Diagnostic and therapeutic strategies following spinal cord and brachial plexus injuries

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Lutaj nehom i traži put do mjeseca i zvijezda. [...] I ne pitaj se da li ti to možeš. Možeš, idi... hodi.

(Za Almu, da nikada ne zaboravi)
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ABSTRACT

Traumatic injuries to the spinal cord and brachial plexus induce a significant inflammatory response in the nervous tissue with progressive degeneration of neurons and glial cells, and cause considerable physical and mental suffering in affected patients. This thesis investigates the effects of the antioxidants N-acetyl-cysteine (NAC) and acetyl-L-carnitine (ALC) on the survival of motoneurons in the brainstem and spinal cord, the expression of pro-apoptotic and pro-inflammatory cell markers, axonal sprouting and glial cell reactions after spinal hemisection in adult rats. In addition, a novel MRI protocol has been developed to analyse the extent of neuronal degeneration in the spinal cord.

Rubrospinal neurons and tibial motoneurons were pre-labelled with the fluorescent tracer Fast Blue one week before cervical C3 or lumbar L5 spinal cord hemisection. The intrathecal treatment with the antioxidants NAC (2.4 mg/day) or ALC (0.9 mg/day) was initiated immediately after injury using Alzet 2002 osmotic mini pumps. Spinal cord injury increased the expression of apoptotic cell markers BAX and caspase 3, induced significant degeneration of rubrospinal neurons and spinal motoneurons with associated decrease in immunoreactivity for microtubule-associated protein-2 (MAP2) in dendritic branches, synaptophysin in presynaptic boutons and neurofilaments in nerve fibers. Immunostaining for the astroglial marker glial fibrillary acidic protein and microglial markers OX42 and ED1 was markedly increased. Treatment with NAC and ALC attenuated levels of BAX, caspase 3, OX42 and ED1 expression after 2 weeks postoperatively. After 4-8 weeks of continuous intrathecal treatment, NAC and ALC rescued approximately half of the rubrospinal neurons and spinal motoneurons destined to die, promoted axonal sprouting, restored the density of MAP2 and synaptophysin immunoreactivity and reduced the microglial reaction. However, antioxidant therapy did not affect the reactive astrocytes in the trauma zone. The inflammation modulating properties of ALC were also studied using cultures of human microglial cells. ALC increased the microglial production of interleukin IL-6 and BDNF, thereby possibly mediating the anti-inflammatory and pro-regenerative effects shown in vivo.

To study degeneration in the spinal cord following pre-ganglionic and post-ganglionic brachial plexus injuries, adult rat models of ventral root avulsion and peripheral nerve injury were used. A novel MRI protocol was employed and the images were compared to morphological changes found in histological preparations. Ventral root avulsion caused degeneration of dendritic branches and axonal terminals in the spinal cord, followed by
significant shrinkage of the ventral horn. Extensive astroglial and microglial reactions were detected in the histological preparations. Peripheral nerve injury reduced the density of dendritic branches but did not cause shrinkage of the ventral horn. Quantitative analysis of MRI images demonstrated changes in the ventral horn following ventral root avulsion only, thus validating the developed MRI technique as a possible tool for the differentiation of pre-ganglionic and post-ganglionic nerve injuries.

Keywords: Spinal cord injury, brachial plexus injury, acetyl-L-carnitine, N-acetyl-cysteine, MRI, motoneurons.
ORIGINAL PAPERS

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

I. Neuroprotective effects of N-acetyl-cysteine and acetyl-L-carnitine after spinal cord injury in adult rats.
   **Karalija** A, Novikova LN, Kingham PJ, Wiberg M, Novikov LN. *PLOS ONE*. 2012;7(7)

II. The effects of N-acetyl-cysteine and acetyl-L-carnitine on neural survival, neuroinflammation and regeneration following spinal cord injury.

III. The effects of acetyl-L-carnitine treatment on neuroinflammation: An *in vitro* study
   **Karalija** A, Kelk P, Wiberg M, Kingham PJ.
   Manuscript

IV. Differentiation of pre- and postganglionic nerve injury using MRI of the spinal cord.
   **Karalija** A, Novikova LN, Orädd G, Wiberg M, Novikov LN.
   Manuscript, submitted to *PLOS ONE*.

