University of Alberta

Strategies to Promote Peripheral and Central Nervous System Regeneration

by

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Abstract

Peripheral nerve injuries are often associated with poor functional recovery, despite the capacity of the peripheral nervous system to support regeneration. This is particularly evident following injuries to large nerve trunks close to the spinal cord and far from the target tissues. The poor functional recovery following such injuries involve the prolonged dennervation of target tissues and the chronic axotomy that neurons undergo as they regenerate long distances necessary to reach and reinnervate target tissues. Here we further investigate the effects of chronic axotomy on the regenerative ability of injured motoneurons. We then investigate mechanisms for enhancing peripheral motor neuron and sensory neuron regeneration following axotomy, looking specifically at pharmacologically manipulating levels of intracellular cyclic adenosine monophosphate. Lastly, we investigate the application of electrical stimulation, previously shown to be successful in enhancing peripheral motoneuron regeneration, on a model of central nervous system sensory axon injury and regeneration.

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Table of Contents

List of Tables

List of Figures

Abbreviations

Arg1:	Arginase I
ANOVA:	Analysis of Variance
BDNF:	Brain-Derived Neurotrophic Factor
cAMP:	Cyclic Adenosine Monophosphate
CL:	Conditioning Lesion
CNS:	Central Nervous System
CP:	Common Peroneal
CREB:	cAMP Responsive Element Binding Protein
dbcAMP:	dibutyryl cAMP
DRG:	Dorsal Root Ganglion
ERK:	Extracellular Signal-Regulated Kinase
Exp.:	Experimental Group
FG:	Fluorogold
GAP-43:	Growth-Associated Protien-43
GDNF:	Glial-Derived Neurotrophic Factor
GFR-α:	GDNF family receptor alpha
IBMX:	3-isobutyl-1-methylxanthine
ICC:	Immunocytochemistry
MAG:	Myelin-Associated Glycoprotien
MU:	Motor Unit
n-n:	Nerve to Nerve Repair
NT:	Neurotrophin
OMgp:	Oligodendrocyte Myelin Glycoprotien
PDE:	Phosphodiesterase Inhibitor
PKA:	Protien Kinase-A
PNS:	Peripheral Nervous System
RAGs:	Regeneration Associated Genes
Regen.:	Regeneration
STIM:	Electrical Stimulation
TrkB:	Tropomyosin Receptor Kinase-B

CHAPTER 1

GENERAL INTRODUCTION: PERIPHERAL AND CENTRAL NERVE INJURY

1.1 INTRODUCTION

The regeneration of injured neurons of the adult mammalian peripheral nervous system (PNS) is generally successful. This is in comparison to the adult mammalian central nervous system (CNS) in which axon regeneration is virtually nonexistent. This difference can be attributed to key differences that exist between the cellular response to injury in both the PNS and CNS: (1) Rapid phagocytosis of myelin debris by macrophages and Schwann cells in the PNS prevent the regenerating axons from exposure to myelin-associated inhibitory proteins and (2) nonneural cells of the PNS, Schwann cells, undergo proliferation and differentiation into growth supportive phenotype that effectively support axonal regeneration in conjunction with the extracellular matrix of the distal nerve stump (Fu and Gordon, 1997a), (3) the formation of a glial scar following injury in the CNS and (4) molecular inhibitors of axonal regeneration found in CNS myelin including myelin-associated glycoprotein (MAG), NogoA and oligodendrocyte myelin glycoprotein (OMgp) (Filbin, 2003).

Despite the capacity for regeneration of neurons in the periphery, functional recovery following peripheral nerve trauma, particularly following injury to nerve trunks close to the spinal cord at great distance from target tissue, is still generally poor. The lengthy distance of axon regeneration that is required to reinnervated target tissue following such injuries necessitates long periods in which the injured neurons remain without target connections. Such extended periods of chronic axotomy and target denervation have been demonstrated to reduce regenerative capacity of these regenerating neurons, compounded by the considerable misdirection of regenerating axons to inappropriate distal nerve stumps (Thomas et al., 1987; Fu and Gordon, 1997; Boyd and Gordon, 2003). Additionally exacerbating the poor functional recovery, the reinnervated muscle fibers do not fully recover from atrophy suffered during chronic denervation (Fu and Gordon, 1995b).

In the present experiments, we investigate axon regeneration in both the PNS and CNS of the adult rat. In our first experiments, we investigate the effect of chronic axotomy on PNS axon regeneration of motoneurons using a cross suture and graft technique of the femoral nerve. Though previous studies have shown that prolonged

axotomy reduces the regenerative capacity of axotomized motoneurons (Fu and Gordon, 1995a), it is unclear what role target deprivation and/or growth conditions play due to limitations of the "frustrated growth" model of chronic axotomy used in those experiments. In these studies we address the question of whether the reduction of regenerative capacity reported by Fu and Gordon (1995a) is due indeed due to the prolonged removal of target tissue, common to both the model and "real-life" peripheral nerve injury or if the reduction is due to the model imposed condition of frustrated growth.

Our second series of experiments investigate the effect of elevating cyclic adenosine monophosphate (cAMP) on the regeneration of motor and sensory neurons of the PNS and resultant functional recovery using the transected and surgically repaired common peroneal branch of the sciatic nerve in the adult rat. Previous studies have shown that cAMP elevating agents including Forskolin, as well as cAMP analogues resulted in a reduction in time to initiation of outgrowth as well as an increased rate of elongation of peripheral sensory neurons by use of the pinch test (Kilmer and Carlsen, 1987). The phosphodiesterase (PDE) inhibitor theophylline, which is a non-specific PDE inhibitor, resulted in only in a reduction in to time to initiation of outgrowth. Phosphodiesterase inhibitors prevent the breakdown of cAMP by inhibiting PDE enzymes responsible for its degradation. Here we investigate the use of a cAMP specific PDE4 inhibitor, rolipram, at a dosage recently reported to enhance regeneration and functional recovery following spinal cord injury (Nikulina et al., 2004), on the axon regeneration of both motor and sensory neurons as well as the resultant functional recovery.

Lastly, we investigate the effect of electrical stimulation (STIM) on the regeneration of ascending CNS axons of sensory neurons using the dorsal root ganglion (DRG) sensory neurons of the adult rat. These neurons are a particularly interesting model for investigating CNS regeneration because they have both a central axon that ascends in the CNS and a peripheral axon that extends into the PNS. It has been shown that while the peripheral axons undergo robust regeneration following injury, the central axons do not regenerate at all (Neumann and Woolf, 1999b; Bradbury et al., 2000).

However, if the peripheral axons are lesioned prior to the central axons being lesioned, in a procedure known as a conditioning lesion of the peripheral axons, the central axons are able to regenerate in the normally inhibitory CNS (Neumann and Woolf, 1999b). This effect was shown to be mimicked by *in vivo* injection of dibutyryl cAMP (dbcAMP), a membrane soluble analogue of cAMP, into the DRG (Neumann and Woolf, 1999b; Qiu et al., 2002a). Results have previously shown that brief periods of STIM of injured motoneurons results in accelerated axonal outgrowth in the PNS, due to an increase in brain derived nerve factor (BDNF) (Al-Majed et al., 2000; Brushart et al., 2002b). BDNF has been shown to act as a cAMP PDE inhibitor through extracellular signal-regulated kinase (ERK) (Gao et al., 2003). Here we investigate the use of STIM of the peripheral axons of the DRG sensory neurons on the regeneration of concomitantly lesioned ascending central axons, answering the questions of whether STIM of the peripheral axons results in increased cAMP in the DRG, and subsequently whether regeneration of central axons is encouraged.

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CHAPTER 2

PROLONGED TARGET DEPRIVATION RESULTS IN A REDUCTION OF THE CAPACITY OF AXOTOMIZED MOTONEURONS TO REGENERATE

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2.1 INTRODUCTION

Functional recovery is generally poor after peripheral nervous system (PNS) nerve injuries despite the innate capacity of the resident Schwann cells to support axonal regeneration (Terzis and Smith, 1990; Kline and Hudson, 1995; Midha, 1997). Lengthy periods of chronic axotomy during which injured neurons remain without target connections and chronic denervation of Schwann cells and target organs occur as injured neurons regenerate their axons over long distances. These conditions progressively reduce regenerative capacity (Fu and Gordon, 1997a,b; Boyd and Gordon, 2003b). In addition, considerable misdirection of regenerating axons to distal nerve stumps not formerly innervated by the parent neurons exacerbates the poor functional recovery (Gillespie et al., 1986; Thomas et al., 1987; Brushart, 1993; Al-Majed et al., 2000c).

In a classic study, Holmes and Young (1942) used a delayed-repair animal model to chronically axotomize sensory and motor neurons and concluded that prolonged axotomy did not compromise axon regeneration. This model involved transecting the tibial nerve and ligating it for prolonged periods, effectively frustrating axonal growth of chronically axotomized neurons. Axons were encouraged to regenerate into a freshly cut distal nerve stump but prevented from remaking functional target connections. The authors found that the numbers of axons that regenerated was not influenced by the duration of the neuronal axotomy. In contrast, in a similar model of frustrated growth, Fu and Gordon (1995a) demonstrated a reduced capacity of chronically axotomized motoneurons to regenerate. Motoneurons were chronically axotomized by cutting the tibial nerve and ligating and suturing the proximal nerve stump to an innervated muscle to effectively curtail axon outgrowth for periods of 2w to one year; a period of at least 4 months was allowed for chronically axotomized motoneurons to regenerate their axons and reinnervate muscle (Fu and Gordon, 1995a). Under these conditions, the number of reinnervated motor units progressively fell to a plateau of ~35% after a 6m period of prolonged axotomy. Further, a reduction of tubulin and actin expression from peak levels at 7 days post-injury (Tetzlaff et al., 1988; Petrov et al., 1996) was consistent with a decline in regenerative capacity after prolonged axotomy.

It is possible that regenerative success may have been exaggerated in the 1942 study by the counting of multiple sprouts emanating from the proximal into the distal nerve stump (Holmes and Young, 1942). As many as 20 daughter axons regenerate from each parent axon (Aitken et al., 1947). Each regenerating axon that crosses the lesion site is exposed to as many as 150 potential distal pathways and each axon branches to enter several vacant Schwann cells tubes (Witzel et al., 2005). Even when regenerating axons make functional target connections, as many as 5 daughter axons per neuron remain for many months (Toft et al., 1988; Mackinnon et al., 1991). This indicates that the axon counting used by Holmes and Young (1942), is likely to overestimate the regenerative ability of axotomized neurons. The enumeration of motoneurons that regenerated their axons by retrograde labeling of regenerated axons (Fu and Gordon, 1995a; Boyd and Gordon, 2001; Boyd and Gordon, 2002) avoids the above mentioned problems of axon counting by counting only the parent neuron.

The frustrated axonal growth model of prolonged axotomy utilized in both Holmes and Young (1942) and Fu and Gordon (1995a) does not fully represent the regenerative events after injury and surgical repair of nerve trunks in human patients. Regenerating axons may succeed in crossing the surgical gap into distal nerve stumps but fail to reach denervated targets. Axons may also fail to cross the surgical gap and thereby the neurons may remain chronically axotomized. In the latter case, axons may grow for variable distances into the proximal nerve stump despite the poor regenerative environment (Cajal, 1991; Amir and Devor, 1993), the epineurial tubes providing some substrate for the axons as do the empty muscular tubes of denervated muscles (Glasby et al., 1986).

In the present study, we have adopted a model which more closely mimics long-term injury in the PNS, where motoneuron axons regenerate over long distances and remain chronically axotomized for extended periods of time before they reach their denervated target muscles. This was achieved by transecting the motor branch of the femoral nerve and suturing it into a long saphenous graft from the contralateral femoral nerve to allow prolonged axon regeneration while preventing target reinnervation. This "continuous growth" model was adopted to address the important unanswered question of whether the reduction in regenerative capacity reported by Fu and Gordon (1995a) was

due to the frustrated growth imposed by the frustrated growth model or target deprivation which is common to both the frustrated growth model and the "real-life" PNS injury that is mimicked by the continuous growth model. We used retrograde fluorogold (FG) labeling and enumeration of motoneurons that regenerated their axons after chronic axotomy in both the frustrated growth and the continuous growth models to demonstrate that chronic axotomy reduced motor axonal regeneration, regardless of whether the axotomized motoneurons regenerate their axons during the period following chronic axotomy. Some of these results have been described previously in abstract form (Xu et al., 2003).

2.2 MATERIALS AND METHODS

2.2.1 Experimental Design

Sixty eight female Sprague Dawley rats, weighing approximately 250 to 300 g, were obtained from Harlan Sprague Dawley, Indianapolis, IN. Rats had access to water and food *ad libitum*, were housed in a flat bottom, woodchip filled cage, and were exposed to a 12 h light/dark cycle. All experimental manipulations were performed strictly adhering to Canadian Council on Animal Care guidelines and approved by the Health Sciences Animal Welfare and Policy Committee (University of Alberta).

Rats were randomly assigned to three surgical groups. The motor and cutaneous sensory branches of the femoral nerve of the rat were used in this study as a cross-suture model of peripheral nerve injury and repair (Fig. 1A).

2.2.2 Surgical Procedures

Anesthesia was induced in all rats by intraperitoneal injection of sodium pentobarbital (0.07mL/100g: Somnotol®; MTC Pharmaceuticals, Cambridge, ON, Canada). All surgical procedures were performed in an aseptic manner. The skin of the groin was shaved and prepared with 70% ethanol prior to surgical incision. The skin incisions were repaired with a continuous 4-O silk suture (Ethicon, Inc. Somerville, NJ, USA) and cleaned with sterile saline. Animals were placed under a heat lamp and observed until they recovered reflex responsiveness and visible consciousness before being returned to normal confines.

In the immediate repair control group (Control), the left femoral motor nerve was transected and the proximal stump sutured to the distal stump of the same nerve with 9-O ethilon® nylon (Ethicon, Inc. Somerville, NJ, USA) within silicone tubing to promote axonal regeneration and reinnervation of the target quadriceps muscle (Fig. 1B). The two nerve stumps were opposed using 9-0 epineurial sutures that were passed through a 3 mm silicone ® tube (Inner diameter 0.76mm, Helix Medical, Inc. Carpinteria, CA). The tube was used to stabilize the suture site (Fig 1E). Two months later, in a second surgery, the left femoral motor branch was transected above the cross-suture site and the saphenous sensory branch cut so as to cross-suture the left motor branch to the previously intact left saphenous sensory branch of the femoral nerve (Fig. 2A).

In the 2m axotomy with no regeneration experimental group 1 (Exp.1), the left femoral nerve motor branch was transected and the proximal stump sutured into a blind-end tube using 9-O nylon epineurial sutures to prevent axon regeneration (Fig. 1C). The denervated distal stump of the motor branch was then deflected to avoid regeneration into the stump. The blind end tube was prepared by closing one end of a 3mm length of the 0.76mm ID silicone tubing with silicone prior to surgical procedures. Two months later the left femoral proximal motor nerve stump was transected and cross-sutured to the distal stump of the freshly transected left saphenous branch as described for the control condition (Fig. 2A).

In the 2m axotomy with regeneration experimental group 2 (Exp.2), the motor branch of the left femoral nerve was cut and the proximal nerve stump was cross-sutured to the distal stump of the right femoral sensory nerve branch that was dissected free as a 4 cm graft from the right hindlimb (Fig. 1D). The proximal stump of the contralateral saphenous graft was opposed to the distal stump of the freshly transected left motor branch using 9-0 ethilon® nylon epineurial sutures that were passed through a 3 mm silicone ® tube as described above (Fig 1E). The distal stump of the graft was deflected and sutured to an innervated muscle to prevent target reinnervation. The denervated distal nerve stump of the left femoral motor branch was deflected to avoid axon regeneration into the stump. Two months later the proximal stump of the femoral nerve motor branch was cross-sutured to the distal segment of the freshly transected left saphenous branch as described for the control and Exp. I groups (Fig. 2A).

2.2.3 Motoneuron Enumeration

Six weeks following the second surgery in all groups, we retrogradely labeled motoneurons by exposing and transecting the cutaneous sensory branch of the left femoral nerve 2.5 cm distal to the cross-suture site. A 3mm length of nerve was removed from the tip of the distal stump for analysis. The tip of the proximal nerve stump was exposed to a pool of 4% FG in 0.1 M cacodylic acid for 1h in a Vaseline® well, after which it was extensively irrigated with sterile saline (Richmond et al., 1994) (Fig. 2B).

Six days following the application of FG, all rats were deeply anesthetized with sodium pentobarbital and perfused with 300mL of room temperature saline followed by 500mL of ice-cold 4% paraformaldehyde (pH 7.4) via the aorta. Following perfusion, the thoracolumbar spinal cord segment T11 to L2 which contains the femoral motoneuron cell bodies, was removed and post-fixed in 30% sucrose in a 4% paraformaldehyde solution overnight. Spinal cord tissues were then embedded in Optimum Cutting Temperature (OCT) Tissue-Tek Liquid (Sakura, Japan, distributed by Cedarlane Laboratories Canada), frozen in liquid nitrogen and stored at -80°C prior to cryostat sectioning.

Longitudinal frozen sections of 50μm thoracolumbar spinal cord segments dissected from each rat were cut using a cryostat at -21°C (Jung CM 3000, Leica, Germany). The sections were serially mounted onto glass slides, allowed to dry, and cover slipped. Femoral motoneurons which regenerated their axons a distance of at least 2.5 cm into the cutaneous sensory distal nerve stump and were exposed to FG were identified and enumerated at 20X magnification under UV fluorescence at barrier filters of 430nM by an investigator who was blind to sample group identity. Cells labeled with FG showed blue fluorescence (Fig. 2C). Multiple counting of split neurons was corrected for by multiplying the total number of cells counted per animal by a correction factor of 0.63, calculated via the method of Abercrombie and Johnson (1946).

2.2.4 Axonal Measurement

A 3mm saphenous distal nerve segment was removed distal to the site of FG application (Fig. 2B). This segment containing regenerated nerves was fixed in glutaraldehyde (3% in 0.1M phosphate buffer), stained with osmium tetroxide (OsO_4, 3% solution in 0.1 M phosphate buffer), dehydrated through a series of ascending alcohols,

and embedded in araldite. Cross-sections of 2μm were made using a glass blade microtome and mounted using Entellan. Cross-sections were then photographed at 40X magnification (see Fig. 3). Five representative sub-sections of each cross-section were analyzed for size and number of myelinated axons using Image-Pro Plus (Media Cybernetics, Inc. Silver Spring, MD). These numbers were averaged and normalized to the total area of the nerve cross-section.

2.2.5 Statistical Analysis

Once normal distribution of data was established, statistical analysis between group nerve fiber data was assessed through one-way analysis of variance (ANOVA) (GraphPAD InStat Software V. 1.13 (1990) Dr. Paton, University of Alberta) using arithmetic means (±SE). For the abnormal distributions of motoneuron counts, differences between geometric means (±SE) were assessed again through ANOVA. Statistical significance was accepted at 5% ($p<0.05$).

2.3 RESULTS

2.3.1 Prolonged target deprivation reduced the number of motoneurons that regenerated axons into the distal nerve stump

We addressed whether a state of frustrated axonal growth, rather than a state of chronic axotomy, reduces the regenerative capacity of motoneurons (Fu and Gordon, 1995c). We compared the number of motoneurons that regenerated axons after chronic axotomy with frustrated growth (Exp.1 – Frustrated Growth, n=26) with the number that regenerated axons after a period of chronic axotomy in which axonal growth was encouraged but target reconnection was prevented (Exp.2 – Continuous Growth, n=22).

As shown in representative nerve cross-sections of a 3 mm segment of nerve taken immediately distal to the point of FG application (Fig. 3), the myelinated regenerated axons were visibly larger in diameter in the control group. In this group the axons regenerated within their original quadriceps motor branch immediately after nerve transection and nerve-nerve resuture. During the 2 m prior to the second surgery, the regenerated axons were permitted to reinnervate muscle (Control, n=21 (Fig. 1B)). In the second surgery, the motor nerve branch of the femoral nerve was cut proximal to the suture site and the proximal nerve stump then cross-sutured to the blind ended saphenous

nerve graft to encourage axon regeneration without target reconnection (Fig. 2A). In the experimental groups, the femoral neurons were chronically axotomized and the regeneration of their axons either encouraged within a contralateral saphenous nerve graft (Exp. 1-No Regen.) or prevented for 2 m (Exp.2 –Regen) (Fig. 1C,D). In the second surgery, the proximal motor nerve branch was sutured into the distal stump of the ipsilateral saphenous nerve to promote axon regeneration for a 6 week period. Exposure of these axons to FG to backlabel their cell bodies in the ventral horn permitted the enumeration of the FG-backlabeled motoneurons that had regenerated their axons into the distal stump (Fig. 2B, C).

Because the motoneuron counts were not normally distributed, geometric means ($\pm$ SE) of the number of femoral motoneurons that regenerated axons after the second surgery were calculated and used for comparison using analysis of variance (ANOVA) between the two experimental groups and one control group. The mean ($\pm$ SE) of the number of motoneurons that regenerated axons after the second surgery after a 2m period of chronic axotomy was significantly lower than in the control group whether the chronically axotomized motoneurons were or were not permitted to regenerate their axons ($p< 0.05$; Fig. 4). The period of 2m chronic axotomy reduced the number of motoneurons that regenerated their axons into a freshly denervated stump to approximately 60%. This is in agreement with our previous observations describing the reduced regenerative capacity of chronically axotomized tibial and CP motoneurons (Fu and Gordon, 1995c; Boyd and Gordon, 2001; Boyd and Gordon, 2002). There was however, a significant difference noted between the regenerative success of the motoneurons whose axons did and did not regenerate during the 2m period of axotomy. Those motoneurons that regenerated their axons within a saphenous nerve graft after prolonged axotomy (Exp.2 – Regen) were significantly more successful in regenerating axons than when axon growth was frustrated (Exp.1 – No Regen.; $p<0.05$; Fig. 4). The number of motoneurons that regenerated their axons during prolonged axotomy and thereafter regenerated axons into the distal saphenous branch was significantly higher than the number that regenerated their axons following a 2m period of prolonged axotomy with frustrated axonal growth. In light of the ability of exogenous BDNF and GDNF to promote axon regeneration in chronically axotomized motoneurons (Boyd and

Gordon, 2002; Boyd and Gordon, 2003a), the exposure of the regenerating axons to sources of neurotrophic factors within the distal nerve stump may account for the difference (See Discussion).

2.3.2 Prolonged motoneuron axotomy reduced the size of regenerated nerve fibers

Along with the total number of motoneurons that regenerated axons into the distal nerve stump, we also compared the number and diameter of the regenerated nerve fibers for the experimental and the control groups (Fig. 5). The average total number of myelinated axons that regenerated into the freshly transected distal nerve segment, though showing a tendency to decline in both of the experimental groups as compared to the control group, showed no significant difference (Control n=14, Exp.1 n=10, Exp.2 n=10 (Fig. 5)). This is in agreement with the data published by Holmes and Young (1942) which were incorrectly interpreted as evidence that chronic axotomy did not reduce axonal regenerative capacity.

The analysis of the diameter of the nerve fibers that had regenerated into the fresh saphenous distal nerve stump after 2m chronic axotomy showed a significant decline in both of the experimental groups when compared to control (Fig. 6) ($p<0.05$). The myelinated axon diameters were normally distributed in all groups and the distributions for both experimental groups overlapped and were not statistically different (Fig. 6A, B). The distributions were however, significantly reduced from controls where there were significantly more large diameter nerve fibers (Fig. 6B, C). In both experimental groups, the average nerve fiber diameter declined by approximately 33% decline as compared to the control group. There was no significant difference in average nerve fiber diameter between the two experimental groups (Fig. 6D) ($p>0.5$). The larger nerve fibers in the control group reflects the recovery of nerve fiber size when regenerating axons remake functional connections (Gordon and Stein, 1982). In the control group, the axons reform functional nerve-muscle connections after the first surgery. Since regenerating axons grow in diameter in direct proportion to the size of their parent axons, the regenerating axons after the second surgery are larger for the control group than for the two experimental groups. In the chronically axotomized neurons, the progressive atrophy of the nerves is not reversed by reconnection with target connections (Milner et al., 1981;

Gillespie and Stein, 1983; Gordon et al., 1991) with the result that the parent axons of the regenerated axons are smaller in the experimental groups.

