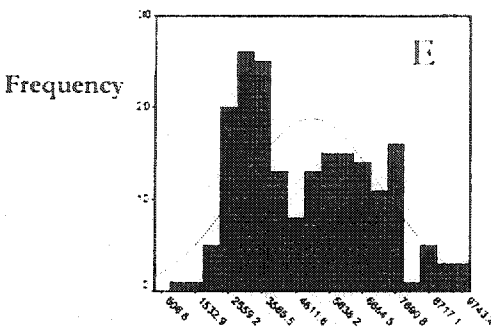
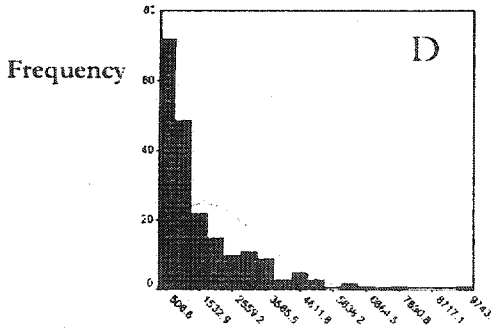
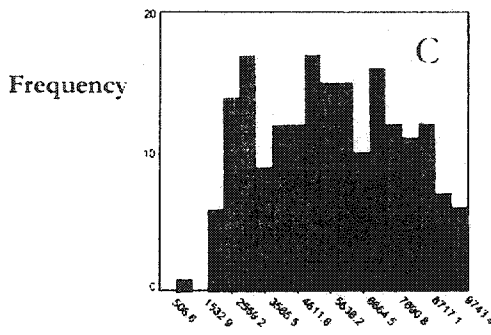
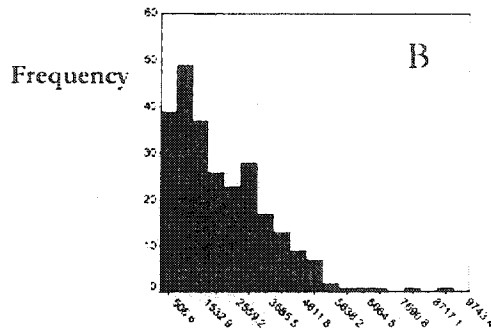
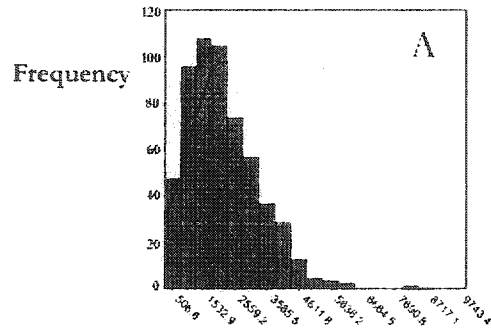
The myofibrillar ATPase-stained cross-sections of normal gastrocnemius muscle featured a mosaic appearance of overlapping fast and slow-twitch fibres with relatively more fast twitch fibres (Figure 8E), in agreement with the literature (Karpati and Engel, 1968). Immediate Repair specimens showed some similarity to normal muscle but with some evidence of fibre type grouping (Figure 8C). The N-M specimens (Figure 8B) demonstrated a strikingly similar mosaic pattern to normal (Figure 8E), among the patchy atrophic regions and small zones of fibre type grouping. The N-N specimen (Figure 8A) showed less mosaicism with slightly more fibre type grouping. Among the sensory protected groups (Figure 8A,B), the relative proportion of fast to slow twitch fibres was similar to Normal (Figure 8E). In contrast, UP muscle (Figure 8D) contained only slow twitch fibres.

Of note was the significantly larger total muscle fibre area in the IR specimens relative to normal (Figure 8F), the larger fast twitch fibre area (Figure 8G) and the almost equal proportion of fast and slow twitch muscle (Figure 8C, Figure 9C). However, the frequency distribution showed a wider variation in muscle fibre areas in the IR group (Figure 9C) compared to normal (Figure 9E) as previously reported (Schmalbruch, 1986). Unprotected control muscle had significantly reduced total muscle fibre area (Figure 8F) and no fast-twitch fibres (Figure 8G). Although the total fibre areas for the N-N and N-M groups were similar (Figure 8F), the fast twitch fibre area in the N-M specimen was significantly larger than that of the N-N group (Figure 8G). The frequency distribution pattern of the total muscle fibre areas showed a more left-skewed distribution for the N-N group (Figure 9A) relative to the N-M group (Figure 9B).









## Discussion

This study corroborates our previous work (Hynes et al, 1997; Bain et al 2001) demonstrating the positive effect that long-term “sensory protection” confers on denervated skeletal muscle. In the present study, the lateral gastrocnemius was used since it has been reported previously that in rabbits and rats, sprouts of a transposed motor nerve form new neuromuscular junctions in an aneural region (contains no motor endplates) of denervated muscle, which has been shown to be the lateral head of the gastrocnemius (Brunelli 1982).

### Maintenance of Existing Distal Endoneurial Sheath Integrity Facilitating Regeneration

This study provides evidence that the integrity of the existing distal endoneurial sheaths is maintained by sensory axons within the sheaths. Collagen levels are similar to normal, an indication of reduced collagenization of the sheaths, as reported by Fu and Gordon (1995a, 1995b) in their study using a motor-motor nerve repair. Negligible apoptotic Schwann cells and a predominance of the normal axon-to-Schwann cell couplings, along with an overall large axon size distribution provides further evidence of preservation of the normal nerve environment by sensory axons within the distal endoneurial sheaths. These features suggest that sensory axons within the sheaths during denervation reduce the long-term denervation changes in the distal nerve stump (Waller, 1850).

Reinnervation capacity in the distal nerve environment is preserved so that regenerating motor axons can elongate along a favorable substrate to reach their target muscle fibres. Evidence of successful regeneration is demonstrated in the N-N group by axons of various sizes in different stages of myelination which reflects concurrent axonal degeneration and regeneration (Gutmann and Young, 1944).

In the absence of the distal endoneurial sheath (N-M group), chronic degeneration changes are more pronounced and regenerative capacity is impaired.

### Maintenance of Existing Muscle Fibre Architecture and Receptiveness to Reinnervation

Impaired motor recovery following denervation has been associated with a lack of muscle fibre

receptiveness to reinnervation due to muscle fibre necrosis (Gutmann, 1948) and fibrous tissue deposition (Gutmann and Young, 1944). In general, sensory nerve protection preserves muscle fibre architecture and these findings are supported by our previous work (Hynes et al, 1997; Bain et al, 2001) and others who report that muscle fibre architecture was maintained following dorsal root ganglia (DRG) implantation in muscle during denervation (Wang et al, 2001).

It has been previously reported that collagen synthesis is increased in muscle following a period of denervation (Savolainen et al, 1988), as we observed in the Unprotected control muscle. Sensory protection minimized the replacement of healthy fibres by collagen, fibrotic tissue and fat when sensory axons directly influenced muscle fibres, i.e. neurotization. Evidence of this is demonstrated by fibre shape, arrangement and negligible intermysial deposits of collagen, fibrin and fat, which is similar to normal. However, preservation of total muscle fibre size occurs to the same extent regardless of the path taken by sensory axons to the muscle (within the endoneurial sheaths as in the N-N, or directly i.e. N-M).

Unprotected control muscle showed a substantial shift in fibre type distribution toward slow-twitch properties in accordance with other studies on denervated muscle (Fu and Gordon, 1995b). This may suggest that slow twitch fibres are more resilient and fast twitch fibres more susceptible to loss of innervation. Both groups of sensory protected muscle showed evidence of reinnervation (fibre type grouping) as shown by Hynes et al and the concurrent preservation of the normal mosaic fibre arrangement which may suggest that sensory axons target fast-twitch fibres to prevent their loss during denervation. This occurs independent of the endoneurial sheath. However, in the absence of the endoneurial sheath, sensory axons exerted a more pronounced effect demonstrated by significantly larger fast-twitch fibres in the N-M group in contrast to the N-N group. This may be attributed to the influence of sensory axons in directly regulating the distribution of factors to the vulnerable fast-twitch muscle fibres, independent of the endoneurial sheath. The "enclosed fibre method" has also been used to define measures of randomness of selecting regions of muscle from which to assess the number of enclosed fibres in individual fascicles throughout the muscle, and allows quantification of the fibre-type arrangements (Lexell et al, 1983; Lexell et al, 1986; Lexell et al, 1989; Lexell and Downham, 1991).

The present study suggests two possible mechanisms of sensory protection. Sensory axons within distal endoneurial sheaths preserve the structural integrity of the sheaths until appropriate reinnervation occurs. Sensory axons may also directly or indirectly support denervated muscle fibres by influencing the supply of substances such as neurotrophic factors, neurotransmitters, or other molecules (Griesbeck et al, 1995). Upon distal nerve transection, sensory neurons release and deliver BDNF to the denervated stump (James et al, 1998) and NGF is reported to influence the release of BDNF (Michael et al, 1997 ). Under these conditions, muscle function has been reportedly improved. Others reported that sensory axons can release acetylcholine (Falempin et al 1989).

The findings of the present study are consistent with our previous work (Bain et al, 2001) confirming the beneficial effect of sensory protection on long-term denervated skeletal muscle. Our preliminary conclusion suggests that sensory-motor nerve repair appears to preserve the distal endoneurial sheath architecture and therefore provide a favourable regeneration substrate. There may also be a direct influence of sensory axons in sustaining the muscle fibres during denervation. This sensory nerve influence appears to preferentially influence fast twitch fibres. Confirmation of improved state of intramuscular axonal anatomical features using alternative methodology as well as the specific changes in neurotrophin factor profiles is under investigation by our group.

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**Figure Legends**

Figure 1 Diagrammatic representation of microsurgical techniques. The distal tibial nerve was exposed in the posterior thigh, mobilized from the popliteal vessels, and transected approximately 13 mm proximal from the nerve entry point into the muscle.