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# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>4-HNE</td>
<td>Hydroxynonenal</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine</td>
</tr>
<tr>
<td>AANS</td>
<td>American Association of Neurological Surgeons</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ALC</td>
<td>Acetyl-L-carnitine</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BAX</td>
<td>BCL-2-associated X protein</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>BCL-2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
</tr>
<tr>
<td>BPI</td>
<td>Brachial plexus injury</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>ChABC</td>
<td>Chondroitinase ABC</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CONT</td>
<td>Control</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CSPG</td>
<td>Chondroitin sulphate proteoglycan</td>
</tr>
<tr>
<td>CT</td>
<td>Computer tomography</td>
</tr>
<tr>
<td>CTM</td>
<td>Computed tomographic myelography</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DICOM</td>
<td>Data Imaging and Communications in Medicine</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPX</td>
<td>Dibutyl phthalate xylene</td>
</tr>
<tr>
<td>DRA</td>
<td>Dorsal root avulsion</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal root ganglion</td>
</tr>
<tr>
<td>DRR</td>
<td>Dorsal root rhizotomy</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>ED1</td>
<td>Anti-CD68 antibody</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>Erk</td>
<td>Extracellular signal–regulated kinase</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>FB</td>
<td>Fast blue</td>
</tr>
<tr>
<td>FOV</td>
<td>Field of view</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-Aminobutyric acid</td>
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</table>
GDNF Glial cell line-derived neurotrophic factor
GFAP Glial fibrillary acidic protein
HMC3 Human microglial clone 3
HRP Horseradish peroxidase
IL-1β Interleukin 1 beta
IL-6 Interleukin 6
LP Lipid peroxidation
LPS Lipopolysaccharide
MAP2 Microtubule associated protein 2
MEM-α Minimum essential medium-alpha
MAPK Mitogen-activated protein kinases
MP Methyl prednisolone
MRI Magnetic resonance imaging
mRNA Messenger ribonucleic acid
MSC Mesenchymal stem cells
NAC N-acetyl cysteine
NeuN Neuronal nuclei antigen
NGF Nerve growth factor
NO Nitric oxide
NOS Nitric oxide synthase
NSC Neural stem cells
NT-3 Neurotrophin-3
O$_2^-$ Superoxide
OX42 Anti-CD11b/c antibody clone
PanNF Pan neurofilament
PBS Phosphate-buffered saline
PCR Polymerase chain reaction
PFA Paraformaldehyde
PIPES Piperazine-N,N'-bis(2-ethanesulfonic acid)
PI3K Phosphoinositide 3-kinase
PNI Peripheral nerve injury
PNS Peripheral nervous system
RDS Radical defense system
ROI Region of interest
ROS Reactive oxygen species
SC Spinal cord
SCs Schwann cells
SCI Spinal cord injury
SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM Standard error of the mean
SNR Signal-to-noise-ratio
SYN Synaptophysin
TBI Traumatic Brain Injury
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>TBS-T</td>
<td>Tris buffered saline with Tween</td>
</tr>
<tr>
<td>TE</td>
<td>Echo time</td>
</tr>
<tr>
<td>TIFF</td>
<td>Tagged image file format</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TR</td>
<td>Repetition time</td>
</tr>
<tr>
<td>TrkB</td>
<td>Tropomyosin receptor kinase B</td>
</tr>
<tr>
<td>TurboRARE</td>
<td>Turbo rapid acquisition with relaxation enhancement</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>WB</td>
<td>Western blot</td>
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Sammanfattning på svenska

Bakgrund


Material & metod

Studierna av antioxidanthandlingens effekter efter ryggmärgsskada genomfördes i rättmodell, tillämpande särskilda läkemedelssumpar som kontinuerligt frisatte substanserna i ryggmärgsvätskan. Studien som undersökte ALC:s inverkan på mikroglia utsöndring av neurotrosiska faktorer genomfördes i cellkulturer av för ändamålet representativa celler från människa. Studien av nivådiagnostik av nervskada med hjälp av MRI genomfördes i rättmodell, tillämpande en kraftfull magnetkamera för underökning av smådjur.

Resultat

Behandling av ryggmärgsskada med NAC och ALC bidrog till ökad överlevnad av både lokala motorneuron och neuron belägna långt från skadeområdet. Behandlingen minskade också kraftigt neuroinflammationen. Effekterna på nervcellsöverlevnad och neuroinflammation var detekterbara redan efter två veckor och kvarstod efter fyra och åtta veckor. ALC och NAC bidrog därtill till förbättrat överlevnad av dendriter och axon och bidrog dessutom till tillväxt av axon i skadeområdet. Behandlingen hade emellertid ingen inverkan på ärret i skadeområdet, och inga nervfibrer kunde ses passera genom ärret in i den friska vävnaden.

Underökningen av ALC:s effekter på humana mikroglia i cellkultur visade att behandling med ALC ökade utsöndringen av flera faktorer associerade med förbättrad nervcellsöverlevnad.

MRI av ryggmärgen för nivådiagnostik av nervskada visade att vår metod med mycket hög grad av säkerhet kunde användas för att skilja de två skadephyerna från varandra. Mätningarna av MRI-bilderna och motsvarande ryggmärgsssegment i vävnadsprover visade hög grad av samstämmighet.