2.4 DISCUSSION

2.4.1 Effect of chronic axotomy on motor axonal regeneration

Many previous studies established that chronically axotomized motoneurons whose axons are prevented from regenerating progressively lose their regenerative capacity (Fu and Gordon, 1995c; Boyd and Gordon, 2002; Sulaiman et al., 2002; Boyd and Gordon, 2003a). In the present study, we demonstrate that the regenerative capacity of chronically axotomized motoneurons is compromised to the same extent whether or not nerve repair is carried out to encourage axon regeneration over long distances without target contact. Hence long periods of time required for axotomized neurons to regenerate their axons to distant denervated targets successively reduce their regenerative capacity. In combination with the chronic denervation of Schwann cells in the distal nerve stumps, chronic axotomy of neurons is a major contributing factor to poor functional recovery after surgical repair of severed nerves in humans, especially when the site of repair is quite distant to the denervated muscle and sensory targets (Fu and Gordon, 1997b; Boyd and Gordon, 2003b; Sulaiman O. et al., 2005).

Counts of regenerated axons did not detect the significant decline in the number of motoneurons that regenerated their axons after chronic axotomy. Similar numbers of myelinated axons in freshly denervated distal nerve stumps after chronic axotomy corroborates with findings of Holmes and Young in the rabbit that regenerated myelinated axon numbers were the same for freshly and chronically axotomized neurons (Holmes and Young, 1942). The number of axon sprouts per neuron was high, an estimated ratio of the numbers of regenerated axons to motoneurons that regenerated axons over the 6 week period being in the order of 10: 1 (Fig. 7). Whether or not the ratio is similar for sensory and motor neurons, it is between the reported high and low estimates of 20 (Aitken et al., 1947) and five (Aitken et al., 1947; Toft et al., 1988; Mackinnon et al., 1991) daughter sprouts per parent axon. The counts are generally higher after nerve section and repair than for regenerated axons after a crush injury (Giannini et al., 1989). While the differences in myelinated axon counts were not

significant, the counts tended to be lower in experimental versus control groups after the 6 week period of axon regeneration. At shorter times of regeneration, axon counting is a better representation of regenerative success (Richardson and Verge, 1987; Hoke et al., 2002). An example is that FK-506-induced significant increases in numbers of chronically axotomized motoneurons that regenerated their axons and of regenerated axons (Sulaiman et al., 2002). These data indicate that axonal sprouting from parent axons increases as a function of time. For freshly axotomized sensory and motor neurons, it may take as long as a month for all the axon sprouts to cross the suture line (Brushart et al., 2002b; Witzel et al., 2005). This process of staggered axon regeneration across the repair site contributes to the long delays in axonal regeneration and the consequent deterioration of regenerative capacity (Gordon et al., 2003; Sulaiman O. et al., 2005).

Regenerated nerve fibers were significantly larger for neurons that experienced a second axotomy and nerve repair after a first axotomy and nerve repair that facilitated reconnection with denervated targets than chronically axotomized neurons that regenerated without target contact (Fig.6). Parent nerve fibers undergo progressive atrophy after axotomy that is reversed when the regenerating nerve fibers make functional target connections (Gordon and Stein, 1982; Gillespie and Stein, 1983); axon diameter varying in direct proportion to neurofilament content that is down- and up-regulated, respectively (Gordon and Stein, 1982; Gillespie and Stein, 1983; Hoffman et al., 1987; Hoffman and Cleveland, 1988; Hoffman et al., 1993).

Regenerating axons and their myelin sheath normally increase in diameter in direct proportion to their parent axons (Devor and Govrin-Lippmann, 1979). Within the 2 month period of axon regeneration after immediate re-suture of the cut muscle nerve branch of the femoral nerve to the quadriceps muscle, regenerating axons make functional nerve-muscle contacts within 2 weeks (Brushart et al., 1998). After a 2 month period of regeneration when the parent axons are re-cut and cross-sutured to a freshly denervated nerve stump of a saphenous nerve graft, the parent and their daughter axons are therefore likely to have made considerable recovery of size. As a result, the caliber of the newly regenerated axons should recover in proportion to the relatively larger dimensions of the parent axons as was found to be the case for the significantly larger

caliber of the regenerated axons of the control group as compared to the chronically axotomized nerve groups.

We conclude that chronic axotomy of motoneurons results in a reduced regenerative capacity, specifically by reducing the number of motoneurons that regenerate axons and by limiting the recovery of the size of the regenerating nerve fibers.

2.4.2 Evaluation of animal models of chronic axotomy

Under conditions in which neurons remain chronically axotomized during a lengthy period of axon regeneration toward denervated targets, as is the case after proximal nerve injuries in humans, axons that succeed in crossing the site of injury after surgical repair may regenerate within the distal nerve stumps for some time. In our experimental models of chronic axotomy we either sutured the proximal nerve stump into a blind-end silicone cap (frustrated growth model) or we cross-sutured the proximal stump to the distal stump of a blind-end sensory nerve graft (continuous growth model). Cut ends of injured human peripheral nerves are most commonly repaired via sensory nerve grafts (Millesi et al., 1972; Midha and Mackay, 1999) the sensory nerve grafts used in this study mimicking the regeneration of axons within these grafts and into distal nerve pathways that are distant from denervated targets for periods of 2 months or more. In the rat model of protracted regeneration of chronically axotomized neurons within saphenous nerve grafts without targets (continuous growth model), we observed the significant reduction in number of motoneurons that regenerated axons as we did under the frustrated growth condition (Fig. 4).

It is possible that axon sprouts bypassed the ligature to grow into the surrounding innervated muscle. However, innervated in contrast to denervated muscles do not support axon regeneration and sprouts fail to grow (Watson, 1970). Progressive atrophy of the nerve fibers proximal to ligation attest to their failure to regenerate and remake functional connections (Gordon et al., 1991). Alternatively, observations of Cajal (1991) of the turning of regenerating axons into the proximal nerve stump after frustrated growth into distal nerve stumps indicated that axons may retrogradely regenerate on relatively poor substrates within the proximal nerve stumps. Nonetheless, this retrograde sprouting is unlikely to be substantial, with only 2% of nerve fibers demonstrating retrograde regeneration from a neuroma (Amir and Devor, 1993) and there being no significant

increase in numbers of nerve fibers in the proximal stump of a cut and ligated rabbit peripheral nerve even after 8 months (Gordon et al., 1991).

It is also unlikely that motoneuron cell death could account for our findings of fewer than normal regenerating motoneurons after chronic axotomy because all motoneurons survive axotomy when nerves are injured at a distance from the cell body in adult rabbits and rats (Swett et al., 1991; Gordon et al., 1991; Vanden Noven et al., 1993). Sensory neurons may be more susceptible to cell death after injury although estimates vary from few to some 20% of neurons. Alternatively in neonatal rats, multiple axotomy may produce significant loss of neurons (Tam et al., 2003).

It has been reported that the use of a sensory nerve graft, which is the current treatment standard for peripheral nerve injuries in human care, is not as effective in promoting axon regeneration of injured motoneurons into the distal motor stump than is a motor or mixed nerve graft (Nichols et al., 2004a). Schwann cells in motor pathways express a motor specific carbohydrate moiety that have been implicated in attracting motor axons to regenerate in appropriate motor pathways (Martini et al., 1992; Martini et al., 1994) and motor and sensory neurons have been demonstrated to show a preference for motor and sensory pathways respectively (Brushart, 1988; Brushart, 1993). It has also been suggested that the target muscle constitutes the primary influence that promotes preferential motor reinnervation, as motor axons will not preferentially regenerate in motor pathways when muscle target connections are prevented (Robinson and Madison, 2004). The issue remains contentious with reports of smaller regenerated motor axons in sensory than motor grafts implicating factors specific to motor and sensory axons within the distal nerve stumps (Ghalib et al., 2001). The fact remains that sensory nerve grafts are routinely used in human peripheral nerve repair. In our experiments a sensory graft was utilized due to the nonexistence of a continuous motor or unbranching mixed nerve branch that was sufficiently long enough to allow two months of continuous axonal regeneration without target muscle reinnervation.

2.4.3 Progressive decline in neurotrophic support for chronically axotomized neurons

Schwann cells play an essential role in supporting axonal regeneration. In their absence, axons fail to regenerate (Hall, 1986a; Hall, 1986b). Axons regenerate within the

bands of Bungner formed by proliferating Schwann cells and their processes within the basement membrane of the distal nerve stump. Findings that freshly axotomized motoneurons regenerate as well in the presence of exogenous brain derived neurotrophic factor (BDNF) or glial derived neurotrophic factor (GDNF) suggest that Schwann cells within the distal nerve stump that synthesize these and other neurotrophic factors provide sufficient amounts of the factors to support axonal regeneration (Boyd and Gordon, 2001). This is strongly supported by the attenuation of axonal regeneration after nerve crush by anti-BDNF antibodies (Zhang et al., 2000) and reduced numbers of motoneurons that regenerate axons after nerve section and repair in TrkB deficient transgenic mice (Boyd and Gordon, 2001). Chronically axotomized motoneurons whose axons were prevented from regenerating within denervated distal nerve terminals responded to exogenous administration of BDNF and GDNF by regenerating their axons in greater numbers than untreated chronically axotomized motoneurons (Boyd and Gordon, 2003a). In addition to the promotion of axon regeneration, exogenous BDNF also reversed the atrophy of the motoneurons, as did exogenous GDNF (Boyd and Gordon, 2002; McPhail et al., 2005)

Normally non-neural cells and Schwann cells in particular in the distal nerve stumps synthesize many neurotrophic factors including BDNF, GDNF and neurotrophic factors 4/5 (NT-4/5) and 3 (NT-3) (Fu and Gordon, 1997b; Boyd and Gordon, 2003b). Although Schwann cells in motor and sensory pathways have been reported to express trophic factors differentially (Hoke et al., 2005) and the time course of expression of these factors differs between factors, most factors including BDNF and GDNF and their receptors, TrkB, and p75 for BDNF, and the ret and GFR,-α receptors for GDNF, are expressed in the Schwann cells within the first month of denervation (Meyer et al., 1992; Funakoshi et al., 1993; Naveilhan et al., 1997). Differences in time course of expression are illustrated in Fig. 8 where the up- and down-regulation of GDNF is much more rapid than that of BDNF (Kobayashi et al., 1996; Hammarberg et al., 2000; Boyd and Gordon, 2003b).

The time course of expression of these factors in Schwann cells and neurons and of their receptors on motoneurons could explain, at least in part, the decline in regenerative capacity of motoneurons that suffer chronic axotomy in the absence of target

muscle connections. Immediately following axotomy, BDNF receptors for BDNF and GDNF are rapidly upregulated in motoneurons. While the increase in BDNF in the motoneuron tapers off and returns to normal by 2 weeks, the elevation of the receptors for BDNF and GDNF, is maintained for 6 weeks following injury (Kobayashi et al., 1996; Hammarberg et al., 2000; Boyd and Gordon, 2003b) (Fig. 8). This autocrine support along with the elevation of the receptors for the factors could sustain sufficient levels of BDNF to allow axonal regeneration to proceed normally. The non-neural cells of the distal nerve stump, specifically the Schwann cells, also dramatically upregulate expression of GDNF and neurotrophins that include BDNF, NT 4/5 and NT-3 at about 1 week post-injury (Ernfors et al., 1989; Meyer et al., 1992; Sendtner et al., 1992; You et al., 1997) (Fig. 8). While the elevation of GDNF is relatively short-lived (2 weeks) the elevation of BDNF in the distal nerve stump continues for up to 28d after injury (Fig. 8). This upregulation may sustain neurotrophic levels and guidance of regenerating axons in the distal nerve stumps even after the return of BDNF expression by the motoneurons to basal levels.

However, we show here that the PNS does not appear to be programmed to allow long-term axon regeneration, and in cases of long-term regeneration or delayed nerve repair, another important consideration is that the denervated distal nerve stumps also begin to degenerate (Sulaiman and Gordon, 2000; Sulaiman O. et al., 2005). There is progressive atrophic loss of Schwann cells (Weinberg and Spencer, 1978; Wood, 1998), a corresponding decline in BDNF, GDNF and NT-4/5 (Hammarberg et al., 2000), and a diminished ability of these denervated Schwann cells to interact with and support regenerating axons (You et al., 1997; Sulaiman and Gordon, 2000; Hoke et al., 2002). The decline in neurotrophin/GDNF release and reception results in a reduced regenerative ability of the motoneurons. This is supported by studies demonstrating the ability of BDNF and GDNF to "rescue" the regenerative ability of motoneurons following prolonged axotomy in the frustrated growth model (Moir et al., 2000; Boyd and Gordon, 2002).

The process of progressive decline in access of the chronically axotomized motoneurons to neurotrophic factors explains the reduction in regenerative capacity in both of our experimental models of chronic axotomy. Here we show that the continuous

growth model of chronic axotomy (Exp Group 2) is somewhat more successful at regenerating axons into the distal nerve stump than the frustrated growth model (Exp Group 1) of chronic axotomy. In both cases, there would be a reduction in regenerative capacity due to loss of neurotrophic factors in the distal nerve stump. In the frustrated growth model of chronic axotomy, a decline in regenerative ability would be expected to occur rapidly following the cessation of motoneuron-generated BDNF due to the removal of the distal nerve stump. Comparatively, in the continuous growth model, a more gradual decline in regenerative ability occurs as the distal nerve stump deteriorates over the 2 months of chronic axotomy. At the two month time point, the continuous growth model has not degenerated to the same point as in the frustrated growth model. In both cases it can be assumed that this decline in regenerative ability may be rescued with exogenous application of BDNF and/or GDNF (Boyd and Gordon, 2002; Boyd and Gordon, 2003a). If indeed this is the explanation, there would be a more pronounced difference between the two models after short periods of axotomy and no difference between the two models of axotomy at longer periods of axotomy.

The physiological response of the motoneuron to chronic axotomy resulting in the decline in regenerative ability is not clear. There is evidence of a decline in the upregulation of actin and tubulin mRNA from peak levels following injury (Tetzlaff et al., 1988; Petrov et al., 1996). It would appear that the decline in neurotrophins by the distal nerve stump and subsequent decline in reception of neurotrophins by the motoneurons results in a down-regulation of genes necessary for axon regeneration, including actin and tubulin but the exact mechanism of interaction is not clear. In the practical context of the regenerating PNS, these findings indicate that the reduction in regenerative capacity of motoneurons over a period of prolonged axotomy is, as outlined above, certainly interconnected with the denervation of the distal stump.

Additionally confounding the important issue of functional recovery is the degeneration of target tissue. Following periods of prolonged denervation, target muscle undergoes denervation atrophy (Anzil and Wernig, 1989; Fu and Gordon, 1995b; Kobayashi et al., 1997b; Aydin et al., 2004). Deficits in muscle mass and maximum tetanic force have been demonstrated to be directly proportional to the time interval of denervation (Aydin et al., 2004). Each of these factors, including reduction in

motoneuron regenerative capacity, distal nerve stump Schwann cell denervation and target muscle denervation, compound in the regenerating PNS during prolonged periods of chronic axotomy to exacerbate poor functional recovery.

2.5 Figures

2.5.1 Figure 1. Surgery 1

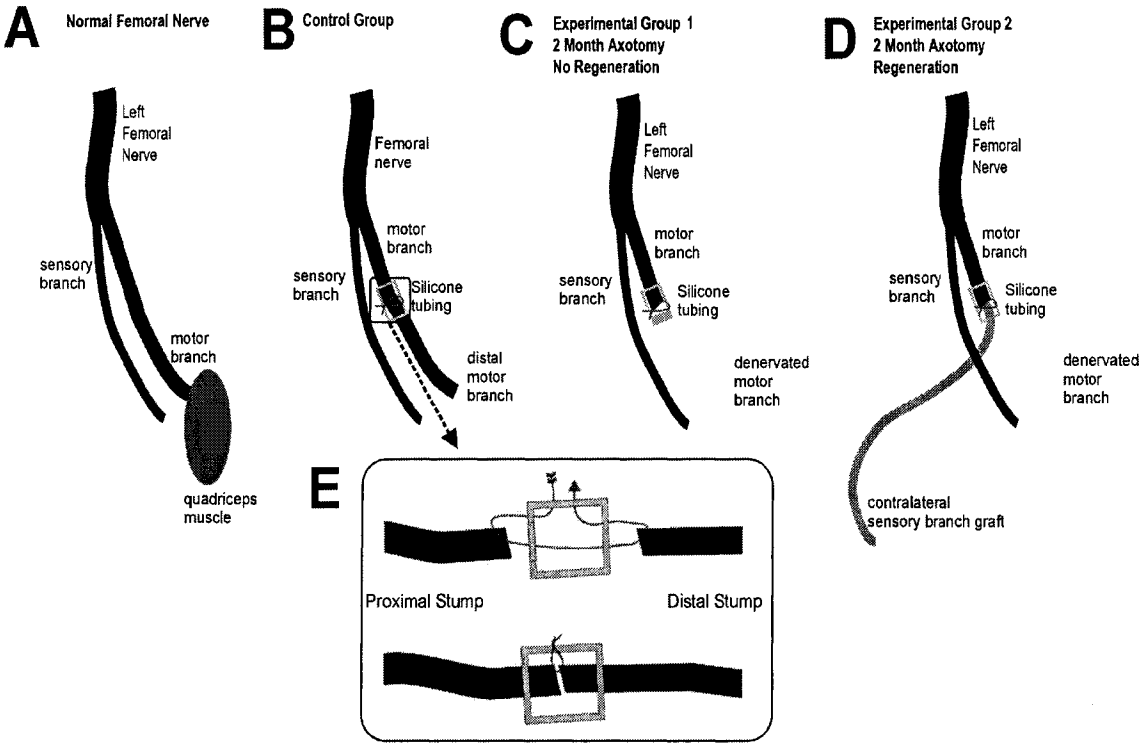

Figure 1: Schematic diagrams of femoral motor branch surgical manipulations in the left hindlimb of the rat to examine the effect of 2m prolonged axotomy on femoral motoneuron regenerative capacity. A) The normal femoral nerve and its branches, B) Control Group (n=21) Surgery 1: The left femoral motor branch was transected and the proximal nerve stump immediately resutured to the distal nerve stump to promote axon regeneration and reinnervation of the target quadriceps muscle. The surgical repair within a segment of silicone tubing is shown. C) Experimental Group 1 (n=26) Surgery 1: The left femoral motor branch was transected and the proximal nerve stump capped in silastic tubing to prevent axon regeneration and target reinnervation for the duration of the 2 m chronic axotomy. D) Experimental Group 2 (n=21) Surgery 1: the Left femoral motor branch was transected and the proximal nerve stump cross-sutured to a 4 cm long graft that was dissected from the right hindlimb saphenous cutaneous nerve branch of the femoral nerve. The end of the graft was sutured to innervated muscle so that reinnervation of target denervated end-organs was prevented and axotomy of the femoral neurons was prolonged for a 2m period. E) Schematic representation of the nerve-nerve suture technique to promote axonal regeneration. In the first stage, a 9-0 nylon suture was threaded through the epineurium of both the proximal and distal stumps of the nerves via the insertion of the needle through the middle of the 5 mm long closed silastic tubing. After 'catching' the two stumps, the needle was threaded out of the tubing. In the second stage, the nerve stumps were drawn together within the tubing, aided by the surface tension of the two opposing nerve stumps and the size of the tubing providing a snug fit of the nerve stumps. The suture was tied on the outside of the silastic tubing to hold the stumps together.

2.5.2 Figure 2. Surgery 2 and labeling of motoneurons

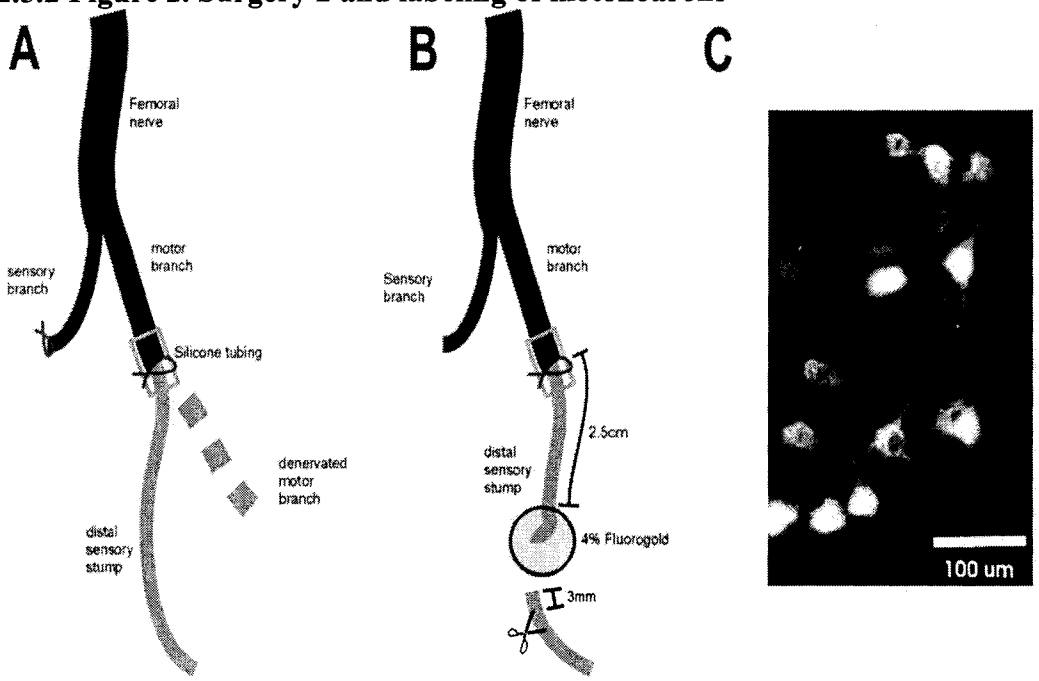

Figure 2: Schematic illustrations of A) the procedures of the second surgeries, 2 m after Surgery 1, B) the retrograde labeling of motoneurons with Fluorogold via the regenerated axons in the distal nerve stump, and C) an example of the backlabeled motoneurons. A) Two months after Surgery 1, the left femoral motor nerve branch was transected just proximal to the transection site at Surgery 1. The proximal nerve stump of the quadriceps motor nerve was sutured to the distal nerve stump of the freshly transected quadriceps sensory stump to encourage axon regeneration. B) Six weeks after Surgery 2, the saphenous distal nerve was transected 2.5 cm from the site of the quadriceps to saphenous nerve-nerve suture. The tip of the cut nerve stump was exposed to 4% Fluorogold in 0.1 M cacodylic acid for 1 hr in a Vaseline well that confined the dye to the tip of the nerve. A 3mm long section of saphenous nerve was removed distal to the site of the Fluorogold application to cut cross-section, stain with osmium tetroxide for enumeration and measurement of the caliber of regenerated and myelinated axons.

2.5.3 Figure 3. Representative nerve fiber photomicrographs

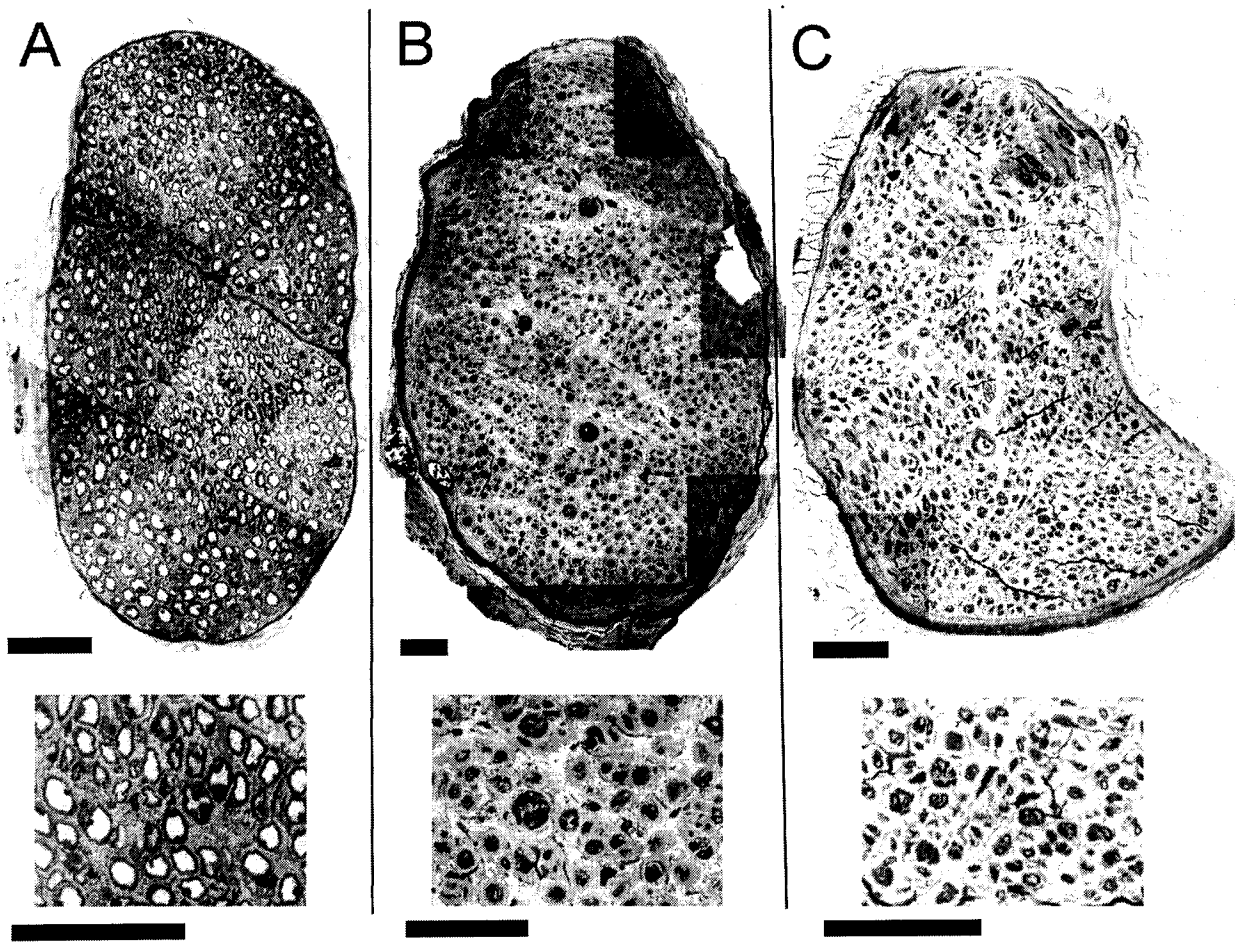

Figure 3: Photomicrographs of representative cross-sections of the saphenous nerve fibers used to count myelinated axons and to measure their cross-sectional areas using Image Pro Plus (see methods for details). A) Immediate repair Control group – (Control), B) Axotomy for 2 m with NO regeneration Experimental Group 1 (Exp.1), C) Axotomy for 2m with regeneration (Exp. 1). Note that the axons are visibly larger after immediate nerve repair.