(SN=saphenous nerve; TN=tibial nerve; Gastroc=gastrocnemius;

CPN=common peroneal nerve)

A. Sensory Protection Techniques. "Sensory protection" was provided by mobilizing the ipsilateral saphenous nerve from the distal aspect of the hindlimb from the medial side of the ankle proximally to just above the popliteal fossa. The proximal saphenous nerve was then transposed from this anterior location through muscle to the posterior aspect where it was sutured to the distal tibial nerve stump in an end-to-end fashion (Nerve-to-Nerve, N-N(SP)) or was directly neurotized to the region above the motor endplate of lateral gastrocnemius muscle (Nerve-to-Muscle, N-M (SP)). The proximal stump of the tibial nerve was further mobilized from the sciatic nerve and the epineurium was sutured onto the anterior surface of the biceps femoris muscle using 10-0 nylon. The duration of sensory protection was 6 months.

B. Controls. (Immediate Motor Repair, IR) The ipsilateral common peroneal nerve was mobilized from the sciatic nerve, transected distally, and the epineurium of the common peroneal nerve stump was immediately sutured to the distal stump (following denervation) of the tibial nerve using 10-0 nylon. The proximal stump of the tibial nerve was further mobilized from the sciatic nerve and the epineurium was sutured onto the anterior surface of the biceps femoris muscle using 10-0 nylon. (Unprotected Control) Following mobilization and transection of the tibial nerve, 10-0 nylon suture was tied around the distal tibial nerve stump to facilitate future identification of the neuroorrhaphy site. The proximal stump of the tibial nerve was further mobilized from the sciatic nerve and the

epineurium was sutured onto the anterior surface of the biceps femoris muscle using 10-0 nylon. The duration of denervation was 6 months.

C. Second Surgery (Motor Reinnervation). A second surgery was performed following the “sensory protection” or denervation interval. The original neurotomy site was re-exposed and examined for evidence of dehiscence and muscle contraction upon nerve stimulation. In “sensory protection” groups, the saphenous nerve was removed from its distal site. The ipsilateral common peroneal nerve was mobilized from the sciatic nerve, transected distally, and the epineurium of the proximal stump was sutured to the distal stump of the tibial nerve using 10-0 nylon.

D. Diagrammatic representation of site of procurement of distal tibial nerve and gastrocnemius muscle. A segment (7mm) of the tibial nerve was harvested from a distal site within the gastrocnemius muscle, placed on a wooden stick, oriented in a proximal to distal direction, and immersed in cold fixative. A portion of the gastrocnemius muscle was harvested from a site just above the motor endplate zone.

Figure 2

Morphometric analysis of axon area. The distal tibial nerve was harvested at a standard distance distal to the neurotomy site, placed in fixative and dehydrated. The nerve segment was oriented to identify the distal and proximal ends then was embedded in plastic, sectioned transversely and mounted on glass slides. The sections were then stained with toluidine blue. Three representative samples of nerve from each experimental group were used in the analysis. Six randomly selected images from each nerve specimen were digitized using a camera (Photometrics Cool Snap HQ) mounted to a computer-assisted light microscope and morphometric analysis was performed by an independent observer using computer-assisted image analysis software (Metamorph, Version 4.6.7, 1992-2001). Mean axon area was calibrated to a standard reference image area. Statistically significant findings ( $p < 0.05$ ) are indicated by asterisks. (“A”=axon) (Scale Bar = 3 mm ) (Final Magnification = 500 X)

\* N-N vs. N-M \*\* Normal vs. IR, N-M, UP \*\*\* N-M vs. IR

A. Nerve-to-Nerve (N-N) B. Nerve-to-Muscle (N-M)

C. Immediate Repair (IR) D. Unprotected Control (UP)

E. Normal F. Graph representing mean values  $\pm$  standard error of the mean (SEM).

Figure 3 Frequency distributions of mean axon areas of the distal tibial nerve (x-axis normalized to  $93 \mu\text{m}^2$ ).

A. Nerve-to-Nerve (N-N) B. Nerve-to-Muscle (N-M)

C. Immediate Repair (IR) D. Unprotected Control (UP)

E. Normal

Figure 4 Morphometric analysis of the mean number of axons. Measurements were based on six randomly selected digitized light microscopic images of toluidine blue stained nerve cross-sections. Statistically significant findings ( $p < 0.05$ ) are indicated by asterisks.

\* IR vs. all groups \*\* UP vs. all groups

("A"=axon; "Bl"=blood vessel; "C"=collagen ) (Scale Bar = 3 mm ) (Final Magnification= 500 X ).

A. Nerve-to-Nerve (N-N) B. Nerve-to-Muscle (N-M)

C. Immediate Repair (IR) D. Unprotected Control (UP)

E. Normal F. Graph represents mean values  $\pm$  standard error of the mean (SEM)

Figure 5 Morphometric analysis of mean area of collagen. The distal tibial nerve was harvested, immersed in fixative and dehydrated. The nerve specimens were oriented to identify the proximal and distal ends. The specimens were then embedded in plastic, sectioned transversely and mounted on glass slides. The sections were then stained with toluidine blue. Ultra-thin transverse sections of representative regions of nerve were mounted on copper/palladium 200 mesh grids and counterstained with uranyl acetate and lead citrate and viewed under a transmission electron microscope (80 kV). Three representative samples of nerve from each experimental group were used in the analysis. Using glossy

electron micrograph images of the nerve sections, morphometric analysis was performed using a stereologic computerized image analysis system (Kontron MOP-Videoplan, Version 5.4).

(Collagen = "C" ) Statistically significant findings ( $p < 0.05$ ) are indicated by asterisks.

(Final Magnification = 600 X)

\* N-N vs. N-M, UP    \*\* UP vs. N-N, IR, Normal

A. Nerve-to-Nerve (N-N)    B. Nerve-to-Muscle (N-M)

C. Immediate Repair (IR)    D. Unprotected Control (UP)

E. Normal    F. Graph represents mean values  $\pm$  standard error of the mean (SEM).

Figure 6

Morphometric analysis of myelin sheath thickness. Measurements were taken from glossy electron micrograph images of ultra-thin cross-sections of distal tibial nerve captured by a transmission electron microscope (80 kV). (M=myelin; A=axon) (Final Magnification= 600 X)

A. Nerve-to-Nerve (N-N)    B. Nerve-to-Muscle (N-M)

C. Immediate Repair (IR)    D. Unprotected Control (UP)

E. Normal    F. Graph represents mean values  $\pm$  standard error of the mean (SEM).

\* Normal vs. all groups

Statistically significant findings ( $p < 0.05$ ) are indicated by asterisk.

Figure 7

Qualitative analysis of muscle fibres harvested from the same region from the lateral gastrocnemius muscle. Muscle was immersed in fixative and dehydrated. The muscle specimens were then embedded in plastic, sectioned transversely and mounted on glass slides. The sections were then stained with toluidine blue. Three representative samples of muscle from each experimental group were used in the analysis. Ultra-thin transverse sections of representative regions of muscle were mounted on copper/palladium 200 mesh grids and counterstained with uranyl acetate and lead citrate and viewed under a

(Scale Bar = 1  $\mu$ m) (Final Magnification = 24,000X)

(Fat= "f"; Collagen= "C"; Muscle Fibre= "mf" )

- A. Nerve-to-Nerve (N-N)      B. Nerve-to-Muscle (N-M)  
 C. Immediate Repair (IR)      D. Unprotected Control (UP)  
 E. Normal

Figure 8

Classification of muscle fibre types and measurement of fibre size in lateral gastrocnemius muscle. Muscle specimens harvested from the same region in all animals were embedded in optimal cutting temperature embedding medium and immersed in liquid nitrogen.. Transverse sections were cut and mounted on glass slides and stained for myofibrillar adenosine triphosphatase (ATPase), with pre-incubation at pH 4.3. Qualitative muscle fibre classification and fibre size measurements were performed by an independent observer and based on digitized images captured by a camera mounted to a light microscope (Olympus BX60) and attached to a desktop computer with morphometric software (Image Pro Plus, Version 4 for Windows, 1993-1998). Muscle fibre cross-sectional areas and relative proportions were measured from non-overlapping light microscopic images selected from several representative muscle regions and based on approximately 200 fibres per group. Only whole fibres contained within each field were included. Dark fibres represent slow twitch motor units and are designated with "s". Light fibres represent fast twitch motor units and are designated with "f".

(Scale Bar = 3 mm). (Final Magnification = 40 X ) [UP contained no fast twitch fibres]  
 [Percentages denote slow twitch (upper parentheses) relative to fast twitch (lower parentheses) fibres]

- A. Nerve-to-Nerve (N-N)      B. Nerve-to-Muscle (N-M)  
 C. Immediate Repair (IR)      D. Unprotected Control (UP)  
 E. Normal  
 F. Graph represents mean values  $\pm$  standard error of the mean (SEM).



\* UP vs. all groups    \*\* IR vs. all groups

G. Graph represents mean values  $\pm$  standard error of the mean (SEM).

\* N-M vs. N-N, all groups    \*\* IR vs. all groups

Statistically significant findings ( $p < 0.05$ ) are indicated by asterisks.

Figure 9

Frequency distributions of the total muscle fibre area (slow and fast twitch fibres) of the gastrocnemius muscle (x-axis normalized to  $9743 \mu\text{m}^2$ ).

A. Nerve-to-Nerve (N-N)    B. Nerve-to-Muscle (N-M)

C. Immediate Repair (IR)    D. Unprotected Control (UP)

E. Normal

**N. Prelude to Chapter 5 (Veltri, Bain, Fahnestock)**

Karen Veltri , James R. Bain, Margaret Fahnestock. A functional assessment of sensory protected muscle following denervation. (submitted to J Appl Physiol)

Results presented in Chapter 4 support our previous work confirming the benefit of sensory protection in preserving muscle histology. Additionally, nerve and muscle morphometric data support the mechanisms whereby sensory axons preserve the neural environment and provide a trophic influence on muscle fibres to ensure fibre “receptiveness”.