Diskussion & slutsatser

Slutsatsen av ryggmärgsskadestudierna är att både ALC och NAC har positiva effekter vid behandling av ryggmärgsskada, och att antioxidanterna skulle kunna vara av värde för klinisk behandling. Det är emellertid osannolikt att endast antioxidanthandling kommer att räcka för att bekämpa samtliga skadliga processer efter ryggmärgsskada. ALC och NAC kan dock tänkas vara intressanta som en del i en framtida kombinationsterapi. Studien som berör MRI-undersökning av ryggmärgen efter olika typer av nervskador visar en helt ny metod för nervskadediagnostik. Metoden skulle kunna vara av nytta för att följa neurologiska sjukdomar och behandlingseffekter i prekliniska studier. Ett anpassat protokoll, tillämpbar för patienter, skulle dessutom kunna bidra till förbättrad diagnostik efter BPI.
Introduction

1. Clinical background

1.1 Spinal cord injury (SCI)

SCI affects 250,000-500,000 people annually worldwide, with traumatic spinal cord injury comprising approximately 90% of the cases (Rubiano et al., 2015). Traumatic SCI is mainly an affliction of the young male, with the mean age at of diagnosis being approximately 30 years (Wyndaele and Wyndaele, 2006). By far the most common causes of injury are traffic accidents, accounting for around 50% of injuries. The mortality rate following SCI is roughly 50% in the early phase, with an additional 15-20% of patients dying in the hospital at a later time point (Harkey et al., 2003). More than half of the injuries occur at the cervical level. Almost 50% of all injuries are considered complete grade A injuries according to the ASIA scoring system, meaning complete lack of motor and sensory function below the level of injury (Sekhon and Fehlings, 2001). The surviving patients are left with permanent motor, sensory and autonomic dysfunction, resulting in a diverse spectrum of co-morbidities. The main reported secondary morbidities are urinary bladder dysfunction with urinary sepsis, atelectasis with pneumonia, decubital sores and deep vein thrombosis (Sekhon and Fehlings, 2001). Currently no generally accepted treatment exists for traumatic SCI, with rehabilitation and treatment of secondary disorders being the only targets for therapy.

Due to the increasing incidence of traumatic SCI, the low mean age of the patients, the serious and potentially lethal associated diseases, and the lack of causal therapy, a great need for therapeutic advances persists.

1.2 Brachial plexus injury (BPI)

BPI principally occurs either in neonates as a complication to childbirth, or in adults, usually as a part of the traffic accident multitrauma (Kaiser et al., 2012, Ouzounian, 2014). Much like in the case of SCI, young males are typically the ones afflicted (Sakellariou et al., 2014). Depending on the type, level and extent of injury, the BPI may result in a more or less severe loss of upper limb function. The appropriate therapeutic approaches and expected prognosis may vary greatly depending on these factors. Early repair is indicated in some cases of BPI, having been shown to improve neuronal survival in preclinical trials as well as functional outcome in clinical studies (Ma et al., 2003, Gu et
al., 2004, Jivan et al., 2009). In other cases, spontaneous return of function is expected, warranting watchful waiting for signs of recovery.

Several diagnostic tools are currently clinically available. The main modalities used are electrodiagnostic testing, various clinical tests and different radiological approaches utilizing computer tomography (CT) and/or magnetic resonance imaging (MRI) technology (Sakellariou et al., 2014). To date however, no single diagnostic method or combination of methods has been shown to be sufficiently reliable. In some cases, surgical exploration is still warranted in order to establish the correct diagnosis and provide appropriate therapy. In order to offer the correct treatment at the appropriate time point, a reliable non-invasive method for early establishment of the type of BPI is needed.
2. Pathophysiology of the SCI

Traumatic SCI, regardless of the type of primary injury, inevitably sets in motion a cascade of reactions detrimental to the morphological and functional integrity of the spinal cord. Termed the secondary injury, and commencing only seconds after initial trauma, these events eventually culminate in the chronic phase of spinal cord injury, which can last for years. The secondary injury is constituted by a wide range of complex and intertwining destructive physiological and biochemical processes. These processes cause a progressive death of neurons and glial cells by way of apoptosis, the formation of a cavity in the spinal cord tissue, and the creation of a glial scar preventing axonal regeneration across the trauma zone (Dumont et al., 2001, Kawano et al., 2012, Silva et al., 2014).

Many of the mechanisms of the secondary injury are self-enhancing and self-propagating, thus causing a downward spiral of parenchymal destruction and loss of function that extends to neighbouring segments initially spared from the primary injury. What may initially be a localized and small spinal cord lesion will over time develop into a cavity, with subsequent progressive loss of function. These events can be described as occurring on a cellular level, but are essentially caused by a set of complex, interconnected and interacting molecular processes.

2.1 Secondary degeneration after SCI

2.1.1 Retrograde degeneration of neurons & death of glial cells

Traumatic SCI inevitably induces the death of spinal neurons and supporting glial cells in the vicinity of the trauma zone. At 3 hours after injury, a penumbra of dead and dying cells can be observed close to the lesion site, with the cells initially succumbing due to trauma-induced necrosis (Dusart and Schwab, 1994). The majority of cells that die will however do so by way of apoptosis as a part of the secondary injury (Donnelly and Popovich, 2008). Apoptosis commences at 4 hours after injury and persists for approximately 3 weeks, occurring both in neurons and in glial cells (Zhang et al., 2012a). However, cells in and around the immediate trauma zone are not the only ones affected by injury. Distant neurons projecting caudal to the injured spinal cord segments, such as motoneurons belonging to descending motor pathways, also undergo apoptotic cell death as a consequence of SCI (Deumens et al., 2005). Thus, an injury that initially causes localised cell death
eventually induces the death of not only nearby cells, but also cells found far away from the injury zone.