2.5.4 Figure 4. Mean number (±SE) of retrogradely labeled femoral motoneurons

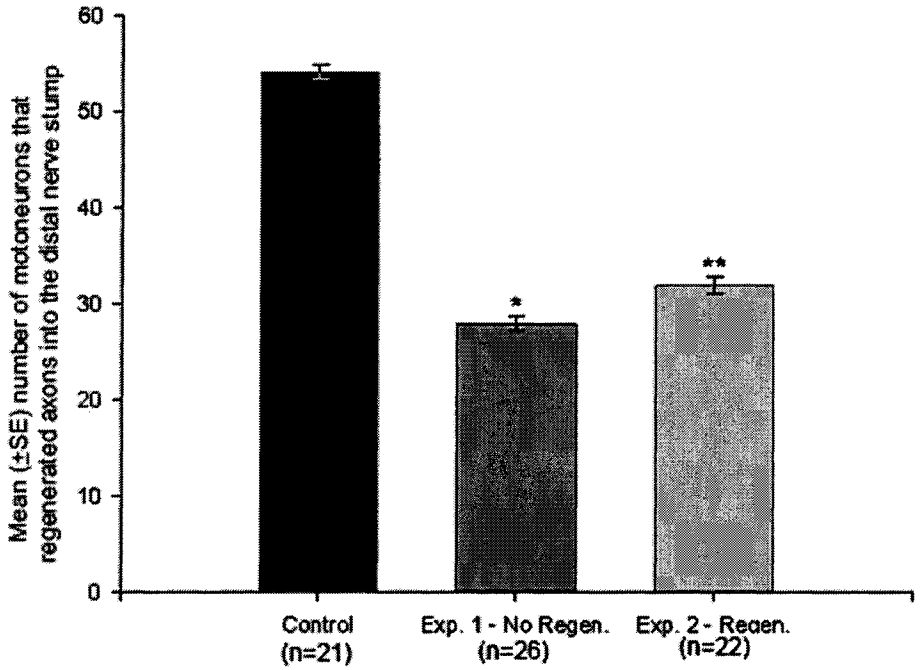

Figure 4: The geometric mean (± S.E.) of the number of femoral motoneurons that regenerate their axons after 2 m chronic axotomy as compared to the number that regenerate their axons after immediate nerve repair. Chronically axotomized motoneurons were either NOT permitted to regenerate their axons (Exp 1 –No Regen.) or encouraged to regenerate their axons without remaking functional nerve-muscle connections (Exp.2 –Regen.). The mean numbers of motoneurons that regenerated their axons was highest in the Control group where motoneurons regenerated their axons immediately into the distal nerve stump and were permitted to remake functional nerve-muscle connections (54±1 motoneurons). These numbers were significantly higher than the chronically axotomized motoneurons that were not permitted to remake functional connections over a 2 m period, whether or not the motoneurons could (Exp.2 - Regen., 32±1 motoneurons) or could not (Exp.1 – No Regen., 28±1 motoneurons) regenerate their axons in the distal nerve stumps during the period of chronic axotomy ($p<0.05$). Interestingly, significantly more motoneurons regenerated axons after chronic axotomy when axon regeneration into distal nerve stumps was encouraged during the 2 m period of chronic axotomy as compared to those motoneurons whose regenerative growth was frustrated. * denotes statistical difference between Control and Exp.1 ($p<0.05$). ** denotes statistical difference between Control and Exp.2 and well as between Exp.1 and Exp.2 ($p<0.05$).

2.5.5 Figure 5. Mean number (±SE) of regenerated nerve fibers

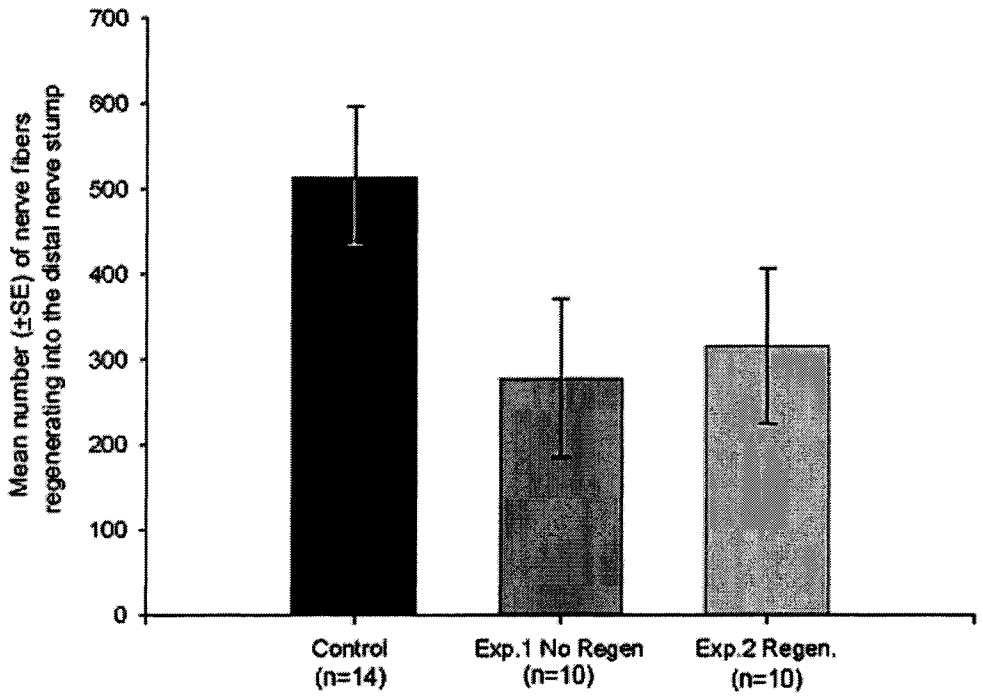

Figure 5: Number (mean ± S.E.) of nerve fibers that regenerated a distance of 2.5 cm into the distal nerve stump of the saphenous nerve following a period of either immediate nerve suture and target reinnervation (Control, 514.8±81.4 nerve fibers) or after chronic axotomy for a 2 m period under conditions in which axon regeneration was NOT permitted (Exp.1 – No Regen., 278±92.7 nerve fibers), or was permitted WITHOUT target reconnection (Exp. 2 – Regen., 280±87.7 nerve fibers). The trend for the chronically axotomized neurons to regenerate fewer nerve fibers was not significant.

2.5.6 Figure 6. Mean diameter (±SE) of nerve fibers regenerated

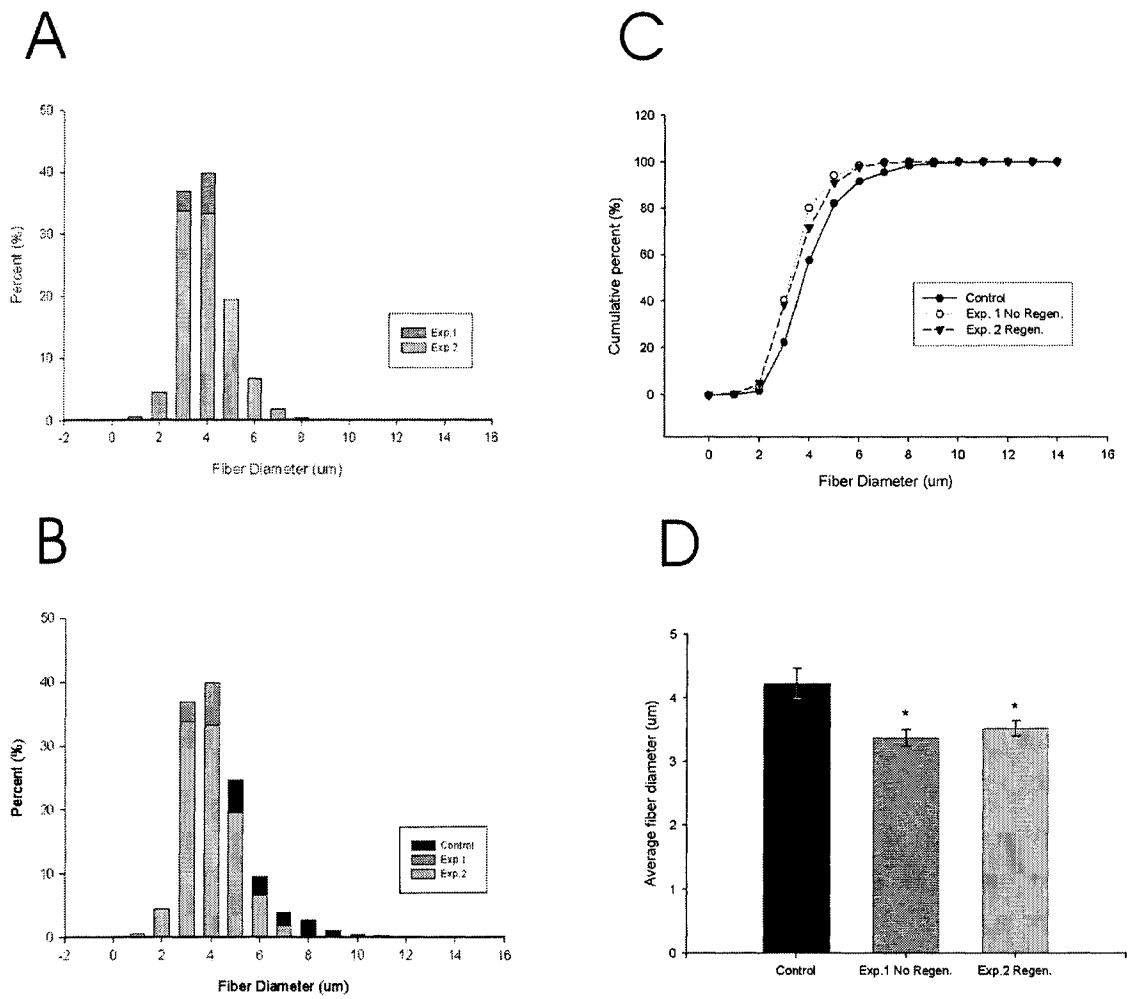

Figure 6: The diameters of nerve fibers that regenerated axons after a second nerve section and repair are shown either as percent frequency histograms (A,B), cumulative histograms (C) or as arithmetic means (± S.E) in D. The three groups are the control, Exp1 and Exp2. The neurons did not experience chronic axotomy in the control group. In the two experimental groups, the femoral neurons were chronically axotomized for 2 months prior to a 6 w period of axon regeneration. Nerve fiber diameters were measured in nerve samples taken 2.5 mm distal to the suture site. In the Exp.1 –No Regen. Group, axon regeneration was prevented during the 2 m period of chronic axotomy. In the Exp. 2 – Regen. Group, axons were encouraged to regenerate within a saphenous nerve graft. Fiber diameters were normally distributed in all 3 groups (A-C). The nerve fibers were significantly bigger in the Control group than in either of the 2 experimental groups ($p<0.05$), whose fiber diameter histograms overlapped and were not significantly different ($p>0.05$). * denotes statistical difference between experimental groups and Control ($p<0.05$).

2.5.7 Figure 7. Mean number (±SE) of retrogradely labeled motoneurons compared to mean number (±SE) of regenerated nerve fibers

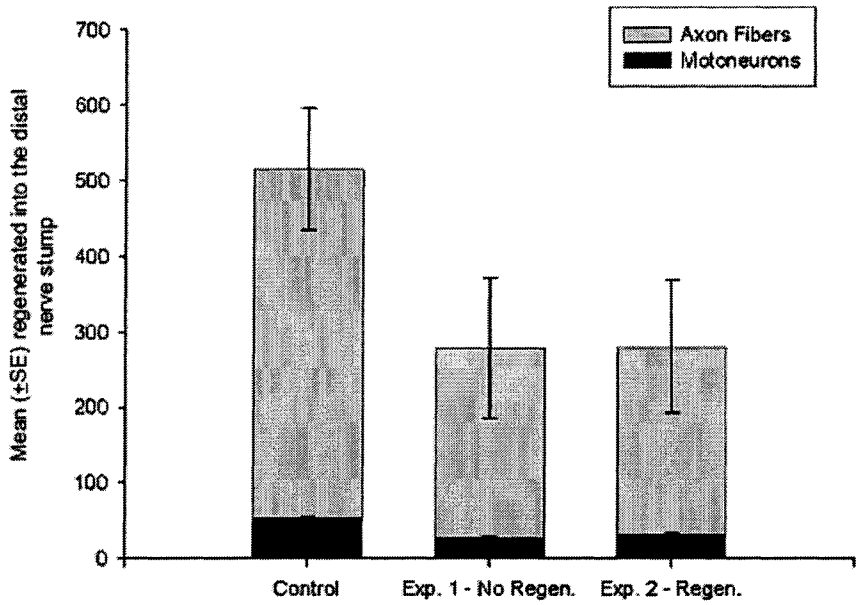

Figure 7: The average number of motoneurons that regenerated into the distal nerve stump and were retrogradely labeled is compared to the average number of axon fibers regenerated into the distal nerve stump. In each group, there is approximately a 10:1 ratio of average axon fibers (±SE) to motoneurons (±SE). Control (514.8±81.4 axon fibers vs. 54±1 motoneurons), Exp. 1 – No Regen. (278±92.7 axon fibers vs. 28±1 motoneurons), Exp. 2 – Regen. (280±87.7 axon fibers vs. 32±1 motoneurons) Though this calculation does not include sensory axons present in the femoral nerve motor branch, if proportionally the same numbers of sensory fibers are assumed to regenerate in each case, each motoneuron can be assumed responsible for the same number of daughter axon sprouts regenerating in to the distal nerve stump in each experimental condition.

2.5.8 Figure 8. Schematic representation of neurotrophic support in the distal nerve stump

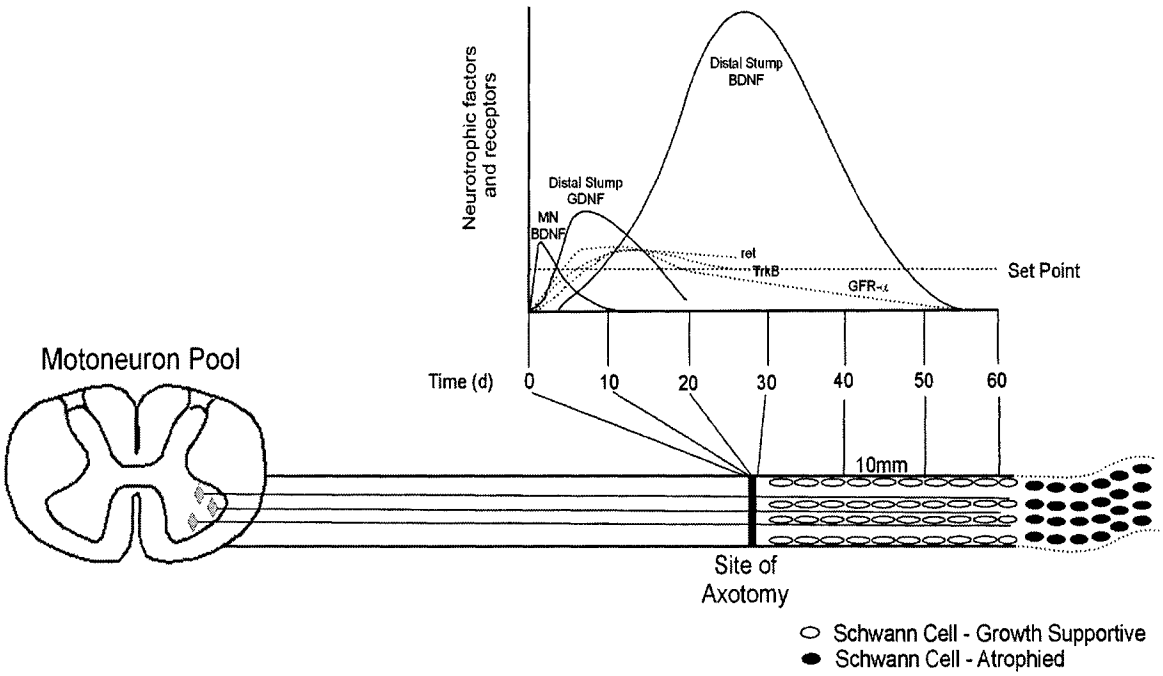

Figure 8: Schematic representation of the neurotrophic support of regenerating axons following axotomy of motoneurons (Modified from Boyd and Gordon, 2003b). BDNF and TrkB are rapidly upregulated by the motoneurons (MN) as are GDNF and its receptors, ret and GFR-α. The neurotrophic factors are downregulated rapidly but the receptors decline more slowly. The upregulation of the neurotrophic factors in the distal nerve stump is greater especially for BDNF but BDNF upregulation occurs more gradually than in the motoneurons. The expression of the neurotrophic factors and their receptors declines slowly within 60d of axotomy. Even after immediate nerve repair, axon sprouts from the proximal nerve stumps 'stagger' across the suture line such that all axons cross within 30d (Brushart et al., 2002b). Hence it is likely that the elevated neurotrophic factors and their receptors in the motoneurons are important in the outgrowth of the axons but play a progressively receding role with time. Axons regenerating at a rate of 1-3 mm/day are also exposed to progressively declining levels of neurotrophic factors in the distal nerve stump although the low levels of BDNF in the distal nerve stump may be sufficient to support axonal regeneration for some weeks before the chronically denervated Schwann cells atrophy (see Discussion for details).

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CHAPTER 3

THE PHOSPHODIESTERASE INHIBITOR ROLIPRAM PROMOTES AXONAL REGENERATION OF PERIPHERAL NERVES ACROSS THE SURGICAL GAP

3.1 INTRODUCTION:

The role of cyclic adenosine monophosphate (cAMP) in peripheral axon regeneration has largely been neglected since the promising studies of Carlsen in the 1980s. Carlsen initially reported that adenylate cyclase, responsible for the catalyzation of cAMP formation from adenosine triphosphate (ATP), underwent transient retrograde transportation following transection of the frog sciatic nerve (Carlsen, 1982). This was the basis for the suggestion that the adenylate cyclase: cAMP system may have an important role in peripheral axon regeneration. Subsequent investigation of the adenylate cyclase: cAMP system found that the previously reported accumulation of adenylate cyclase activity did in fact result in a dramatic increase in cAMP concentration in the regenerating nerve stump. This elevation of cAMP was temporally coincident with the initiation and elongation of regenerative nerve sprouts (Kilmer and Carlsen, 1984). It was also reported that supplementing this accumulation of adenylate cyclase activity with Forskolin, a potent activator of adenylate cyclase, resulted in a sustained increase in rate of sensory nerve regeneration *in vivo* (Kilmer and Carlsen, 1984). Forskolin was shown to increase levels of cAMP *in vitro*, and this suggested that the growth promoting effects of Forskolin *in vivo* may be mediated through elevated cAMP levels. Evidence for this suggestion were provided by the last experiments published by this group, examining the effects of cAMP elevating agents including Forskolin, cAMP analogues, and a phosphodiesterase (PDE) inhibitor on mammalian peripheral nerve regeneration (Kilmer and Carlsen, 1987). Chronic infusion of Forskolin or cAMP analogs resulted in a reduction in time to initiation of outgrowth as well as an increased rate of elongation. However, infusion of the PDE inhibitor theophylline was reported to produce a decrease in time to initiation of outgrowth, but decrease the rate of axonal outgrowth. The effects of theophylline were mimicked by caffeine, which is less potent PDE inhibitor and suggested that this effect may not be due entirely to an increase in cAMP concentration.

Similar to the effects of elevated cAMP reported by Carlsen and colleagues, previous investigations had reported that a conditioning lesion (CL), which is a crush injury or transection of the nerve distal to and temporally prior to a test lesion, results in a significant increase in the outgrowth of regenerating neurons (McQuarrie et al., 1977; McQuarrie, 1978; McQuarrie et al., 1978; Jacob and McQuarrie, 1993). The effects of

dbcAMP on axonal outgrowth were at that time reported to have no effect on the numbers or rate of regenerating axons (McQuarrie et al., 1977). This appeared to rule out an elevation of cAMP in explaining the mechanism of the "conditioning lesion effect" (CLE). However, this disagreed with earlier reports by Pichichero et al. (1973) observing an increased rate of reflex recovery following treatment of crush injured sciatic neurons with dbcAMP, as well as the aforementioned Carlsen experiments. As a possible explanation to resolve these contradictory reports, the respective increases in cAMP were not quantified, and it is possible that a minimum level of cAMP elevation is required to affect regenerative rates. This point may be supported indirectly by the observations of Kilmer and Carlsen (1987), where by increasing the concentration of exogenously administered cAMP increased the regeneration promoting effect. This could indicate a minimum level of cAMP elevation is required to noticeably affect regeneration rates, a minimum level not met by the administration of dbcAMP by McQuarrie et al. (1977), but met by the conditioning lesion as well as by Pichichero (1973) and Carlsen (1982, 1984, 1987).

Investigation into the mechanisms of enhancement of regeneration by a conditioning lesion reported that *in vitro*, the conditioning lesion resulted in earlier neurite initiation, greater outgrowth and a reduction in initial neurite branching, resulting in straighter neurites extending further from the cell body (Lankford et al., 1998). This appears to be quite similar to the effects described by Kilmer and Carlsen (1987) whereby chronic infusion of Forskolin or cAMP analogs resulted in a reduction in time to initiation of outgrowth as well as an increased rate of elongation of neurites. However, the exact molecular mechanisms of the conditioning lesion effect were never understood.

Since that time, evidence for the role of cAMP in peripheral regeneration has come indirectly from studies involving central nervous system (CNS) regeneration. Initially, a role for cAMP in the CNS was suggested following observation that growth cones of cultured neurons turned towards a gradient of dbcAMP (Lohof et al., 1992). Following this, a series of experiments from different groups reported a central role of neuronal cytosolic cAMP levels in determining whether particular gradients of growth factors and guidance molecules would be attractive or repulsive for growth cone turning and elongation (Ming et al., 1997; Song et al., 1997; Song et al., 1998). Importantly, it

was reported that modulating growth cone turning via electrical activity was dependent on Ca^{2+} influx, cAMP increase and PKA activation, as blocking of either of these resulted in ablation of the stimulation effect (Ming et al., 2001b). This suggests that an influx of Ca^{2+} leads to an increase in cAMP, presumably through an activation of Ca^{2+} dependent adenylate-cyclases (Xia and Storm, 1997) and thus activates PKA.

Cyclic AMP was directly implicated in central axon regeneration a short time later, when priming of cerebellar neurons with dbcAMP or neurotrophins, shown to raise cAMP, resulted in these neurons being able to regenerate on potent inhibitors of axonal regeneration normally present in the CNS (MAG/myelin) (Cai et al., 1999a). In this same study it was shown that the presence of a protein kinase A (PKA) inhibitor during priming completely ablated the effect. This suggested that this priming effect was indeed operating through activation of PKA.