Histologic parameters, axon density, axon area, myelin thickness, collagen content, and axon-to-Schwann cell coupling are indicators of neural integrity but do not measure the integrity of the connection between the axon and target fibre.

In the next Chapter an identical model to that in Chapter 4 was used to elucidate the mechanisms of sensory protection and evaluation included electrophysiological measurements of nerve and muscle and analysis of isometric twitch and tetanic tension.

The following work has been prepared for submission to J Applied Physiology and is included herein as a manuscript.

## O. CHAPTER 5

### A Functional Assessment of Sensory Protected Muscle Following Denervation

Karen Veltri, James R. Bain, Margaret Fahnestock

Abbreviated Title: Function of Sensory Protected Muscle

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This paper was presented in part at Neuroscience Research Day, Department of Neurology, Hamilton Health Sciences Corporation, Royal Botanical Gardens, Hamilton, Ontario, October 11, 2002 and Annual Meeting of the Group for the Advancement of Microsurgery (GAM), Jasper, Alberta, June 6, 2001 and St. John, New Brunswick, June 12, 2002.

## Abstract

Multiple mechanisms of “sensory protection” have recently been suggested (Veltri et al, unpublished data). The existing distal nerve architecture is preserved by sensory axons within endoneurial sheaths and muscle integrity is maintained when sensory axons are neurotized to muscle, independent of the endoneurial sheath.

The purpose of this study was to determine if these mechanisms are consistent with preservation of muscle electrophysiologic and contractile properties. Following transection of the tibial nerve, rats were assigned to one of the following groups: saphenous to tibial nerve neurotization (Nerve-to-Nerve sensory repair)(SP), saphenous to gastrocnemius neurotization (Nerve-to-Muscle sensory repair)(SP), Unprotected Controls, or immediate common peroneal to tibial nerve neurotization (Immediate motor Repair) or unoperated control (Normal).

After a 6-month period of denervation followed by motor reinnervation, electrophysiologic and force measurements were performed on the gastrocnemius muscle. Mean compound muscle action potential (CMAP) amplitude in the Nerve-to-Nerve (SP) group was significantly higher than the Nerve-to-Muscle (SP) and Unprotected control groups. This may suggest a preservation of endoneurial sheaths and possibly the original motor endplates by sensory axons (as demonstrated by improved ultrastructure, Veltri et al, unpublished data). These electrophysiological data appear to support our previous work whereby we demonstrated improved distal nerve ultrastructure with Nerve-to-Nerve sensory protection. In this paper, we note that this improvement in electrophysiological function (i.e. CMAP) did not translate into improved muscle contractile force. This was shown by similar twitch and tetanic forces between both sensory protected groups. However, in support of our previous work (Bain et al 2001), sensory protection resulted in a significant increase in twitch force compared to Unprotected controls. Of note is that sensory protection does not appear to influence muscle properties of time to peak (TTP) given similarities in these values among the groups. However, these findings contrast the finding that half relaxation time ( $\frac{1}{2}$  RT) and total twitch duration (TTD) was significantly reduced in both sensory protected groups compared to normal, suggesting a possible selective influence of sensory axons on fast twitch motor units, however further

investigation must be undertaken. This study is consistent with our previous conclusions (Bain et al 2001) that sensory protection improves muscle electrophysiology and twitch force compared to no protection. However, despite the preservation of the endoneurial sheath and endplates (Nerve-to-Nerve), twitch force does not improve. This study also raises questions concerning whether sensory axons influence muscle properties, given the variable findings in TTP and  $\frac{1}{2}$  RT. Further studies are necessary to address these issues.

**Key Words:** contractile force, electrophysiology, foreign nerve innervation

## Introduction

Peripheral nerve injury causing muscle denervation is usually associated with severe muscle atrophy leading to functional changes. This is amplified by increasing denervation time as occurs in proximal nerve injuries (Sunderland and Bradley, 1950). A period of denervation greater than 6 months results in serious irreversible muscle characteristics including fibrosis, loss of fibre quantity and reduced cross-sectional area (Irintchev et al, 1990; Helgren et al, 1994), and diminished percentage of motor endplates (Fu and Gordon, 1995a,b).

Causes of impaired muscle function after denervation may include decreased number of neurons or impaired motor axon regeneration into appropriate muscle fibres, loss of substrate and trophic support for regenerating axons in the distal nerve stump, diminished receptiveness of muscle fibres to motor reinnervation possibly resulting from inhibition of Schwann cell proliferation (Love and Thompson, 1998), fibrosis (Sunderland, 1991), reduced activity of the motor endplate enzyme, choline acetyltransferase and biochemical composition (Ribaric et al, 1991), and depletion of the satellite cell pool (Irintchev et al, 1990; Schmalbrach et al, 1991).

Deterioration of the distal nerve endoneurial sheaths due to collagenization impairs motor axon regeneration along the endoneurial sheath toward its target muscle fibre (Morris et al, 1972). Motor axon regeneration directly into muscle fibres (Fu and Gordon, 1995a,b), has been associated with less effective trophic support (Helgren et al, 1994).

Functional correlates of denervated gastrocnemius muscle include reduced nerve conduction velocity, compound muscle action potential (CMAP) amplitude, and isometric contractile twitch and tetanic force (Dumitru, 1995). Normal gastrocnemius muscle consists of mixed fibre types (Guth et al, 1968) with specific contractile rates and duration (Buller and Buller, 1980; Close 1989) consisting predominantly of fast twitch fibres (Gillespie et al, 1986). Denervation causes alterations in distribution of fast and slow-twitch motor units coinciding with changes in contractile properties (Buller and Buller, 1980). Despite the increase in muscle fibre innervation due to collateral sprouting after nerve injury, a decrease in CMAP amplitude is attributed to immature endplates or a decrease in mean axon diameter of damaged axons.

Wallerian degeneration causes disruption of the motor endplate components (Waller, 1850) followed by spontaneous discharge of motor end-plate potentials (MEPPs) (Dumitru, 1995). Within 8 hours of denervation, electrical stimulation of the axon results in normal neuromuscular transmission since synaptic vesicles maintain normal quantal release of acetylcholine (ACh). After 8 hours, however, MEPPs cease and, upon axon stimulation, muscle contraction is absent due to disruption of the endplate.

Atrophic and sparsely distributed myofibrils are unable to generate force at a normal rate resulting in reduced twitch and tetanic force and increase in time to peak (Finkelstein et al, 1993). Twitch speed is decreased due to decreased nerve conduction velocity attributed to an increased and prolonged active state after a single stimulus (Dumitru, 1995). A decrease in CMAP amplitude and duration is also a reflection of muscle fibre depletion (Dumitru, 1995). A feature of reinnervated muscle fibres is increased CMAP amplitude due to hyperinnervation. Original neurons regenerate into their native endplates concurrently with collateral sprouts already established (Frank et al, 1974). Furthermore, normal motor unit recruitment pattern (Henneman, 1957) may be altered in reinnervated muscle whereby recruitment may instead begin with large motor units (Milner-Brown et al, 1974). This results in an initially large MUAP and twitch force and increased rate of twitch.

Different strategies have been employed to diminish the changes associated with denervation atrophy. These include the exogenous application of factors such as ciliary neurotrophic factor (CNTF) (Helgren et al, 1994), pan-neurotrophin-1 (PNT-1) (Funakoshi et al, 1998), exercise (Hie et al, 1982), electrical stimulation (Kanaya and Tajima, 1992; Al-Majed et al, 2000); and microsurgical repair using a motor nerve donor (Gutmann, 1945), a sensory nerve (Hynes et al, 1997; Bain et al, 2001) or dorsal root ganglia implantation (Ochi et al, 1992; Ochi et al, 1996). Despite these efforts to minimize denervation time, recovery of muscle function is incomplete. Although muscle fibre size and number is preserved, fewer motor units exist (Close, 1969; Fu and Gordon, 1995a,b). The latter studies agree that sensory axons can prevent muscle atrophy but cannot mimic a motor nerve in restoring muscle contractile function.

Various modalities whereby sensory axons exert their effect on denervated muscle have been attempted but none have provided conclusive evidence of the mechanism of sensory protection (Weiss and Edds, 1945;

Hynes et al, 1997; Wang et al, 2001;; Bain et al, 2001; Papakonstantinou, et al, 2002). Sensory axon-derived Schwann cells may preserve the endoneurial sheath structure or neurotrophic factors secreted by or stimulated by sensory-derived Schwann cells and axons may maintain muscle integrity (Veltri et al, unpublished data).

Our recent study indicated a contribution of the endoneurial sheath of the distal nerve stump and also the direct influence of the sensory nerve on the preservation of muscle ultrastructure (Veltri et al, unpublished data). However, the contribution of both the distal nerve stump and the sensory axon to the maintenance of function has not been assessed. The purpose of the present study was to determine the role of sensory axons on the distal nerve stump and on the muscle fibre during long-term denervation and whether sensory axons affect electrophysiological and muscle contractile function.



approximately 13 mm from the nerve entry point in the lateral head of the gastrocnemius (Figure 1A). The free distal stump was ligated with 9-0 nylon suture to minimize extraneous innervation from other axons. The proximal stump of the nerve was sutured with 9-0 nylon onto the superficial surface of the biceps femoris muscle to avoid reinnervation from proximal tibial nerve axons.

Sensory-to-motor repair (N-N) following denervation was performed as described in Bain et al (2001) and shown here in Figure 1A. The saphenous nerve was chosen as a sensory nerve donor to avoid potential ectopic innervation by motor axons in a mixed nerve. The site at which the saphenous nerve was transected and transposed into the popliteal fossa was distal to the bifurcation of the femoral nerve and where the nerve divides into its purely sensory branch to the medial thigh (Harsh et al, 1991), avoiding motor axon influences.