Among the best-known triggers of apoptotic cell death are various oxidants present in the injury zone. One of the more extensively studied oxidants is H$_2$O$_2$, which is believed to directly induce apoptosis. It does so both through the death receptor signalling pathways of the cells and through mitochondrial signalling pathways (Zhang et al., 2012a). The noxious effects of other oxidants such as nitric oxide (NO) and reactive oxygen species (ROS) on the mitochondria, membranes and DNA also contribute to the induction of programmed cell death. Indeed, a decrease of the oxidant-protective glutathione levels inside the cells has been shown to increase apoptotic activity, whereas an increase of glutathione is linked to a stronger production of the anti-apoptotic B-cell lymphoma 2 (Bcl-2) protein (Zhang et al., 2012a).

While triggers such as oxidants induce the cell's apoptotic cascade, the subsequent process is mediated by a complex set of proteins. Caspases, a group of proteolytic enzymes, are some of the main mediators of apoptotic events in mammals. Various caspases exist, with caspase 3 being one of the chief executioners of apoptosis. Caspase 3 is capable of inducing apoptosis by fragmenting DNA and mediating the release of cytochrome c from the mitochondria (Porter and Janicke, 1999, Zhang et al., 2012a). As such, it plays an important role in the apoptotic processes in the central nervous system (CNS), being upregulated early after SCI (Springer et al., 2001, Hagberg, 2004). Blocking of caspase 3 has indeed previously been shown to ameliorate the loss of neurons (Charriaut-Marlangue, 2004). The actions of caspase 3 are in turn modulated by the two proteins BCL-2-associated X protein (BAX) and Bcl-2 (Adams and Cory, 1998, Rudel, 1999). It is the ratio between the pro-apoptotic BAX and the anti-apoptotic Bcl-2 protein (Bcl-2/BAX) that regulates the apoptotic process. An increased ratio, i.e. increased expression of Bcl-2 in relation to BAX, is linked to greater survival of cells (Hou et al., 2003, Vukojevic et al., 2008, Song et al., 2013). The early ensuing and prolonged degeneration of neurons and glial cells is an important obstacle to overcome as part of future neuroprotective and proregenerative therapies. The caspases in general and caspase 3 in particular, with additional proteins controlling its function, may be crucial targets of treatment.

Apart from the death of neurons and glial cells, SCI will also cause the destruction of dendrites and axons. Inflammation and edema following SCI causes swelling of axons and shedding of myelin early after injury (Donnelly and Popovich, 2008). In the chronic phase of SCI Wallerian degeneration occurs, causing a loss of axons in the proximity of the trauma zone. Furthermore, SCI causes a general loss of dendritic branches and synaptic boutons (Bernstein et al., 1984, Nacimiento et al., 1995, Novikova et al., 1996).
The inevitable result of spinal cord injury is therefore a diminished functional connectivity of the spinal cord circuitry.

2.1.2 Neuroinflammation

Neuroinflammation is one of the foremost hallmarks of the SCI, commencing almost immediately after the primary injury. Unlike inflammation in many other organs, the inflammatory response after SCI is not self-limiting, and can persist for years (Alexander and Popovich, 2009). The local dormant microglial cells are the resident macrophages of the CNS, and are some of the first cells to react to injury, being activated and infiltrating the trauma zone. The activated microglia secrete a variety of different cytokines which, among other actions, contribute to the recruitment of leukocytes from the bloodstream. Neutrophils are the first of the peripheral cells to infiltrate the trauma zone, doing so already after 2 hours. They are later followed by monocytes, which start to infiltrate the injury zone after 2 days. The infiltrating monocytes will soon differentiate into macrophages (Fig. 1). While neutrophils will eventually disappear from the SCI trauma zone, the macrophages remain for years in the human spinal cord, continuously exerting their immunological effects (Alexander and Popovich, 2009). B- and T-lymphocytes are also present in the injury zone, although little is known about their role and importance in the secondary injury (Trivedi et al., 2006, Alexander and Popovich, 2009).

The presence of macrophages in the vicinity of the trauma zone may in its complexity be described as a double-edged sword, exhibiting both destructive and pro-regenerative features. Principally, two functionally different types of macrophages are present after SCI.

The M1 macrophages are considered pro-inflammatory and neurotoxic. They are well known to secrete the highly reactive NO and O$_2^-$, as well as a variety of ROS. M1 macrophages also produce the pro-inflammatory and pro-apoptotic cytokines tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) (Tang and Le, 2016). Subsequently, they contribute to the gradual breakdown of myelin, destruction of dendrites and axons, aggravated mitochondrial dysfunction and progressive death of motoneurons and glial cells (Bareyre and Schwab, 2003).

The M2 macrophages, in contrast, are generally considered to exert anti-inflammatory and pro-regenerative actions. They secrete various cytokines associated with the process of regeneration, among others brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and the potentially pro-regenerative interleukin 6 (IL-6) (Heese et al., 1997, Coull et al., 2005,
Scheller et al., 2011, Zhang et al., 2012a). This subset of microglial cells has, among other beneficial features, been shown to promote the regeneration of axons after SCI (Kigerl et al., 2009).