In a further investigation of the molecular mechanisms of the priming effect, Cai et al. (2002) showed that the priming resulted in the synthesis of polyamines. This occurred through a transcription-dependent upregulation of Arginase I, a key enzyme in their synthesis. Inhibiting this polyamine synthesis resulted in the blocking of the cAMP effect on regeneration. Overexpression of Arginase I or delivery of exogenous polyamines were found to be each sufficient to overcome inhibition by MAG/myelin (Cai et al., 2002). This suggests the pathway presented in Figure 1; increased levels of cAMP, activation of PKA, transcription-dependent upregulation of Arg I, resulting in polyamine synthesis and ultimately the ability of these central axons to regenerate on inhibitors of regeneration in the CNS.

Tying these cAMP studies in the CNS more closely to peripheral nervous system (PNS) axon regeneration, it was shown that if the peripheral branches of DRG neurons were lesioned before the dorsal column central axons (conditioning lesion) the central axons which normally do not regenerate, undergo extensive regeneration (Qiu et al., 2002b). This is similar to the growth occurring following priming. Following peripheral lesion, DRG-cAMP levels were shown to triple and Myelin/MAG no longer inhibited growth. This was PKA dependent, as inhibitors of PKA at the time of peripheral lesion prevent this effect. Furthermore, injection of dbcAMP into the DRG completely mimicked the effect of the conditioning lesion, allowing extensive regeneration of the

dorsal column axons (Neumann et al., 2002; Qiu et al., 2002b). This suggests that the conditioning lesion is resulting in an increase in cAMP, identical to that which occurs following priming, and results in a similar initiation of enhanced axon regeneration in the normally growth inhibitory environment of the CNS (Snider et al., 2002; Qiu et al., 2002a) perhaps through the same molecular mechanism. Additionally, it has been demonstrated that either stimulation of the peripheral axons, or injection of dbcAMP elevated levels of tubulin and GAP-43 (Han et al., 2004; Al-Majed et al., 2004).

It is on this basis of evidence that we intend to investigate the molecular mechanism of the conditioning lesion effect on enhancing peripheral regeneration. It has as discussed above, been reported that following the conditioning lesion of peripheral axons, DRG-cAMP levels are increased and this increase is initially PKA dependent (Qiu et al., 2002a). As well, artificially enhancing cAMP levels resulted in an increased rate of peripheral axonal elongation (Kilmer and Carlsen, 1987), in effect mimicking the conditioning lesion's effect. It remains to be seen if the pathway elucidated in central regeneration is in fact the pathway responsible for enhancing peripheral regeneration. This pathway is readily testable *in vivo* and could perhaps provide possible clinical targets to promote peripheral regeneration.

In this experiment, we explore the aforementioned pathway (Figure 1). We tested the effect of rolipram, a cAMP specific PDE inhibitor, on the regeneration of axotomized common peroneal (CP) sensory and motor axons. PDE inhibitors were shown previously to increase initiation of outgrowth but not elongation of sensory axons using the pinch test (Kilmer and Carlsen, 1987), however we investigate the cAMP specific PDE inhibitor rolipram in both motor and sensory neuron regeneration at a dosage recently reported to enhance regeneration and functional recovery following spinal cord injury (Nikulina et al., 2004). If indeed cAMP enhances axon regeneration in the PNS, rolipram should by reducing cAMP breakdown, promote regeneration of both motor and sensory axons.

3.2 MATERIALS AND METHODS:

3.2.1 Experimental Design

Forty female Sprague Dawley rats, weighing approximately 180 to 210 grams at the beginning of the experiment, were obtained from University of Alberta Biological Sciences Animal Services. Rats had access to food and water ad libitum, were housed in a flat bottom, woodchip filled cage and were exposed to a 12 hour light/dark cycle. All experimental manipulations were performed adhering to Canadian Council on Animal Care guidelines and approved by the Health Sciences Animal Welfare and Policy Committee (University of Alberta).

Rats were randomly assigned to either of the two experimental groups, Group 1 - control saline treatment or Group 2 - rolipram treatment. The CP branch of the sciatic nerve was used as our model of peripheral neuron injury and regeneration (Figure 2). In each of the groups, the CP nerve was exposed at the level of the knee, transected and the proximal and distal sumps were repaired to each other (n-n repair). This afforded direction of the regenerating motor axons into the epineurial sheath of the distal stump. Immediately following this initial surgery, a second surgery was performed to implant an Alzet pump, containing either saline (Group 1 – Control) or rolipram, the PDE inhibitor (Group 2 – Experimental). We investigated the regeneration of both motor and sensory neuron axons comparing rolipram treatment to a control saline treatment in a series of 4 experiments (Table 1); Experiment 1: a two week time period of regeneration following CP n-n repair and pump implantation, with retrograde Fluorogold (FG) labeling at a point 10mm into the distal nerve stump, Experiment 2: a one week time period of regeneration following CP n-n repair and pump implantation with FG labeling 1.5mm distal into the distal nerve stump, Experiment 3: four and a half weeks following CP n-n repair and pump implantation, we examined recovery of whole muscle and motor unit recovery following CP n-n repair and pump implantation, and finally Experiment 4: measurement of cAMP levels of dorsal root ganglion lumbar 4 and 5 removed at a period of 3 days post CP n-n repair and pump implantation.

3.2.2 Surgical Procedures

Anesthesia was induced in all animals by intraperitoneal injection of a ketamine hydrochloride (60mg/kg - Ketalean, Bimeda, Cambridge Ont.) and xzlazine (8mg/kg -

Rompun, Bayer AG, Germany) cocktail. Animals were operated on only when judged to be at surgical plane of anaesthesia. Surgical plane was assessed by limb withdrawal in response to light hind toe pinch normally sufficient to elicit the reflex. All surgical procedures were performed in an aseptic manner. The lateral aspect of the right hind limb and upper back was shaved and prepared with 70% ethanol. Each animal was prepared with opthalmic ointment to prevent cornea damage (Duratears Naturale, Alcon, Ontairio).

Surgery 1A

In both groups of all experiments, an incision was made along the lateral right hindlimb and the common peroneal nerve branch of the sciatic nerve was exposed and dissected. At a point 10mm from the entrance of the common peroneal nerve into the tibialis anterior muscle, the common peroneal nerve was transected. The transected common peroneal nerve was then repaired (n-n repair) using 9-O Ethilon® nylon (Ethicon, Inc. Somerville, NJ, U.S.A.) epineurial sutures and a 3mm segment of 0.76mm interior diameter (ID) silicone Silastic ® tubing (Helix Medical, Inc. Carpinteria, CA). The overlying muscles were sutured back into place using a continuous 5-O silk suture (Ethicon, Inc. Somerville, NJ, U.S.A.) and cleaned with saline. Finally, the skin incision was repaired using a series of 4-O silk sutures (Ethicon, Inc. Somerville, NJ, U.S.A.).

Surgery 1B

Immediately following the first surgery, the Alzet pump was implanted. An incision of 2cm was made between the shoulder blades of the rat. A pocket was formed between the skin and underlying muscles along the right flank of the animal using large blunt nosed scissors. The Alzet osmotic minipump (Pump model:2ML2, flow rate: 5.0ul/hr - Durect Corporation, Cupertino, CA) was then filled with the appropriate solution to allow delivery of either 0.4μmol/kg/hr of rolipram in saline/DMSO (Nikulina et al., 2004) or saline/DMSO alone and then placed into this pocket.

The 2.0mL/5.0μL/hr flow rate pumps were prepared by filling with either 2ml of 0.0160 M rolipram in 50% Saline and 50% dimethyl sulfoxide (DMSO) or 2mL of 50% Saline and 50% DMSO. The rolipram solution was prepared by dissolving 0.00881g of rolipram per 1 mL DMSO, then adding 1mL of saline.

These pumps were then incubated in saline at 37°C for approximately 1 hr prior to implantation. This incubation was suggested by manufacturer to allow the pump to achieve steady-state flow rate before being implanted. Once the pump was implanted, the overlying area of incision was cleaned using sterile saline and the skin was repaired with 4-O silk suture.

3.2.3 Retrograde Labeling of Motor and Sensory Neurons

Experiment 1

In the first group of experiments, a two week period of rolipram delivery and recovery was allowed following surgery 1. The common peroneal nerve was then dissected under surgical plane anesthesia as previously described. At a point 10mm distal to the point of common peroneal nerve repair, the nerve was transected and the tip of the proximal nerve stump exposed to 4% Fluorogold (FG; Fluorochrome Inc., Denver, CO)in 0.1 M cacodylic acid for 1h in a Vaseline® well (Richmond et al., 1994) (Figure 3A1). Animals were allowed to recover for 7 days to allow for retrograde transport of the applied fluorescent dye to the neuronal cell bodies.

Experiment 2

In the second group of experiments, a one week period of rolipram delivery and recovery was allowed following surgery 1 (Figure 3A2). The common peroneal nerve was then dissected under surgical planed anesthesia. At a point 1.5mm from the point of common peroneal nerve repair, the nerve was crushed and a micropipette was used to inject approximately 300ul of FG into the crush area using a picospritzer (Intercel Picospritzer III). Animals were allowed to recover for 7 days before perfusion.

3.2.4 Motor and Sensory Neuron Enumeration

In all groups, rats were deeply anesthetized with sodium phenobarbital (Somnotol®: 30 mg/kg, intraperitoneal injection) and perfused with 100mL of saline followed by 500mL of ice-cold 4% paraformaldehyde (pH 7.4) through the aorta. Following perfusion, thoracolumbar spinal cord segment T11 to L2 containing the common peroneal nerve motoneuron cell bodies (Swett et al. 1986), as well as the right DRGs L4 and L5 were removed and post fixed in 30% sucrose in 4% paraformaldehyde solution overnight. Spinal cord tissue and DRGs were then embedded in Optimum Cutting Temperature (OCT) Tissue-Tek Liquid (Sakura, Japan, distributed by Cedarlane

Laboratories Canada), frozen in liquid nitrogen and stored at -80°C prior to cryostat sectioning.

Thoracolumbar spinal cord segments harvested from each of the animals were sectioned into 50μm transverse longitudinal sections, while dorsal root ganglion tissue was sectioned into 10μm cross-sections using a cryostat (Jung 3000). These sections were serially mounted onto glass slides, allowed to dry and cover slipped. Backlabeled common peroneal motoneurons and sensory neurons were identified and enumerated at 20X magnification under UV fluorescence at barrier filters of 430nm. Cells labeled with FG showed blue fluorescence (Figure 3B). Multiple counting of split neurons was corrected for by the method of Abercrombie and Johnson *(Abercrombie and Johnson, 1946)* with a correction factor of 0.63 for motoneurons and 0.25 for sensory neurons.

3.2.5 Functional Evaluation – Muscle Tetanic Force and Motor Unit (MU) Recording

Experiment 3

At a period of 4.5 weeks from surgery 1, animals were anesthetized using a ketamine hydrochloride and xzlazine cocktail as described above. The trachea was cannulated for mechanical ventilation if necessary. Maintenance doses of anesthetic were supplied via a cannula inserted into the right external jugular vein. To maintain blood volume, 0.5-1 ml of the 5% dextrose-saline solution was administered via the intravenous cannula approximately once per hour throughout the experimental procedure.

A laminectomy was performed from lumbar vertebrae 1 to 6 (L1 to L6). The tibialis anterior (TA) muscle in both the right and left hindlimb was isolated by denervating all hip, tail and limb muscles except the TA. This was achieved by transecting the femoral nerve and the tibial nerve. The distal tendon of the TA muscle was isolated, cut and a securely tied with 2-0 silk for force recording. The EDL and peroneal group tendons were cut to prevent inadvertent muscle forces. Finally, two Teflon-coated fine silver wires with bared ends (5mm) were inserted along the sciatic nerve to allow direct stimulation.

The hindlimb was then immobilized at the knee and ankle using a stereotaxic frame and the distal TA tendon was secured to a Kulite Force Transducer (10N to 0.1mN for whole-muscle and MU recordings, respectively). Isometric muscle twitch and tetanic

forces were measured in response to suprathreshold (2X threshold) stimulation of the sciatic nerve.

Isometric MU twitch forces of the experimental leg were recorded in response to suprathreshold (2X threshold) stimulation of teased filaments of L4 and L5 ventral roots. A single MU was isolated by the all-or-none recruitment of a twitch contraction by steadily increasing the stimulation voltage applied to the rootlet. Representative MU mean twitch forces were obtained from each animal. The total number of MUs in each muscle was estimated by dividing the whole muscle twitch force by the mean MU twitch force. Procedures of muscle and MU force recording have been previously described in detail (Tam et al., 2001).

3.2.6 cAMP Measurement

Experiment 4

At a period of 3d after surgery 1, animals were again anesthetized using sodium phenobarbital (Somnotol®: 30 mg/kg, intraperitoneal injection) and the right lumbar dorsal root ganglion 4 and 5 (DRG L4 and L5) were removed and immediately frozen in liquid nitrogen. Samples were homogenised in 0.1N hydrochloric acid (HCl) and 0.5mM 3-isobutyl-1-methylxanthine (IBMX) and then cAMP levels analyzed following manufacturer's instructions (R&D cAMP (low pH) Immunoassay, R&D Systems Inc., MN U.S.A.).

3.2.7 Statistical Analysis

Statistical analysis between group mean values ($\pm$SE) was assessed through one-way analysis of variance (ANOVA) (GraphPAD InStat Software V. 1.13 (1990) Dr. Paton, University of Alberta). Statistical significance was accepted at 5% ($p<0.05$).

3.3 RESULTS:

3.3.1 Rolipram Resulted in Increased Motor and Sensory Axon Regeneration into the Distal Nerve Stump

We addressed whether the elevation of cAMP through sustained delivery of rolipram, a cAMP PDE inhibitor, resulted in increased motor and sensory axon regeneration following transection and n-n repair in the PNS in experiments 1 and 2. In the first experiment, we investigated the number of motor and sensory neurons that were

able to regenerate their axons 10mm into the distal nerve stump over a 2 week period of recovery. Following CP nerve transection, n-n repair and 2 week rolipram treatment, the mean number (±SE) of motor and sensory neurons that regenerated axons into the distal nerve stump was significantly greater than in the saline treated group ($p<0.05$) (Figure 4, Table 2). Sensory neuron regeneration was increased by 55.9% with rolipram treatment compared to saline treatment, while motoneuron regeneration was increased by 38.5% with rolipram treatment compared to saline treatment.

3.3.2 Rolipram Resulted in Increased Motor and Sensory Neuron Regeneration across the Site of Surgical Repair

To assess whether rolipram was promoting axonal regeneration by accelerating overall axonal regenerative rate or by promoting the outgrowth of axons across the surgical site, a recovery period of 1 week and a regenerative distance of 1.5mm from the site of n-n repair was investigated in experiment 2. The administration of rolipram continually for a one week period immediately following n-n repair resulted in significantly more motor and sensory neurons regenerating their axons 1.5mm across the surgical repair site than in the saline treated group ($p<0.05$) (Figure 5, Table 3). Rolipram treatment resulted in sensory neuron regeneration increasing by 188.3% compared to saline treatment, while motoneuron regeneration was increased by 82.5% compared to saline treatment.

3.3.3 Rolipram Treatment Resulted in Recovery of a Larger Number of Small Motor Units

To assess the effects of long term administration of rolipram had on recovery of function, we investigated recovery of muscle tetanic force and MU force and number, at a time period of 4.5 weeks following surgical n-n repair in experiment 3. The administration of continuous rolipram treatment for a four week period of recovery resulted in more motoneurons reinnervating significantly MUs (saline, 78.1 ± 7.5 (n=9) vs. rolipram, 103.3 ± 7.9 (n=9), Figure 6A) with significantly smaller average MU force (saline, 14.1 ± 1.7 (n=9) vs. rolipram, 8.9 ± 0.7 (n=9), Figure 6B). The relative increase in MN force in the saline treated compared with rolipram treated group compensated for the decreased number of MU in the saline treated compared with rolipram treated group with the result that the overall percent recovery of muscle tetanic force was the same

(saline, 35.2 ± 1.5 (n=10) vs. rolipram, 31.5 ± 2.9 (n=9), Figure 6C, Table 4). The recovery of only 35% of muscle tetanic force, when compared to contralateral control muscle, is most likely due to reduced recovery of muscle fiber cross-sectional area following denervation (Fu and Gordon, 1995a).

3.3.4 Rolipram Resulted in Increased cAMP Levels in the DRG

We hypothesized that the elevation of cAMP was having a direct effect on the regenerative program in the cell body of the injured neurons, as suggested by previous data indicating an increase in cAMP in the DRG results in the ability of these neurons to regenerate their central axons (Qui et al., 2002), as well as upregulate tubulin and GAP-43 (Han et al., 2004; Al Majed et al., 2004). However, it is also possible that the elevation of cAMP is affecting the growth supportive myelinating Schwann cells as suggested by recent research, showing the mytogenic properties of rolipram on this population of cells (Walikonis and Poduslo, 1998). To ascertain whether cAMP was acting directly on the cell body or indirectly through SC replication, the levels of cAMP were investigated in the DRG L4 and L5 3 days following surgery 1 and pump implantation in experiment 4. Rolipram treatment resulted in significantly increased levels of DRG intracellular cAMP compared to Saline treated controls (Figure 8, Table 5). This was 138% of the saline treated control group. Both groups had significantly elevated cAMP when compared to normal levels, 300% and 414%, saline and rolipram respectively. This approximately agrees with previous data from Qui et al. (2002) which demonstrated a 250% increase in cAMP 24 hours following a conditioning lesion, similar to the transection and repair in our experiment. This clearly demonstrates that rolipram elevated cAMP levels up to 4X "normal" uninjured levels and significantly more than that of CP-transection alone with saline treatment.

Interestingly, following the prolonged period of rolipram administration (4 week), we also recorded significantly lower animal body weight in the rolipram treated group (saline: 314.3 ± 6 (n=8) vs. rolipram: 279.4 ± 6.5 (n= 7), Figure 7, Table 4). It is suspected that the prolonged period of rolipram treatment (4 week) had suppressive effects on appetite.

3.4 DISCUSSION:

3.4.1 Effect of Rolipram Treatment on Motor and Sensory Regeneration and Functional Recovery

The present results indicate that subcutaneous delivery of the cAMP specific PDE inhibitor rolipram results in significantly increased motor and sensory axonal regeneration. We demonstrate that at a two week period of recovery there are significantly more motor and sensory neurons extending axons into the distal nerve stump in the rolipram treated group compared to the saline treated control group. Further, we demonstrate that the increase in regeneration of both motor and sensory axons seen in the rolipram treated group is present and in fact more pronounced at a period of one week directly across the site of surgical repair.

We interpret this as indicating that the acceleration of axon outgrowth following rolipram treatment is due primarily to an increase in neurite outgrowth immediately across the site of surgical repair. This is supported by observing the percentage increase in the number of regenerating sensory and motor axons: percentage wise there was a greater increase in axonal regeneration of both the motor and sensory neurons with rolipram treatment at one week immediately across the surgical site than at two weeks 10mm into the distal nerve stump (rolipram /saline*100) (Table 2 and 3). This is in agreement with previously published data of Kilmer and Carlsen (1987), who reported that the nonspecific PDE inhibitor theophylline produced a faster initiation of sensory axon outgrowth, while not accelerating overall regenerative rate by means of the pinch test.

These results are also in agreement with previous data demonstrating the ability of electrical stimulation to increase motor axon regeneration by promoting the onset of axon regeneration without increasing overall regenerative speed (Al-Majed et al., 2000c; Brushart et al., 2002b). Electrical stimulation has been shown to result in the elevation of both BDNF and its receptor TrkB (Al-Majed et al., 2000b). BDNF has been demonstrated to act as a PDE inhibitor and increase intracellular cAMP (Cai et al., 1999b; Gao et al., 2003a), and in fact, electrical stimulation has been shown to result in increased levels of cAMP in the DRG (Gordon lab, unpublished data). The upregulation of BDNF through electrical stimulation has been linked to downstream upregulation of regeneration

associated genes (RAGs), including an upregulation of tubulin and GAP-43 (Al-Majed et al., 2000a; Al-Majed et al., 2004). GAP-43 has been associated with neurite extension as well as effective pathfinding to appropriate targeting of regenerating axons (Strittmatter et al., 1995; Aigner and Caroni, 1995; Aigner et al., 1995; Aigner and Caroni, 1993; Caroni et al., 1996). Indeed, electrical stimulation of injured motoneurons not only accelerated outgrowth of injured motoneurons, but increased target specificity (Al Majed et al., 2000). Further, it has been reported that exogenous delivery of dbcAMP to primary sensory neurons of the DRG results in increased expression of growth-associated tubulin isotypes, but does not increase the velocity at which tubulin is delivered to the tips of regenerating axons by slow component b transport (Han et al., 2004) (Figure 1). Slow component b transport rate is identical to the rate of regeneration (Wujek and Lasek, 1983). We demonstrate here that rolipram treatment results in an elevation of intracellular cAMP in the DRG above the elevation of intracellular cAMP found in the saline treated group at 3 days post-injury. The application of rolipram at the time of injury resulted in a 4X increase in cAMP when compared to baseline levels, and an approximate 38% increase in cAMP above saline treated control animals. This indicates that rolipram is mediating its regenerative effects through its effect of elevating cAMP in the cell body of effected neurons. This line of evidence suggests that elevating cAMP through either electrical stimulation (BDNF) or pharmacological manipulation (dbcAMP or rolipram) results in upregulation of downstream RAGs, including tubulin and GAP-43, resulting in accelerated axonal outgrowth.

Though not accelerating the overall regenerative rate of injured axons, the acceleration of axon outgrowth is an important advantage following axonal injury. For freshly axotomized sensory and motor neurons, it may take as long as a month for all the axonal sprouts to cross the suture line due to "staggered" asynchronous regeneration (Al-Majed et al., 2000c; Brushart et al., 2002b; Witzel et al., 2005). Following nerve crush injury just 25% of axons regenerating across the repair site within the first week (Witzel et al., 2005). The process of staggered axon regeneration across the repair site contributes to the long delays in axonal regeneration and a consequential deterioration of regenerative capacity (Fu and Gordon, 1995a; Fu and Gordon, 1995b; Gordon et al., 2003; Sulaiman O. et al., 2005; Furey et al., 2005).The physiological basis of axonal

stagger is not fully understood. Witzel et al. (2005) demonstrated neurons growing laterally across the repair site before entering distal nerve stump basal lamina tubes, while others enter directly, which may contribute to the staggered regeneration. In fact, it has been reported that a CL, shown to result in elevated cAMP, resulted outgrowth of neurites that elongated with fewer branching points (Lankford et al., 1998). Stagger may also be due to intrinsic differences in ability of individual neurons to mount a successful regenerative response. Indeed differences exist in axonal ability to regenerate in the presence of proteoglycan inhibitors present in the distal nerve stump (Krekoski et al., 2001; Groves et al., 2005). Groves et al (2005) demonstrated that the application of proteoglycan degrading enzymes resulted in a greater proportion of axons regenerating in the first week of regeneration into a nerve graft. This indicates not only that removal of proteoglycan inhibition results in enhanced axonal regeneration, but that a select group of neurons regenerate initially even in the presence of such inhibitors. This indicates intrinsic differences in neuronal susceptibility to proteoglycan inhibition, inhibition which may be responsible for axonal stagger. As nerves degenerate during Wallarian degeneration following injury, proteoglycan degrading enzymes, including matrix metalloproteinases MMP-2 and MMP-9 are upregulated and result in increased growth-promoting properties of degenerated nerve (Ferguson and Muir, 2000). We hypothesize based on this evidence that once Wallarian degeneration occurs in the distal nerve stump, inhibitory proteoglycans are removed by upregulated degrading enzymes which subsequently allows previously inhibited neurons to begin axonal regeneration. This could form the basis for staggered axonal regeneration. The elevation of cAMP and its downstream effects of upregulated RAGs could be due to elevated cAMP allowing previously inhibited neurons to overcome proteoglycan inhibition and regenerate axons into a freshly denervated, not yet degenerated distal nerve stump. This is similar to elevated levels of intracellular cAMP allowing ascending sensory axons to grow on normally inhibitory substrates, including MAG, and regenerate in the CNS (Neumann and Woolf, 1999a; Qiu et al., 2002b). This would reduce staggered regeneration to allow a greater proportion of neurons to regenerate their axons across the suture site earlier.

