NEUROPROTECTION AND AXONAL REGENERATION AFTER PERIPHERAL NERVE INJURY

DAG WELIN

Department of Integrative Medical Biology
Section for Anatomy
Department of Surgical and Perioperative Sciences
Section for Hand & Plastic Surgery
Umeå University, Sweden, 2010
Till Tuva
# TABLE OF CONTENTS

**ORIGINAL PAPERS** ................................................................. 7  
**ABBREVIATIONS** ................................................................. 8  

**INTRODUCTION** ................................................................. 9  
Clinical background ................................................................. 9  
Classification of nerve injuries ................................................ 10  
Factors influencing functional recovery after peripheral nerve injury ... 10  
Degeneration and regeneration after nerve injury .......................... 11  
Surgical repair after peripheral nerve injury ............................... 14  
Neuroprotection after peripheral nerve injury ............................. 15  

**SPECIFIC AIMS** ................................................................. 18  

**MATERIALS AND METHODS** .................................................. 19  
Experimental animals ............................................................... 19  
Labeling with retrograde fluorescent tracers. ............................... 19  
Sciatic nerve injury and repair ................................................ 20  
Ventral root rhizotomy and avulsion ......................................... 20  
Experimental groups ............................................................... 21  
N-acetyl-cysteine treatment ..................................................... 22  
Tissue processing ................................................................. 23  
Neuronal counts ................................................................. 24  
Laser microdissection ............................................................. 24  
RNA isolation and quantitative RT-PCR .................................... 24  
Image processing ................................................................. 25  
Statistical analysis ............................................................... 25  

**RESULTS** .............................................................................. 27  
Peripheral nerve injury induces delayed loss of cutaneous sensory neurons ... 27  
Spinal motoneurons degenerate after ventral rhizotomy and avulsion ... 27  
Nerve repair improves survival of axotomized sensory neurons ........ 27  
Regeneration of sensory neurons and motoneurons after nerve repair ... 28  
Regenerating sensory neurons up-regulate peripherin mRNA expression ... 28  
Sensory neurons down-regulate expression of NF-H mRNA after injury ... 28  
Regenerating sensory neurons up-regulate ATF3 mRNA expression ....... 29  
N-acetyl-cysteine promotes survival of spinal motoneurons ............. 29  
N-acetyl-cysteine promotes survival of cutaneous sensory DRG neurons ... 29  
Effects of N-acetyl-cysteine and nerve grafting on neuronal regeneration ... 29  
Effect of N-acetyl-cysteine and nerve grafting on axonal sprouting ....... 29
DISCUSSION .................................................................................................................. 31
Fluorescent retrograde tracers for neuronal quantification ................................. 31
Mechanism of retrograde cell death after peripheral nerve injury .................. 31
Response of spinal motoneurons to distal and proximal axotomy ................. 32
Sensory neurons after peripheral nerve injury ..................................................... 33
Neuroprotective effect of nerve grafting ............................................................. 34
Regulation of peripherin and ATF3 genes in primary sensory neurons .......... 34
N-acetyl-cysteine supports neuronal survival ................................................. 36

CLINICAL IMPLICATIONS ...................................................................................... 40

CONCLUSIONS ......................................................................................................... 42

ACKNOWLEDGEMENTS ......................................................................................... 43

REFERENCES ........................................................................................................... 44

PAPERS I-IV ............................................................................................................... 59
ABSTRACT

Following microsurgical reconstruction of injured peripheral nerves, severed axons are able to undergo spontaneous regeneration. However, the functional result is always unsatisfactory with poor sensory recovery and reduced motor function. One contributing factor is the retrograde neuronal death which occurs in the dorsal root ganglia (DRG) and in the spinal cord. An additional clinical problem is the loss of nerve tissue that often occurs in the trauma zone and which requires “bridges” to reconnect the injured nerve ends. The present thesis investigates the extent of retrograde degeneration in spinal motoneurons and cutaneous and muscular afferent DRG neurons after permanent axotomy and following treatment with N-acetyl-cysteine (NAC). In addition, it examines the survival and growth-promoting effects of nerve reconstructions performed by primary repair and peripheral nerve grafting in combination with NAC treatment.

In adult rats, cutaneous sural and muscular medial gastrocnemius DRG neurons and spinal motoneurons were retrogradely labeled with fluorescent tracers from the homonymous transected nerves. Survival of labeled neurons was assessed at different time points after nerve transection, ventral root avulsion and ventral rhizotomy. Axonal regeneration was evaluated using fluorescent tracers after sciatic axotomy and immediate nerve repair. Intraperitoneal or intrathecal treatment with NAC was initiated immediately after nerve injury or was delayed for 1-2 weeks.

Counts of labeled gastrocnemius DRG neurons did not reveal any significant retrograde cell death after nerve transection. Sural axotomy induced a delayed loss of DRG cells which amounted to 43-48% at 8-24 weeks postoperatively. Proximal transection of the sciatic nerve at 1 week after initial axonal injury did not further increase retrograde DRG degeneration, nor did it affect survival of corresponding motoneurons. In contrast, rhizotomy and ventral root avulsion induced marked 26-53% cell loss among spinal motoneurons. Primary repair or peripheral nerve grafting supported regeneration of 53-60% of the motoneurons and 47-49% of the muscular gastrocnemius DRG neurons at 13 weeks postoperatively. For the cutaneous sural DRG neurons, primary repair or peripheral nerve grafting increased survival by 19-30% and promoted regeneration of 46-66% of the cells. Regenerating sural and medial gastrocnemius DRG neurons upregulate transcription of peripherin and activating transcription factor 3. The gene expression of the structural neurofilament proteins of high molecular weight was significantly downregulated following injury in both regenerating and non-regenerating sensory neurons. Treatment with NAC was neuroprotective for spinal motoneurons after ventral rhizotomy and avulsion, and sural DRG neurons after sciatic nerve injury. However, combined treatment with nerve graft and NAC had significant additive effect on neuronal survival and also increased the number of sensory neurons regenerating across the graft. In contrast, NAC treatment neither affected the number of regenerating motoneurons nor the number of myelinated axons in the nerve graft and in the distal nerve stump.

In summary, the present results demonstrate that cutaneous sural sensory neurons are more sensitive to peripheral nerve injury than muscular gastrocnemius DRG cells. Moreover, the retrograde loss of cutaneous DRG cells taking place despite immediate nerve repair would still limit recovery of cutaneous sensory functions. Experimental data also show that NAC provides a highly significant degree of neuroprotection in animal models of adult nerve injury and could be combined with nerve grafting to further attenuate retrograde neuronal death and to promote functional regeneration.

Key words: Dorsal root ganglion; Motoneuron; Axonal reaction; Peripheral nerve graft; Nerve regeneration; N-acetylcysteine.
ORIGINAL PAPERS

This thesis is based on the following papers which are referred in the text by Roman numerals.


All published papers are reproduced with the permission of the copyright holders.
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATF3</td>
<td>Activating transcription factor 3</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal root ganglion</td>
</tr>
<tr>
<td>FB</td>
<td>Fluorescent tracer Fast Blue</td>
</tr>
<tr>
<td>FG</td>
<td>Fluorescent tracer Fluoro-Gold</td>
</tr>
<tr>
<td>FR</td>
<td>Fluorescent tracer Fluoro-Ruby</td>
</tr>
<tr>
<td>i.m</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>i.p</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>i.t</td>
<td>Intrathecal</td>
</tr>
<tr>
<td>L4/L5/L6</td>
<td>Lumbar segments 4/5/6</td>
</tr>
<tr>
<td>MG</td>
<td>Medial gastrocnemius nerve</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetyl-cysteine</td>
</tr>
<tr>
<td>NF-H</td>
<td>Neurofilament proteins of high molecular weight</td>
</tr>
<tr>
<td>NG</td>
<td>Peripheral nerve graft</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer solution</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SC</td>
<td>Spinal cord</td>
</tr>
<tr>
<td>S.E.M</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SUR</td>
<td>Sural nerve</td>
</tr>
<tr>
<td>TB</td>
<td>Fluorescent tracer True Blue</td>
</tr>
</tbody>
</table>
INTRODUCTION

Clinical background
Peripheral nerve lacerations are common injuries and often cause long lasting disability (Jaquet et al. 2001) due to pain, paralyzed muscles and loss of adequate sensory feedback from the nerve receptors in the target organs such as skin, joints and muscles (Lundborg and Rosen 2007). Normal function will not be regained even if the nerve is repaired (Lundborg 2000a). Nerve injuries typically affect young adults and the majority of injuries is domestic or occupational accidents with glass, knives of machinery (McAllister et al. 1996; Rosberg et al. 2005) but road traffic accidents, iatrogenic injuries, assault, self-inflicted injuries are other known causes. The severity of the injuries varies from minor, such as digital nerve injury to major, such as brachial plexus injury. Nerve injuries affect both the individual patient and the society, for example, an injury to the ulnar or median nerve in the forearm has major social consequences (Jaquet et al. 2001) and results in a sick leave for a median time of 157 or 273 days, respectively. The total cost per patient with ulnar or median nerve injury has been estimated to be EUR 51 238 and EUR 31 186, respectively (Rosberg et al. 2005).

Classification of nerve injuries
The typical peripheral nerve is composed by efferent motor axons originated from spinal motoneurons and afferent sensory axons from dorsal root ganglion (DRG) neurons. An injury to the peripheral nerve, therefore, will affect both motor and sensory function and induce retrograde (axon) reaction in spinal cord and DRGs. There are different types of nerve injury and the most common include nerve transection, compression, crush, traction and avulsion.

To facilitate clinical work, Seddon et al. (1943) classified nerve injuries into three main groups: neurapraxia, axonotmesis and neurotmesis. Neurapraxia is the mildest type of nerve injury and is often associated with transient ischemia without physical disruption of the axons or myelin sheath. Although this form of nerve injury blocks nerve impulse transmission, it does not require any surgical intervention and recovery usually occurs within a few weeks. Axonotmesis refers to the disruption of several axons and myelin sheath but without significant damage to the connective tissue layers (Seddon et al. 1943). Axons distal to the injury degenerate but there is possibility for spontaneous regeneration without surgical treatment. Neurotmesis is usually associated with partial or complete severance of the nerve with disruption of the axon, myelin sheath and the connective tissue elements. It is the most severe type of nerve injury and no functional recovery is possible without surgical nerve reconstruction. Another common grading system is the Sunderland’s classification.
which contains five degrees of severity of the nerve injury. However, it requires histological analysis of the injured nerve (Birch 2005).

There are different techniques used to repair injured peripheral nerves. For example, “epineural repair” indicates that micro sutures were placed in the epineurium to align the nerve ends whereas in “fascicular repair” individual nerve fascicles are repaired. Nerve reconstructions could be also subdivided into “primary repair” if it is performed within 48 hours after injury, “early secondary repair” if performed between 2 days and 6 weeks and “late secondary repair” (Green 1998). In this thesis, all nerve injuries are complete transections of the peripheral nerves and, therefore, can be classified as “neurotmesis”. For nerve reconstruction and grafting only primary repairs with epineural suturing technique were used.