Both M1 and M2 macrophages are present early in the secondary injury. However, the M1 response is considered to be the much stronger one. Moreover, contrary to what is generally observed in wound healing elsewhere, M2 macrophages disappear early, while M1 macrophages may remain indefinitely after SCI (Kigerl et al., 2009). This feature accounts, at least in part, for the chronic nature of the neuroinflammatory response after injury. Therefore, it may be a possible target for therapeutic manipulation.

![Diagram](image)

**Fig. 1** Illustration showing the time of arrival of different immunologically active cells to the SCI zone. Macrophages derived from monocytes will not be separable from activated microglia, contributing to the total pool of activated macrophages. While other immunologically active cells will eventually clear from the injury zone, the macrophages can remain indefinitely, causing chronic inflammation.

### 2.1.3 Lesion scar formation

Much like microglia, the astroglia act as local maintainers of homeostasis in the spinal cord. Subsequently, they are some of the first cells to respond to SCI. Among the factors triggering astroglial activity are IL-1 and fibrinogen, both derived from extravasated plasma after damage to the blood-brain-
barrier (BBB) (Cregg et al., 2014). The subsequent inflammatory response, elicited by injury and mediated by microglia and neutrophils, drives the astrocytes away from the immediate injury zone. The astrocytes will settle in the periphery of the trauma zone, forming a thin layer of reactive astrocytes in a process termed gliosis (Fitch et al., 1999, Silver et al., 2014). The scar formation involves only limited proliferation of astroglia, with the cells mainly undergoing hypertrophy and forming a mesh-like structure. The gliotic layer will eventually be restructured, with the astroglial cells aligning themselves perpendicularly to the long axis of the spinal cord, thereby very efficiently blocking the passage of regenerating axons (Silver et al., 2014). Activated astroglial cells produce a variety of specific intermediate filament proteins, among others the glial fibrillary acidic protein (GFAP). In parallel with the astroglial reaction, fibroblasts from the damaged meninges also infiltrate the trauma zone. The fibroblasts proliferate and secrete a variety of extracellular matrix molecules such as fibronectin, laminin and type IV collagen, resulting in the formation of the fibrotic scar tissue (Kawano et al., 2012). The glial and fibrotic components together form the lesion scar, which impedes regeneration through the trauma zone.

Apart from acting as a physical barrier, the glial cells of the scar produce and accumulate chondroitin sulphate proteoglycans (CSPGs) in the extracellular matrix. The deposition of CSPGs commences within 24 hours after injury, with considerable concentrations being found in the scar tissue months after trauma. The CSPGs efficiently prevent axonal regeneration through the scar (Cregg et al., 2014). Apart from the CSPGs, other proteins in the glial scar also contribute to the inhibition of axonal regeneration. One of the more efficient growth-inhibiting proteins worth mentioning is reticulon 4 (Nogo), which is a potent blocker of neurite outgrowth in the CNS (GrandPre et al., 2000).

Much like the inflammatory reaction, the glial scar formation is a complex process with both beneficial and detrimental implications for recovery.

In the early stages of the secondary injury the immense inflammatory response, including the presence of ROS, NO and O₂⁻, threatens the integrity of surrounding spinal cord segments. By closing of the injury zone, the astroglia prevent further spreading of noxious compounds and subsequent parenchymal destruction (Rolls et al., 2009). Additionally, the reactive astrocytes provide protection from NO (Chen et al., 2001), scavenge glutamate (Cui et al., 2001), contribute to the reparation of the BBB (Lee-Liu et al., 2013), and support regeneration by secreting BDNF (Lee-Liu et al., 2013).

In the later stages of the secondary injury, the solid astroglial scar sealing of the injury zone, becomes an obstacle for nerve regeneration. Chemically
rather than physically the CSPGs, Nogo and other molecules interfere with axonal regeneration, causing a collapse of the growth cone and retraction of the neurite (Rolls et al., 2009, Schwab, 2010) (Fig. 2). The astrocytes also prevent the differentiation of neural stem cells and progenitor cells, further contributing to the prevention of regeneration (Lee-Liu et al., 2013). Despite different strategies to remove or alter the scar in order to facilitate the crossing of growing neurites, no clinically applicable methods have yet been found. Thus, the glial scar poses one of the greatest challenges in the process of spinal cord regeneration and return of function.

**Fig. 2** Schematic illustration of the secondary events following SCI. The initial traumatic injury is followed by an inflammatory response, expanding the lesion cavity in the rostral and caudal directions. Neurons found in the vicinity of the trauma zone undergo neurodegeneration, while their axons sustain secondary damage caused by the inflammatory response, contributing to axonal loss and demyelination. The influx of inflammatory cells causes astroglial cells to align almost perpendicularly to the direction of the axons, and secrete a variety of inhibitory proteins. The astroglia also enter the lesion cavity, forming a glial scar. The axons sprouting from surviving neurons are prevented from crossing the injury zone, thereby impeding functional recovery. Adapted from (Fitch and Silver, 2008).
2.2 Oxidative damage after SCI

Traumatic SCI induces a series of extremely complex and interacting chemical processes, resulting in a gradual destruction of the spinal cord parenchyma. These processes are intimately associated with the detrimental part of the neuroinflammatory response. While described separately, the chemical processes are essentially intimately associated, and are often mutually reinforcing.