An alternative explanation for cAMP elevation resulting in accelerated motor and sensory neuron regeneration is the mitogenic effect cAMP has on Schwann cells

(Walikonis and Poduslo, 1998; Kim et al., 2001). Following nerve injury, Schwann cells normally begin to proliferate, with nonmyelinating Schwann cells reaching a peak of proliferation by 2 days after injury, 1 day earlier than myelinating Schwann cells (Clemence et al., 1989). The proliferation of Schwann cells is temporally correlated to the invasion of the distal nerve stump by macrophages, which release Schwann cell mitogenic factors such as cAMP-dependent Platelet Derived Growth Factor-B (PDGF-B), acidic and basic fibroblast factors (FGFs) as well as TGF-β (Reynolds and Woolf, 1993; Chandross et al., 1995). Schwann cell migration has been demonstrated to precede axon regeneration, as the axons regenerate along the Schwann cells; indeed, following surgical repair with a silicone tube, Schwann cells migrate along the tube prior to axon regeneration (Williams et al., 1983; Williams and Varon, 1985; Liu, 1992; Karlsson et al., 1993; Dahlin et al., 1995). The accelerated proliferation of Schwann cells could result in increased migration of Schwann cells across the micro-gap of the surgical repair site, providing the substrate necessary for axonal regeneration. Additionally, Schwann cells upregulate a number of axonal regeneration supportive molecules, such as neurotrophic factors, including BDNF, nerve growth factor (NGF), and glial-derived growth factor (GDNF) (Fu and Gordon, 1997a). The early proliferation of Schwann cells due to artificially increased cAMP may accelerate upregulation of such regeneration supportive molecules by the Schwann cells to result in accelerated outgrowth of injured axons across the repair site. In light of evidence for elevated cAMP in the DRG, as well as temporally coincident elevated tubulin and GAP-43 production in the cell body of injured neurons, it seems unlikely that the proliferation of Schwann cells is solely responsible for the ability of rolipram to accelerate the outgrowth of axons. It seems more likely that a combination of both situations is responsible for the accelerated regeneration.

In either scenario, the ability of elevated cAMP, either through electrical stimulation or pharmacological manipulation, to accelerate the regeneration of injured axons by reducing the amount of time required for injured axons to successfully cross the suture site and enter the distal nerve stump, reduces the amount of deterioration of regenerative capacity caused by prolonged denervation of the distal nerve stump and chronic axotomy of injured neurons (Fu and Gordon, 1995a; Fu and Gordon, 1995b). This is measured not only by a significantly increased number of motoneurons

regenerating into the distal nerve stump at both one and two weeks post-injury, but also by an acceleration of functional recovery. We demonstrate the continuous application of rolipram results in accelerated reinnervation of the muscle in a physiological "normal" pattern of MU and size. By 4.5 weeks, the saline treated control group and the rolipram treated group were equal in terms of recovered muscle tetanic force. However, the rolipram treated group regained a greater number of MUs which developed significantly lower forces. This is indicative not only of a greater number of motoneurons regenerating and reinnervating muscle, but of a regaining of fine control of movement by MU recruitment that is contingent on a large number of MUs (Fu and Gordon, 1995a).

3.4.2. Clinical Applicability of Rolipram Treatment

Undoubtedly, the acceleration of onset of axonal regeneration has beneficial effects on functional recovery as outlined above. However, we noted through the course of rolipram treatment that there was a significant decline in rat body weight compared to saline controls (Table 5). This suggests that prolonged system application of rolipram results in appetite suppression and corresponding body weight loss. This could be due to the common side-effects of nausea and emesis noted in clinical trials of rolipram as an anti-depressant (Hebenstreit et al., 1989).

If indeed we are correct in assuming that the acceleration of axon elongation occurs primarily across the surgical site and within the first week, treatment longer than 1 week or perhaps even shorter would be sufficient in order to accelerate the onset of outgrowth of regenerating axons. This would avoid unwanted side-effects of longer term rolipram administration including nausea and emesis noted in clinical trials of rolipram for depression, and related effects reported here of appetite suppression and weight loss. Additionally, site specific administration of rolipram to sensory and motor cell bodies using a catheter could allow a reduced dosage of rolipram to be effective in accelerating onset of axonal regeneration and perhaps alleviate unwanted side-effects.

In clinical cases of peripheral nerve injury and surgical repair, the acceleration of axonal regeneration across the surgical site and into the distal nerve stump offers undeniable benefits. By reducing the stagger of axonal regeneration and thereby the time required for all axons to cross the surgical site, the period of time in which the distal nerve stump and target organs remain denervated is reduced, a period of time which has

been shown to result in reduced regenerative capacity of the injured neurons (Fu and Gordon, 1995a; Fu and Gordon, 1995b; Furey et al., 2005). By accelerating axonal outgrowth from the proximal nerve stump across the surgical site and into the distal nerve stump, not only are the harmful effects of prolonged axotomy and denervation minimized, but most importantly, the clinical goal of functional recovery is accelerated and improved.

3.5 Tables

3.5.1 Table 1. Experimental Design

Experiment	Group 1 – Control	Group 2 – Experimental
1	CP N-N, Saline Pump 2 week regen., FG 10mm from N-N repair	CP N-N, Rolipram Pump (0.40μM/kg/hr) 2 week regen., FG 10mm from N-N repair
2	CP N-N, Saline Pump 1 week regen., FG 1.5mm from N-N repair	CP N-N, Rolipram Pump (0.40μM/kg/hr) 1 week regen., FG 1.5mm from N-N repair
3	CP N-N, Saline Pump 4.5 week regen., Muscle and MU recording	CP N-N, Rolipram Pump (0.40μM/kg/hr) 4.5 week regen., Muscle and MU recording
4	CP N-N, Saline Pump 3d later, removal of DRG L4 and L5 tissue and measurement of cAMP	CP N-N, Rolipram Pump (0.40μM/kg/hr) 3d later, removal of DRG L4 and L5 tissue and measurement of cAMP

CP = Common peroneal
FG = Retrograde Fluorogold Labeling
N-N = Nerve to Nerve repair
MU = Motorunit
DRG L= dorsal root ganglion lumbar

3.5.2 Table 2. Motor and Sensory Neuron Regeneration Following 2 Week Recovery

Group	Mean (±SE)	N	% of Saline
Motor			
Saline	250.4±35.6	7	
Rolipram	390.3±48.3	6	155.9
Sensory			
Saline	277.9±38.5	6	
Rolipram	384.8±22.3	7	138.5

3.5.3 Table 3. Motor and Sensory Neuron Regeneration Following 1 Week Recovery

Group	Mean (±SE)	N	% of Saline
Motor			
Saline	162.7±50.4	6	
Rolipram	469.1±101.8	5	288.3
Sensory			
Saline	191.0±35.0	5	
Rolipram	348.5±56.3	6	182.5

3.5.4 Table 4. Motor Unit Size and Number 4.5 Weeks Following CP Transection and Repair

Group	Motor Unit Size (mN)	Motor Unit Number	% Recovery Tetanic Force	Body Weight (g)	N
Rolipram	8.9 ± 0.7	103.3 ± 7.9	31.5 ± 2.9	314.3 ± 6	9
Saline	14.1 ± 1.7	78.1 ± 7.5	35.2 ± 1.5	279.4 ± 6.5	9

3.5.5 Table 5. DRG cAMP Levels

Group	Mean (±SE) (pmol/500µL)	N
Rolipram	37.3 ± 2.8	9
Saline	27.1 ± 3.0	9
Normal	9.01 ± 1.67	7

3.6 Figures

3.6.1 Figure 1. Model of cAMP Enhancement of PNS/CNS Regeneration

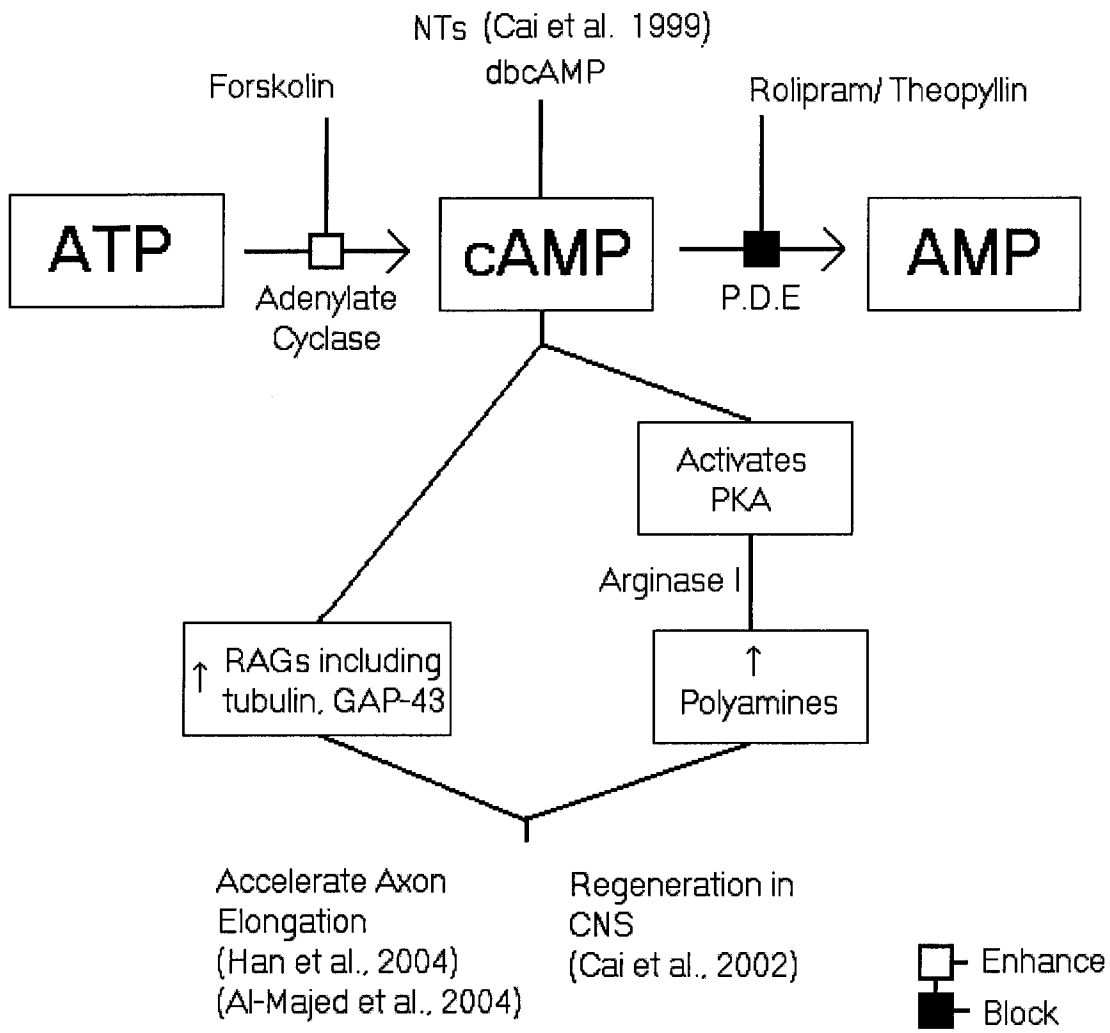


Figure 1: Adenosine triphosphate (ATP) is converted to cyclic adenosine monophosphate (cAMP) by adenylate cyclase. Artificially, adenylate cyclase is activated by Forskolin, resulting in an increase in adenylate cyclase and a corresponding increase in cAMP. cAMP can also be elevated by neurotrophins and cAMP analogues. cAMP is broken down into adenosine monophosphate (AMP) by a cAMP-specific phosphodiesterase (PDE). This breakdown is inhibited by PDE inhibitors such as rolipram and theophyllin, resulting in elevated levels of cAMP. cAMP then causes activation of protein kinase A (PKA). PKA results in the transcription dependent gene upregulation of Arginase I, and finally, Arginase I upregulation results in the formation of certain polyamines, and ultimately growth of central axons on normally inhibitory substrates such as myelin/myelin associated glycoprotein (MAG) (Cai et al., 2002). Alternatively, with increased cAMP, regeneration associated genes (RAGs).

3.6.2 Figure 2. Experimental Design

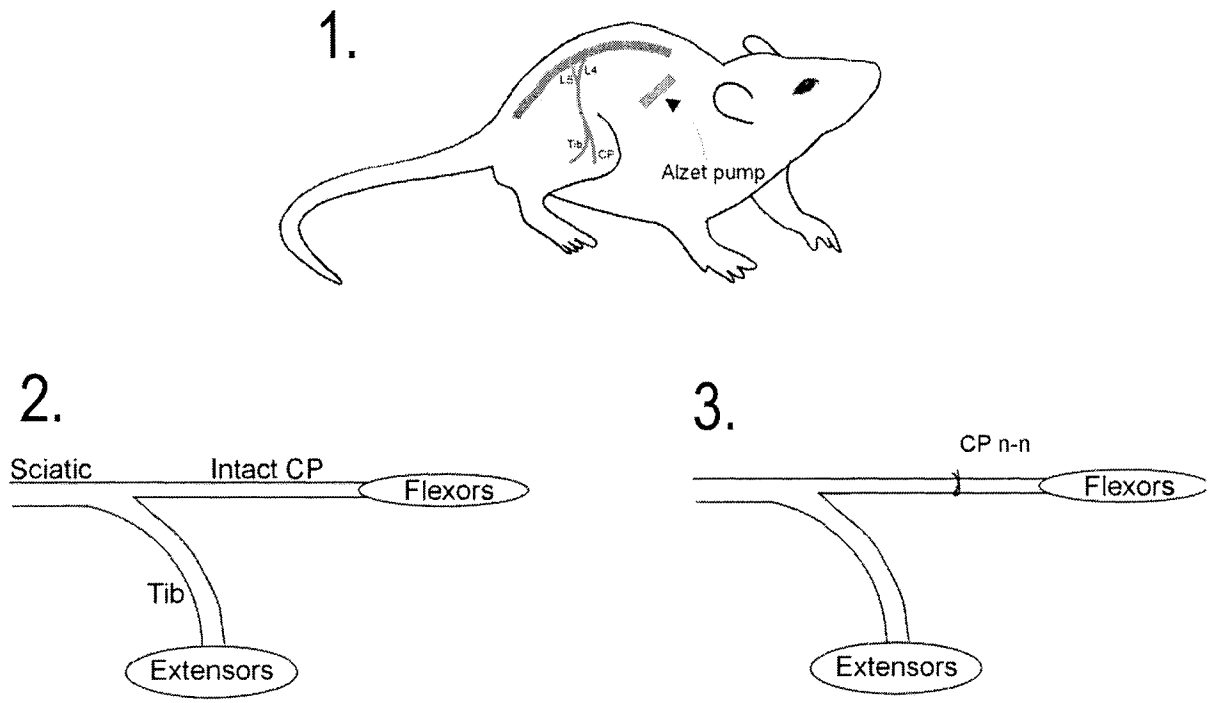


Figure 2: Model for pharmacological investigation of rolipram on peripheral nerve regeneration, indicating the subcutaneous implantation of the Alzet pump and the transection and repair of the common peroneal nerve 10mm from nerve-muscle interface.

3.6.3 Figure 3. Schematic representation of fluorescent backlabeling of common peroneal motoneurons.

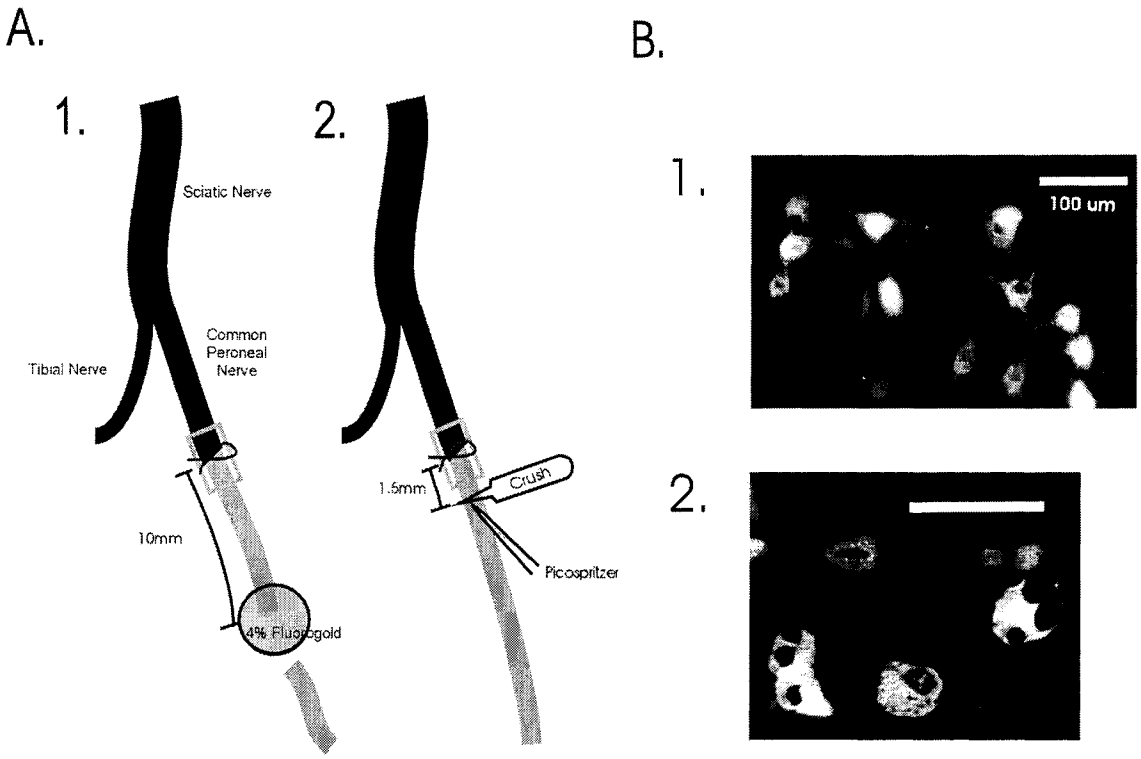
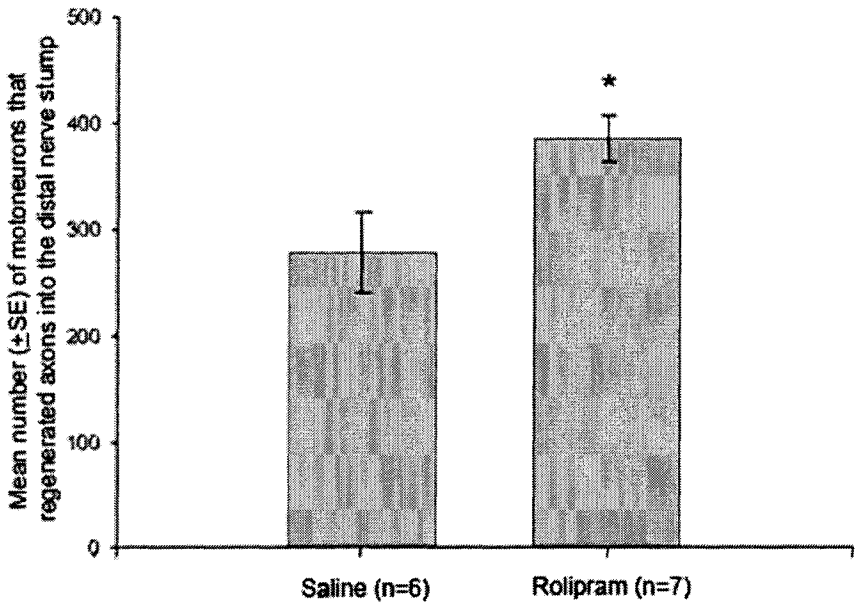


Figure 3: A1: Experiment 1: The common peroneal nerve was transected and repaired, and a period of two weeks was allowed for motor and sensory axon regeneration. At the end of two weeks, the nerve was transected again 10mm distal to nerve repair. The proximal stump was exposed to 4% Fluorogold (FG) in cacodylic acid for 1 hr in a Vaseline® well. A2: Experiment 2: The common peroneal nerve was transected and repaired, and a period of one week was allowed for motor and sensory neurons regeneration. At the end of one week, the nerve was crushed 1.5mm from the site of surgery 1, and 300ul of FG injected using a picospritzer. B: Photomicrographs of motoneurons backlabeled with FG. Fluorescently labeled motoneurons were identified by their blue white fluorescence under barrier filters of 430μm. B1 is a representative spinal motor neuron image, while B2 is representative of DRG sensory neurons.

3.6.4 Figure 4. Motor and Sensory Nerve Axonal Regeneration at 2 Weeks

A.



B.

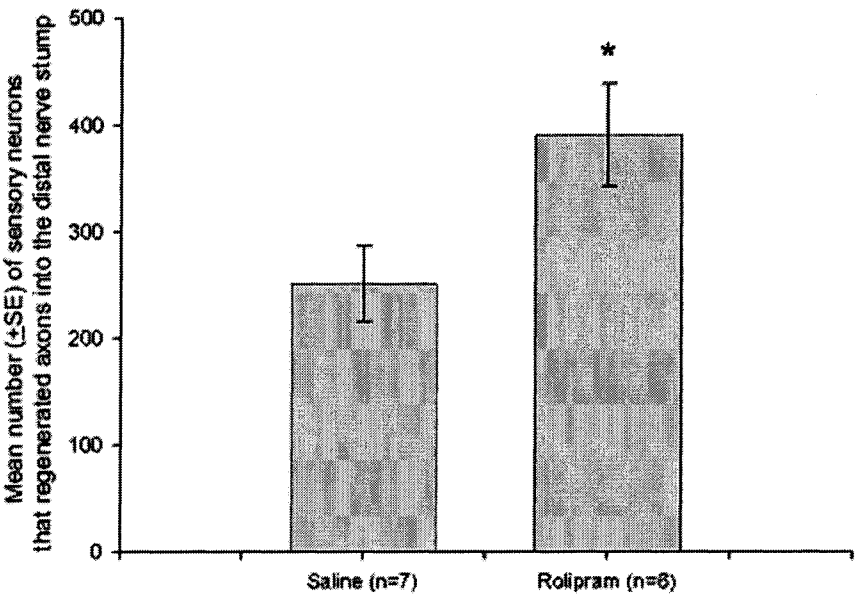
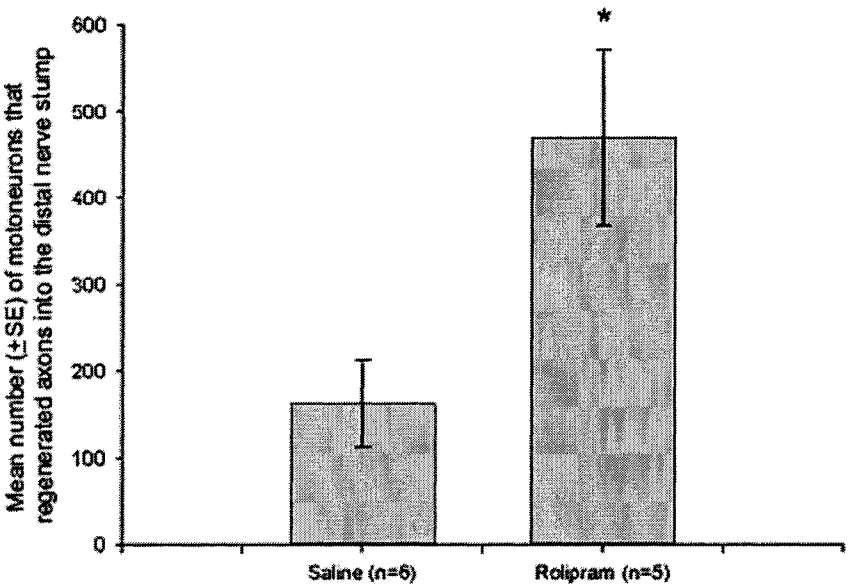


Figure 4: The arithmetic mean number (±SE) of motor (Fig. 4A) and sensory (Fig. 4B) neurons that regenerated axons 10mm into the distal nerve stump at a time period of 2 weeks in either the saline treated group or rolipram treated group. * denotes significant difference ($p<0.05$).

3.6.5 Figure 5. Motor and Sensory Nerve Axonal Regeneration at 1 Week

A.



B.