Factors influencing functional recovery after peripheral nerve injury

The current management of a lacerated peripheral nerve is based on either direct microsurgical repair where the cut ends are approximated with sutures, or autologous nerve grafting where an existing defect is bridged with a harvested donor nerve. Even if the injured nerve is meticulously repaired the outcome is often unpredictable and disappointing (Jaquet et al. 2001; Ruijs et al. 2005; Lundborg and Rosen 2007). Several factors have been identified that influence the outcome after nerve repair. Young age is a main factor for recovery and could be explained by shorter regeneration distance, greater regeneration potential and brain plasticity (Lundborg 2000a; Ruijs et al. 2005). It has also been shown that delay until surgical repair is unfavorable for recovery (Ruijs et al. 2005) which is suggested to result from neuronal loss, distal nerve stump fibrosis and Schwann cell atrophy (Gordon et al. 2003; Lundborg and Rosen 2007). The proximity of the injury is a significant predictor for motor recovery due to the long regeneration distance leading to irreversible muscle atrophy before target reinnervation (Ruijs et al. 2005). Good cooperation and motivation of the patient, cognitive capacity and specialized hand therapy are other factors contributing to the outcome after nerve repair (Ruijs et al. 2005; Lundborg and Rosen 2007). A vital component for normal motor control is adequate proprioceptive feedback (Westling and Johansson 1984). Therefore, sensory regeneration is important for the recovery of motor function. Future modifications in surgical techniques are not likely to significantly improve the outcome after a nerve injury. Thus, attention has been focused on the neurobiology following nerve injury and how various components of axon reaction and Wallerian degeneration can be modulated.
Degeneration and regeneration after nerve injury

Axon reaction

After nerve injury signaling through injury-induced discharge of axonal potentials, interruption of target-derived factors and retrograde injury signals transported from the site of injury to the cell body enables the neuron to respond to the trauma. Proposed injury signals of importance consist of microtubule-dependant axonal transport of mitogen-activated protein kinases (MAPK) including Erk and JNK, and local release of cytokines LIF, IL-6 and CNTF leading to activation of the JAK-STAT pathway (Abe and Cavalli 2008). Axotomized neurons respond by up-regulation of regeneration-associated genes in association with conversion of the neuron from a transmitting to a growth state (Boyd and Gordon 2003).

Nerve transection produces morphological changes in the neuronal perikarya known as chromatolysis or axon reaction. The changes include swelling of the cell body, nucleolar enlargement, displacement of the nucleus to the periphery and dissolution of Nissl bodies (Kreutzberg GW 1995). Chromatolysis is explained as a change in the neurons metabolism aiming at an increased regenerative potential but may also be a sign of severe trauma with loss of a large axoplasmic volume. Axon reaction could result in both neuronal survival and regeneration or neuronal death (Lundborg 2000a). The principal determinant of the extent of neuronal death after axotomy seems to be the loss of target-derived neurotrophic factors (Terenghi 1999).

For many years it was recognized that experimental nerve injuries result in a loss of primary sensory neurons and more recently it was confirmed by direct observation of DNA fragmentation in vivo (Groves et al. 1997; Oliveira et al. 1997; Hart et al. 2002a). The extent of the loss ranges from 7 to 51% (Rich et al. 1989; Liss et al. 1994; Liss et al. 1996; Groves et al. 1997; Tandrup et al. 2000; Hart et al. 2002a; Ma et al. 2003; Jivan et al. 2006), depending on the experimental model used. Since the first prerequisite for axonal regeneration is the survival of the neuron following injury, it is likely that this neuronal cell death is of great importance for the outcome of the axonal regeneration and target organ reinnervation (Fu and Gordon 1997). Motoneurons seem to be more resistant to peripheral axotomy (Novikova et al. 1997) however, 20 to 30% of motoneurons die after proximal C7 axotomy or lumbar rhizotomy (Gu et al. 1997; Ma et al. 2001; Zhang et al. 2005; Jivan et al. 2006), and more than 50% degenerate after ventral root avulsion at 4-8 weeks after injury (Koliatsos et al. 1994; Novikov et al. 1995).

Posttraumatic neuronal death is believed to be an apoptotic response comprising pathways and mediators that are activated in various neuropathological conditions (Becker and Bonni 2004). It is possible that different cell types encode different apoptotic and survival genes, and also that cells may activate different apoptotic
pathways in response to different external stimuli (Pettmann and Henderson 1998). Following axotomy, apoptosis might be initiated by alterations in electrical activity, neurotoxic inflammatory products and loss of target derived neurotrophic support (Ambron and Walters 1996; Fu and Gordon 1997). Central components in the present context are the mitochondria. Pro-apoptotic mediator Bax and pro-survival Bcl-2 interact at the level of the mitochondria and it has been suggested that their ratio determines the fate of the cell by governing mitochondrial outer-membrane permeability (Gillardon et al. 1996). Bcl-2 has been shown to heterodimerize with Bax which counteracts the death-repressor activity of Bcl-2. When Bax exerts dominance over Bcl-2, pores are formed in the mitochondrial membrane which allows release of apoptotic molecules (cytochrome-c) into the cytosol, thus triggering the execution caspase-cascade. This culminates in the activation of caspase-3, an ‘effector caspase’ which is directly involved in the proteolytic action on cells, e.g. digestion of structural proteins in the cytoplasm and degradation of chromosomal DNA (Becker and Bonni 2004). Recently, a different response in regulation of Bax, Bcl-2 and caspase-3 has been indentified in cutaneous and muscular subpopulations of sensory DRG neurons after injury. The Bcl-2:Bax ratio in sensory DRG neurons projecting to muscles increases whilst the ratio for sensory DRG neurons projecting to the skin decreases. Simultaneously the levels of caspase-3 have been markedly down-regulated in the muscular afferent neurons and progressively up-regulated in the cutaneous afferent neurons (Reid et al. 2009).

Wallerian degeneration

After a nerve transection, a well-defined cascade of cellular changes occurs in the distal nerve segment and to the first node of Ranvier in the proximal nerve stump. The process is called Wallerian degeneration and involves degeneration of all affected axons, degradation of their myelin sheaths and invasion of macrophages to remove debris. Wallerian degeneration creates a favorable microenvironment for regeneration of surviving neurons (Navarro et al. 2007). The Schwann cells divide by mitosis and line up within each basal lamina tube to form the bands of Büngner, providing guidance for regenerating nerve fibers (Frostick et al. 1998; Terenghi 1999; Hall 2001). During the initial phase of regeneration an axon in the proximal stump sends out multiple sprouts, averaging five axons, which regenerate into multiple endoneurial tubes in the distal stump, potentially leading to complex mismatch in reinnervation of denervated targets. If the axons lack a supportive structure they will, together with connective tissue, form a neuroma. After target reinnervation all axons from a neuron, but one, die-back (Gordon et al. 2003). Regenerating axons in the distal nerve stump enlarge in diameter and reach normal size when they make connections with the target organs (Sulaiman and Gordon 2000).
**Schwann cells and peripheral nerve injury**

The Schwann cells promote survival and regeneration by increased synthesis of surface cell adhesion molecules (CAMs), by providing a basement membrane with extracellular matrix proteins laminin and fibronectin, and by producing various growth factors (Frostick et al. 1998; Lundborg 2000a; Lundborg and Rosen 2007). After injury, Schwann cells in the distal stump proliferate and convert their function from myelination of electrically active axons to growth support for regenerating nerve fibers (Fu and Gordon 1997). Optimal expression of growth-supportive molecules by Schwann cells is associated with macrophages infiltrating the distal stump from peripheral circulation (Gordon et al. 2003). The presence of viable Schwann cells in the distal nerve stump is essential for regeneration after nerve injury (Fu and Gordon 1997). It has been demonstrated that prolonged axotomy leads to rather limited regeneration due to atrophy of Schwann cells and decline in expression of neurotrophic factors (Sulaiman and Gordon 2000; Hoke et al. 2002; Gordon et al. 2003; Jivan et al. 2006).

**Neurotrophic factors and their receptors after peripheral nerve injury**

It is mainly agreed that neurons rely on neurotrophic support for survival. Trophic factors can be divided into neurotrophins (NGF, BDNF, NT-3 and NT-4/5), neuropoetic cytokines (IL-6, LIF and CNTF) and the Glial cell-line derived neurotrophic factor family (GDNF, Neurturin, Persephin and Artemin). In the uninjured neuron the trophic factors are produced by the target organ and delivered to the neuron by retrograde axonal transport. Neurons sensitive to neurotrophins express the high-affinity tyrosine kinase (Trk) receptors and the low-affinity p75 receptor. Neurons sensitive to the GDNF family express the GFRα receptors and the common signal transduction unit, ret receptor. Neuropoetic cytokines binds to specific receptors and the common signal transduction receptor subunit, gp130 (Boyd and Gordon 2003). Different subpopulations of neurons have been identified and characterized by overlapping but yet distinct trophic requirements (McMahon et al. 1994). A large proportion of the primary sensory afferents expresses the TrkA receptors for NGF, whereas trkC receptors for NT-3 are mainly expressed by large diameter proprioceptive neurons (Lindsay 1996). Motoneurons express TrkB and TrkC receptors and bind BDNF, NT-4/5 and NT-3. A part of the neurons in the dorsal root ganglion lacks Trk receptors and express GFRα receptors which bind members of the GDNF family. After a sciatic crush, the Schwann cells in the distal end up-regulate their expression of neurotrophins NGF, BDNF and NT-4/5 and trophic factors GDNF, LIF and IL-6. Motoneurons up-regulate trkB and GFRα1 receptors. Neither intact nor injured motoneurons express GDNF (Boyd and Gordon 2003). In primary sensory afferents all Trk receptors are down-regulated after axotomy (Bergman et al. 1999) but the GFRα1 receptor is dramatically up-regulated in large-diameter sensory afferents (Bennett et al. 2000). It is possible that surviving
peripheral axotomy sensory and motor neurons express receptors which match the profile of neurotrophic factors produced by Schwann cells in the distal nerve stump.

**Regenerative response after axonal injury**

The functional goal of regeneration is to replace the distal nerve segment lost after injury, to reinnervate the target organs and to restitute their function. A peripheral nerve injury activates a complex molecular response in the neuron. The biochemical changes develop within hours after axotomy, triggered by the signaling mechanism mentioned previously, and is overall thought to induce a decreased synthesis of products for neurotransmission and increased synthesis of growth associated proteins and components of the membrane. Signaling through the axotomy-activated kinases lead to up-regulation or activation of several transcription factors including c-Jun, cAMP responsive element binding protein (CREB), STAT-3, Akt and Nuclear Factor kB (NFkB). This upregulation leads to changes in gene expression in a large number of genes, many of which the function is unknown but involves changes in transcription factors, cytoskeletal proteins, cell adhesion and guidance molecules, trophic factors and receptors, cytokines, neuropeptides and neurotransmitter synthesizing enzymes, ion channels and membrane transporters (Navarro et al. 2007).