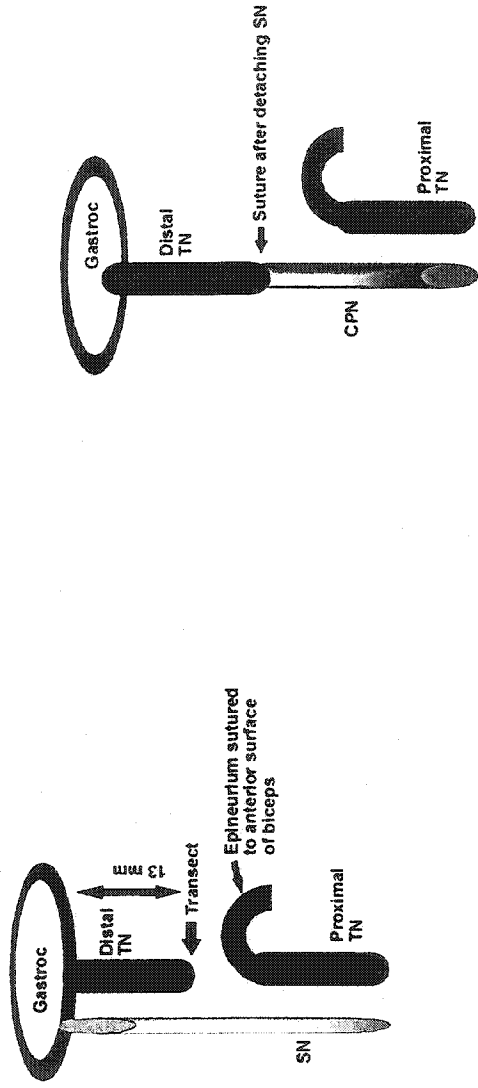
Sensory-to-muscle repair (N-M) (SP) was performed by suturing the proximal segment of the transposed saphenous nerve to the aneural zone of the lateral gastrocnemius muscle (Brunelli, 1982) using 9-0 nylon (Figure 1A).

Immediate motor repair (IR) was performed as described in Bain et al (2001) and shown here in Figure 1B. Muscle reinnervation started after approximately 14 days and was mature 3 months following this repair. In the Unprotected Control group (UP), during the 6-month denervation period, no sensory repair was performed (Figure 1B).

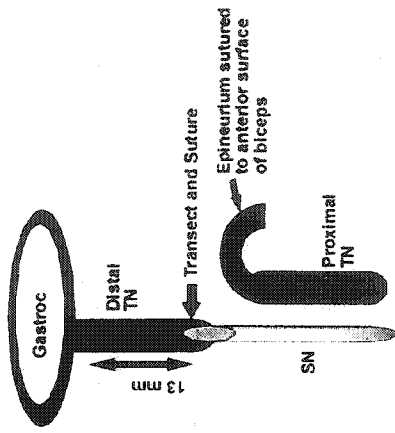
In the two sensory protected (N-N, N-M) and Unprotected (UP) control groups, motor reinnervation was re-established after 6 months by suturing the freshly transected proximal stump of the common peroneal nerve as described in Bain et al (2001) (following detachment of the saphenous nerve) to the distal tibial nerve stump (in sensory protected groups) (Figure 1C). A 6-month denervation period maximized the effect of axon sprouting *in vivo* (Kobayashi et al, 1997).

In all groups, the tibial nerve was stimulated using a portable surgical nerve stimulator (Model 85-62010, C-Line, Vary-Stim III, Xomed-Treace) prior to the second surgery, and the gastrocnemius muscle was examined for evidence of muscle contractions which would have indicated ectopic motor innervation,

C.



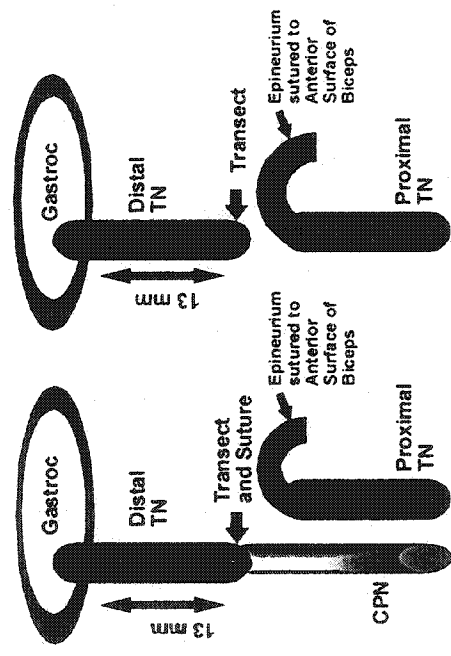
A.



Nerve-to-Muscle

Nerve-to-Nerve

B.



Immediate Motor Repair      Unprotected Control

resulting in the animal being excluded. Similarly, any sign of dehiscence of the nerve repair would have led to animal exclusion.

#### Electrophysiological and Isometric Contractile Function Assessments

Final assessments were performed on all animals 3 months following the second reinnervation surgery in sensory protected and Unprotected Control groups. The Immediate Repair group animals were assessed 3 months after the initial surgery.

#### Electrophysiologic Study

Under general anaesthesia, without muscle paralysis, electrophysiological studies were performed with a Neuromax portable electromyography machine (XLTEK Inc, Oakville, Ontario). A supramaximal stimulus was delivered to the sciatic nerve through a monopolar needle electrode placed at a standard location above the trifurcation where the nerve divides into its common peroneal, tibial and sural branches. This was done to ensure adequate distance between stimulating and recording electrodes giving a more accurate electrophysiologic recording .

Compound muscle action potential (CMAP) recordings were obtained from a monopolar needle electrode (G1) inserted into the gastrocnemius with the reference (G2) electrode inserted into the Achilles tendon. Recorded, amplified and displayed signals from treated and control hindlimbs were automatically measured by the same electromyography machine. As described in Bain et al, compound muscle action potential (CMAP) amplitude (baseline to peak) and distal motor latency was recorded automatically from the lateral gastrocnemius.

The muscle and sciatic nerve were kept damp in optimal physiologic condition by preventing drying and cooling, with lactated Ringers solution and warmed mineral oil was used to maintain muscle surface temperature (31-33 C°). Distal motor latencies were measured from stimulus artifact to initial negative onset, and compound muscle action potential amplitudes were measured from baseline-to-peak (Dumitru,

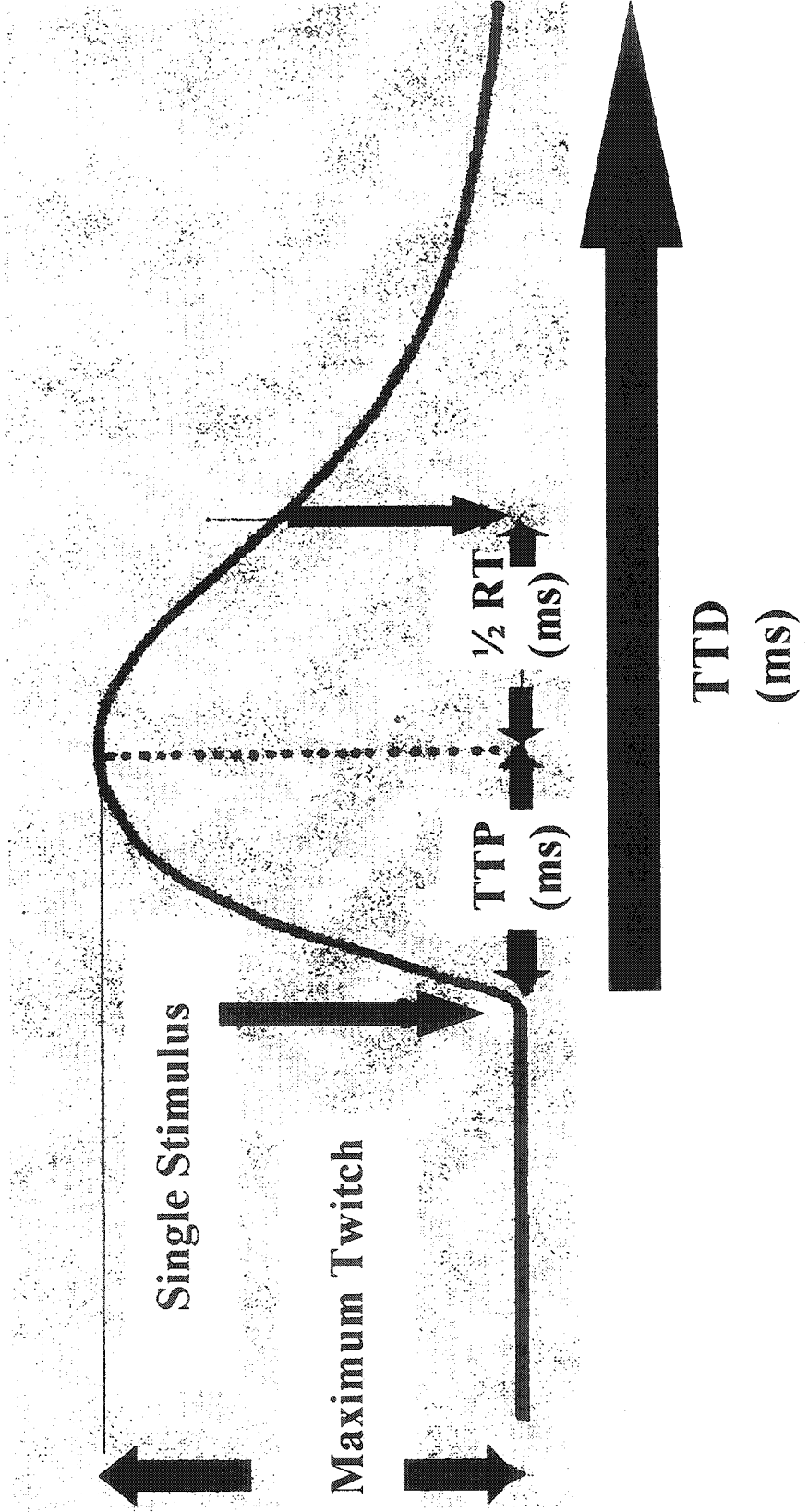
1995). Nerve conduction velocity (NCV) was calculated using a standard distance between stimulating and recording electrodes (43 mm).