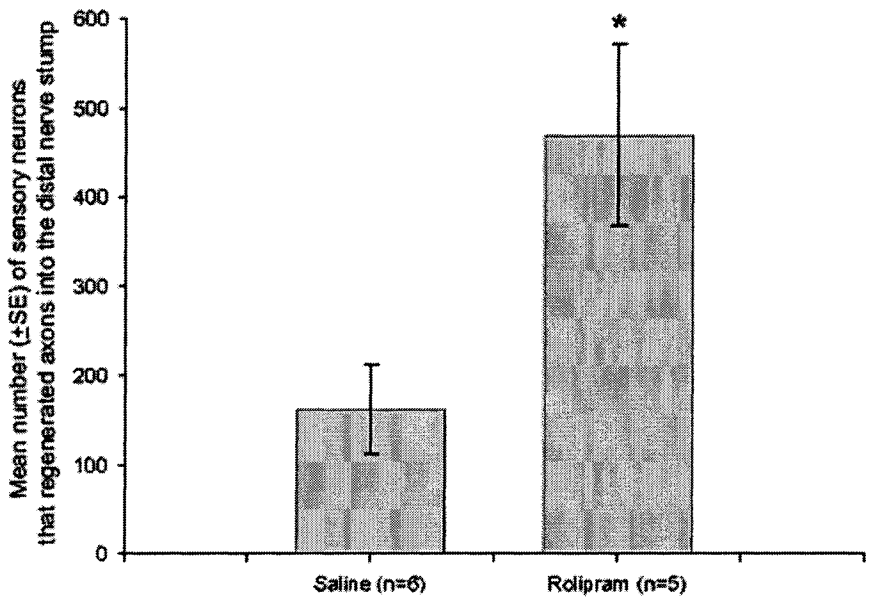
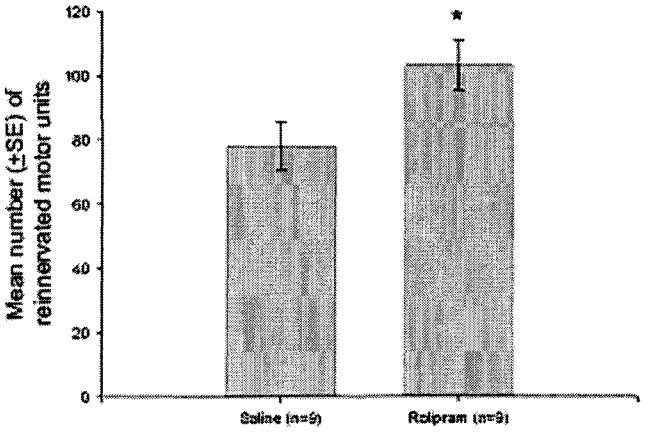


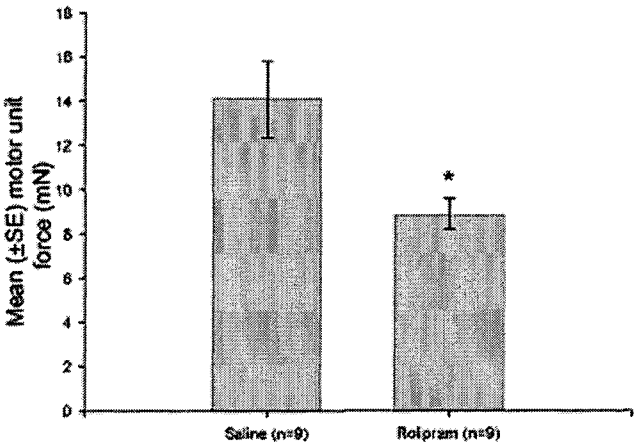
Figure 5: The arithmetic mean number (±SE) of motor (Fig. 5A) and sensory (Fig. 5B) neurons that regenerated axons 1.5mm across the site of surgical repair at a time period of 1 weeks in either the saline treated group or rolipram treated group. * denotes significant difference ($p<0.05$).

3.6.6 Figure 6. Reinnervated Motor Unit Size and Number

A.



B.



C.

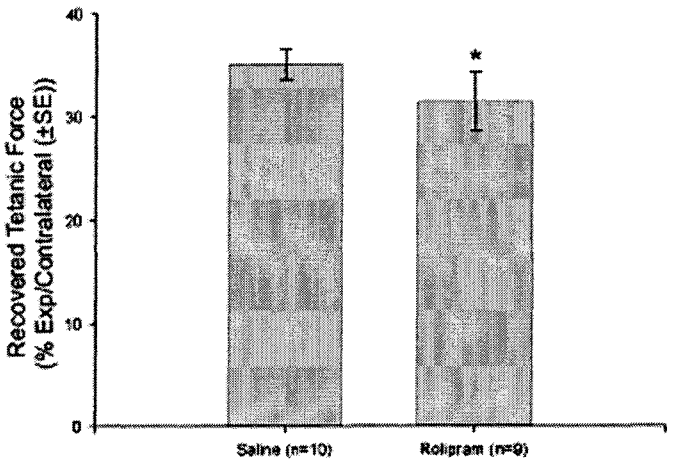


Figure 6: 4.5 week recovery of the tibialis anterior muscle; mean (±SE) number of MUs (A), mean (±SE) MU force (B), and % Force Recovery (experimental/contralateral control limb) (C) in either the saline treated group or the rolipram treated group. * denotes significant difference ($p<0.05$).

3.6.7 Figure 7. 4.5 Week Body Weight

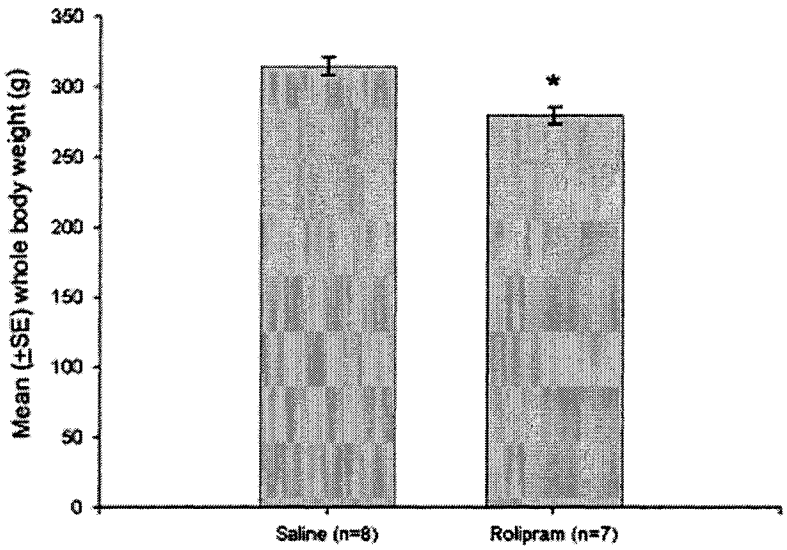


Figure 7: Mean (±SE) body weight following 4.5 week treatment with either saline or rolipram.

3.6.8 Figure 8. cAMP Elevation Following Rolipram Administration

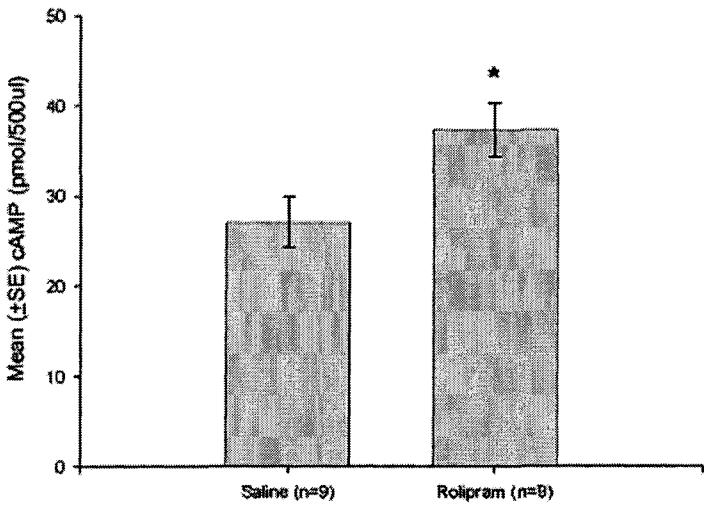


Figure 7: Arithmetic mean (±SE) of cAMP elevation 3 days following rolipram or saline treatment. * denotes significant difference ($p<0.05$).

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CHAPTER 4

THE EFFECT OF ELECTRICAL STIMULATION ON AXONAL REGENERATION IN THE CENTRAL NERVOUS SYSTEM

4.1 INTRODUCTION:

Following axotomy in the central nervous system (CNS), there is generally failure of the injured neurons to regenerate axons and reform functional connections (Cajal, 1991; Schwab and Bartholdi, 1996). This is in contrast to the robust regeneration and in most cases functional reinnervation seen following axotomy in the peripheral nervous system (PNS) (Cajal, 1991).

The difference in ability to regenerate axons and restore functionality between the CNS and PNS has been largely attributed to: 1) formation of a glial scar following injury in the CNS - a glial scar being a physical barrier to regeneration consisting predominately of reactive astrocytes and proteoglycans and a molecular barrier of inhibitory molecules upregulated in the core of the lesion including semaphorin 3, ephrin-B2 and its receptor EPHB2, and Slit proteins (Silver and Miller, 2004); and 2) molecular inhibitors of axonal regeneration found in CNS myelin - these inhibitory molecules including myelin-associated glycoprotein (MAG), NogoA and oligodendrocyte myelin glycoprotein (OMgp), which induce growth cone collapse and inhibit neurite outgrowth (Filbin, 2003).

The effect of these different environments on axonal regeneration is particularly evident in the case of the primary sensory neuron of the dorsal root ganglion (DRG). These sensory neurons possess bipolar axons that project both into the CNS and PNS (Figure 1), and have strikingly different regenerative capacities following injury. While regeneration of the peripheral axons following injury is well established there is a complete lack of regeneration of the central axons following dorsal column lesions (Neumann and Woolf, 1999b; Bradbury et al., 2000). Of particular interest however, is that the differential ability of these sensory neurons to regenerate axons can be modified. This modification of central axon growth capacity was first reported by Richardson and Issa (1984), showing that if a CL of the peripheral axons was inflicted concomitantly with a lesion of the central axons, the central axons were 100 times more likely to regenerate into a peripheral nerve graft. As well, it was reported that regeneration of the central axons between the DRG and the spinal cord, though not into the spinal cord, was accelerated by a CL occurring one week prior to the central axon lesion at the site of the DRG (Richardson and Verge, 1987).

These studies indicated that the sensory neurons could be coerced into increasing regeneration of central axons on peripheral grafts and in the transition zone from PNS to CNS, but an effect on the regeneration of the central axons within the inhibitory environment of the CNS had not been reported. Since these initial reports, it has subsequently been shown that a CL of the peripheral axons concomitant with or previous to the central axon lesion resulted in massive axonal regeneration into and even past the lesion site within the CNS (Neumann and Woolf, 1999b). This regeneration occurred through the normally inhibitory environment of the CNS, in contrast to a peripheral nerve graft and was thus a dramatic shift in regenerative capacity of these central axons. The noted regeneration indicated not only an ability to regenerate axons given favorable substrate (peripheral nerve graft), but an ability to resist growth inhibition present in the CNS.

Recent investigation into the mechanism behind the shift in axonal regenerative capacity showed that the CL was enabling regeneration in the presence of the normally inhibitory molecules MAG/myelin (Qiu et al., 2002a). Multiple streams of evidence have subsequently linked the change in axonal regenerative capacity to changes in genetic expression occurring at the cell body. The change in genetic expression was triggered by an elevation of cyclic adenosine monophosphate (cAMP) in the DRG cell bodies following the CL, demonstrated by pharmacological manipulation of intracellular cAMP levels; Blocking Protein Kinase A (PKA, a downstream effector of cAMP) ablated the CL effect (Qiu et al., 2002a), while priming cultured neurons with dibutyryl cAMP (dbcAMP, a membrane soluble cAMP analogue), neurotrophins (Brain Derived Neurotrophic Factor [BDNF]/ Glial Derived Neurotrophic Factor [GDNF]) (Cai et al., 1999b), or *in vivo* injection of dbcAMP into the DRG (Qui et al. 2002; Neumann et al. 2002) mimicked the CL effect. This elevated cAMP interacted with PKA to cause an upregulation of certain polyamines through transcription dependent upregulation of Arginase I (ArgI), an enzyme responsible for their synthesis (Cai et al., 2002). In fact, endogenous application of the end-product polyamines or inducing an overexpression of ArgI were each found to be sufficient in replicating the CL effect on central axon regeneration.

This series of investigations implicates the following pathway as the basis for alteration of the regenerative capacity of the central axon: CL of peripheral axon raises cAMP in the cell body in the DRG which leads to a change in growth phenotype of their cell body. This change to a growth/regenerative state involves an upregulation of growth necessary polyamines through upregulation of their synthesizing enzyme, ArgI by cAMP-PKA signaling (Figure 2). When the central axons are then subsequently injured, the upregulated growth program normally responsible for peripheral axon regeneration is already active and is parlayed to the injured central axons, conferring a resistance to molecular inhibition normally effective in retarding their regeneration. Consistent with this is the report that the developmental loss of regenerative capacity of CNS neurons is mediated by a developmentally regulated decrease in endogenous neuronal cAMP levels (Cai et al., 2001). Interestingly, the CL effect was originally described by its ability to enhance PNS regeneration. Investigations previous to those described above had reported that a conditioning lesion (CL), in this case a sciatic nerve crush injury or transection of the nerve distal to and temporally prior to a peripheral axotomy test lesion, resulted in a significant increase in the outgrowth of regenerating peripheral axons (McQuarrie and Grafstein, 1973; McQuarrie et al., 1977; McQuarrie, 1978; Jacob and McQuarrie, 1993). The mechanism of action of this peripheral CL was also attributed to an elevation of cAMP, as injection of cAMP analogs into the cell body of these neurons (DRG) had shown similar enhancement of peripheral axonal regeneration; a reduction in time to initiation of outgrowth, as well as an increased rate of elongation (Carlsen, 1982; Kilmer and Carlsen, 1984; Kilmer and Carlsen, 1987; Lankford et al., 1998). Additionally, as we have reported in chapter 3, our recent investigations show a significant increase in regeneration of both motor and sensory neurons following treatment with rolipram, a cAMP elevating agent. Although the effect in the PNS has never been conclusively found to be due to cAMP-PKA signaling and downstream polyamine synthesis, it has been strongly inferred from these original studies as well as the later investigation of CL in CNS regeneration, that cAMP elevation is the basis of the effect.

The effect of a CL on peripheral axonal regeneration is similar in physical response to the effect found with electrical stimulation following a peripheral nerve

injury. Electrical stimulation (STIM) was initially reported to result in accelerated onset of sprouting from intact axons following partial denervation and electrical stimulation (Hoffman, 1952). Studies since then have shown that electrical stimulation accelerated sprouting and functional recovery (Manivannan and Terakawa, 1994; Nix and Hopf, 1983; Pockett and Gavin, 2005). Our laboratory has reported the stimulation induced acceleration of regeneration of axotomized peripheral axons of the femoral nerve (Al-Majed et al., 2000a; Al-Majed et al., 2000b; Brushart et al., 2002a). A stimulation length of one hour immediately following transection of the nerve was successful in accelerating regeneration, and was found to be due to changes occurring within the cell body, as blockage of anterograde excitation of the neurons using tetradotoxin (TTX) resulted in no effect of STIM. This suggested an enhancement of growth program similar to that which occurs following a CL. Further investigation into this enhanced growth program found that STIM resulted in a dramatic increase in mRNA of BDNF and its receptor, tyrosine receptor kinase B (TrkB) within hours of the stimulation. This neurotrophic elevation was persistent for about a week following electrical stimulation. As discussed above, it was shown that neurotrophins, specifically BDNF, elevated cAMP and resulted in central axon growth on intrinsic inhibitors of regeneration found within the CNS (MAG/myelin) in a cAMP dependent mechanism (Cai et al. 1999). It has been more recently shown that BDNF actually acts as a cAMP phosphodiesterase inhibitor through extracellular signal-regulated kinase (ERK) (Gao et al., 2003b). This results in a blockage of cAMP breakdown, and thus an elevation of cAMP. The authors also show that this blockage of cAMP breakdown is sufficient to push the intracellular levels of cAMP above a threshold to overcome inhibition by MAG.

BDNF has also been shown to regulate neuronal survival through cyclic AMP response element binding protein (CREB) transcription factor gene regulation (Finkbeiner et al., 1997; Finkbeiner, 2000b). This effect involves BDNF interacting with its TrkB receptor to set a cascade of intermediates to affect the CREB promoter resulting in upregulation of survival factors, which are potential contributing factors responsible for increased regeneration in the PNS and expected resistance to inhibitors and enhanced regeneration in the CNS.

Another possible mechanism of stimulation is a Ca^{2+} influx mediated by electrical activity. Electrical stimulation has been shown to cause an increase in intracellular Ca^{2+} (Ming et al., 2001c). Influx of calcium has been shown to increase intracellular cAMP through calcium dependent adenylate cyclases (Ming et al., 2001a). As well, an influx of calcium has been shown to result in an upregulation of BDNF through CREB transcription factor (Finkbeiner, 2000a), indicating many possible pathways that stimulation could be exciting to effect the regeneration summarized in Figure 2. Consistent with this possibility is the report of voltage-gated calcium channel agonists themselves being able to elicit spinal cord regeneration (Unlu et al., 2002). Indeed many studies have shown the beneficial effects of BDNF on central survival and regeneration (Lindsay, 1988; Kobayashi et al., 1997a; Oudega and Hagg, 1999; Ramer et al., 2000; Hiebert et al., 2002; Sayer et al., 2002). As well, BDNF has been shown to be upregulated in the DRG following a lesion of the peripheral nerve as well (i.e. following a CL) (Meyer et al., 1992). Additionally, TrkB immunoreactivity has been demonstrated in the DRG following sciatic nerve lesion (Foster et al., 1994). Specifically, an upregulation of BDNF was shown in the large sensory neurons (Zhou et al., 1999). This specific upregulation in only the large diameter sensory fibers is particularly interesting because it has been shown that following peripheral nerve lesion, there is a shift towards large afferent fiber domination of primary fiber afferent input into the spinal cord (Coggeshall et al., 1997). It would correlate then that the upregulation of BDNF is responsible for the differential survival of large fiber sensory afferents and smaller fiber ones. This would further indicate the importance of BDNF in neuronal survival and regeneration, but would perhaps indicate that the stimulation effect might have a preferential effect on only the larger diameter sensory neurons. It is with this evidence that it can be hypothesized that electrical stimulation at the time of central axon lesion may result in a similar increase in regenerative capacity to that seen following a CL.

There are however, some key differences which may be important in the success of STIM of peripheral axons in CNS axon regeneration. These include differences in the observed effect on regeneration between a CL and STIM. A CL resulted in earlier neurite initiation, greater outgrowth and a reduction in initial neurite branching, resulting in straighter neurites extending further from the cell body (Lankford et al., 1998). STIM

however, has been reported only to advance the initial onset of outgrowth, without increasing regeneration speed (Brushart et al., 2002b). This could perhaps be showing a lack of specificity in observations made in the CL investigations, as overall regeneration rate was measured, compared to the actual rate of regeneration following initial neurite sprouting measured following STIM.

It is on this basis of evidence that we intend to investigate the hypothesis that STIM, like a CL (Neumann and Woolf, 1999b), will result in promoting the regeneration of lesioned central axons of the DRG primary sensory neurons. In this experiment, we use the model of primary sensory neurons with axons extending into the CNS and PNS and cell bodies in the DRG to explore the possibility that STIM of the peripheral axons concomitant with a lesion of the central axons results in similar ability of the central axons to overcome inhibition by myelin and MAG and regenerate into the site of lesion. This will be compared to a lesion of the central axons with sham treatment of the peripheral axons, as well as lesion of the central axons concomitant with a condition lesion of the peripheral axons, as investigated by Neumann and Woolf (1999). In addition, our protocol of STIM will be investigated in terms of cAMP elevation in the DRG cell bodies of the stimulated axons through immunocytochemistry for cAMP.

4.2 MATERIALS AND METHODS:

4.2.1 Experimental Design

Fourteen female Sprague Dawley rats, weighing approximately 220 to 260 grams at the beginning of the experiment, were obtained from University of Alberta Biological Sciences Animal Services. Rats had access to food and water ad libitum, were housed in flat bottomed, woodchip filled cage and were exposed to a 12 hour light/dark cycle. All experimental manipulations were performed adhering to Canadian Council on Animal Care guidelines and approved by the Health Sciences Animal Welfare and Policy Committee (University of Alberta).

As discussed above, we selected the DRG sensory neuron as our model to investigate the role of stimulation on CNS regeneration. This allowed us to compare the response of lesioned central axons with a concomitant CL of the peripheral axons (Neumann and Woolf, 1999b) (Group B - CL) to the response of lesioned central axons

with concomitant STIM of the peripheral axons (Group C – STIM 20Hz and Group D – STIM 200Hz). Two frequencies of stimulation, STIM 20Hz and STIM 200Hz were used because the original stimulation protocol of 1 hour electrical stimulation at 20Hz previously shown to enhance regeneration of peripheral motoneurons (Al-Majed et al., 2000c) was physiologically relevant frequency of hindlimb motoneuron discharge but perhaps not for sensory neurons (Hoffer et al., 1987; Loeb et al., 1987). The 200Hz rate is a frequency that is more applicable to sensory neurons, being the maximum firing rate of hindlimb primary spindle afferents during locomotion (Prochazka and Gorassini, 1998). A control group was included in which there was no treatment other than the central axons lesioned (Group A – Sham). Rats were randomly assigned to one of the four groups.

4.2.2 Surgical Procedures

Anesthesia was induced in all animals by intraperitoneal injection of sodium pentobarbital (0.07mL/g: Somnotol (MTC Pharmaceuticals, Cambridge, ON). Animals were operated on only when judged to be at surgical plane of anaesthesia. Surgical plane was assessed by limb withdrawal in response to light hind toe pinch normally sufficient to elicit the reflex. All surgical procedures were performed in an aseptic manner. All regions operated on were shaved and prepared with 70% ethanol. Each animal was prepared with opthalmic ointment to prevent corneal damage due to drying (Duratears Naturale, Alcon, Ontairio). Postoperative pain relief was supplied by buprenorphine HCl (Buprenex; 0,05mg/kg) following animal recovery from anesthetic.

Surgery 1 – Dorsal Hemisection at T8

In each of the experimental groups all animals except one underwent a dorsal hemisection at Thoracic vertebra 8 (T8). T8 was located by removing the mutilocular adipose tissue from above the mid thoracic region and locating the blood vessel that enters between T4 and T5. One can then count down the vertebral column to T8. At T8 a laminectomy was performed, removing the lower half of the vertebrae. This was achieved by making an incision of length no more than ½ inch above the location of T8. The incision was directed down to the cord on both sides of the spinal processes and the muscles were separated from the vertebral column on both sides. The animal was positioned on a rolled paper towel so that a flexion of the torso allowed the vertebral

column between T8 and T9 to be slightly separated, allowing access to clip T8 with the bone roungers. The spinal processes on T8 were removed using bone roungers, and the lower half of the vertebrae removed. Only half of the vertebra was removed to retain as much stability of the vertebral column as possible following surgery.

The laminectomy was followed by a dorsal hemisection at T8, transecting the corticospinal tract including the sciatic component of the fasciculus gracilis, which contains axons arising from dorsal root ganglia T7 and below, and extends all the way up the spinal cord ipsilateral to the DRG from which it initiated (Figure 3) (Neumann and Woolf, 1999b). The fasciculus gracilis conveys information on two point discrimination, vibration and conscious proprioception to the ipsilateral nucleus gracilis. The dorsal hemisection was performed using iridectomy scissors and the lesion extended from the tractus spinocerebellaris dorsalis to just above the central canal. The muscles overlying the spinal cord were then repaired using 5-O silk (Ethicon, Inc. Somerville, NJ, U.S.A.), and the incision closed using 9mm wound clips (Autoclip, Clay Adams, MD, U.S.A.).

Surgery 2 – Sciatic STIM/CL/Sham

In all animals, immediately following surgery 1, the sciatic nerve treatments were performed. In Group A (SHAM), the sciatic nerve was dissected but no conditioning performed. In Group B (CL), the sciatic was transected and sutured to underlying innervated muscle. This was done to prevent reinnervation during the period of regeneration.

In Group C (STIM 20Hz) the sciatic nerve was subjected to a intra-operative 1 hour electrical stimulation protocol previously described on the femoral nerve (Al-Majed et al., 2000c). The sciatic nerve was left intact, and stimulation was delivered intraoperatively at 20Hz (100μsec, 3-5 V) for 1 hr (72,000 pulses/hr) using a Grass (Quincy, MA) SD-9 stimulator and two insulated "Cooner" which were bared of insulation for 2-3mm on either side and one side was twisted to form a small loop to secure the wire on either side of the nerve. The cathode was sutured in place alongside the sciatic nerve and the anode was sutured in place distal and contralateral to the cathode to muscle close to the sciatic (Figure 4).

In Group D (STIM 200Hz) the sciatic nerve was again subjected to similar electrical stimulation protocol for a period of 1 hr with the exception of a stimulation

frequency of 200Hz. The overall number of electrical pulses was identical to that delivered in Group C to control for number of pulses and investigate frequency only. This was achieved by intermittent firing of 200Hz for 25ms every 250ms (72000 pulses/hr).

In all groups, the animals were then rehydrated though subcutaneous injection of 4ml of warm (~30°C) saline and observed to recovery of consciousness under a heat lamp. Once the animals had recovered from anesthetic, post-operative pain was controlled with subcutaneously injected buprenorphorine HCl as stated above for management of postoperative pain.