2.2.1 Oxidative stress, ROS formation and the radical defense system

Mitochondria are the cellular power plants, and are principally composed of two membrane layers. The outer membrane is permeable to nutrients, adenosine diphosphate (ADP), adenosine triphosphate (ATP) and ions. The inner membrane, on the contrary, only allows free passage of carbon dioxide, oxygen and water molecules, while regulating the permeability for all other molecules. The mitochondria produce more than 95% of the ATP in mammalian cells through oxidative phosphorylation. Oxidative phosphorylation utilizes carbohydrates, fatty acids and proteins as substrates for ATP production. The process is dependent on the electron transport chain (ETC), in which oxygen is required to produce ATP, with carbon dioxide and water being the by-products (McEwen et al., 2011). With the tricarboxylic cycle and β-oxidation occurring in the mitochondria, and a majority of oxygen being consumed in mitochondrial reactions, this organelle accounts for a large part of the total oxygen consumption (Jia et al., 2012).

While the oxygen molecule is central for all mammalian life, incompletely excited or reduced forms of oxygen (i.e. ROS) are highly reactive and damaging to the organism. ROS, if not efficiently neutralised by the cell’s radical defense system (RDS), may cause great damage. In total, at least ten different sources of ROS have been identified in the mammalian mitochondria (Andreyev et al., 2005, Adam-Vizi and Chinopoulos, 2006), while numerous other sources can be found in the cell in general. Some of the main oxidants present in the cell are superoxide ($\mathrm{O}_2^-$) and the compounds generated by combining oxygen with the abundantly present hydrogen, namely the hydroxyl radical ($\mathrm{OH}^-$) and hydrogen peroxide ($\mathrm{H}_2\mathrm{O}_2$). The hydrogen radicals are some of the most potent radicals in nature, capable of oxidising many compounds. $\mathrm{H}_2\mathrm{O}_2$ is not a radical per se, but is highly reactive and capable of yielding free radicals.
Under normal physiological and metabolic conditions, the cell is capable of controlling the burden of oxidative stress by employing a set of enzymes belonging to the RDS. Three of the main enzymes are the superoxide dismutase enzymes (SOD 1-3), glutathione peroxidase, and catalase (Starkov, 2008, Jia et al., 2012).

SOD 1 is present in the mitochondria, while SOD 2 and 3 are found in the cytosol. SOD is the catalyst of the reaction in which $O_2^-$ is dismutated to $H_2O_2$ and $O_2$ ($O_2^- + O_2^- + 2 H^+ \rightarrow H_2O_2 + O_2$).

The function of catalase is to further degrade hydrogen peroxide to water and oxygen ($2 H_2O_2 \rightarrow 2 H_2O + O_2$) (Jia et al., 2012). Glutathione is present in a reduced state (GSH) and in an oxidized state, called glutathione disulphide (GSSG).

Glutathione contains a thiol group, capable of donating an electron. When donated to a free radical, the electron neutralizes the radical, while GSH itself becomes reactive. Two reactive GSH molecules combine, forming a stable GSSG molecule (Kaplowitz, 1981, Jia et al., 2012). Apart from neutralizing free radicals GSH is, among other reactions, capable of degrading $H_2O_2$ through the glutathione-ascorbate cycle (Noctor and Foyer, 1998). Altogether, the diverse actions of GSH make the molecule an instrumental tool for the control of oxidative stress in all living cells.

In a cell under homeostasis the previously described defense mechanisms against ROS will be sufficient to control oxidative stress. However in the case of neurotrauma, the ability of the cell to maintain homeostasis and efficiently protect itself from ROS is greatly diminished. This is due to a number of complicated and intertwining reactions, the sum of which plunges the cell into a downward spiral of oxidative stress and tissue injury. Injury will, among other things, interrupt the blood supply to the tissue, preventing the delivery of oxygen and elimination of CO$_2$. This in turn may lead to mitochondrial dysfunction with a dysfunctional oxidative phosphorylation and build-up of ROS, as well as release of calcium from the damaged mitochondria (Dumont et al., 2001). Mitochondrial dysfunction in combination with excitotoxicity, lipid peroxidation, iron dependent hydroxyl radical formation and other injury mechanisms, cause a rampant production of ROS.

Several studies have demonstrated the exceptional fragility of the spinal cord in relation to several of the mentioned mechanisms of ROS production. Spinal mitochondria have been shown to be 10-fold more sensitive to hydroxynonenal (4-HNE), the by-product of lipid peroxidation (LP), than brain mitochondria (Vaishnav et al., 2010). Mitochondria of the spinal cord produce more ROS, cause more oxidative damage and have a decreased ability of performing mitochondrial respiration compared to mitochondria found in
the brain. Spinal mitochondria also produce less ATP than brain mitochondria (Yonutas et al., 2015). Furthermore, the capacity of spinal cord mitochondria to sequester calcium is only 10-20% of the capacity of brain mitochondria, while the total amount of tissue calcium is 8 times greater (Panov et al., 2011). As such, the spinal cord is a tissue exceptionally susceptible to oxidative stress.