Recently, the roles of neuronal intermediate filaments and activating transcription factor 3 (ATF3) in the regenerative response have been examined. Neuronal intermediate filaments are crucial components of the neuronal cytoskeleton comprising the neurofilament triplet proteins of low (68 kDa), medium (150 kDa), and high (NF-H, 200 kDa) molecular weight, in addition to α-internexin (66 kDa) and peripherin (57 kDa) (Helfand et al. 2004; Lariviere and Julien 2004). Peripherin and NF-H define two distinct subpopulations of sensory DRG neurons (Fornaro et al. 2008). It has been shown that after nerve injury sensory neurons in DRGs and spinal motoneurons up-regulate peripherin (Oblinger et al. 1989; Troy et al. 1990; Wong and Oblinger 1990; Terao et al. 2000) and down-regulate NF-H (Wong and Oblinger 1990; Muma et al. 1990).

**Surgical repair after peripheral nerve injury**

The gap between the two ends of a divided nerve can vary from a few millimeters to several centimeters depending on the type of injury and the timing of surgery. A small nerve gap is traditionally repaired with primary nerve suturing. However, if the peripheral nerve injury is associated with a significant tissue loss, bridging strategies are needed to provide a physical substrate for axonal growth. Defects in the peripheral nervous system are most commonly bridged by autologous nerve grafts obtained from “less important” sensory nerves from the patient’s legs. So far, nerve grafts have been superior to other substitutes since they provide appropriate
alignment and contain the cellular constituents of normal peripheral nervous tissue. The technique is not optimal, however, since the patient will suffer from loss of sensation, scarring and sometimes pain in the donor region (Wiberg and Terenghi 2003). In case of dorsal and ventral root avulsion from the spinal cord, transfer of previously uninjured nerves may be considered. If the distal nerve end is removed from the muscle, direct implantation of the nerve back into the muscle, a so called neurotisation, can be performed (Birch 2005). Retrograde neuronal degeneration after peripheral nerve injury can experimentally be reduced by early nerve repair (Ma et al. 2003; Jivan et al. 2006; Zhang et al. 2006) possibly by reestablishment of endogenous neurotrophic support from the distal nerve stump (Boyd and Gordon 2003; Low et al. 2003). Clinical results from repair of the brachial plexus also demonstrate that early nerve reconstruction provides better functional outcome when compared to delayed nerve repair (Jivan et al. 2008).

Recent studies have also demonstrated that in contrast to sensory nerve autografting, more efficient axonal regeneration could be obtained with motor nerve grafts (Moradzadeh et al. 2008; Chu et al. 2008). Improved regeneration with motor grafting may be a result of the nerve's Schwann cell basal lamina tube size (Moradzadeh et al. 2008) or reflect different expression of neurotrophic factors from Schwann cells in the sensory and motor nerve grafts (Hoke et al. 2006; Chu et al. 2008). However, the obvious problems from the donor region limit the potential clinical use of grafts from motor nerves.

**Neuroprotection after peripheral nerve injury**

Several experimental studies have shown that permanent axotomy induces significant but delayed retrograde cell death of sensory neurons projecting mainly to skin and does not affect survival of sensory neurons projecting to muscle (Tandrup et al. 2000; Hu and McLachlan 2003; Welin et al. 2008). Therefore, a therapeutic window exists where immediate neuroprotective treatment could be initiated to attenuate retrograde degeneration before nerve repair could be performed.

**Neurotrophic factors**

It is well known that neurotrophic factors regulate the development, maintenance and function of the vertebrate nervous system. Studies *in vitro* have shown that activation of PKA, Ras/PI-3K/Akt (PI-3K/Akt), or Ras/Raf/ MAPK/ERK (MAPK/ERK) signaling pathway by neurotrophins and GDNF could promote both neuronal survival and enhance neurite growth in neuronal populations (Hetman et al. 1999; Soler et al. 1999; Liot et al. 2004; Chierzi et al. 2005). In contrast, the JAK-STAT signaling pathways are activated by cytokines and can mediate regenerative sensory axon growth (Liu and Snider 2001). The depletion of neurotrophic factors from target organs and Schwann cells plays a significant role in the induction of
retrograde degeneration (Boyd and Gordon 2003). Administration of exogenous neurotrophic factors has shown to reduce neuronal loss in sensory neurons (Bennett et al. 1998; Ljungberg et al. 1999) and motoneurons (Novikov et al. 1995). Neurotrophic factors can also promote axonal regeneration (Boyd and Gordon 2002; McKay et al. 2003) and counteract development of axon reaction (Boyd and Gordon 2003). However, the specificity of neurotrophic factors for neuronal subpopulations (Terenghi 1999), unpredictable interactions (Novikova et al. 2000a) and side effects (Martin et al. 1996; McArthur et al. 2000; Apfel 2002) makes clinical use difficult.

**Antioxidants**

During past decades, significant number of various neuroprotective agents has been tested both in vitro and in vivo. For example, treatments with acetyl-L-carnitine (Hart et al. 2002b; Wilson et al. 2007), the monoamine oxidase inhibitor deprenyl (Hobbenaghi and Tiraihi 2003), the cytokine modulator linomide (Ekstrom et al. 1998) and thyroxine (Schenker et al. 2003) have been shown to protect sensory DRG neurons from retrograde cell death.

It is well established that following injury to the nervous system, mitochondrial impairment could lead to generation of reactive oxygen species and activation of apoptotic cascades. Given the important role that oxidative stress plays in promoting neuronal death, administration of antioxidants may be potentially attractive as clinically applicable neuroprotective agents (Merenda and Bullock 2006). N-acetyl-cysteine (NAC), a thiol-containing compound, has a broad range of actions which includes antioxidant activity, enhancement of intracellular glutathione levels, inhibition of proliferation, and stimulation of transcription (Holdiness 1991; Yan and Greene 1998; Arakawa and Ito 2007). NAC has been used in clinical practice for many years as a mucolytic agent for treatment of congestive and obstructive lung diseases and as the drug of choice for paracetamol intoxication. Recently it has been shown that NAC can rescue sensory and motor neurons from retrograde degeneration (Hart et al. 2004; Zhang et al. 2005; West et al. 2007b). NAC has a broad range of actions potentially relevant to its neuroprotective effects. Most of the results are from in vitro experiments and the mechanism of NAC neuroprotective effect in vivo is largely unknown. NAC has a direct reductant and free radical scavenging effects, interacting with reactive oxygen species (ROS) and is neuroprotective in multiple neuronal models in vitro (Yan et al. 1995; Ferrari et al. 1995). NAC can also enhance neuronal biosynthesis of glutathione (Dringen and Hamprecht 1999), the principal renewable free radical scavenger within neurons (Cooper and Kristal 1997; Heales et al. 1999). Depletion of intracellular, and especially mitochondrial glutathione leads to neuronal death in vitro (Wullner et al. 1999). Increased glutathione levels by NAC prevented cytochrome c release from the mitochondria, thereby preventing the apoptotic cascade (Kirkland and Franklin 2001). However, the neuroprotective effect of NAC has also been shown to be
glutathione-independent (Yan et al. 1995) and other antioxidants fail to mimic its neuroprotective effect (Ferrari et al. 1995). It has been reported that NAC can signal through the neurotrophic factor-activated Ras-ERK pathway and stress-activated JNK pathways but does not activate the PI3-K/Akt survival pathway (Xia et al. 1995; Park et al. 1996; Yan and Greene 1998). These findings strongly suggest that NAC can share with neurotrophic factors the capacity to maintain neuronal survival and could be used as a neuroprotective agent to rescue sensory and motor neurons after peripheral nerve injury.
SPECIFIC AIMS

The aims of this study were:

- To compare the retrograde degeneration and axonal regeneration of sensory DRG neurons and spinal motoneurons projecting to different target organs (Paper I).

- To examine the gene expression of neuronal intermediate filaments and activating transcription factor 3 (ATF-3) in cutaneous and muscular sensory DRG neurons (Paper II).

- To evaluate the neuroprotective effect of N-acetyl-cysteine treatment on spinal motoneurons after ventral rhizotomy and ventral root avulsion (Paper III).

- To evaluate the neuroprotective and growth-promoting effects of N-acetyl-cysteine treatment and nerve grafting on sensory DRG neurons and spinal motoneurons after peripheral nerve injury (Paper IV).
MATERIALS AND METHODS

Experimental animals
The experiments were performed on adult (8-12 weeks; \(n=228\)) female Sprague-Dawley rats (Møllegaard Breeding Center, Denmark). The animal care and experimental procedures were carried out in accordance with the standards established by the NIH Guide for Care and Use of Laboratory Animals (National Institutes of Health Publications No. 86-23, revised 1985) and the European Communities Council Directive (86/609/EEC). The study was approved by the Northern Swedish Regional Committee of Ethics in Animal Experiments. All surgical procedures were performed under general anesthesia with iterated intraperitoneal injections using a mixture of ketamine (Ketalar, Parke-Davis) and xylazine (Rompun, Bayer). Benzylpenicillin (Boehringer Ingelheim; 60 mg i.m.) was given after each surgical procedure.

Labeling with retrograde fluorescent tracers
In order to study neuronal survival and regeneration after peripheral nerve injury, DRG neurons and spinal motoneurons projecting to the sural or medial gastrocnemius nerves were retrogradely labeled with fluorescent tracers (Novikova et al. 1997). Under an operating microscope, the nerves were transected at the same level in the popliteal fossa of the hind limb and the proximal nerve end was introduced into a small polyethylene tube containing two microliters of Fast Blue (FB, 2% aqueous solution, EMS-Chemie GmbH, Germany; Papers I, II and IV) or True Blue (TB, 2% aqueous solution, Molecular Probes; Paper III). The tube was fixed to the surrounding muscles using Histoacryl® glue (B.Braun Surgical GmbH, Germany) and sealed with a mixture of silicone grease and vaseline to prevent leakage. Two hours later the tube was removed, the nerve rinsed in saline and the wound closed in layers. Fast Blue and True Blue produces a very efficient retrograde labeling of neurons at 1 week after tracer application and the staining remains constant for at least 6 months (Novikova et al. 1997; Houle and Ye 1999). However, since it has been reported that axotomized adult DRG neurons may start to die already after 1 week (Hart et al. 2002a), we also used the fluorescent tracer Fluoro-Gold (FG, 2% solution in saline, Fluorochrome) in experiments with short-term survival (Paper I) since this dye produces very rapid neuronal labeling within 2-3 days (Novikova, unpublished observation) but gradually disappears from the labeled neurons after 4 weeks (Novikova et al. 1997; Akhavan et al. 2006). In the experiments dealing with the time course of retrograde degeneration (Paper I), the sural and medial gastrocnemius nerves were labeled on different sides. To identify sural and medial gastrocnemius neurons which had regenerated across the repair site...
and into the distal stump of the sciatic nerve the fluorescent tracer Fluoro-Ruby (FR, 10% solution in normal saline, Molecular Probes) was used (Papers I, II and IV).

Fast Blue tracer was also injected into the medial gastrocnemius muscle at four different sites using a 10-µl Hamilton syringe (Hamilton, Switzerland) and into the sural nerve using a fine-glass microelectrode (Paper II). To test the presence of non-specific labeling, axotomized sural nerve was divided and capped proximal to the site of Fast Blue administration to prevent dye uptake. In addition, contralateral DRG were also examined. These control experiments confirmed that there was no leakage of the tracer.