### Isometric Contractile Force

At the completion of the electrophysiological studies, the animal was placed prone and the tibia fixed to a recording frame with a 22-gauge needle. The lateral gastrocnemius muscle tendon was isolated atraumatically from remaining tendonous components of the Achilles tendon, and tied with 2-0 silk to a force transducer (Model FT10, Grass Instruments, Quincy, Massachusetts) for measurement of isometric contractile force. The gastrocnemius muscle was set to optimal resting length by adjusting the transducer position to that at which the twitch tension ( $P_t$ ) no longer increased with extended length. The "twitch" tension was measured after delivering a single supramaximal stimulus using a monopolar needle electrode with current pulse duration  $50 \mu s$ , to ensure current is of sufficient duration to stimulate / recruit all motor units (Finkelstein et al, 1993). Stimuli were delivered to the sciatic nerve every 10 seconds (Gillespie et al, 1986), using the Neuromax portable electromyography machine (XLTEK Inc., Oakville, Ontario).

The muscle tensions were digitized and analyzed automatically using Windaq software (DATAQ Instruments, Akron, Ohio). Maximum "twitch force" was measured from the mean of 4 consecutive twitch contractions. The maximum twitch force is defined as the peak amplitude of the force trace of single isometric twitch at optimal muscle length (Figure 2).

From the twitch response trace, time to peak (TTP), half relaxation time ( $\frac{1}{2}$  RT), and total twitch duration (TTD) was measured (Figure 2). Time to peak (TTP) is defined as the time from the commencement of the rise of force to a peak force of a single isometric twitch at optimal muscle length. Half relaxation time is defined as the time taken for the twitch contraction to decay to half its maximum force (Buller and Buller, 1980). Total twitch duration is measured from the time at which the muscle contraction commences to the time the trace reaches baseline. The rate of isometric twitch contraction is characterized by its time to peak (TTP), defined as the time from contraction onset until the peak of maximum tension (Alway, 1985). Half relaxation time ( $\frac{1}{2}$  RT) is the time from the peak of maximum



tension until the time at which force has decreased by one half. The TTP and  $\frac{1}{2}$  RT are shorter for fast-twitch muscle relative to slow-twitch muscle. Time to peak is an indicator of the distance between the recording tip of the electrode and the discharging muscle fibres, with a shorter TTP indicating a closer proximity of the motor unit. The duration of a motor unit potential is a measure from the initial take-off of the negative peak until its return to baseline and is an indicator of the synchrony of the muscle fibre action potentials (Kimura, 1983).

Maximum tetanic force ( $f_0$ ) was analyzed automatically for each stimulation frequency. Tetanic force is defined as the maximum force produced in response to a train of pulses at which force no longer increases linearly with an increase in frequency of pulses.

To determine maximum tetanic force, increasing frequency of stimuli were applied beginning at a low frequency (10 Hz) to induce slow twitch features, up to a frequency of 120 Hz to induce fast twitch features (McComas, 1996) with appropriate recovery intervals between stimuli.

### Statistical Analysis

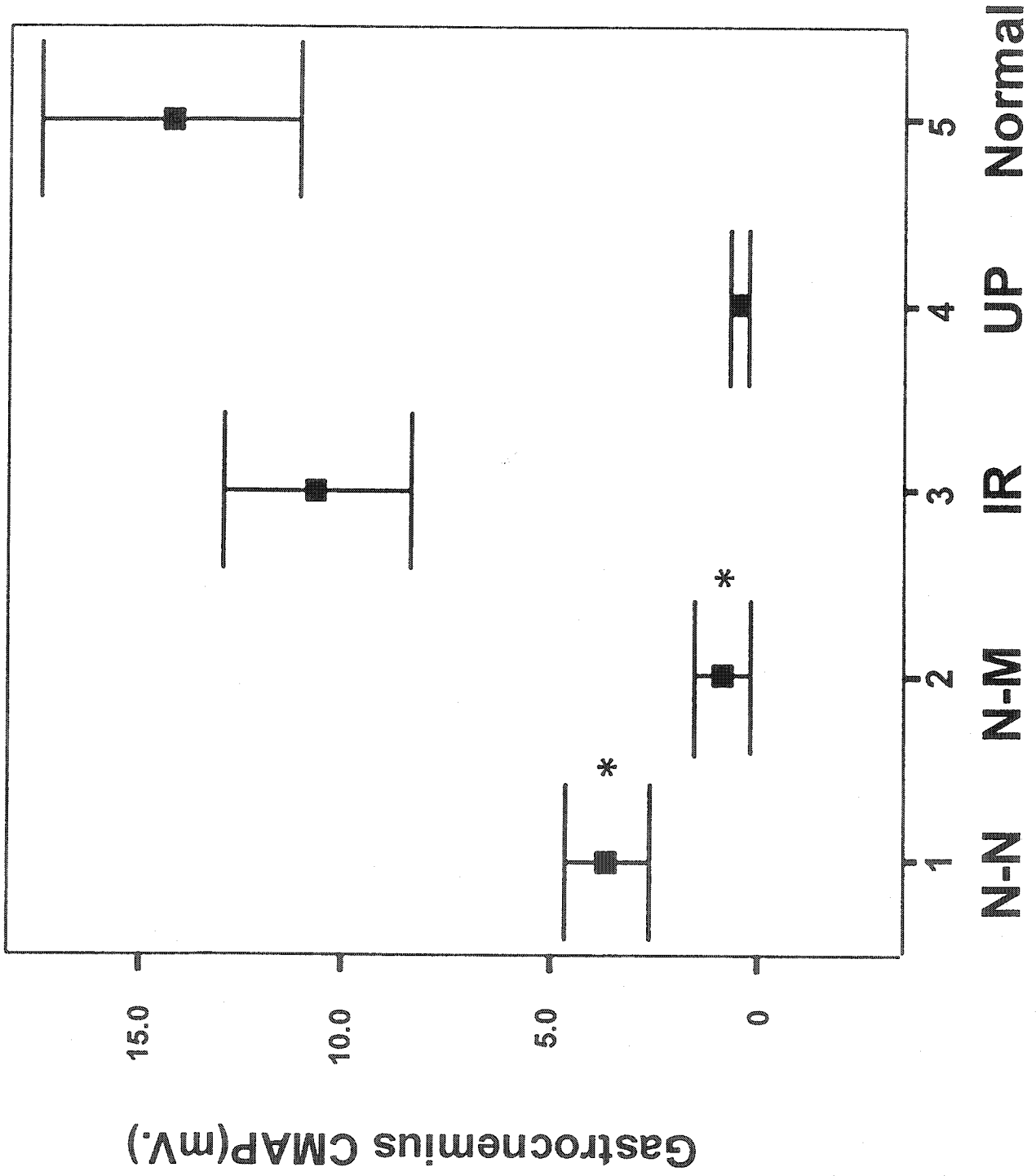
Compound muscle action potential amplitude, distal motor latency, nerve conduction velocity, peak isometric twitch and tetanic force, twitch time to peak, twitch half relaxation time, and twitch total duration, were expressed as mean  $\pm$  standard error. Parametric data were compared with a one-way analysis of variance (ANOVA) using SPSS Version 8 statistical software and  $p < 0.05$  was considered statistically significant. A *post hoc* Tukey's procedure was also performed for intergroup comparisons if indicated.

### **Results**

One animal in the N-N (SP) group died during the initial surgery and 4 animals in the UP group died during the second surgery. Thus, 33 of the initial 38 animals were used for final assessments. No evidence of ectopic reinnervation or nerve dehiscence was observed.

Compound muscle action potential amplitude (CMAP) in the N-N (SP) group was significantly higher than the N-M (SP) and UP group (Figure 3). CMAP amplitude in the N-M (SP) group was similar to that of