In all of the animals that underwent surgery 1, the bladder had to be manually voided for the first 2-3 days following surgery, until voiding of the bladder returned within animal control. Rats were rehydraded for two days post-surgery with daily subcutaneous injection 4mL of warm saline.

4.2.3 Labeling of Sensory Spinal Tracts

Following a 14 week recovery period, all rats in each group other than the 3 omitted from T8 lesion underwent sensory tract labeling with Cholera Toxin B as previously described (Oudega et al. 1994). The right sciatic nerve was again dissected under surgical plane anesthesia as described above. The sciatic was then crushed using fine tipped forceps. A ligature (5-O Ethicon) was loosely tied around the dissected sciatic nerve just distal to the crush. A second ligature was tied loosely around the sciatic distal to the first ligature and this ligature was pulled gently to supply a level of stiffness to the nerve necessary to insert the Hamilton syringe. The Hamilton syringe was then inserted through the epineuria distal to the crush and then smoothly inserted to the point of crush (Figure 5). The first ligature was then tied firmly around the needle tip distal to the crush site, and 3ul of 1% Cholera Toxin subunit B solution was injected (CTB; in distilled water; List Biological laboratories Inc., Campbell CA). The syringe was then withdrawn from the nerve and the first ligature was tightened around the nerve. This was done to prevent leakage of the CTB subunit distal to the crush site. The second ligature was removed and the overlying muscle and skin was repaired using 5-O and 4-O silk, respectively. Animals were left to recover for 5 days to allow for retrograde transport of the applied dye to the neuronal cell bodies and anterograde transport of the dye from the cell bodies through the central axons.

4.2.4 Immunocytochemistry

Following the 5 day period, all rats were anesthetized with sodium phenobarbital (Somnotol®: 30 mg/kg, intraperitoneal injection) and were intracardially perfused using a peristaltic pump (Peri-Star, World Precision Instruments, Aston, Stevenage) with warm (~30°C) saline solution (250ml/rat) consisting of 1L saline, 2.5g NaNo2 (vasodilator), 0.02g Heparin (anticoagulant), spatula tip of Phenol Red (pH marker) and 0.170g NaHCO3 (base) followed by a room temperature 4% paraformaldehyde, 5% sucrose solution (250ml/rat).

The spinal cord was then cut into sections. A lesion section (approximately 1 inch in length with the lesion in the center) as well as a 1 inch caudal section was retained and frozen in OCT (Optimum Cutting Temperature Liquid, Sakura, Japan) by 2-methylbutane cooled to -60 to -80°C by dry ice (solid phase CO).

Tissues were sectioned into 40μm longitudinal saggital sections using a cryostat (Jung CM 3000, Leica, Germany) and serially mounted (Fisherbrand Microscope slide, Fisher Scientific, U.S.A). The tissue was then fixed to the slides for semi-free floating ICC (Herzog & Brosamle, 1997).The spinal cord tissues from all animals were then processed with immunocytochemistry (ICC) for CTB to reveal anterograde labeled sensory axons as described previously with slight modification (Oudega et al., 1994). The processed tissue could then be visualized using a light microscope at 20X.

The spinal cord tissues from all animals were then processed with immunocytochemistry (ICC) for CTB to reveal anterograde labeled sensory axons as described previously with slight modification (Oudega et al., 1994). Tissues were sectioned into 40μm longitudinal saggital sections using a cryostat (Jung CM 3000, Leica, Germany) and serially mounted (Fisherbrand Microscope slide, Fisher Scientific, U.S.A). The tissue was then fixed to the slides for semi-free floating ICC (Herzog & Brosamle, 1997). This was achieved by first air-drying the sections to the slide overnight and then fixing the tissue to the slide using a droplet of Krazy Glue© on the most proximal tip of the tissue. The slide was then transferred to a staining jar with tris buffered saline (TBS) with the glued tip up. Sections usually then spontaneously detached from the slide except for the glued tip, and in cases where this did not occur, a fine paintbrush was used to coerce it from the slide. This method allows more

penetration of the 40μm thick sections than having the tissue fixed on the slide completely, and offers advantage over the completely free-floating method; maintenance of serial order of the sections, reduction of amount of reagents necessary (and thus a reduction in cost of tissue processing) as well as the removal of tissue transferring steps necessary in completely free-floating methodology (and thus a reduction in tissue processing time and possible loss or damage of tissue).

The tissue was then rehydrated in TBS for 10 minutes twice at room temperature (RT). Following this, the tissue was incubated with TBS and 0.05% Triton-X (TBS-TX) for 45 minutes at RT. Next, the sections were incubated with 5% normal rabbit serum in TBS-TX (TBS-TX-NRS) for 30 minutes at RT. The sections were then incubated for 65hrs with goat anti-cholera toxin B subunit antibodies (1:8,000; List Biological Laboratories, Inc.) in TBS-TX-NRS at 4°C. Sections were then washed twice for 10 minutes in TBS, followed by incubation for 2hrs with rabbit anti-goat IgG (H+L) antibodies (1:200; Vector Labs, Burlingame, CA) in TBS-TX-NRS at RT. Sections were again washed in TBS (2X10 minutes). This was followed by 2 hour incubation with avidin-biotin complex (ABC Elite, Vector Labs) at RT. This was followed by another washing in TBS (2X10 minutes), and finally developed for 5-10minutes in 0.04% 3,3 – diaminobenzidin-4 HCL (DAB), 0.06% NiCl2 and 0.006% H2O2 in Tris-HCL buffer. Tissue was then stained using Cresyl Violet (0.05%) for 1 minute and washed with distilled water for 2X 5 minutes. Sections were air dried for 1 hr, dehydrated through an ethanol dehydration series (50%, 75%, 99%, and 2 X Xylene) and coverslipped with Permount (Fisher Scientific, U.S.A.). The processed tissue could then be visualized using a light microscope; images were captured using Image Pro Plus ® (Figure 6).

4.2.5 Quantification of the Regeneration of Sciatic Dorsal Column Sensory Fibers

The regenerative response of the dorsal column sensory fibers regenerated by each of the experimental conditions was quantified by constructing collage images of each section showing labeled fibers using images taken at 20X magnification (Leica DMRBE upright microscope, DAGE MTI DC-330 3CCD Color Camera) in an imaging program (Image Pro Plus, Media Cybernetics). These reconstructions of the lesion site were then analyzed using commercially available digital image measuring software (Adobe Photoshop CS, Adobe Systems Inc. U.S.A.) analyzing fiber regenerative

distances into the lesion area. Quantitative measurements of the number and length of the regenerating fibers into and beyond the lesion site were recorded and compared between groups.

4.2.6 cAMP Measurements

Postnatal day 16-21 Sprague-Dawley rats were divided into four groups: Sham, CL, STIM 20Hz and STIM 200Hz. The animals were anesthetized and the right sciatic nerve exposed. Depending on group, the sciatic nerve condition was performed as described above. 24 hrs later, the animals were again anesthetized and the right lumbar dorsal root ganglion 4 and 5 (DRG L4 and L5) were removed and immediately frozen in liquid nitrogen. Samples were homogenised in 0.1N HCl and 0.5mM IBMX and then analyzed following manufacturer's instructions in an R&D cAMP low pH Elisa Assay Kit.

4.2.7 Statistical Analysis

Statistical analysis between group mean (±SE) was assessed through one-way analysis of variance (ANOVA) (GraphPAD InStat Software V. 1.13 (1990) Dr. Paton, University of Alberta). Statistical significance was accepted at 5% ($p<0.05$).

4.3 RESULTS:

These experiments are currently at an intermediate stage.

4.3.1 Conditioning Lesion Results in Greatest Regeneration of Central Axons

Analysis of our intermediate data indicates that a CL (Group B) of the peripheral axons concomitant with a central axon lesion resulted in a statistically significant increase in regeneration of ascending sensory fibers into the lesion area compared to sham treatment (Group A). This is in agreement with previous data published by Neumann and Wolff (1999). Our data also demonstrates that 200Hz stimulation (Group D) results in slightly better corticospinal axon regeneration than 20Hz stimulation (Group C) when compared to sham treatment, though not as effective as the CL, and not statistically significant from sham (Group B)(Figure 7, Table 1). Because of the low sample size in the sham group as well as the 200Hz, statistical significance was not reached between groups.

4.3.2 Stimulation Results in Significant Increases in cAMP in the DRG

There was a significant increase in average cAMP (±SE) measured 24hrs following CL (15.72±2.27 pmol/500ul) as well as 200Hz (17.9±2.07 pmol/500ul) as compared to baseline levels (9.02±1.67 pmol/500ul) (Figure 8, Table 2). There was no significant difference between CL and 200Hz. However, the 20Hz stimulation resulted in a non-significant increase in cAMP (11.3±1.59 pmol/500ul) compared to normal levels.

4.4 DISCUSSION:

The present intermediate results indicate that both frequencies of STIM as well as the conditioning lesion appear to be successful at increasing DRG sensory axon regeneration within the CNS above that seen in control animals. At this intermediate point, statistical analysis between groups suggests that only the conditioning lesion results in significantly increased neurite extension into the lesion site above control levels. The ability of a CL of the peripheral axons of primary sensory neurons to promote the regeneration of their central axons when subsequently injured is in agreement with previous data (Neumann and Woolf, 1999a; Neumann et al., 2002). However, with the addition of samples, it is likely that 200Hz STIM will also result in significantly increased neurite extension into the lesion site.

The increase in regenerative capacity of central ascending sensory axons following a CL or 200Hz STIM is temporally correlated with significantly elevated levels of cAMP in the DRG L4 and L5. This agrees with previously published data demonstrating the ability of exogenous dbcAMP injected into the DRG to mimic the effect of a CL ((Neumann et al., 2002; Qiu et al., 2002a). In fact, both 200Hz STIM and CL are statistically indistinguishable in their ability to increase intracellular cAMP levels, roughly 1.8X that of sham cAMP levels. 20Hz STIM was not successful at statistically elevating cAMP above sham levels.

From our intermediate results it appears that either frequency of stimulation is not as effective as a conditioning lesion in promoting axon regeneration of DRG in the CNS. This is contrary to our hypothesis, and surprising especially in light of the cAMP data indicating that the 200Hz stimulation raised cAMP as much as a conditioning lesion in the DRG cell bodies. If future data continues in this trend it would seem that merely

increasing cAMP in the DRG is not as successful in promoting DRG central axon regeneration as the conditioning lesion. This in turn would imply that the CL mediates it effect via mechanisms in addition to the elevation of cAMP. This conclusion is supported by recent data demonstrating that injection of dbcAMP into the DRG results in increased tubulin production without increasing regenerative rate or axonal growth capacity in the PNS or allowing central branches to regenerate as long as a CL (Han et al., 2004). Additionally, though elevation of cAMP has been shown to block the inhibition of axon elongation by CNS myelin (Cai et al., 1999b), exogenous cAMP is not able to block additional inhibitors of elongation, such as semaphorins and chondroitin sulfate proteoglycans (CSPGs) also present in the injured spinal cord (Song et al., 1998; Pasterkamp and Verhaagen, 2001; Dontchev and Letourneau, 2002).

If our hypothesis that STIM increases ascending central sensory axon regeneration is in fact shown to be substantiated, it would indicate a clinically feasible means to improve axonal regeneration following dorsal column lesion injury. It is stressed that this may be a clinically feasible mechanism because the CL effect, though from our intermediate data is more effective at promoting central axon regeneration than either of the STIM frequencies, is not a feasible strategy to promote regeneration, as further nervous system damage is not desirable. However, to maximize the success of regeneration, it may be necessary to couple electrical stimulation with the blockage of additional inhibitors of elongation.

4.5 Tables

4.5.1 Table 1. Average Central Axon Neurite Regeneration

Group	Average Neurite Outgrowth (um)	S.E.	N
Group A – Sham	307.83	119.13	3
Group B – CL	624.23	94.07	6
Group C - 20Hz	469.48	76.44	5
Group D - 200Hz	492.86	64.69	3

4.5.2 Table 2. cAMP Levels 24hr Following Treatment

Group	cAMP (pmol/500ul)	S.E.	N
Group A – Sham	9.02	1.67	7
Group B – CL	15.72	2.27	7
Group C – 20Hz	11.3	1.59	8
Group D – 200Hz	17.9	2.07	8

4.6 Figures

4.6.1 Figure 1. The primary sensory neuron of the dorsal root ganglion.

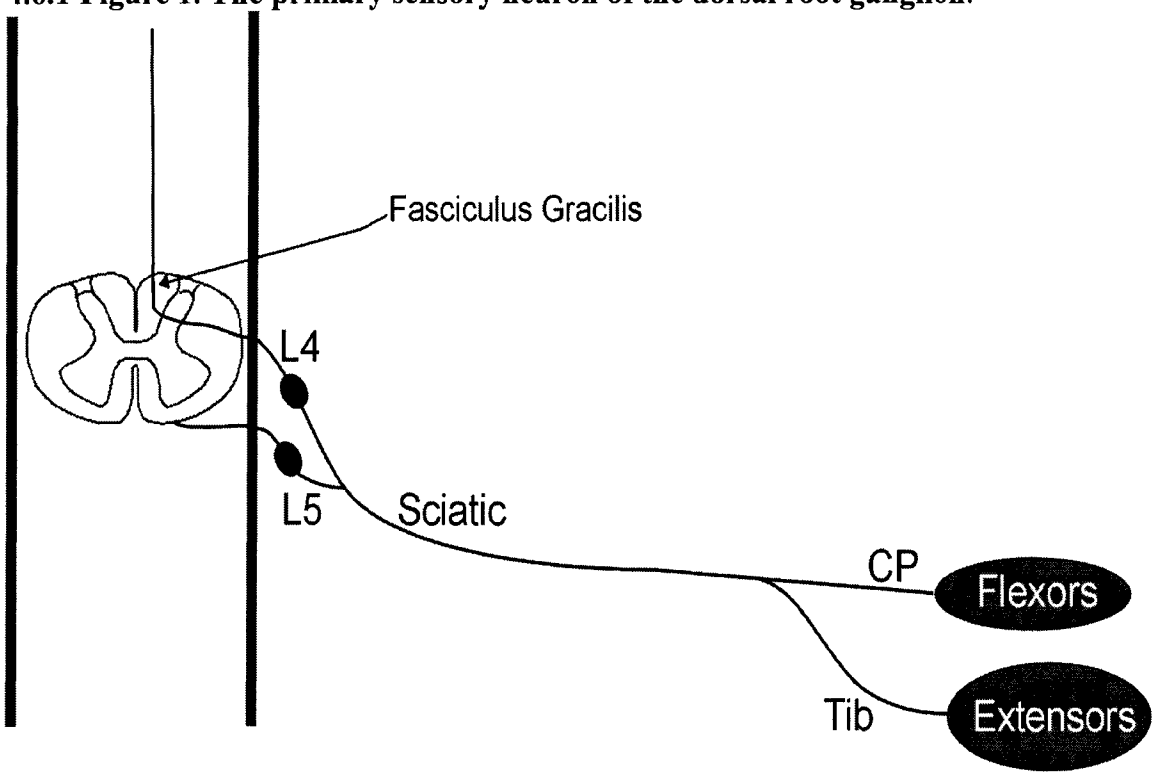

Figure 1: Schematic of primary sensory neurons of the sciatic nerve, which branch peripherally into the common peroneal nerve as well as the tibial nerve. These sensory neurons possess bipolar axons that project both into the CNS (fasciculus gracilis) and PNS (sciatic nerve) from the cell body in the DRG. The major afferent contributors to the sciatic are DRG Lumbar 4 and 5 (DRG L4 & L5).

4.6.2 Figure 2. Combined Molecular Mechanisms of Conditioning Lesion and Electrical Stimulation on Enhancing Central Axonal Regeneration

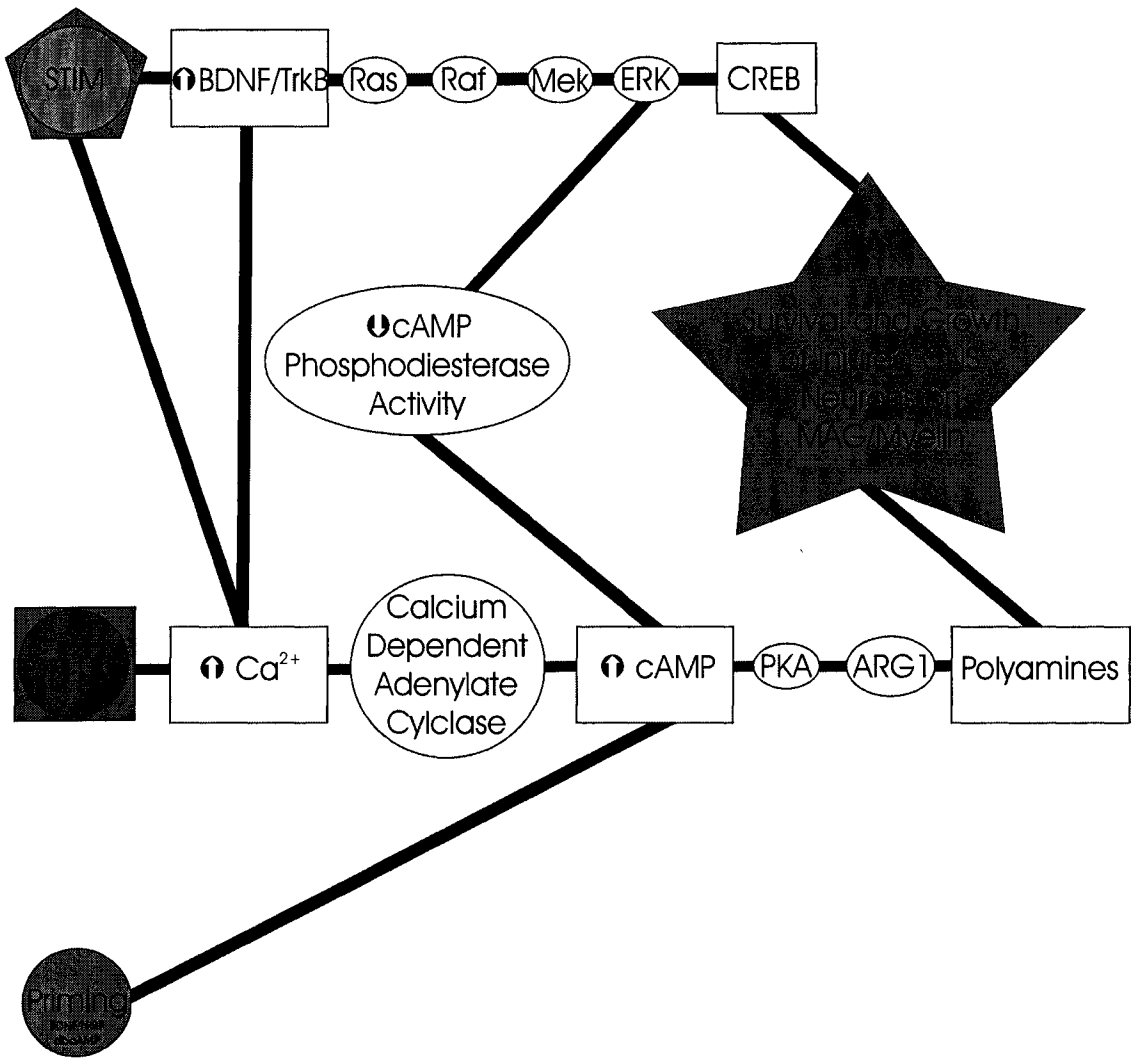

Figure 2: Following electrical stimulation of the sciatic nerve, brain derived neurotrophic factor (BDNF) and its tyrosine receptor kinase (TrkB) are upregulated. BDNF has been shown to act through a Ras-Raf-Mek-ERK pathway to activate cyclic AMP response element binding protein (CREB) which upregulated genes responsible for survival and perhaps growth on the inhibitory substrate of the central nervous system. As well, BDNF acts as specific phosphodiesterase inhibitor through extracellular signal-regulated kinase (ERK), causing an increase in cAMP, as it inhibits the phosphodiesterase responsible for breaking down cAMP. This blockage of cAMP breakdown is sufficient to push the intracellular levels of cAMP above a threshold to overcome inhibition by MAG. Following a conditioning lesion (CL) of the peripheral axons of the primary sensory neurons, cAMP in the cell body is increased. This is suggested to be due to intracellular calcium increases interacting with calcium dependent adenylate cyclases, responsible for converting ATP to cAMP. BDNF has also been shown to cause an increase in intracellular calcium (Ca2+), as has electrical stimulation in general.

4.6.3 Figure 3. Experimental Design

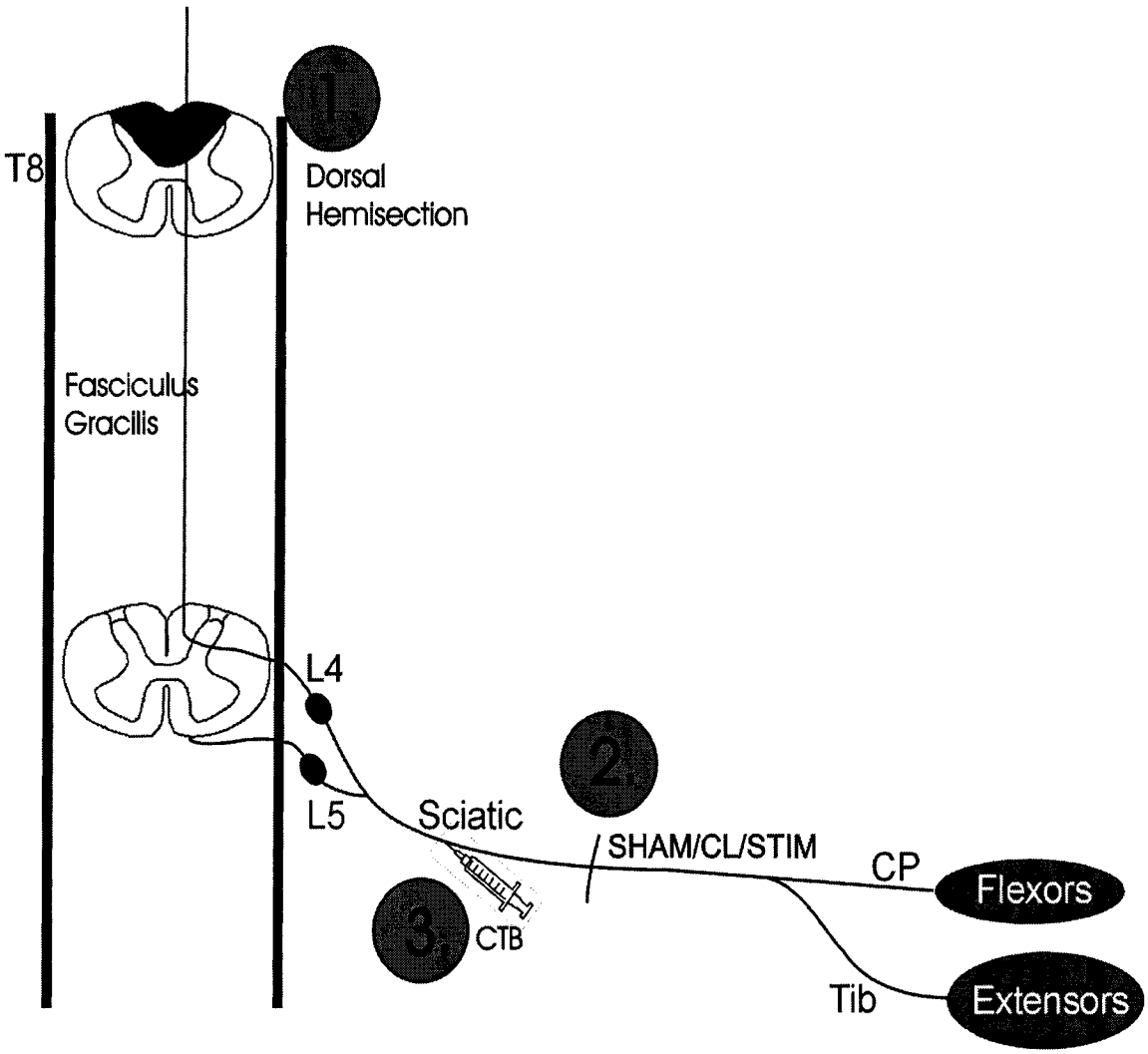

Figure 3: In all animals but one from each group, a dorsal hemisection at thoracic vertebrae 8 was performed (1), transecting the ascending gracilis funiculus, which contains the ascending central axons of the primary sensory afferents of dorsal root ganglia lumbar 4 and 5 (DRG L4 and L5). Concomitant with surgery 1 in all animals of all groups, the sciatic nerve (2) was exposed and either just isolated (Group A – SHAM), isolated and transected (Group B – Conditioning Lesion (CL)) or isolated and electrically stimulated (STIM) for 1 Hr at 20Hz (Group C – 20Hz STIM) or 1Hr at 200Hz (Group D – 200Hz STIM). Animals were allowed to recover and regeneration was allowed for a 12 week period following 1 and 2. At that time, the sciatic nerve of all animals that underwent 1 and 2 was crushed and injected with Cholera Toxin Subunit B (CTB) for retrograde tracing of the peripheral primary sensory axons and anterograde tracing of the ascending primary sensory axons. Five days after the CTB injection, animals were perfused and tissues removed.