Sciatic nerve injury and repair
Sciatic nerve transection and repair were performed at 1 week after Fast Blue labeling of the sural and medial gastrocnemius nerves. The sciatic nerve was exposed via a muscle splitting incision and the wound held open with retractors, taking care at all times not to traumatize the nerve. The surgery on the nerve was performed using sterile micro instruments and an operating microscope (Zeiss, Carl Zeiss, Germany). The nerve was divided at mid-thigh level. To produce permanent axotomy, the proximal nerve stump was ligated with 6-0 Prolene, capped with polyethylene tube or wrapped in Spongostan® (Johnson & Johnson Medicals, UK) to prevent spontaneous regeneration. For nerve grafting, a 10 mm piece of the sciatic nerve was excited, reversed and interposed between the nerve stumps. Primary repair and nerve grafting of the divided sciatic nerve were performed using four 10/0 Ethilon® epineural sutures (S&T Marketing AG, Switzerland) and fibrin glue (Tisseel®, Immuno, Vienna, Austria) to secure the graft into position. Skin and fascia were closed with interrupted 3-0 silk veterinary sutures.

Ventral root rhizotomy and avulsion
One week after tracer application to the medial gastrocnemius nerve, a lumbo-sacral laminectomy was performed and L5-L6 ventral roots of the left side were identified and transected close to the corresponding DRGs (ventral rhizotomy). To perform ventral root avulsion, the proximal stump of the root was grasped with jeweller’s forceps and slowly pulled until it ruptured and came out in its entire length. The root was found to regularly rupture at its site of exit from the spinal cord. Spinal roots were covered with Spongostan® and the wound was closed in layers. Survival of labeled motoneurons was assessed four week after injury (Novikov et al. 2000).
Experimental groups

Paper I
To study the time course of neuronal loss, three groups of animals with FG-labeled nerves were killed at 3 days (n=6), 7 days (n=5) and 14 days (n=5). Five groups of animals with FB-labeled nerves were killed at 1 week (n=5), 4 weeks (n=6), 8 weeks (n=5), 13 weeks (n=5) and 24 weeks (n=6). To study nerve regeneration, three groups of animals with FB-labeled medial gastrocnemius nerve (MG) and three groups of animals with FB-labeled sural nerve (SUR) were used. One week after retrograde FB-prelabeling of the SUR or MG nerves in the the popliteal fossa, a sciatic axotomy was performed proximally in the thigh. In the first group (n=6 for SUR, n=6 for MG) the sciatic nerve was capped during the entire survival period. In the second group (n=5 for SUR, n=7 for MG), a primary suture of the sciatic nerve was performed. In the third group (n=6 for SUR, n=5 for MG), the sciatic nerve was repaired using a peripheral nerve graft. At 12 weeks after sciatic nerve transection and repair, the nerve was again transected 10 mm distal to the repair site and the retrograde tracer Fluoro-Ruby was applied to the proximal nerve stump (Jivan et al. 2006). To achieve optimal FR-labeling of the DRG neurons, the animals were left to survive for one week before termination of the experiment.

Paper II
To study the expression of regeneration-associated genes after nerve injury and repair, four groups of experimental animals were used: 1) control animals with FB injection into medial gastrocnemius muscle (n=5), 2) control animals with FB injection into sural nerve (n=5), 3) animals with FB-labeled medial gastrocnemius neurons, sciatic nerve transection and immediate primary repair (n=5) and 4) animals with FB-labeled sural neurons, sciatic nerve transaction and immediate primary repair (n=5). At 8 weeks postoperatively the sciatic nerve was transected 10mm distal to the repair site and the proximal stump was labeled with Fluoro-Ruby to reveal regenerating neurons.

Paper III
The medial gastrocnemius (MG) motorneuron pool was unilaterally prelabeled with a retrograde tracer. The effect of NAC (given either intraperitoneally, or intrathecally by infusion into the cerebrospinal space) on survival of those motorneurons was then determined after ventral rhizotomy, or root avulsion. To study the effects of delayed NAC treatment separate groups of animals were used. Numbers of animals in each experimental group are detailed in Tables 1-3.

Paper IV
To study whether NAC could protect sural sensory neurons from retrograde cell death, sciatic nerve was transected at 1 week after neurons were labeled with Fast
Blue and treatment was initiated. The proximal stump was covered with Spongostan® (Johnson & Johnson Medicals, UK) to prevent regeneration. Controls (n=5) and treated animals (n=4) were allowed to survive for 8 weeks.

To study NAC effect on nerve regeneration, three groups of animals were used (control, n=5; nerve grafting, n=6; nerve grafting with NAC treatment, n=8). One week after Fast Blue application to the transected sural nerve, the sciatic nerve was divided and repaired with peripheral nerve graft. In the treated group, NAC infusion was initiated immediately after nerve repair. At 12 weeks postoperatively, sciatic nerve was again transected 10 mm distal to the graft-nerve anastomosis and the nerve stump was introduced into a polyethylene tube containing Fluoro-Ruby. The animals were left to survive for 1 week before the termination of the experiment.

**N-acetyl-cysteine treatment**

The clinically available L-stereoisomer of N-acetyl-cysteine (NAC, Tika, 200mg/ml) was used in all experiments. In the experimental groups receiving *intraperitoneal injections*, treatment was commenced immediately after the experimental injury (1ml bolus). The rats were given injections once daily until termination of the experiment. In the ventral root avulsion group of animals, the dose tested was 150mg/kg/day, but after ventral rhizotomy, we used 150mg/kg/day and 750mg/kg/day to evaluate any dose response relationship. In order to exclude any possible toxic effect of NAC on motorneurons, treatment was also given to control animals for 4 weeks (n=5) and 8 weeks (n=2). Normal controls included non-injured rats labeled with FG for 5 weeks and with TB for 8 weeks.

In the experimental groups receiving *intrathecal infusion*, an Alzet 2002 osmotic mini-pump (Alza Corp., CA, USA) containing NAC was implanted subcutaneously in the neck and connected to a polyethylene catheter (Intermedic, PE-60, Clay Adams, NJ) which was inserted into the lower lumbar subarachnoid space after L5-L6 laminectomy (Novikov et al. 1997; Zhang et al. 2005). The catheter tip was gently advanced rostral to the level of L3-L4 DRGs, fixed to the S1 vertebral bone by Histoacryl® glue, and additionally secured by several sutures to the back muscles. The implantation site was covered with Spongostan® and the wound closed in separate layers. The mini-pump infusion speed corresponded to 2.4 mg/day of NAC. At intervals of 14 days, the emptied mini-pump was replaced by a second pump containing the same solution. The total time of intrathecal NAC infusion was 8 weeks.

To establish whether NAC would give neuroprotective benefit if treatment would be commenced well after injury, four further groups underwent ventral root avulsion and mini-pump implantation. Minipumps and catheters in the first two groups contained either PBS (“avulsion + intrathecal vehicle”) or NAC (200 mg/ml,
“avulsion + intrathecal NAC, immediate treatment”), and the mini-pumps were replaced after two weeks. The third group’s first minipump contained NAC (200mg/ml), but its catheter was primed with PBS, and its length calibrated at 37°C such that delivery of NAC into the subarachnoid space was delayed by one week. The pump was replaced two weeks later to continue delivery of NAC (avulsion + intrathecal NAC, 1-week delayed treatment). In the final group, the first mini-pump contained PBS, but was replaced after one week by another pump with NAC (200mg/ml), such that drug would reach the end of the catheter a week later, beginning active treatment two weeks after injury (avulsion + intrathecal NAC, 2-week delayed treatment). A group of non-injured rats, labeled for 7 days with TB, served as baseline controls.

**Tissue processing**

At the end of the survival period, the animals were given an intraperitoneal overdose of sodium pentobarbital (240mg/kg, Apoteksbolaget, Sweden) and transcardially perfused with Tyrode’s solution (37°C) followed by 4% paraformaldehyde (PFA, pH 7.4). Spinal cord segments L4-L6 and homonymous DRGs were harvested and post-fixed in 4% PFA overnight. The spinal cord segments were cut in serial 50 µm thick parasagittal sections on a vibratome (Leica Instruments, Germany), mounted onto gelatin-coated slides and coverslipped with DPX. The DRGs were cryoprotected in 20-30% sucrose for 2-3 days at 4°C, embedded in Tissue-Tek (O.C.T., Miles Inc., Elkhart, IN, USA), frozen at -80°C, cut in serial 40µm thick sections on a cryomicrotome (Leica Instruments), mounted on gelatin-coated slides and coverslipped with DPX(Novikova et al. 1997). For morphometric analyses and axon counts (Paper IV), 2 mm long nerve specimens were excised at 3-5 mm distance proximal and distal to the implantation site and from the middle of the nerve graft. The nerves were additionally fixed in 3% glutaraldehyde overnight, postfixed in 1% OsO4 in 0.1 M cacodylate buffer (pH=7.4), dehydrated in acetone, and embedded in Vestopal. Semithin transverse sections were cut on a 2128 Ultratome (LKB, Sweden) and counterstained with Toluidine Blue. For laser microdissection (Paper II), L4 and L5 DRG were rapidly harvested without perfusion and frozen in liquid nitrogen. Serial 10 µm cryosections of DRG were cut using Bright (UK) 5040 microtome and mounted onto RNase-free UV light-treated membrane slides: 1 mm polyethylene-tetraphthalate (PET) membrane, PALM (Zeiss, Germany). Slides were air-dried in the cryostat before being fixed in ice-cold methacarn (8 parts methanol, 1 part glacial acetic acid) for 10 minutes, dipped into ice-cold RNase-free PBS to remove excess OCT, and then in serial ethanol (70/96/100% for 2 minutes/2 minutes/3 minutes) respectively for tissue dehydration.
Neuronal counts
Nuclear profiles of labeled neurons were counted in all sections at x250 magnification in a Leitz Aristoplan fluorescence microscope using filter block A (Fast-Blue and Fluoro-Gold) and N2.1 (Fluoro-Ruby). The total number of nuclear profiles were not corrected for split nuclei, since there was a uniformity in nuclear size and since the nuclear diameters were small in comparison with the section thickness (Ma et al. 2001). Furthermore, in estimations of retrograde cell death the accuracy of this technique is similar to that obtained by using physical disector (Ma et al. 2001) or by counting neurons reconstructed from serial sections (Novikova et al. 1997). In Paper III, the cross-sectional soma area of the labeled neurons was measured with a Eutectic Neuron Tracing System (Raleigh, North Carolina) at x250 magnification. In Paper IV, myelinated axons in the proximal and distal nerve stumps, and in the middle of the nerve graft were counted at x1000 final magnification using Stereo Investigator™ 6 software (MicroBrightField, Inc., USA).

Laser microdissection
Laser capture microdissection was performed on PALM Microlaser Technologies microbeam microdissection system (Zeiss, Germany). Retrogradely labeled fluorescent neurons were visualized, marked under fluorescence illumination (FB - UV filter, 350 nm excitation; FR – Cy3 filter, 555 nm excitation) and cut. The PET membrane of the slide is cut simultaneously and provides a ‘back-bone’ to facilitate laser pressure catapulting - a precisely aimed but defocused high energy laser beam to catapult the area against gravity into the collecting plastic tube. Individual neurons were catapulted contact-free into a PALM AdhesiveCap with a total of 90-100 cells captured for each group. This procedure allows collection of material in a contact-free manner, minimizing the risk of contamination which is particularly important in sensitive downstream applications.