Fig. 3

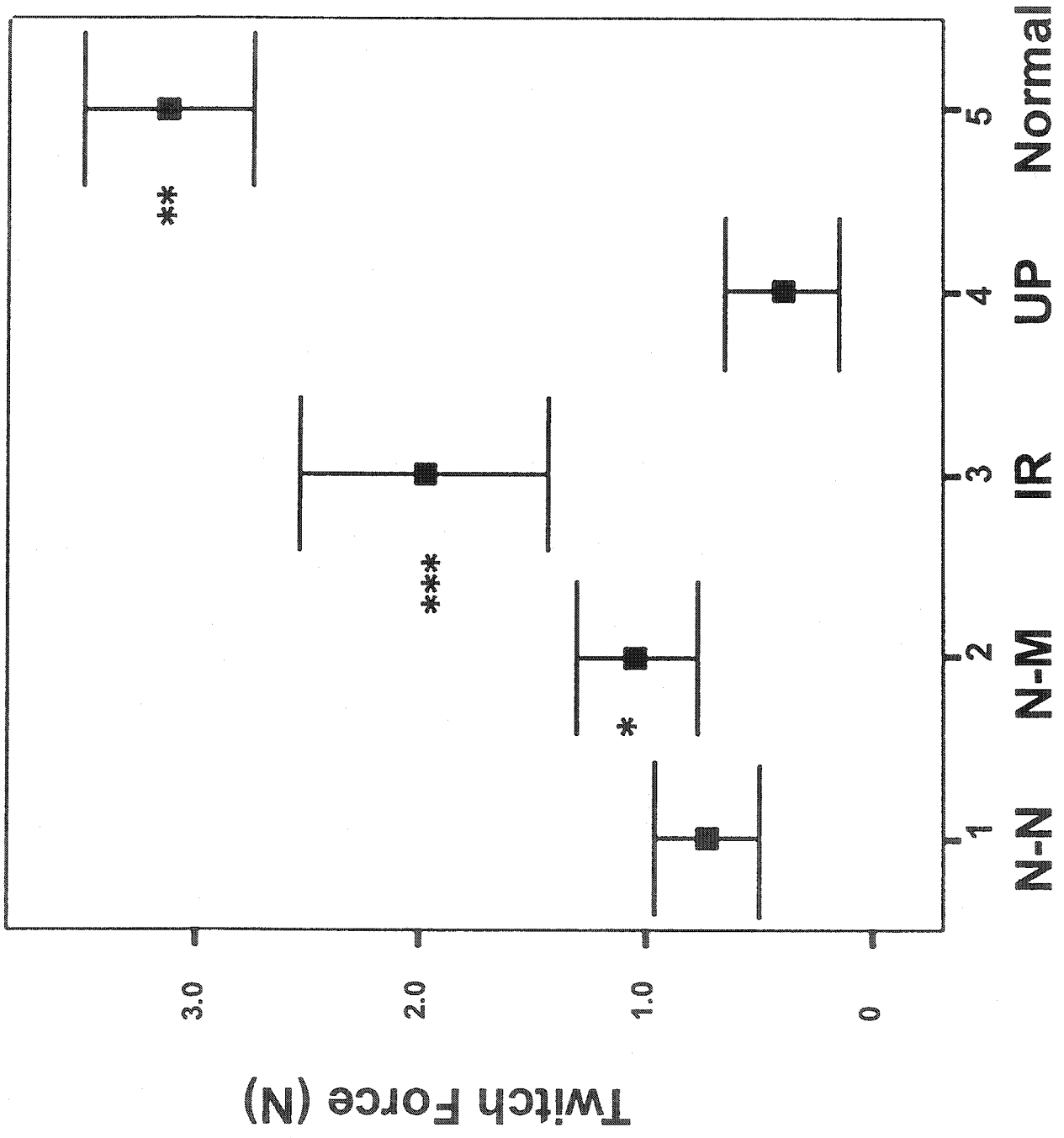


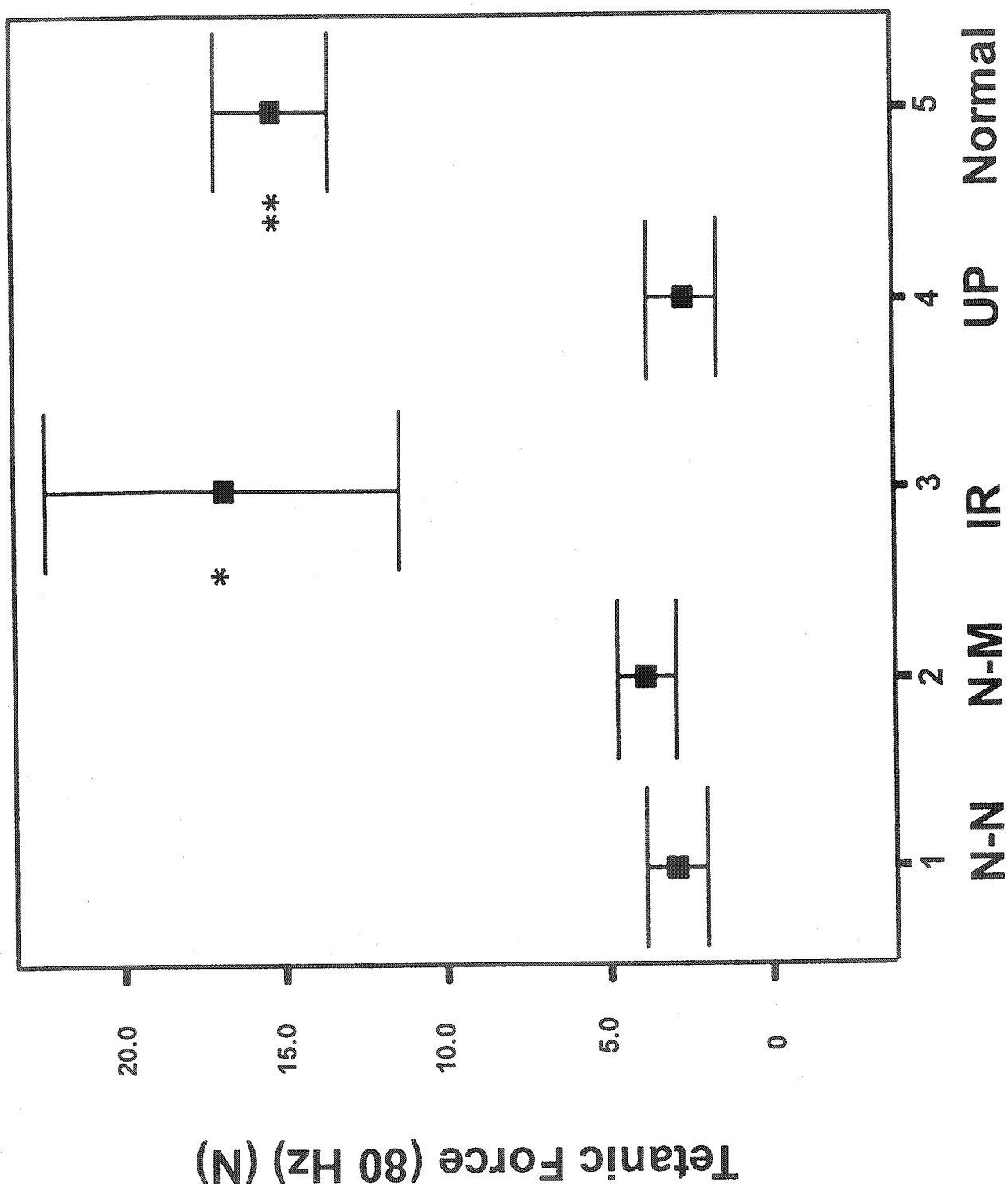
the UP group (Figure 3). As expected, the CMAP amplitude of the IR and normal group was significantly higher than all other experimental groups (Figure 3), although no significant difference existed between these 2 groups. There was no significant difference in nerve conduction velocity among the experimental groups (Table 1). The mean twitch force in the N-M (SP) group was significantly higher than the UP group (Figure 4). However, no significant difference in twitch force existed between the sensory protection (SP) groups (N-N, N-M) (Figure 4). Twitch force in both sensory protection groups (SP) was significantly lower than the IR and normal group (Figure 4) consistent with our previous findings (Bain et al 2001). As expected, the normal group displayed a significantly higher twitch force relative to all experimental groups (Figure 4). Tetanic force was similar in the sensory protection (SP) groups and UP group (Figure 5). Normal and IR groups displayed similar tetanic forces (Figure 5) which were significantly higher than the other experimental groups (Figure 5) consistent with our previous findings (Bain et al 2001).

There was no significant difference in twitch time to peak (TTP) among the experimental groups (Table 1). The normal group displayed a significantly higher half relaxation time ( $\frac{1}{2}$  RT) than the other experimental groups except for the IR group (Table 1). Total twitch duration (TTD) was significantly higher in the normal group than all other experimental groups except for the IR group (Table 1).



Fig. 4





**Table 1. Electrophysiologic and Properties of Gastrocnemius Muscle (mean (standard error))**

Group	NCV (m/s)	TTP (ms)	½ RT (ms)	TTD (ms)
Nerve-to-Nerve (SP)	16.8 (2.5)	55.8 (3.8)	40.7 (2.6)	102.6 (6.9)
Nerve-to-Muscle (SP)	14.0 (0.7)	50.8 (2.4)	45.0 (1.3)	99.2 (5.8)
Immediate Repair (IR)	15.3 (0.6)	48.0 (2.6)	48.5 (3.4)	114.0 (7.1)
Unprotected (UP)	12.9 (1.1)	46.0 (3.8)	42.2 (2.6)	85.8 (10.6)
Normal	15.8 (0.4)	55.1 (1.6)	50.4 (1.0) *	122.3 (3.2) **

NCV=nerve conduction velocity; TTP = time to peak; ½ RT = half relaxation time;

TTD = total twitch duration

\* significant difference ( $p < 0.05$ ) for Normal vs. Nerve-to-Nerve, Unprotected

\*\* significant difference ( $p < 0.05$ ) for Normal vs. Nerve-to-Nerve, Nerve-to-Muscle, Unprotected

## Discussion

A substantial decline in contractile force was reported in mouse soleus muscle that was denervated up to 7 months due to muscle fibre atrophy and reduced axon number (Irintchev et al, 1990). The present study suggests that sensory protection can reverse the decline in contractile force. Evidence of this is shown by significantly improved electrophysiological and muscle contractile function in both sensory protection groups relative to the UP group, also consistent with our previous work (Bain et al, 2001).

Unlike previous studies which used subjective measures of functional return in terms of gait analysis (Bora 1967), withdrawal to pain stimulus and return of reflexes (Mayer et al, 1964; Allpress and Pollock, 1986), all of which are subject to bias (Cabaud et al, 1980), the present study used objective methods of assessing the integrity of muscle reinnervation (McNamara et al, 1987). These include electrophysiological measures of nerve conduction velocity, compound muscle action potential amplitude and isometric contractile force. These measures are useful in assessing the differential return of muscle function particularly in denervated muscle, which is very difficult to assess (Kline et al, 1975).

These data suggest that a possible mechanism of sensory protection may involve sensory axons within distal endoneurial sheaths preserving the structure of the sheaths facilitating reinnervation of the target muscle through preservation of the original motor endplates. Sensory axons may also possible directly influence other factors which sustain the muscle and maintain its receptiveness to motor reinnervation.