4.6.4 Figure 4. Electrical Stimulation.

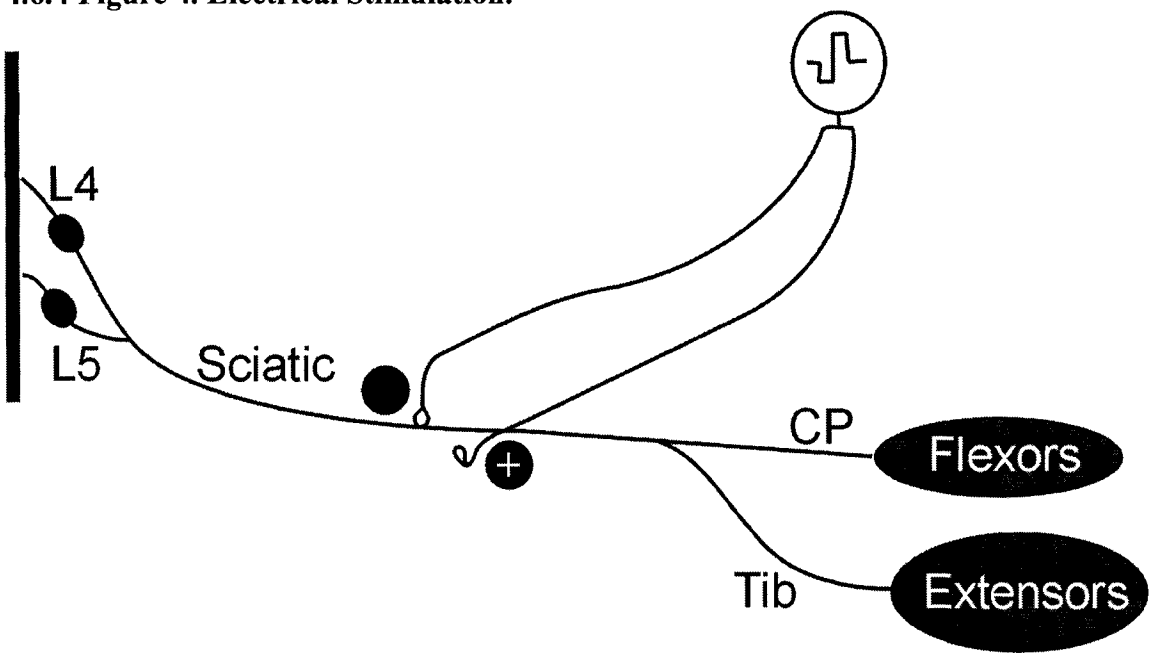

Figure 4: In Group C – STIM, the sciatic nerve was isolated and electrical stimulation was delivered intraoperatively at 20Hz (100μsec, 3-5 V) or 200Hz intermittently for 1 hr using a stimulator and two insulated silver wires. The cathode was sutured in place alongside the sciatic nerve and the anode was sutured in place distal and contralateral to the cathode to muscle close to the sciatic nerve.

4.6.5 Figure 5. Cholera Toxin B Injection Method

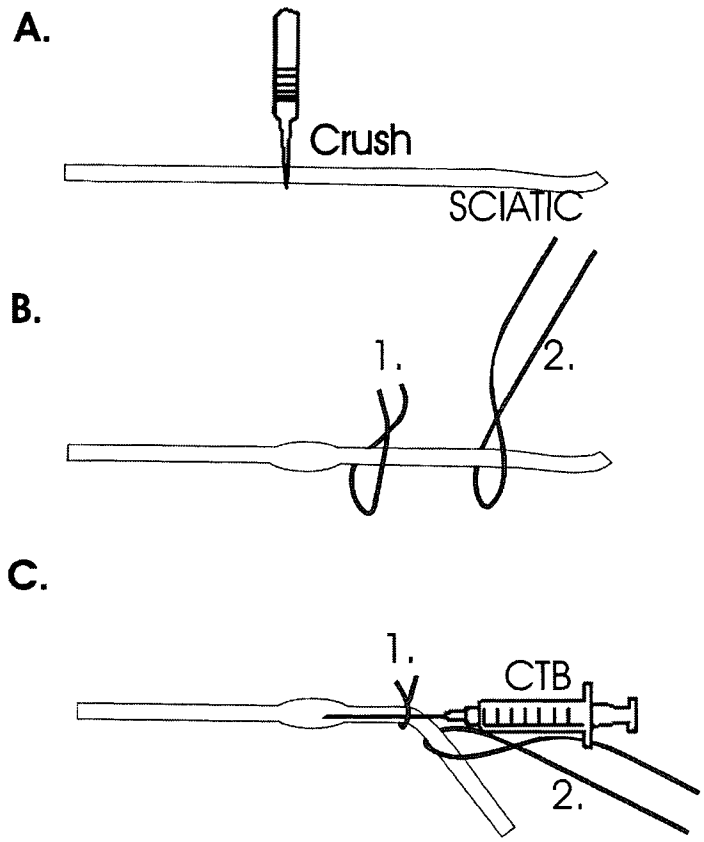

Figure 5: Schematic of the Cholera Toxin subunit B (CTB) injection method. (A.) The sciatic nerve is exposed at mid thigh and fine tipped forceps are used to crush the nerve. (B). Two ligatures of 5-O Ethicon were then tied loosely around the dissected sciatic nerve, one (1) just distal to the crush, and the other (2) about 1cm or so distal to the first. (C.)The second ligature (2) was pulled gently to supply a stiffening tension on the nerve. The Hamilton syringe was then through the epineuria distal to the crush and under the first ligature (1), and was then inserted to the point of crush. The first ligature (1) is then tightened, and 2ul of CTB was injected through the Hamilton syringe into the crush site. The Hamilton syringe was then withdrawn and ligature 1 fastened around the nerve to prevent flow of the CTB distal to the crush site. Animals were then repaired and recovered for 5 days to allow central labeling of the primary afferents.

4.6.6 Figure 6. Representative Images of CTB Immunocytochemistry

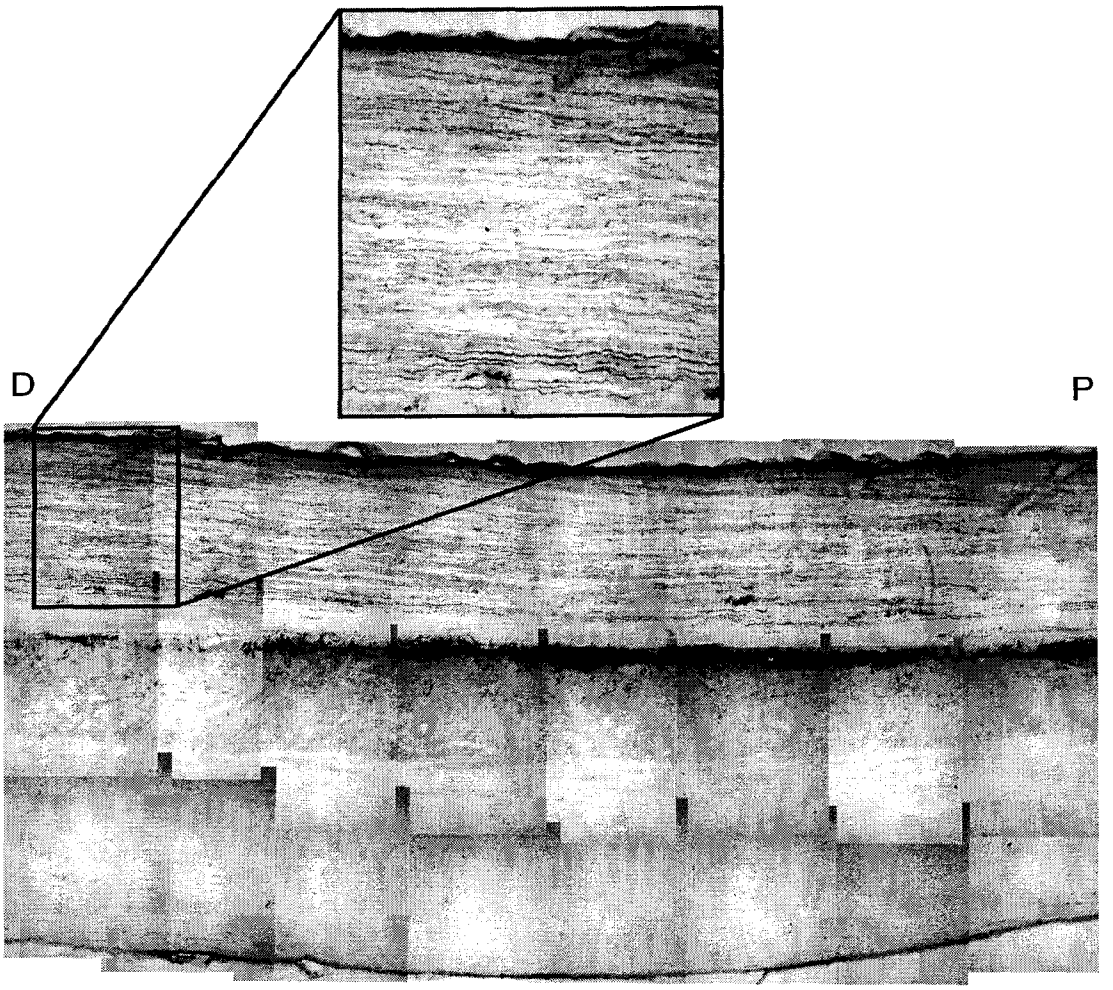

Figure 6: Representative image of cholera toxin subunit B immunocytochemistry showing the labeled central ascending axons of the primary sensory neurons of the dorsal root ganglion lumbar 4 and 5. D=distal, P=proximal spinal cord.

4.6.7 Figure 7. Mean Axon Outgrowth Following Sciatic Treatment

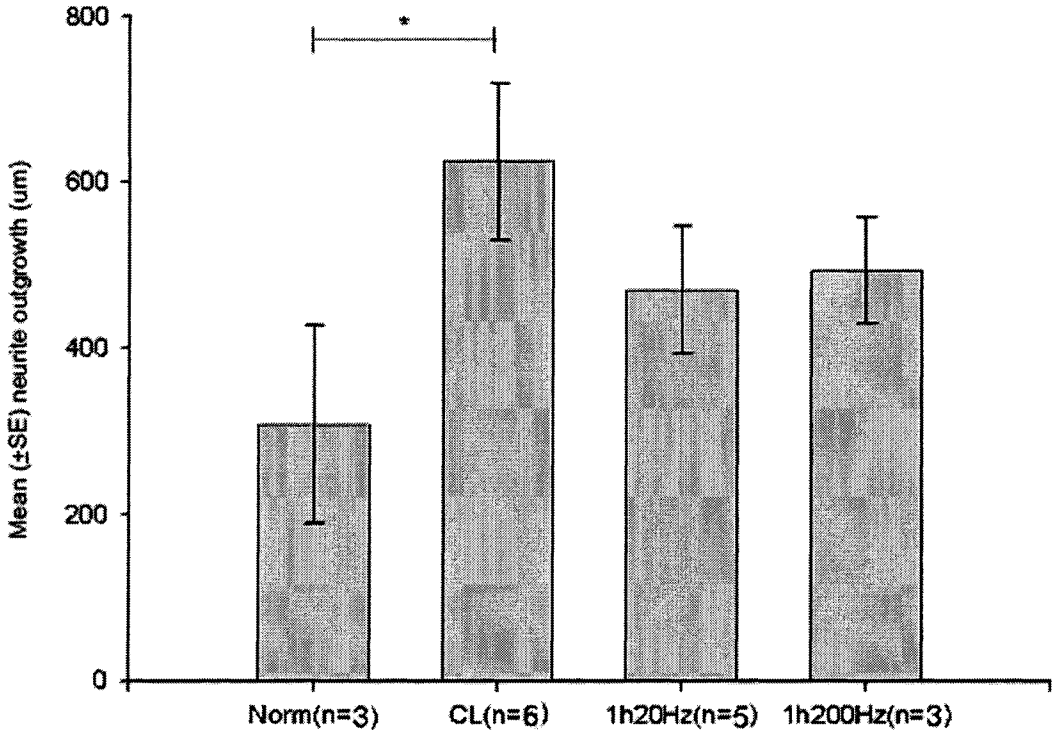

Figure 7: Mean (±SE) central axonal outgrowth 14 weeks following initial central axon injury and sciatic nerve treatment.

4.6.8 Figure 8. cAMP Elevation in DRG Following Sciatic Treatment

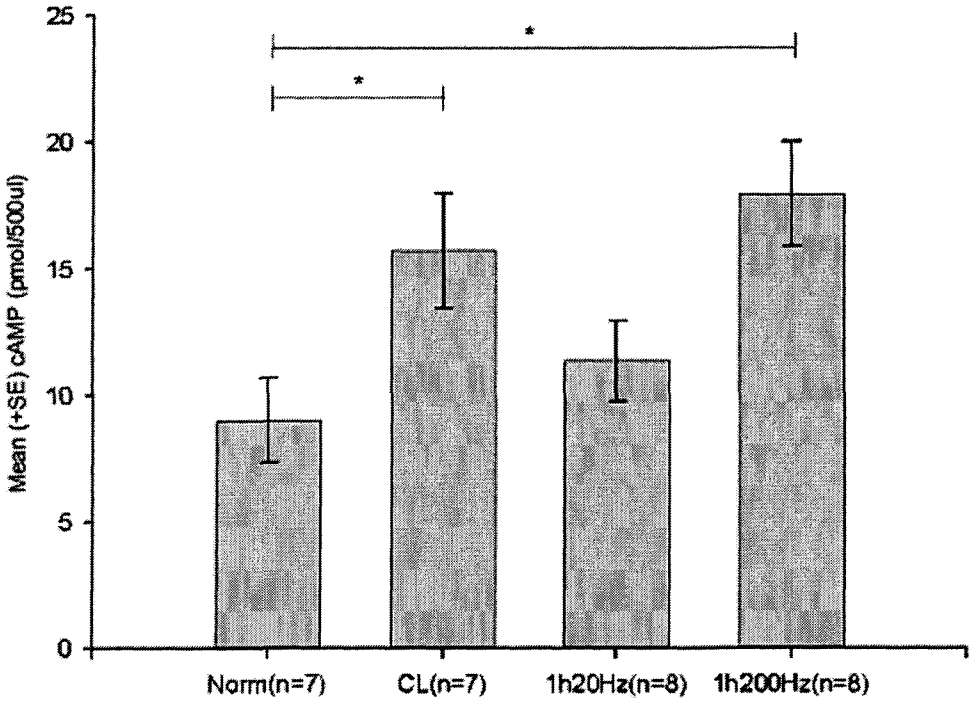

Figure 8: Mean (±SE) DRG cAMP levels 24hr following sciatic nerve treatment of either a conditioning lesion, or stimulation at either 20Hz or 200Hz. * denotes statistical significance ($p<0.05$).

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CHAPTER 5

GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Experiment 1: Prolonged target deprivation results in a reduction of the capacity of axotomized motoneurons to regenerate

Following peripheral nerve injury, particularly injury to large nerve trunks close to the spinal cord and distant from target tissue, there is generally poor recovery of function. This is due in large part to the chronic axotomy suffered by regenerating motor and sensory neurons as they regenerate the long distances to target tissue required in such instances (Fu and Gordon, 1995c). Here we explored further the effect of prolonged axotomy on the motoneurons regenerative ability. Particularly, we investigated whether model specific frustrated growth or "real-life" injury and model common target deprivation were responsible for the previously reported loss of regenerative capacity. This was achieved by comparing two models of chronic axotomy versus a control model in which neurons regained functional connections. The two models were: (1) "frustrated growth" in which the motor branch of the femoral nerve was capped to prevent regeneration and reinnervation of the distal nerve stump, and (2) "continuous growth" in which the motor branch was sutured into a long saphenous graft to facilitate prolonged target deprivation while allowing continual growth. The results demonstrate that regardless of growth into distal stump or no growth into distal stump, there is a significant decline in regenerative capacity when compared to control motoneurons that were able to reform functional connections and target reinnervation. Regenerative capacity was measured in terms of backlabeled motoneuron counts and axon diameter and total number. There were significant declines in both motoneuron count and axon diameter in both the experimental models of axotomy. However, while there was a distinct trend towards a decline in prolonged axotomy, no significant difference was found between total axon counts existed. This is due to a large variance. We attribute this variance to the ability of parent axons to sprout daughter axons -- as many as 20 daughter axons that regenerate from each parent axon in the proximal stump (Aitken et al., 1947). As well, we attribute this variance as the reason Holmes and Young (1942) found no appreciable decline in regenerative capacity of chronically axotomized motoneurons. However, our results are somewhat blurred by the inclusion of a sensory graft rather than a motor graft in our "continuous growth" model of chronic axotomy. This was done due to a nonexistence of a sufficiently long unbranching motor branch that would allow

contained axonal regeneration for the 2 month period while preventing target reinnervation. It has been reported that the use of a sensory graft is not as effective in promoting regeneration of injured motoneurons than is a motor or mixed nerve graft (Nichols et al., 2004b). This is presumably due to the presence of "motor" Schwann cells which may be more effective in supplying neurotrophic support to regenerating motor axons. We feel that this does not invalidate our results, as by the two month period both a sensory or motor graft would be at a similar level of atrophy and not be supplying very much neurotrophic support. In fact it a progressive atrophic loss of Schwann cells (Weinberg and Spencer, 1978; Wood, 1998), a corresponding decline in BDNF, GDNF and NT-4/5 (Hammarberg et al., 2000), and a diminished ability of these denervated Schwann cells to interact with and support regenerating axons (You et al., 1997; Sulaiman and Gordon, 2000; Hoke et al., 2002) has been reported over the course of prolonged distal nerve stump denervation. This would lead us to the assumption that in both cases, with the absence of neurotrophic support, there would be a decline in regenerative capacity.

5.1.1 Future Directions:

We here provide evidence for a reduction in regenerative capacity of motoneurons following prolonged axotomy. It would be interesting to further investigate differences between the two models of prolonged axotomy. In our discussion of the above experiments, we hypothesize that the continuous growth model is receiving trophic support from the graft that it is growing through that gradually tapers off as the graft atrophies. On the basis of this hypothesis, we suggest that at a shorter period of axotomy, there would be less reduction in regenerative capacity in association with less atrophy of the graft, and vice versa, with a longer period of axotomy, there would be a greater reduction in regenerative capacity. It would be interesting and fairly simple to test this hypothesis using the current model with varying time courses of chronic axotomy.

Additionally though our hypothesis was that during prolonged period of axotomy, the distal nerve stump (i.e. the graft) atrophies to a point where it is not supplying adequate levels of neurotrophins to support regenerative capacity, it would be interesting to look at different types of grafts (i.e. motor or mixed grafts) in terms of the

continuous growth model of prolonged axotomy. A suggested graft could be a 4cm segment of the sciatic nerve with branches ligated from a donor animal.

5.2 Experiment 2: The phosphodiesterase inhibitor rolipram promotes axonal regeneration of peripheral nerves across the surgical gap

In the second of the studies, we utilized the transected and repaired common peroneal nerve to answer the question of whether elevated cAMP results in increased motor and sensory nerve regeneration, and whether that translates into benefits in functional recovery. The results demonstrate that administration of rolipram, a cAMP specific phosphodiesterase (PDE) inhibitor, results in elevated levels of cAMP in the sensory cell body, and results in increased motor and sensory neuron axonal regeneration. This increase in regeneration was particularly evident immediately across the surgical site and we attribute rolipram's success in increasing regeneration to primarily accelerating axon outgrowth across the surgical site. Additionally, we demonstrate that functional recovery is accelerated in terms of recovery of a larger number of motor units in the rolipram treated group compared to saline treated controls. However, we also demonstrate that with longer periods of rolipram treatment (4 weeks), rats underwent a significant decline in body weight from saline treated controls. We hypothesize that this is due to appetite suppression, as rolipram treatment in humans for depression was suspended due to side-effects of nausea and vomiting.

5.2.1 Future Directions:

The effect of shorter term rolipram treatment on motor and sensory regeneration

Here we supply evidence demonstrating that increasing intracellular cAMP accelerates the regeneration of both motor and sensory axons. We report that the effect of rolipram was present one week into treatment and 1.5mm across the surgical repair site, indicating that rolipram may be having its effect by accelerating axon outgrowth across the surgical repair site. If this is indeed the case, shorter periods of rolipram treatment may be as effective as the two week protocol we investigated in this report. It would be interesting to investigate whether shorter periods of rolipram treatment, such as one or two days or even for a couple hours following nerve injury would be as effective in promoting regeneration. This could be accomplished utilizing the same model employed

here, with varying lengths and dosages of rolipram treatment. Additionally, lower dosages of rolipram treatment could be attempted with localized delivery of rolipram to the motor and sensory cell bodies via a catheter implanted into the subdural space. Again, depending on the success of treatment with catheterized delivery of rolipram, varying lengths of rolipram treatment could be attempted.

Investigation of the mechanism of action of increased cAMP.

Additionally it would be interesting to investigate further the actual mechanism by which rolipram acts. It has been demonstrated that elevated cAMP caused regeneration of sensory axons in the normally inhibitory CNS (Neumann et al., 2002; Qiu et al., 2002b). This elevated cAMP was shown to interact with protein kinase A (PKA) to cause an upregulation of certain polyamines through transcription dependent upregulation of Arginase I, an enzyme responsible for their synthesis, to allow regeneration of axons on normally inhibitory MAG/myelin (Cai et al., 2002). It would be interesting to investigate our proposed mechanism of action, involving the ability of cAMP to permit axons to regenerate on inhibitory proteoglycans found in the PNS, as well as the replication of Schwann cells on peripheral nerve regeneration and functional recovery. This could be accomplished by replicating the current experiments with the addition of the administration of an inhibitor of mitogenesis, such as mitomycin-C (Hall, 1986b), chondroitinase ABC (Groves et al., 2005) or *in vitro* experiments with or without exogenous cAMP on media containing proteoglycans similar to that done by Steinmetz et al. (2005).

More evidence for cAMP effect in cell body

Though we have shown here that the administration of rolipram results in elevated levels of cAMP in the sensory cell bodies of the DRG compared to saline treated controls at 3 days post injury, we have not completely ruled out the effects of elevated cAMP on non-neural Schwann cells as a contributing factor in the accelerated regeneration. Though it seems unlikely that replication of Schwann cells would be playing a major role in the acceleration of axonal regeneration immediately across the surgical site, it is unknown and must be considered. This could be perhaps investigated by injecting and analyzing a marker of cell division such as Bromodeoxyuridine (Bertuzzi et al., 2002) in Schwann cells at the site of surgical repair.

5.3 Experiment 3: The effect of electrical stimulation on axonal regeneration in the central nervous system

Here we supply preliminary answers to the question of whether electrical stimulation of dorsal root ganglion (DRG) sensory neurons peripheral axons results in promotion of concomitantly injured central axons of the same neurons. As discussed in the study's introduction and discussion, previous studies have demonstrated that the normally nonexistent regeneration of DRG sensory neuron central axons following transection could be promoted to regenerate if the peripheral axons were previously or concomitantly injured (Neumann and Woolf, 1999b). This effect was shown to be due to increased cAMP (Qiu et al., 2002a). Electrical stimulation has previously been shown to increase BDNF and result in increased peripheral regeneration (Al-Majed et al., 2000b; Al-Majed et al., 2000c). BDNF has been shown to increase cAMP, acting as a PDE inhibitor (Gao et al., 2003a). Here we demonstrate that the stimulation of peripheral axons of DRG sensory axons results in intracellular cAMP increases in the DRG sensory neuron cell body statistically similar to that found following a conditioning lesion of the same axons. We go on to present preliminary data showing an increase in the regeneration of the central axons of the DRG sensory neurons following STIM of the peripheral axons similar to that found following a CL of the peripheral axons. Further data will be necessary to establish whether this similarity is statistically significant.

5.3.1 Future Directions: Completing the study of electrical stimulation on central sensory axon regeneration

As mentioned above, further experiments will be necessary to ascertain the significance of our present results. This will be accomplished by replicating the experimental methodology outlined in the study.

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