### 2.2.2 Lipid peroxidation

Lipids are an essential part of the mammalian organism, being the main component of the human cell membranes. The integrity of the cell membrane is a prerequisite for continued homeostasis and survival of the organism. However, lipids are sensitive to ROS, and the process in which lipids are degraded by ROS is termed lipid peroxidation (LP).

The LP process has three principal phases of chemical reactions; **initiation**, **propagation** and **termination** (Butterfield and Reed, 2016).

Initiation occurs when a free radical species (R•), such as for example OH• or HOO•, attacks a polyunsaturated fatty acid (LH). In this process, the free radical species removes a hydrogen atom (i.e. a proton and an electron) from an allylic carbon (a carbon surrounded by two carbons bound via double bonds) belonging to the polyunsaturated fatty acid. This reaction converts the fatty acid to a lipid radical (L•), after which the propagation phase commences.

The lipid radical attacks an oxygen molecule O2, thus creating a lipid peroxyl radical (LOO•). This reaction is followed by a reaction in which LOO• attacks a polyunsaturated fatty acid, tearing an electron from it, thus creating another lipid radical. In the process, LOO• is converted to a lipid hydroxyl radical (LOOH) (Fig. 3). Essentially, a self-propagating chain reaction occurs, causing progressive damage mainly to the cell membrane. The only way of breaking the vicious cycle of lipid peroxidation is by neutralising the free radicals. This occurs in the stage of termination (Ayala et al., 2014, Butterfield and Reed, 2016).

Termination is achieved by combining a free radical with another radical, thus converting both to a stable compound. For this reaction to occur spontaneously with significant frequency, the concentration of free radicals must be high. This is, for apparent reasons, not an option in the living organism, which is why the cell employs a number of enzymes that catalyse the neutralisation of free radicals. Among these are the previously mentioned RDS enzymes SOD, peroxidase and catalase. The organism can also utilize
many exogenously produced antioxidants that can act in different ways to counteract the destructive LP process. Among these are vitamin A and E, acetyl-L-carnitine and a variety of other naturally occurring or artificially synthetized antioxidants (Mylonas and Kouretas, 1999, Sepand et al., 2016).

![Initiation and Propagation of LP](image)

**Fig. 3** Schematic illustration of the initiation and propagation stages of LP. $R^*$, free radical; $L^*$, lipid radical; LH, polyunsaturated fatty acid; LOO$^*$, lipid peroxyl radical; LOOH, lipid hydroxyl radical.

With the spinal cord containing plenty of fat, while also being highly vulnerable to oxidative stress, the perquisites for the LP reaction to occur are optimal. Indeed, LP has been shown to play a key role in the oxidative stress reaction following CNS injury, particularly in the case of SCI (Oyinbo, 2011, Bains and Hall, 2012, Borgens and Liu-Snyder, 2012, Smith et al., 2013).

### 2.2.3 Excitotoxicity

Excitotoxicity is a term generally used to describe the neurotoxic effects of excessive stimulation caused by certain neurotransmitters, as well as similar chemical compounds. In the context of neuropathology, it specifically refers to the harmful effects of the neurotransmitter glutamate. The neurotoxic actions of glutamate were first noted in retinal neurons, and were later observed in the entire CNS (Olney, 1969). Abundantly present throughout the brain and spinal cord, the absolute majority of glutamate is stored intracellularly. Free glutamate is rapidly converted by astrocytes to glutamine, and stored in an inactive state inside the astrocytes (Mehta et al., 2013). Rising extracellular levels of glutamate will prompt the neurons to employ special pumps to move the excess glutamate from the extracellular space into the cell (Gottlieb et al., 2003). However, this regulating mechanism has its limits. The system is unable to cope with fast and/or great increases in extracellular glutamate levels. Excess extracellular glutamate will overly excite the cells, causing great influx of the ions Na$^+$ and Ca$^{2+}$, thereby causing some cells to
lyse due to osmotic changes. Apart from osmotic destruction, calcium build-up in the cells interferes with many important cellular processes. Perhaps most importantly, increased calcium levels disrupt mitochondrial function, thereby causing increased ROS formation (Mehta et al., 2013). Indeed, calcium influx into the mitochondria has been shown to interfere with energy production and cause the formation of ROS (Lipton, 2008). Furthermore, increased calcium levels affect calcium-dependent enzymes and nitric oxide synthase (NOS), causing additional formation of ROS and aggravating the oxidative stress reaction.

Due to the promotion of ROS build-up through a variety of mechanisms, excitotoxicity is considered by many researchers to be the main mediator of oxidative stress intracellularly in CNS injury (Mehta et al., 2013). Indeed, glutamate excitotoxicity plays a central role in the pathophysiology of SCI, contributing significantly to the secondary injury both in the grey and white matter (Park et al., 2004).