Compound muscle action potential (CMAP) magnitude is a function of the number and size of the axons stimulated as well as neuromuscular transmission of the impulse along the axon to the target muscle. CMAP measures the integrity of presynaptic occurrences and excitability of the muscle fibre (Jacobsen and Guth, 1965). In the present study, evidence of the preservation of endoneurial sheath structure and improved regenerative capacity is demonstrated by a significant increase in CMAP amplitude in the N-N (SP) group relative to the UP group and the N-M (SP) group, consistent with the reported reduced number of regenerating axons after delayed nerve repair (Fu and Gordon, 1995a,b). In this study, the reduced CMAP amplitude (3.64 mV) relative to that reported by Bain et al (2001) with a CMAP amplitude of 9.5 mV may

be attributed to the fact that recordings were obtained from the lateral head of gastrocnemius in this study rather than from both heads (lateral and medial).

The differences in CMAP amplitude between the sensory protection groups may be related to the type of collateral sprouting. In the N-M (SP) group, collateral axon sprouts may have arisen more proximally from the nodes of the regenerating axons (nodal sprouting) and terminated directly onto the denervated muscle fibre plasmalemma, instead of more distally (terminal axon sprouting) from preserved motor endplates which may be the case in the N-N (SP) group (Lubischer and Thompson, 1999). The preservation of original motor endplates is associated with an increase in CMAP amplitude and synchrony of motor unit activation, and evidence of this is demonstrated in the N-N (SP) group. Synchrony is influenced by the variable distances between axon branch points and the endplate. Variable conduction velocities resulting from variable distances of axon branching lead to asynchrony, and this is evident in the reduced CMAP amplitude of the N-M (SP) group.

Regardless of whether the structure of the endoneurial sheath is preserved, twitch and tetanic force was similar in both sensory protection (SP) groups. These findings may suggest that although sensory axons may preserve the original motor endplates, this does not translate into improved contractile force. The absence of sensory innervation in the UP group is associated with a significant decrease in muscle force likely resulting from excessive collagenization and atrophy (Al-Amood et al, 1991).

Measures of muscle isometric contractile force are related to frequency voltage and duration of stimulus. Although supramaximal stimulation can only be performed experimentally and cannot be accomplished under normal physiological conditions, the measurement of muscle force remains an adequate correlate of reinnervation (Kanaya et al, 1996). It is important to note here the low force values for the unoperated control (normal) group and the higher values (than those reported in our previous study, Bain et al) for the Unprotected control group. This may be due to incomplete denervation and possible ectopic innervation resulting from damage to the nerve or muscle during the surgery. This may have resulted in the only slight differences between the UP control values and the sensory protected (SP) group values. Reinvestigation of the Unprotected group is currently underway to confirm these data.

In accordance with Bain et al, the present study reports that in the IR group, the mean twitch force was approximately 50% of the normal group. Also in agreement with Bain et al (2001), the twitch force exhibited by both sensory protection groups was lower than the IR group but improved relative to the UP group. Consistent with Bain et al, this demonstrates that sensory nerve repair is less effective than immediate motor repair and more effective than no repair. The direct influence of sensory axons on the muscle is demonstrated by the superior twitch force in the N-M (SP) group relative to the UP group. This may possibly suggest an upregulation by sensory axons of components involved in muscle contraction. Tetanic forces for all experimental groups were consistently lower than those reported in our previous study (Bain et al, 2001), most likely due to the measurement from only the lateral head of the gastrocnemius in this study.

Assessment of twitch contractile properties did not reveal any significant findings with regard to alterations in fibre type distribution with sensory protection. Of note is that sensory protection does not appear to influence muscle properties of time to peak (TTP) given similarities in these values among the groups. However, these findings contrast the finding that half relaxation time ( $\frac{1}{2}$  RT) and total twitch duration (TTD) was significantly reduced in both sensory protected groups compared to normal, suggesting a possible selective influence of sensory axons on fast twitch motor units, however further investigation must be undertaken.

In the present study, the lateral gastrocnemius was used since it has been reported previously that in rabbits and rats, sprouts originating from a transposed motor nerve form new neuromuscular junctions in an aneural region (contains no motor endplates) of denervated muscle, which has been shown to be the lateral head of the gastrocnemius (Brunelli 1982). The lateral gastrocnemius muscle is also a predominantly fast twitch muscle, containing roughly 70% fast twitch and 30% slow twitch units (Gillespie et al, 1986). The proportions of fast- to slow-twitch fibres in normal muscle in the present study are consistent with these values.

Variation in muscle fibre area and contractile force exists when nerves consisting of variable axon numbers are used (Kalliainen et al, 1999). Peroneal nerve contains 600 axons while tibial nerve ranges from

1000 to 1300 axons (Schmalbruch, 1986). This discrepancy in axon content between these nerves may have contributed to an underestimation of force. Indeed, the tetanic and twitch force values for the unoperated controls (normal) and IR group observed in the present study which are lower than literature values (Gillespie et al 1986) may be attributed to this variation in nerve type. However, since the same nerve was used for all experimental groups, consistent density of reinnervating axons was achieved allowing for intergroup comparisons. Use of the common peroneal nerve prevented undue tension and stretch injury to the tibial nerve.

McNamara et al (1987) reported poorer twitch force in their motor neurotized group, claiming that this was due to inaccessibility of axons to distal endoneurial tubes. In contrast, the present study reports similar force generation regardless of the accessibility of sensory axons to the endoneurial sheaths. However, we have not considered the fact that there may be differences in the influence of motor versus sensory axons on the endoneurial tube.

Denervation changes involve disruption of myofibrils and specifically, the sarcoplasmic reticulum (Alway, 1985). A loss of twitch tension in the UP group may be due to myofibril structure disruption and reduced cross-bridging of actin and myosin. Sensory axons may have prevented this disruption by upregulating factors which influence second messenger pathways (Manthorpe et al, 1986; Oh and Markelonis, 1980; McComas, 1996).

Although a motor nerve can contain a high proportion of sensory fibres (30% to 55% ) (Zhang et al, 2000) which are thought to receive stimulatory signals from intrafusal muscle fibres (muscle spindles) and Golgi tendon organs, the fact that they cannot form neuromuscular synapses (Gutmann, 1945) excludes any contribution by these sensory axons to force measurements in the present study. We are therefore confident that the twitch force is due to the sole contribution of regenerated motor axons.

In contrast to the present findings, Zalewski (1970) found no benefit of sensory or sympathetic neurons in preserving muscle mass or contractility. However, motor reinnervation was not provided in their study and these results are debatable. When motor reinnervation was provided, a beneficial effect of sensory

axons was identified as expressed by minimal muscle atrophy and improved muscle function (Papakonstantinou et al, 2002).

This study is consistent with our previous conclusions (Bain et al 2001) that sensory protection improves muscle electrophysiology and twitch force compared to no protection. However, despite the preservation of the endoneurial sheath and endplates (Nerve-to-Nerve), twitch force does not improve. This study also raises questions concerning whether sensory axons influence muscle properties, given the variable findings in TTP and  $\frac{1}{2}$  RT. Further studies are necessary to address these issues.

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**Figure Legends**

- Figure 1** Diagrammatic representation of microsurgical techniques. The distal tibial nerve was exposed in the posterior thigh, mobilized from the popliteal vessels, and transected approximately 13 mm proximal from the nerve entry point into the muscle.
- A. (Sensory Protected) "Sensory protection" was provided by mobilizing the ipsilateral saphenous nerve from the distal aspect of the hindlimb from the medial side of the ankle proximally to just above the popliteal fossa. The proximal saphenous nerve was then transposed from this anterior location through the muscle to the posterior aspect where it was sutured to the distal tibial nerve stump in an end-to-end fashion (Nerve-to-Nerve, N-N) or was directly neurotized to the region above the motor endplate of the lateral gastrocnemius muscle (Nerve-to-Muscle, N-M). The proximal stump of the tibial nerve was further mobilized from the sciatic nerve and the epineurium was sutured onto the anterior surface of the biceps femoris muscle using 10-0 nylon. The duration of sensory protection was 6 months. (SN=saphenous nerve; TN=tibial nerve; Gastroc=gastrocnemius)
- B. (Immediate Repair, IR) The ipsilateral common peroneal nerve was mobilized from the sciatic nerve, transected distally, and the epineurium of the common peroneal nerve stump was immediately sutured to the distal stump of the tibial nerve using 10-0 nylon. The proximal stump of the tibial nerve was further mobilized from the sciatic nerve and the epineurium was sutured onto the anterior surface of the biceps femoris muscle using 10-0 nylon. (TN=tibial nerve; CPN=common peroneal nerve; Gastroc=gastrocnemius)
- (Unprotected Control, UP) Following mobilization and transection of the tibial nerve, 10-0 nylon suture was tied around the distal tibial nerve stump to facilitate future identification of neurorrhaphy site. The proximal stump of the tibial nerve was further mobilized from the sciatic nerve and the epineurium was sutured onto the anterior surface

of the biceps femoris muscle using 10-0 nylon. The duration of denervation was 6 months. (TN=tibial nerve; Gastroc=gastrocnemius)

C. (Motor Reinnervation) A second surgery was performed following the “sensory protection” or denervation interval. The original neurotomy site was re-exposed and examined for evidence of dehiscence and muscle contraction upon nerve stimulation. In “sensory protection” groups, the saphenous nerve was removed from its distal site. The ipsilateral common peroneal nerve was mobilized from the sciatic nerve, transected distally, and the epineurium of the proximal stump was sutured to the distal stump of the tibial nerve using 10-0 nylon. (TN=tibial nerve; CPN=common peroneal nerve; Gastroc=gastrocnemius).