RNA isolation and quantitative RT-PCR
Laser captured samples were submerged in 350 µl lysis buffer (RLT buffer, QIAGEN, Germany) and 3.5 µl β-mercapotethanol (Sigma-Aldrich, UK) was added alongside 20 ng carrier RNA before homogenization. The samples were incubated upside down for 30 minutes, then vortexed thoroughly. RNeasy (Qiagen) Micro protocol for isolation of total RNA from microdissected cryosections was undertaken including the optional DNase step. The purified RNA was eluted in 14 µl RNase-free water. The RNeasy (Qiagen) Mini protocol was used for isolation of total RNA from whole L4 and L5 DRG in the HPRT validation group. In this group, total RNA was analysed on a NanoDrop™ 1000 spectrophotometer (Thermo Scientific, USA) for quality and quantity of product. Five micrograms of each sample was then used for qRT-PCR to ensure standardized starting total RNA
quantity. Unfortunately, the quantities of laser-microdissected sample RNA were < 2ng/µl and therefore could not be accurately quantified; however, the quality of RNA was satisfactory.

All total RNA samples were converted to cDNA using a First Strand cDNA Synthesis Kit (Superarray, U.S.A.). qRT-PCR was performed with a Rotor-Gene 6000 (Corbett Life Science, Australia) using SYBR™ Green fluorescence master-mix (Superarray, U.S.A.) and analysed using Rotor-Gene 6000 Series Software version 1.7.61 (Corbett Life Science, Australia). Primers were pre-designed by Superarray - housekeeping gene HPRT (Genbank Accession No. NM012583, Catalogue No. PPR44247E); genes of interest peripherin (Genbank Accession No. NM012633, Catalogue No. PPR45223A), neurofilament triplet proteins of high molecular weight, NF-H (Genbank Accession No. NM012607, Catalogue No. PPR42491A) and activating transcription factor 3, ATF3 (Genbank Accession No. NM012912, Catalogue No. PPR44403A). All reactions had been optimised to work under the same conditions – initial denaturation/HotStart DNA Polymerase activation: 95°C for 15 minutes; PCR cycles: 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds repeated for 40 cycles. One PCR run was performed for each named gene and contained a standard curve, generated from serial dilutions of cultured DRG neuron cDNA over 3 orders of magnitude; all experimental samples were assayed in duplicate. A negative control assay was always included where cDNA template was replaced with RNase-free water. From the standard curves described above, the C(t) values for the three genes of interest were used to calculate mRNA levels (arbitrary units) in each sample. For every animal, the expression level of each gene was normalised to that of HPRT (Reid et al. 2009). Confirmation of the amplified products was established by performing a melting curve analysis: 95°C for 1 minute, 65°C for 2 minutes, then 65 - 95°C, reading every 0.2°C, holding for 1 sec between reads.

**Image processing**
Preparations were photographed with a Nikon DXM1200 digital camera attached to a Leitz Aristoplan microscope. The captured images were resized, grouped into a single canvas and labeled using Adobe Photoshop CS3 software. The contrast and brightness were adjusted to provide optimal clarity.

**Statistical analysis**
In Papers I, III and IV, one-way analysis of variance (ANOVA) followed by a post hoc Newman-Keuls test or Tukey test (Prism®, GraphPad Software, Inc; San Diego, California) were used to determine statistical differences between experimental groups. In Paper II, ANOVA followed by Bonferroni’s Multiple Comparison test
and unpaired student $t$-test were used to determine differences in gene expression. All data was expressed as mean ± S.E.M. A value of $p < 0.05$ was considered to be statistically significant.
RESULTS

Peripheral nerve injury induces delayed loss of cutaneous sensory neurons
In control rats, 3,340 (± 176 S.E.M.) cutaneous sural DRG neurons and 216 (± 8 S.E.M.) muscular gastrocnemius DRG neurons were labeled by Fast Blue (FB) at 1 week after application of the dye to the homonymous nerves (Table 1, Fig. 1, Paper I and Tables 1 and 2, Fig. 1, Paper IV). Counts of Fluoro-Gold-labeled neuronal profiles revealed no significant loss of sural or gastrocnemius DRG neurons at 3 days, 1 week and 2 weeks after axotomy (Table 1, Paper I). Therefore, the numbers of FB-labeled sural and gastrocnemius neurons at 1 week after tracer application were used as baseline controls in the subsequent experiments (“Control” in Tables 1-5, Paper I). Counts of FB-labeled sural DRG neurons after axotomy demonstrated 22% cell loss after 4 weeks (P<0.01, Table 1, Paper I) and 45% loss at 8-24 weeks post-operatively (P<0.001; Table 1, Paper I). In contrast, no significant retrograde cell loss was found among the gastrocnemius DRG neurons up to 24 weeks after nerve transection (P>0.05; compare Table 1 and Table 2, Paper I), nor did it affect survival of the medial gastrocnemius DRG neurons (Table 3, Paper I). There was no loss of spinal motoneurons after sciatic axotomy (Tables 4, 5, Paper I and Tables 1, 2, Paper IV).

Spinal motoneurons degenerate after ventral rhizotomy and avulsion
In control rats, 187 (± 10 SEM) medial gastrocnemius motoneurons were labeled by True Blue (TB) at 1 week after application of the dye to the transected peripheral nerve (Table 1, Paper III). Tracing with Fluoro-Gold (FG) labeled 172 (± 5 SEM) spinal motoneurons. There was no difference in efficacy of these retrograde tracers (P>0.05). Both ventral rhizotomy and ventral root avulsion induced significant retrograde degeneration among the axonally injured motoneurons. Ventral rhizotomy resulted in the death of 26% of medial gastrocnemius motorneurons within 8 weeks (Figure 1B, Paper III) and a 21% reduction in soma area (rhizotomy + no treatment, Table 1, Paper III). The more severe avulsion injury resulted in the death of 53% spinal motoneurons and a 31% reduction in soma area within 4 weeks (avulsion + no treatment, Table 2, Paper III).

Nerve repair improves survival of axotomized sensory neurons
Neither primary repair (p<0.05) nor peripheral nerve grafting (p<0.01) prevented neuronal degeneration among the sural DRG neurons (Table 1, Paper I). However, primary nerve repair increased survival rate of the sural DRG cells by 30% (P<0.001; Table 2, Paper I), whereas nerve grafting increased their survival by 19%
(P<0.01; Table 2, Paper I; Table 1, Paper IV). However, there was no statistical difference between the two nerve repair techniques (P>0.05; Table 2, Paper I). The numbers of medial gastrocnemius DRG neurons and sural and gastrocnemius motoneurons were unaffected by nerve repair (Tables 3-5, Paper I; Table 2, Paper IV).

**Regeneration of sensory neurons and motoneurons after nerve repair**

Neuronal regeneration across the nerve repair site was quantified using second labeling with Fluoro-Ruby (FR). At 13 weeks after sciatic axotomy and nerve grafting, 32% of the sural DRG neurons had succeeded in regenerating their axons 10 mm into the distal stump of the sciatic nerve (Table 2; Fig. 2, Paper I; Table 1; Fig 1, Paper IV). Primary repair was more efficient and promoted axonal regeneration of 54% of the sural DRG cells (P<0.001; Table 2, Paper I). This difference between the two nerve repair methods was not seen among the gastrocnemius DRG cells, where about 50% of the neurons regenerated across the lesion site after both primary repair and nerve grafting (Table 3, Paper I). When calculating the ratio between the number of regenerating (FB+FR)-labeled DRG cells and the number of FB-labeled DRG neurons remaining at 13 weeks after axotomy and nerve repair, we found that the proportion of regenerating neurons (FR+FB/FB ratio in Table 2, Paper I and FR/FB ratio in Table 1, Paper IV) after primary nerve repair and nerve grafting was similar between the sural and gastrocnemius DRG populations. With respect to regeneration of the sural and gastrocnemius motoneurons, 53-59% of these cells became labeled by FR from the distal sciatic nerve stump (Tables 4, 5; Fig. 2, Paper I and Table 2, Paper IV).

**Regenerating sensory neurons up-regulate peripherin mRNA expression**

Peripherin mRNA expression in sensory DRG neurons with regenerating axons (FB+FR double-labeled neurons) was significantly higher than in control non-injured sural and gastrocnemius neurons. Axotomized sural and gastrocnemius sensory neurons that failed to produce axonal outgrowth (FB-labeled cells) did not significantly upregulate peripherin and transcript levels were similar to that seen in non-injured neurons (Fig. 2, Paper II). The relative value of peripherin mRNA was significantly higher in regenerating sural sensory neurons than in regenerating gastrocnemius neurons (P<0.05). However, the sevenfold increase in transcripts of gastrocnemius neurons is a proportionally greater response to injury than the fourfold increase in sural neurons.

**Sensory neurons down-regulate expression of NF-H mRNA after injury**

The relative expression of NF-H in control non-injured neurons was significantly higher in gastrocnemius subpopulation of sensory neurons (Fig. 3, Paper II). Injured
sural and gastrocnemius sensory neurons with established axonal regeneration (FB+FR cells) significantly down-regulated NF-H transcripts, whereas injured non-regenerating sensory neurons (FB cells) reduced transcripts levels even further. There were no significant differences between the regenerating and non-regenerating groups in neither sural nor gastrocnemius neuronal subpopulations.

**Regenerating sensory neurons up-regulate ATF3 mRNA expression**

There was a significant up-regulation of ATF3 in regenerating sural and gastrocnemius sensory neurons (Fig. 4; Paper II). There was no significant difference between the control and non-regenerating groups in the MG neuronal subpopulation. These results highlight a difference in ATF3 expression between neurons with proven regeneration and neurons that had failed to regenerate in both subpopulations of sensory neurons.

**N-acetyl-cysteine promotes survival of spinal motoneurons**

Following *ventral rhizotomy*, intraperitoneal N-acetyl-cysteine (NAC) maintained soma area, and exhibited dose-dependent neuroprotection (Table 1, Paper III). The effect being statistically significant at the higher dose tested (750mg/kg/day, 7% neuron death, P<0.05). Intrathecal NAC was slightly more neuroprotective (5% neuron death, P<0.05; Figure 1C), and also preserved soma area (P<0.001).

When compared to untreated controls intraperitoneal NAC (150mg/kg/day) was also significantly neuroprotective (40% neuron death, P<0.01) after *ventral root avulsion* (Table 2, Paper III), and had some effect to maintain soma area (81% of normal). Intrathecal delivery was even more effective, reducing neuron death to only 30% (P<0.001), and significantly maintaining soma area (92% of normal, P<0.001). Even when intrathecal NAC treatment was delayed by two weeks after avulsion (Table 2, Paper III), it still conferred a highly significant degree of neuroprotection (42% neuron death vs. 56% death with sham treatment, P<0.001). The protective effect was significantly greater (P<0.05 vs. 2-week delay) when treatment was delayed for only one week and interestingly there was no loss of neuroprotection as compared to immediate treatment.

No neurotoxic effect of intrathecal or intraperitoneal treatment with NAC was demonstrated (Tables 1 and 2, Paper III). Similarly, the mean soma area of spinal motoneurons neurons was unaffected by either route of treatment (Tables 1 and 2, Paper III). No adverse effects attributable to treatment or minipump implantation were encountered during this study.
N-acetyl-cysteine promotes survival of cutaneous sensory DRG neurons
Eight weeks of continuous intrathecal treatment with NAC resulted in 78% survival of the sural sensory DRG neurons (P<0.01; Table 1; Fig. 1A-C, Paper IV). However, when nerve grafting was combined with intrathecal infusion of NAC for 8 weeks, survival of DRG neurons was increased by 38% (p<0.01; Table 1; Fig. 1B,D,G, Paper IV) and was not different from the baseline control at 1 weeks after FB labeling (p>0.05, Table 1, Paper IV). Therefore, nerve grafting and intrathecal NAC infusion have highly significant additive effects on survival of sensory DRG neurons after peripheral nerve injury. Survival of sural motoneurons was not affected by nerve grafting and NAC treatment (p>0.05; Table 2, Paper IV).

Effects of N-acetyl-cysteine and nerve grafting on neuronal regeneration
When nerve grafting was combined with intrathecal NAC treatment, the proportion of regenerating neurons was statistically increased to 42% (p<0.05; Table 1; Fig. 1E, H; Paper IV). Thus, improved survival of the sural sensory neurons resulted in better outcome of neuronal regeneration across the nerve graft. However, when calculating the ratio of regenerating FR-labeled and remaining FB-labeled sural sensory neurons at 13 weeks postoperatively, we found that the proportions of regenerating neurons were similar after nerve grafting alone (46.3%; Table 1, Paper IV) and nerve grafting followed by intrathecal NAC treatment (45.6%; Table 1, Paper IV). NAC treatment had no growth-promoting effect on axotomized spinal motoneurons (p>0.05; Table 2, Paper IV).

Effect of N-acetyl-cysteine and nerve grafting on axonal sprouting
All examined specimens from the sciatic nerve and graft contained numerous myelinated fibers. However, there were marked differences in appearance and axon number between the experimental groups as well as between specimens taken from the nerve stumps and grafts (Figure 2; Table 3, Paper IV). Proximal nerve stumps contained 11,041 ± 302 (mean ± S.E.M.) and 11,092 ± 543 myelinated fibers in the groups with nerve graft and nerve graft with NAC treatment, respectively. Although the number of myelinated axons was significantly increased in the middle of the graft by 54.2% (p<0.001) and 47.1% (p<0.001) in untreated and NAC-treated animals, respectively, there was no statistical difference between the experiments (p>0.05). The numbers of myelinated fibers in the distal nerve stump were significantly decreased (p<0.001) and were comparable with the counts in the proximal stump of the nerve (105-109%; Table 3, Paper IV). The axons in the graft and in the distal stump also appeared smaller in diameter and had thinner myelin sheath. Thus, NAC treatment had no effect on sprouting of myelinated axons in the graft and distal nerve stump.
DISCUSSION

Fluorescent retrograde tracers for neuronal quantification
It has been suggested that an “assumption-free” stereological method is one of the most accurate techniques for neuronal quantification (Coggeshall and Lekan 1996; West 1999). However, there are also opposing views indicating that 3-D studies could have potentially huge biases (Guillery and Herrup 1997; von Bartheld 2001; Guillery 2002). For example, using “unbiased” counts of DRG neurons after sciatic nerve transection highly significant variability in the timing and amount of cell loss has been reported (Tandrup 1993; Tandrup et al. 2000; Hart et al. 2002a). In previous investigations in our laboratory, physical disector has been compared with both long-term retrograde Fast Blue-labeling (Ma et al. 2001) and serial reconstructions of labeled profiles (Novikova et al. 1997) and we have found that Fast Blue-labeling provides accurate estimates of retrograde cell death. The existence of different cell populations in DRG (Papers I, II and IV) should also be taken into account when designing experiments to study retrograde degeneration after peripheral axotomy. If only certain subpopulations of sensory neurons degenerate after axotomy, then quantification of total number of DRG neurons even with modern stereological methods (Coggeshall and Lekan 1996; West 1999) will underestimate the degree of retrograde cell death. In addition, only about 50% of all neurons in the L4 and L5 DRGs project their axons to the sciatic nerve (Swett et al. 1991).

Moreover, in the present thesis, regeneration could be examined only by using a double labeling technique. For this purpose, Fast Blue (FB) was used as a long-term marker to assess neuronal survival (Novikova et al. 1997), while Fluoro-Ruby (FR) was used to evaluate axonal regeneration across the injury site (Novikova et al. 1997; Ma et al. 2003). Since Fast Blue requires about one week to provide effective retrograde labeling of neurons, the more rapidly transported tracer Fluoro-Gold (FG) was used to study possible retrograde cell degeneration during the first couple of days after peripheral nerve injury (Novikova et al. 1997). To study gene expression in DRG neurons (Paper II), Fast Blue was also injected into the medial gastrocnemius muscle and sural nerve. Although this technique labels only limited number of sensory and motor neurons, the main goal was to minimize axon reaction in control neurons.

Mechanism of retrograde cell death after peripheral nerve injury
Neurons may die by either passive necrosis, or an active metabolic process akin to apoptosis whose intracellular events show a high degree of homology irrespective of the initiating event (Koliatsos and Ratan 1999). In traumatic injury necrosis is more
associated with direct trauma to the cell body, and occurs too rapidly to be amenable to therapeutic intervention, but active cell death tends to be a more progressive secondary phenomenon, giving a potential therapeutic window. Apoptosis is a complex process involving a variety of different signaling pathways and after peripheral axotomy it could be initiated by interrupted retrograde transport of target-derived neurotrophins, GDNF family members and neuropoietic cytokines signaling through the Ras-ERK pathway, the PI3-K pathway and the JAK-STAT pathway (Kaplan and Miller 2000; Cui 2006; Abe and Cavalli 2008). Initially, axotomy induces transcription of regenerative as well as cell death genes (Nunez and del Peso 1998), the neuron’s fate lying in the ultimate balance of these gene products (Gillardon et al. 1996). How the neuron finally commits to cell death rather than regeneration is not fully understood, but has been associated with abortive entry into the cell cycle (Freeman 1999), and mitochondria are thought to play a key role (Al Abdulla and Martin 1998; Budd and Nicholls 1998). It is likely that the normal electron transport mechanisms are damaged by excess reactive oxygen species (ROS), possibly in response to unphysiological elevation of intramitochondrial nitric oxide (Heales et al. 1999; Elliott and Snider 1999). As a result oxidative metabolism is impaired, mitochondrial homeostasis fails, and they release pro-apoptotic molecules (Budd and Nicholls 1998) that trigger a caspase cascade culminating in active cell death.

**Response of spinal motoneurons to distal and proximal axotomy**

Several factors including the age of the experimental animals, the distance from the lesion site to the cell body and the type of the injury can determine the severity of the retrograde axon reaction. Injury to the sciatic nerve in newborn rodents almost completely eliminates the corresponding spinal motoneurons (Snider et al. 1992; Vejsada et al. 1995; Li et al. 1998), while the same type of injury in adult animals has no effect on motoneuron survival (Carlson et al. 1979; Vanden Noven et al. 1993; Novikova et al. 1997) although it induces cell atrophy, synaptic shedding and degeneration of dendrites (Kreutzberg GW 1995; Carlstedt and Cullheim 2000). The present results are in line with these observations and show that neither sural nor gastrocnemius motoneurons degenerate after a division of the distal nerve branch or permanent sciatic axotomy. However, proximal injuries do induce retrograde motoneuronal death, such that 20-30% die within 16 weeks after spinal nerve transection (Ma et al. 2001; Ma et al. 2003; Jivan et al. 2006) and more than 50% die six weeks after C7 ventral rhizotomy (Gu et al. 1997), and after ventral root avulsion (Wu and Li 1993; Koliatsos et al. 1994; Novikov et al. 1995; Elliott and Snider 1999). The cell bodies of motoneurons become atrophic as a reaction to various types of axonal injury, and this change correlate with the severity of trauma. After proximal axotomy, an early hypertrophy (12% after 4 weeks) is followed by atrophy (20% at 16 weeks)(Ma et al. 2001) while after ventral root avulsion,
significant 50% cell atrophy occurs at 4 weeks postoperatively (Novikov et al. 2000). The present results (Paper III) are consistent with these findings.

**Sensory neurons after peripheral nerve injury**

In contrast to motoneurons, adult DRG neurons undergo significant retrograde cell death also after distal peripheral nerve injury. Recent studies have shown that permanent sciatic transection mainly induces degeneration of small diameter DRG neurons projecting to skin and expressing neurotrophin receptors trkA and trkC as well as GDNF receptors (McMahon et al. 1994; Bennett et al. 1998; Karchewski et al. 1999; Tandrup et al. 2000; Hu and McLachlan 2003). Studies with fluorescent tracers have shown that permanent sciatic axotomy induces significant but delayed retrograde cell death of sural sensory neurons projecting mainly to skin and does not affect survival of medial gastrocnemius sensory neurons projecting to muscle (Hu and McLachlan 2003). The loss of cutaneous afferent neurons is likely to contribute to the poor sensory recovery observed clinically after nerve lesions, since neuronal survival is essential for regeneration (Fu and Gordon 1997), and since the quality of sensation depends upon the number of primary neurons and the size and degree of overlap of their receptive fields (Lundborg 2000b). It is not known why cutaneous afferent neurons are more vulnerable than muscular afferent neurons, since peripheral nerve injury up-regulates a variety of neurotrophic factors both in the proximal and distal nerve stumps (Gillen et al. 1997; Terenghi 1999; Boyd and Gordon 2003). One explanation could be a mismatch between the neurotrophic factors expressed after injury and the receptors present in the axotomized sensory neurons. It is also possible that the amounts of retrogradely transported neurotrophic factors (Curtis et al. 1998) are insufficient for activating the intracellular pathways responsible for survival of cutaneous DRG neurons. When peripheral axotomy is treated with exogenous neurotrophins, almost complete survival of sensory DRG neurons can be achieved (Ljungberg et al. 1999; Kuo et al. 2005).

Our findings are in agreement with previous observations that DRG neurons undergo delayed degeneration after peripheral nerve injury (Tandrup et al. 2000; Jivan et al. 2006), although there are conflicting reports about the time course of degeneration (Tandrup et al. 2000; Hart et al. 2002a). Although the mechanism for delayed neuronal degeneration remains unclear, this phenomenon has been described in cervical motoneurons after brachial plexus injury (Ma et al. 2001; Jivan et al. 2006), in the red nucleus after cervical spinal cord injury (Houle and Ye 1999; Novikova et al. 2000b) and in the cerebral cortex after traumatic brain injury (Smith et al. 1997). One possible explanation for the delayed onset of retrograde reaction could be that during the first 3-4 weeks after injury, neurons convert from a “transmitting mode” to a “growth mode”, which results in axonal sprouting and establishment of transient synaptic contacts. It has been shown that peripheral axotomy induces formation of supernumerary intramedullary axons in spinal motoneurons (Havton and Kellerth 1987) as well as collateral sprouting of
myelinated axons in the dorsal horn (Bennett et al. 1996) and dorsal roots (Lekan et al. 1997). Other possibilities include retrograde transport of neurotrophic factors from spinal motoneurons to primary afferents via mechanism of transsynaptic transcytosis (Rind et al. 2005). However, the latter assumption does not agree with observations that combined transection of peripheral nerve and dorsal roots induces a similar retrograde cell loss as peripheral nerve injury alone (Coggeshall and Lekan 1996).