- Figure 2 Isometric contractile muscle twitch analysis. (TTP=time to peak;  $\frac{1}{2}$  RT=half relaxation time; TTD=total twitch duration]
- Figure 3 Compound muscle action potential (CMAP) amplitude (Graph represents mean values  $\pm$  standard error of the mean).  
Asterisks indicate statistically significant differences ( $p < 0.05$ )  
(mV=millivolts) \* N-N vs. N-M, UP, IR, Normal  
\*\* N-M vs. N-N, IR, Normal
- Figure 4 Isometric contractile twitch force (Graph represents mean values  $\pm$  standard error of the mean)  
(N=newtons) \* N-M vs. UP  
\*\* Normal vs. all groups \*\*\* IR vs. all groups
- Figure 5 Isometric contractile tetanic force (80 Hertz) (Graph represents mean values  $\pm$  standard error of the mean)  
(Hz=hertz, N=newtons)  
\* IR vs. N-M, N-N, UP \*\* Normal vs. N-M, N-N, UP

## **P. Overview of Sensory Protection and Conclusion**

Poor motor recovery following denervation is thought to be associated with unsuccessful axonal regeneration along the distal nerve stump due to collagenization and fragmentation of Schwann cell basal lamina (Gutman and Young, 1944; Sunderland and Bradley, 1950). It is also thought to arise from diminished receptiveness of denervated muscle fibres to regenerating motor axons due to a loss of muscle fibres, satellite cell depletion and fibrosis (Irintchev et al, 1990). The studies described in Chapters 2, 3, 4, and 5 support the hypotheses stated in this thesis and conclude that long-term sensory protection is beneficial in restoring muscle histology and function.

Sensory protection may occur via two possible mechanisms. One is where sensory axons and their Schwann cells may possibly interact with the distal nerve environment to maintain a favourable substrate (reduce fragmentation and collagenization) for regenerating axons. Another involves the possible trophic influence of sensory axons on denervated muscle fibres to reduce atrophy and restore contractile function. The size of fast twitch muscle fibres is significantly increased when sensory axons elongate along the muscle fibres, independent of the endoneurial sheaths, suggesting a possible trophic mechanism.

In the present study, muscle fibre size was not directly related to contractile force. This is consistent with previous reports of almost complete functional recovery without an increase in muscle fibre size (Papakonstantinou et al, 2002). This was attributed to an increase in axon sprouting into the denervated muscle which increased the motor unit size and force tension. In diseases such as Muscular Dystrophy, despite muscle hypertrophy, muscle function is negligible. Perhaps non-contractile fibre content including fat, connective tissue, and inflammatory cells contribute substantially to muscle bulk and not function. For this reason, measurement of whole muscle area is less accurate than the measurement of muscle fibre area, performed in the present study.

The structural influence may be explained by sensory nerve Schwann cells within the distal endoneurial sheath which may function as scaffolding to support regenerating motor axons when they regenerate along the nerve sheaths (Bradley et al, 1998). Sensory-derived Schwann cells may also supply extracellular matrix



(ECM) proteins and myelin-associated glycoprotein (MAG) (Jessen and Mirsky, 1999) or factors involved in maintaining Na<sup>+</sup> channel clustering (Black et al 1990), which are integral endoneurial sheath structural components (Fu and Gordon, 1997). Sensory Schwann cell-derived factors may possibly upregulate other cell-derived neurite-outgrowth-promoting factors such as laminin, present in Schwann cell basal laminae (Lundborg et al, 1994) thus rendering the distal nerve stump the “temporary target” for regenerating axons.

Schwann cell signals elicited by sensory axons within the sheaths may regulate a “neurofilament phosphorylation pathway” (DeWaegh et al, 1992) possibly accounting for the large axons in the N-N group.

Sensory-derived Schwann cells may be a source of neurotrophic factors such as insulin-like growth factor (IGF)-1, IGF-II and ciliary neurotrophic factor (CNTF) (Lundborg et al, 1994; Fu and Gordon, 1997) as well as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and NT-4/5 which are known to support axonal regeneration (Fu and Gordon, 1997; Griesbeck et al, 1995).

Improved neural integrity may also be explained by stimulation by sensory-derived Schwann cells to upregulate neurotrophin receptors associated with axonal regeneration. These may include the p75 receptor to which NGF binds and the tyrosine kinase (Trk) receptor to which BDNF binds. These receptors are upregulated in Schwann cells during acute denervation (Fu and Gordon, 1997). During chronic denervation, axonal regeneration is impaired due to downregulation of the p75 and the Trk receptors (Hoke et al, 2002). Sensory-derived Schwann cells may maintain the upregulation of these growth factor receptors to enhance the neural integrity.

Alternatively, sensory axon-derived neuregulin may support the Schwann cell to axon coupling seen in the N-N group, which prevents Schwann cell apoptosis. In one study (Muller and Stoll, 1998) apoptosis of terminally differentiated Schwann cells was inhibited after axonally-derived neuregulin administration *in vitro* and this finding was also noted *in vivo* (Grinspan et al, 1996).

When neurotized to muscle, multiple sensory fascicles may branch out to contact more of the denervated muscle fibres and increase the direct supply of chemotactic / trophic substances to the fibres or stimulate other factors which bind to muscle receptors to activate a “second messenger pathway” (McComas, 1996). This pathway subsequently activates genes encoding muscle proteins and results in

sufficient fibre receptiveness to motor reinnervation.

Sensory axons may secrete factors which block collagen synthesis and decrease muscle protein catabolism to bring about a reversal of atrophy and fibrosis (Savolainen et al 1988).

Schwann cell-derived myotrophic factors such as ciliary neurotrophic factor (CNTF) and its receptor expressed in skeletal muscle may be supplied by sensory axons (Manthorpe et al 1986). Indeed, one study reported positive sciatic immunocytochemical staining in cultured sensory neurons suggesting the potential of these axons to exert a myotrophic influence on muscle cells (Oh and Markelonis, 1980).

To differentiate between the structural and trophic mechanisms of sensory protection, the studies presented in this thesis have employed a model in which the endoneurial sheath is intact and sensory axons occupy the sheath or, alternatively, sensory axons bypass the sheath. These studies support the conclusion that sensory protection via an end-to-end sensory-motor repair and sensory neurotization prevents denervation atrophy in the rat. However, in terms of the mechanisms of sensory protection, the conclusions are limited due to a number of factors. This model is not without its limitations. Furthermore, the findings of these studies may also support alternate hypotheses such as a trophic mechanism acting independent of the endoneurial sheath as shown by the similarity in contractile twitch and tetanic force measurements in both types of sensory protection (SP).

Another limitation is that motor and sensory axons were not distinguishable without the use of specific stains. Future studies involving double-staining to detect activity of motor-specific (i.e. acetylcholinesterase) or sensory-specific (i.e. carbonic anhydrase) neurotransmitters may differentiate between these axon types.

The primary outcome of interest was motor function, and this was based on compound muscle action potential (CMAP) amplitude and latency, isometric twitch and tetanic force. However, the conclusions based solely on these measurements are limited. Since sensory nerves are initially affected by nerve injury, a more representative measurement may have been sensory function. Furthermore, twitch force is not reported as commonly as is maximum isometric force production, maximum power output and muscle endurance to measure the "clinical" manifestation of muscle function. In Chapter 5, due to the unusually low Unprotected control twitch and tetanic forces, it was not possible to make firm conclusions. Therefore,

to confirm the results, denervation surgery has been repeated in an additional 10 animals with a 6 month denervation interval. Completion of the functional assessments is expected within 9 months of the initial surgery.

Since the animals were not perfused with fixative prior to ultrastructural examination, artifactual remnants such as blood cells inside vessels, were present in the nerve samples. Perfusion would have facilitated the visualization of organelles, glycogen molecules and inflammatory cells. However, in this study, these were not considered, and only the structures such as axons, myelin sheaths, Schwann cells and collagen were considered and were easily identifiable. Furthermore without the identification of intramuscular nerve anatomy using techniques such as silver staining or immunohistochemical staining, it is difficult to quantify the effect of either mechanism of sensory protection. Thus, the morphologic data presented here can only be used as preliminary tools to identify a sensory protection effect, followed by more specific staining methods to further identify the mechanisms.

In the experimental studies described herein, nerve injury was carried out in a standard, precise fashion, with minimal tissue and nerve trauma. As a result, infection and inflammation was minimized. Therefore, the outcomes measured may represent over-estimates of the actual outcomes that would be expected in the clinical situation. In peripheral nerve injury such as obstetric brachial plexus injury where the nerve is avulsed, or in other nerve crush or stretch injuries, functional recovery is often complicated by infection or more extensive tissue and nerve damage. Evaluation of the microsurgical techniques described herein would involve assessment of muscle function as well as muscle biopsy analysis at various stages post-injury.

Another limitation is that, by controlling for some factors (i.e. isolation of an aneural zone within lateral gastrocnemius to identify newly-formed endplates) precluded the direct extrapolation of the findings to whole muscle. Future studies may therefore consider examining both medial and lateral heads of gastrocnemius.

The benefit of sensory protection merits further investigation in humans to determine whether patients with proximal nerve injuries due to avulsion, lesion or rupture, who undergo either sensory neurotization or sensory-motor coaptation, exhibit better muscle function than those who do not. In the clinical scenario, the

first surgery would involve separating the fascicles of a secondary sensory nerve donor, in proximity to the denervated muscle, and neurotizing the sensory nerve fascicles to the target muscle or performing a sensory-motor coaptation. The sensory nerve would act as a temporary “babysitter” of the target muscle until the motor nerve has regenerated distally. The second surgery would involve replacing the sensory donor nerve with the original nerve which would be coapted to its distal stump. The short distance which the original motor nerve would have to proceed to reach the target muscle would improve the capacity to appropriately innervate the muscle.

In the majority of peripheral nerve injuries, a donor sensory nerve is readily available in the vicinity of the denervated muscle, and this sensory donor nerve would circumvent the need to sacrifice a motor nerve. This type of surgery would hopefully reduce morbidity, minimizing the use of scarce healthcare resources and result in an earlier return to work/activities of daily living.

**Q. Future Direction**

One of the main concerns of the reconstructive microsurgeon in treating peripheral nerve injury is to minimize donor site morbidity. When the donor nerve is affected, function of the muscle it innervates is compromised. This necessitates further studies to examine whether similar sensory protection mechanisms identified in this thesis occur in an end-to-side model which would not disrupt the donor nerve and obviate the need for a second surgery. The end-to-side model has also been shown experimentally to be beneficial in preserving muscle function (Noah et al, 1997).

**R. References**

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