**Neuroprotective effect of nerve grafting**

After peripheral nerve injury, Schwann cells in the distal nerve stump divide and up-regulate expression of various neurotrophic factors including NGF, BDNF, NT-4/5, GDNF and LIF (Boyd and Gordon 2003). Although it has been demonstrated that Schwann cell proliferation in the distal nerve stump is not required for functional recovery after nerve injury (Yang et al. 2008), it is well known that expression of neurotrophic molecules by Schwann cells supports both neuronal survival and axonal regeneration (Terenghi 1999; Boyd and Gordon 2003). The latter findings has lead to numerous experimental and clinical studies using peripheral nerve grafts to prevent retrograde cells death and to direct axonal growth toward peripheral targets (Rhrich-Haddout et al. 2001; Ma et al. 2003; Bertelli and Ghizoni 2003; Wu et al. 2004). In previous investigations we have described neurotrophic and neurotropic effects of primary nerve repair and peripheral nerve grafting on the survival and axonal regeneration of cervical DRG neurons and spinal motoneurons (Ma et al. 2003; Jivan et al. 2006). In the latter experiments delayed nerve grafting promoted motoneuron regeneration more efficiently than regeneration of DRG cells. In the present study, however, the proportion of regenerating neurons was rather similar between cutaneous afferent cells, muscular afferent cells and spinal motoneurons. The reason for improved regeneration is unclear but could be influenced by the nature of the graft and the timing of nerve repair. Today there is no real substitution for nerve grafting in patients after peripheral nerve injuries with long nerve gaps. However, despite the advantages in microsurgical reconstruction of injured peripheral nerves, the technique of nerve grafting is not optimal and patients suffer from loss of sensation, scarring and sometimes pain in the donor region (Wiberg and Terenghi 2003). Moreover, neuroprotection provided by peripheral nerve graft for sensory DRG neurons in the present study seems to be inferior to the reported neuroprotective efficacy of systemically administered exogenous neurotrophic factors (Matheson et al. 1997; Ljungberg et al. 1999; Groves et al. 1999).

**Regulation of peripherin and ATF3 genes in primary sensory neurons**

Peripheral nerve injury induces a coordinated molecular response which determines the intrinsic growth state of axotomized neurons through regulation of numerous transcription factors, growth factors, adhesion molecules and structural components
Neuroprotection and axonal regeneration after peripheral nerve injury

(Navarro et al. 2007; Raivich and Makwana 2007). It has been shown that transcription is necessary for successful axon growth (Smith and Skene 1997) and changes observed have been likened to a recapitulation of the developmental phenotype (Costigan et al. 2002; Xiao et al. 2002). In our second study (Paper II), we demonstrated that, when regenerating across the lesion site, cutaneous sural DRG neurons and muscular medial gastrocnemius DRG neurons up-regulated gene expression of peripherin and ATF3.

In the peripheral nervous system, peripherin is expressed mainly in small DRG neurons with unmyelinated axons (Fornaro et al. 2008) and is necessary for the proper development of non-peptidergic small unmyelinated sensory cells (Lariviere et al. 2002). In the present study, we found that peripherin mRNA was expressed in greater quantities in the sural sensory neurons, which are predominantly small neurons with unmyelinated axons, than in the medial gastrocnemius sensory neurons, which are mainly large cells with myelinated axons. Our results suggested that following nerve injury both subpopulations of sensory neurons significantly up-regulate peripherin gene expression, and this is contrary to a previous report demonstrating peripherin transcript up-regulation mainly in large DRG neurons (Oblinger et al. 1989). This obvious discrepancy in expression pattern could be due to differing methodologies since in the previous report authors used axotomy of the sciatic nerve and in situ hybridization with a labeled cDNA probe to quantify mRNA in small neurons throughout L5, many of which may not have been axotomized. In contrast, the present study was based on laser microdissected cells obtained from an identified subpopulation of sensory DRG neurons. It is also possible that the response in the sural subpopulation was potentiated by the conditioning injury 8 weeks prior. However, when compared to corresponding control neurons, the sevenfold increase in medial gastrocnemius sensory cells was greater than the fourfold increase observed in sural neurons. It has been shown previously that peripherin is important for the initiation, extension and maintenance of neurites in PC12 cells (Helfand et al. 2003). Although the role of peripherin is unknown, it is clearly a highly dynamic structure that interacts with the other neural intermediate filaments during development and regeneration (Helfand et al. 2004). The exact mechanism of action of peripherin also remains elusive, although it may be a substrate for Akt in potentiating regeneration and phosphorylation is an important post-transcriptional control (Konishi et al. 2007). Our results demonstrating low peripherin transcript levels in the neurons that failed to regenerate may be explained by the previous reports that peripherin expression is maintained in an up-regulated state for about 8 weeks in axotomized DRG neurons (Wong and Oblinger 1990). Since our experiments took 9 weeks, it is possible that we have missed an early response to injury which has subsequently returned to control levels. Alternatively, the lack of regenerating axons in this population may be a result of failure to up-regulate peripherin in the initial regenerative response.
Our findings regarding the regulation of NF-H mRNA after nerve injury in DRG subpopulations are in accordance with previous reports examining whole DRG after sciatic nerve axotomy which have demonstrated an immediate down-regulation in expression of this neuronal intermediate filament (Wong and Oblinger 1990; Muma et al. 1990; Fornaro et al. 2008).

The transcription factor ATF3 is rapidly induced in all axotomized sensory DRG neurons and immunohistochemistry expression persists for many months if regeneration is prevented (Tsujino et al. 2000; Kataoka et al. 2007). It has been speculated that ATF3 may promote neuronal survival (Tsujino et al. 2000; Nakagomi et al. 2003). Recent studies utilizing over-expression of ATF3 have demonstrated that ATF3 can promote neurite outgrowth both in vitro and in vivo (Seijffers et al. 2006; Seijffers et al. 2007). Moreover, it has been found that ATF3 expression following injury is differentially regulated in sensory and motor neurons (Kataoka et al. 2007) and in neurochemically defined sensory subpopulations (Averill et al. 2004). However, our results demonstrated that gene expression is much reduced in sensory neurons that failed to regenerate compared to neurons with proven regeneration. Neurons with regenerating axons profoundly up-regulated ATF3 transcripts at 1 week after injury, and it might have been expected that neurons failing to produce axonal outgrowth would maintain a high ATF3 transcript levels 9 weeks after injury. The dramatic difference in expression may suggest that this neuronal population is doomed to regenerative failure because of inadequate ATF3 levels of expression; however, it is also possible that ATF3 expression in non-regenerating neurons decreases more rapidly, after initial induction, as a survival response (Kataoka et al. 2007). The exact mechanisms are not entirely understood but it is believed that ATF3 is involved in determining the cell’s fate after injury (Hua et al. 2006).

**N-acetyl-cysteine supports neuronal survival**

Following an injury to the nervous system, mitochondrial impairment could lead to generation of reactive oxygen species and activation of apoptotic cascades (Merenda and Bullock 2006). With respect to axon reaction, it has been reported that degeneration of spinal motoneurons after ventral root avulsion could evolve with oxidative stress (Martin et al. 1999) and often resemble necrosis (Li et al. 1998). In vitro experiments also show that an increase of mitochondrial-derived reactive oxygen species occurs in NGF-deprived sympathetic neurons undergoing apoptotic death (Kirkland and Franklin 2003).

Since mitochondria and the generation of reactive oxygen species are thought to be important in the mechanism behind cell death, agents that can stabilize oxidative
metabolism, or enhance mitochondrial protection from reactive oxygen species could be neuroprotective. Glutathione is the principle species responsible for this within neurons, and its depletion, particularly within mitochondria, does increase susceptibility to toxic stimuli including neurotrophin withdrawal (Cooper and Kristal 1997; Wullner et al. 1999). Based on these findings, it has been proposed that glutathione replacement could be neuroprotective (Cooper and Kristal 1997). However, this agent cannot cross the blood-brain barrier and its principle rate-limiting precursor cysteine is unstable in the CSF (Pan and Perez-Polo 1996; Wang and Cynader 2000). In contrast, L-stereoisomer of N-acetyl-cysteine (NAC) can cross the blood-brain barrier, accumulate in the brain and increase intracellular levels of glutathione (Arakawa and Ito 2007). It has been reported previously that intraperitoneal treatment with NAC (150mg/kg/day) provides significant rescue of sensory DRG neurons via mitochondrial preservation (Hart et al. 2004). The protection of sensory neurons by NAC is dose dependent and effective over a wide therapeutic range (West et al. 2007b).

N-acetyl-cysteine’s mechanism of action remains unclear, and further investigation will be required, although putative mechanisms have been proposed (Yan and Greene 1998; Hart et al. 2004). One possible mechanism is as a glutathione substrate, thereby acting to maintain intramitochondrial defences against reactive oxygen species. Other possibilities include a direct reductant effect that may either equilibrate the increased oxidative stress within the injured neuron, or modulate signal transduction, including that for neurotrophic pathways (Xu et al. 2002; Droge 2002). Such an effect could alter the cells interpretation of reduced neurotrophic support, or modulate the response in favor of survival and regeneration, and away from cell death. This latter effect may explain the action of the D-stereoisomer of NAC, which cannot be metabolised into glutathione, yet still confers a comparable degree of neuroprotection to the L-stereoisomer in certain in vitro models (Ferrari et al. 1995).

Our study on motoneurons (Paper III) demonstrates that immediate systemic treatment with NAC is neuroprotective for adult motorneurons after ventral rhizotomy, and that a dose response effect is evident in that survival rose to 93% when 750mg/kg/day was given. Higher doses would have required the i.p. injection of an excessive volume and were not tested. Intrathecal (i.t.) administration was used to test the efficacy of higher tissue levels of NAC, but proved comparable to the 750mg/kg i.p. dose, suggesting that this dose is supra-maximal, as probably is the 2.4mg/day intrathecal dose. Although systemic treatment (NAC 150mg/kg/day i.p.) was less neuroprotective than intrathecal, it still provided a highly significant degree of neuroprotection, and given the dose response evident in the rhizotomy model it is likely that a higher dose could be comparably effective. Thus, it is apparent that NAC can reduce, or possibly eliminate, active motoneuronal death.
after proximal axotomy in adults, assuming sufficient tissue levels are attained. The clinical experience of NAC during the management of paracetamol poisoning is that it is safe and easily administered systemically even at high doses, making it clinically applicable even for nerve injuries.

However, for NAC to be an applicable therapy for severe nerve injuries, such as the brachial plexus injury, it must also be effective if treatment is not commenced immediately after injury, since that will never be clinically feasible. For sensory neurons neuroprotective therapy can be delayed for 24 hours after distal axotomy without any loss of effect, and we found no loss of neuroprotective effect when NAC treatment was delayed until one week after ventral root avulsion (Table 3, Paper II), presumably reflecting the slower time-course of active motoneuronal death. Some loss was found with a two-week delay of therapy, but that will still leave a highly clinically appropriate therapeutic window for NAC therapy.

Our study on sensory neurons (Paper IV) demonstrates that both nerve grafting and continuous intrathecal infusion of NAC could attenuate retrograde cell death of sural DRG neurons after sciatic nerve injury. However, combined treatment with NAC and nerve grafting significantly improves survival rate and increases the number of regenerating sural sensory neurons. However, the results also indicate that NAC has no growth-promoting effect on spinal motoneurons and does not affect axonal sprouting in the peripheral nerve graft and in the distal nerve stump.

In this investigation we administered NAC via continuous infusion (2.4 mg/day) into subarachnoid space from osmotic mini-pump since this delivery route and concentration have been more efficient in rescuing spinal motoneurons than intraperitoneal NAC injections (Zhang et al. 2005). In contrast to our previous studies, however, we obtained somewhat lower efficacy of neuroprotection (22% versus 32%). One possible explanation is that we used different experimental models of retrograde degeneration, administration routes for NAC and techniques to quantify the extent of retrograde cell death.

The main finding of this study is that NAC and nerve grafting displayed additive neuroprotective effect which resulted in nearly 90% survival of axotomized sural sensory neurons and improved regeneration into the distal nerve stump. Although the mechanism of this effect is unknown, it has been shown that NAC could work in synergy with cytokine CNTF to prevent killing of oligodendrocytes by TNF-alpha (Noble and Mayer-Proschel 1996). Several research groups have also reported that NAC not only works as an antioxidant, but also causes rapid activation of the Ras-ERK signaling pathway and induces immediate early genes in trophic factor-deprived PC12 cells and sympathetic neurons (Yan and Greene 1998), and in amyloid-beta peptide-stressed neuronal cell cultures (Kuperstein and Yavin 2002;
Hsiao et al. 2008). Moreover, NAC can suppress p38 kinase activation, caspase-9 and caspase-3 cleavage and block apoptosis in vitro (Floyd 1999; Junn and Mouradian 2001). Activation of the ERK signaling pathway and suppression of the caspases cleavage by NAC is similar to the effects exerted by various neurotrophic factors on sensory neurons and spinal motoneurons in vitro and after peripheral nerve injury in vivo (Liu and Snider 2001; Boyd and Gordon 2003). It has also been demonstrated that inhibition of ERK and protein kinase A at the time of injury can significantly impair the capacity of injured dorsal root ganglia axons to re-initiate growth cones (Liu and Snider 2001; Chierzi et al. 2005). These observations demonstrate that NAC could add to neurotrophic factors effects to maintain neuronal survival through the Ras-ERK signaling pathway.

However, NAC does not completely mimic the effects of neurotrophic factors and it has been shown that unlike the neurotrophin NGF, NAC does not activate the PI3-K pathway and has no effect on neurite outgrowth in vitro (Yan and Greene 1998). The latter findings could explain the lack of growth-promoting effect on spinal motoneurons and the inability to influence axonal sprouting in the graft and in the distal nerve stump. Nevertheless, NAC has been shown to attenuate Wallerian degeneration and hyperalgesia after peripheral nerve injury (Wagner et al. 1998) and to inhibit functional and structural abnormalities of the peripheral nerve in streptozotocin-induced diabetic rats (Sagara et al. 1996).
CLINICAL IMPLICATIONS

In current medical practice, the focus on treatment of nerve injuries has been on the technical aspects of the repair, and much has been written about different techniques of nerve suturing, but no clear advantage has been shown, for example, between epineural and fascicular suturing. The goal of nerve repair is still to align the cut ends as exactly as possible with minimal manipulation. The general idea is that an end-to-end suture is preferable as long as it can be made without tension. In a fresh nerve injury the nerve ends elasticity remains, often making it possible to perform an end-to-end suture as long as no nerve tissue is lost.

The most severe type of nerve injury is the traction injury where an end-to-end repair is not possible. In those cases, for example brachial plexus injuries, the use of nerve grafts to bridge the gap are mandatory even in the situation of acute nerve repair. In the clinical situation, peripheral nerve surgery is often delayed due to late referrals to specialized units. Surgery can also be further delayed by additional injuries or due to difficulties making a correct diagnosis. For example, in closed brachial plexus injuries it can be difficult to distinguish between neurapraxia, axonotmesis and neurotmesis.

Nerve grafts are used when the cut nerve ends cannot be opposed without undue tension, whether that is a result from reduced elasticity due to scaring or actual loss of a segment of nerve tissue. The most commonly used donor nerve is the sural nerve, but other cutaneous nerves can be considered. The harvested nerve is inserted into the defect as cable graft to connect the different fascicles. One of the main difficulties is to find and match the fascicles in the proximal and distal nerve ends, which is specifically difficult after scar and neuroma formation.

The general results of nerve repair are poor but significant improvement in outcome has been seen if the repair has been performed early after injury (Jivan et al. 2008). This improvement may be a result of reduced technical difficulties when nerves are repaired acutely but is more likely a result from a biological point of view as a nerve injury initiates a complex pattern of events of anterograde and retrograde degeneration affecting neuronal cell bodies, axons and myelin sheath. Neurons in the dorsal root ganglion and in the spinal cord can undergo cell death and atrophy and the process can only be reduced by reconnecting the injured neurons with their peripheral target organs.

This thesis has focused on the degenerative changes that occur proximal to the lesion site after nerve injury. Since the survival of neurons projecting into the peripheral nerve is a prerequisite for regeneration and target organ reinnervation, understanding of this process is of importance to improve the results after nerve
injuries. In the thesis we investigated the survival response of spinal motoneurons and sensory DRG neurons to different types of nerve injuries and the ability for these neurons to regenerate their axons after nerve reconstruction.

One of the main and realistic goals of brachial plexus surgery today is to promote functional motor reinnervation of the shoulder and the arm. Sensory recovery after these injuries is very poor which could be due to the high vulnerability of cutaneous sensory DRG neurons to axonal injury. Thus, prevention of retrograde cell death in sensory ganglia could therefore possibly improve further clinical results after nerve reconstructions.

In this study we demonstrated experimentally that NAC is an effective neuroprotective agent which can reduce post-traumatic cell loss in both DRG and spinal cord. Furthermore, we showed that by reducing the neuronal loss we can achieve a higher number of regenerating cutaneous sensory neurons. Although neuroprotective therapy is not used in today’s clinical practice in peripheral nerve surgery, it has a great potential as neuroprotective treatment with NAC could be initiated at the first suspicion of a nerve injury and before referral of the patient to a specialized unit, thereby reducing the negative effect of a delayed nerve repair.

In the present study we used different routes of drug administration including inthrathecal NAC infusions. However, in a clinical setting, treatment with either intravenous or oral administration resembling the treatment for paracetamol intoxication seems to be more realistic. The neuroprotective dose in humans has not yet been established, but in rats a dose of 30mg/kg/day has been shown to provide complete protection of sensory neurons in the DRG (Hart et al. 2004). The dose given for paracetamol intoxication in humans is approximately 200mg/kg/day. The duration of neuroprotective treatment in humans is not known, however, it seems reasonable to consider treatment during the period of the most extensive neuronal death or at least until nerve repair has been performed.

Future evaluation of possible neuroprotective effects of NAC in humans will also need non biased techniques to evaluate neuronal survival in the DRG and spinal cord. One promising strategy could be based on recent MRI studies (West et al. 2007a) demonstrating the possibility, by the use of a non-invasive technique, to quantify retrograde neuronal death following nerve injury.
CONCLUSIONS

The present thesis investigates the efficacy of N-acetyl-cysteine treatment, primary nerve repair and nerve grafting on the survival and regeneration of sensory DRG neurons and spinal motoneurons after peripheral nerve injury in adult rats.

On the basis of the experimental data, the following conclusions can be made:

• Permanent transection of the peripheral nerve induces delayed retrograde degeneration of sensory neurons projecting mainly to skin but does not affect survival of sensory neurons projecting to muscles. Distal axotomy does not lead to any significant cell death among spinal motoneurons.

• Immediate repair of the divided sciatic nerve with primary suture or peripheral nerve grafting attenuates retrograde cell death in cutaneous afferent neurons and supports axonal regeneration. Although the regenerative capacity of surviving cutaneous afferent neurons is not reduced by the axotomy, recovery of sensory function could be significantly affected by the cell loss which occurs also after nerve repair.

• Subpopulations of regenerating sural and medial gastrocnemius DRG neurons after immediate primary nerve repair up-regulate transcription of peripherin and ATF-3, however, expression of the structural neurofilament proteins of high molecular weight was significantly down-regulated following injury in all subpopulations of sensory neurons.

• Continuous intrathecal and intraperitoneal treatment with N-acetyl-cysteine provides a highly significant protection of spinal motoneurons after ventral rhizotomy and avulsion injury, and sensory neurons after peripheral nerve injury.

• Combined treatment with N-acetyl-cysteine and nerve grafting significantly improves survival rate and increases the number of regenerating sensory neurons. However, N-acetyl-cysteine has no growth-promoting effect on spinal motoneurons and does not affect axonal sprouting in the peripheral nerve graft or in the distal nerve stump.
ACKNOWLEDGEMENTS

During the years of research quite a few people have crossed my way and I would like to thank you all for contributing to this research.

Especially I would like to thank:

Dr Lev Novikov for being a superb supervisor. Without all your help this work would not have been possible. Thank you for everything.

Professor Mikael Wiberg for supervision and for leading me into research and hand surgery.

Professor Jan-Olof Kellerth for being my supervisor and for giving me support and advice.

Dr Liudmila Novikova for teaching me all the practical methods.

Gunnel Folkesson, Maria Brohlin and Gunvor Hällström, Anna-Lena Tallander and Göran Dahlgren for all the technical assistance.

All colleagues working in parallel in the lab, with nurturing parties and coffee breaks: Chris West, Sharmila Jivan, Cheng-Gang Zhang, Aleksandra McGrath, Jonas Pettersson, Christina Ljungberg, Amar Karalija, Paul Kingham, Daniel Kalbermatten, Frankie and Samuel Jonsson.

I also would like to express my thanks to the people from Manchester University: Professor Giorgio Terenghi for your helpful comments and suggestions and also Andy Hart, Adam Reid, Andy Wilson and Alex Hamilton.

And finally, to my dear Tuva for always supporting me.

This study was supported by the Swedish Medical Research Council, Umeå University, EU, County of Västerbotten, Åke Wibergs Stiftelse, Magn. Bergvalls Stiftelse, Clas Groschinskys Minnesfond and the Gunvor and Josef Anér Foundation.
REFERENCES


10. Bennett DL, French J, Priestley JV, McMahon SB (1996) NGF but not NT-3 or BDNF prevents the A fiber sprouting into lamina II of the spinal cord that occurs following axotomy. Mol Cell Neurosci 8: 211-220.


40. Green, B. Repair and grafting of the peripheral nerve. Plastic Surgery. 630-695, 1998. Ref Type: Generic


47. Hart AM, Brannstrom T, Wiberg M, Terenghi G (2002a) Primary sensory neurons and satellite cells after peripheral axotomy in the adult rat -
Dag Welin


49