### 2.2.4 Iron dependent hydroxyl radical formation

Traumatic SCI damages the supplying blood vessels, causing oxygen and nutrient deprivation - with subsequent ROS formation – as well as haemorrhage. A perhaps overlooked but relevant part of the secondary injury is the role of extravasated iron (Fe$^{2+}$) in the formation of ROS. Iron is abundant in the spinal cord, being principally present in two forms.

Iron in its redox active form, i.e. Fe$^{2+}$, is bound to the protein transferrin in the blood plasma and ferritin inside the cell. The iron is tightly bound under physiological conditions. However, when pH falls below 6.0, or when the protein-iron complex comes in contact with O$_2^-$, the affinity for iron drops and the highly reactive iron ion is released. Low pH and the presence of oxygen radicals are both hallmarks of the spinal cord injury, creating all the conditions needed for release of protein-bound iron in the injured tissue (Hall, 2011).

Iron is also present as a part of the haemoglobin protein. Normally an integral part of the protein, the iron is readily released when haemoglobin comes in contact with lipid hydroperoxides or H$_2$O$_2$, both abundantly present in injured the spinal cord. With lipid peroxidation being a rich source of both compounds, the release of iron from haemoglobin is a constantly occurring process (Hall, 2011).

Free iron contributes to aggravation of oxidative stress and lipid peroxidation, and subsequently increased tissue damage. This occurs through two reactions.
• The Fenton reaction, when iron reacts with hydrogen peroxide, yielding a hydroxyl radical: \( \text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \cdot\text{OH} + \text{OH}^- \).

• The simple reaction of iron with oxygen yielding the reactive oxygen molecule \( \text{O}_2^- \): \( \text{Fe}^{2+} + \text{O}_2 \rightarrow \text{Fe}^{3+} + \cdot\text{O}_2^- \) (Liu et al., 2004, Hall, 2011).

While not being the primary source of ROS, the iron-dependent hydroxyl radical formation strongly potentiates injury in the damaged spinal cord. Indeed, the sensitivity of different parts of the CNS to oxidative stress is associated with iron levels found in the tissue (Zaleska and Floyd, 1985).

### 2.3 Regeneration after SCI

The potential of the spinal cord to spontaneously regenerate following traumatic injury varies greatly between different species. The mammalian spinal cord, compared to fish and amphibians, has poor regenerative abilities. Neurogenesis, i.e. the *de novo* creation of glial cells and neurons, may occur in certain parts of the CNS such as the hippocampus and the olfactory bulb (Lee-Liu et al., 2013). However, the ability of the spinal cord to regenerate after severe injury in a way that enables reconstitution of function is very limited. This is particularly true following hemisection/transection of the spinal cord. However, only a minority of patients afflicted by SCI suffer a hemisection/transection, with most injuries being incomplete. Previous studies have found that the preservation of small portions of certain spinal tracts may be sufficient for the recovery of locomotor functions, such as hind limb function in rats (Ballermann and Fouad, 2006). A treatment capable of inducing neural sprouting may therefore be of great value in the functional recovery of motor functions.

### 2.4 Therapeutic strategies after SCI

With the dire consequences of SCI being recognized by physicians for centuries, a great number of potential therapeutic approaches have been investigated. The therapeutic strategies can principally be divided in one of the three following categories: surgical, cellular or pharmacological treatments. The objective(s) of a successful therapy essentially address the basic pathological processes of the secondary injury. An optimal therapy includes: protection of neurons and glia, combating of the destructive components of neuroinflammation, preservation of the neural circuitry, induction of axonal sprouting and removal/manipulation of the glial scar, thereby permitting axonal regeneration through the trauma zone and enabling functional recovery.
The corticosteroid methylprednisolone has previously been the drug of choice in the treatment of SCI, showing significant improvement on sensory and motor function if administered early after injury (Bracken, 2012). Methylprednisolone has however generally fallen out of favour due to high complication rates, predominantly infections (Witiw and Fehlings, 2015). Despite extensive research conducted for many decades, there are today no clinically approved pharmacological treatments for spinal cord injury (Hawryluk et al., 2008, Silva et al., 2014).

2.4.1 Surgical therapy

Trauma to the vertebral column may disrupt the spinal anatomy, causing compression of the spinal cord. Untreated compression may lead to a prolonged and aggravated injury, causing extensive damage. Surgical decompression after spinal cord injury has clearly shown beneficial effects in a number of animal studies, decreasing the extent of secondary injury and improving functional outcome (Silva et al., 2014). Data from clinical studies of patients has however not been equally convincing. Currently, there is no clear consensus regarding the general benefit of decompression surgery. The possible value of surgery is dependent on timing, region of injury and overall health status of the patient. Unlike SCI in experimental animals, the heterogeneity of traumatic SCI in humans complicates assessment, and a consensus regarding appropriate indications for decompression demands larger high-quality studies (Witiw and Fehlings, 2015).

2.4.2 Cell therapy

Great effort has been directed towards identifying an appropriate line of cells for regenerative clinical purposes. A number of different cell types have been studied, including Schwann cells, neural stem cells, mesenchymal stem cells, and olfactory ensheating cells.

Schwann cells (SCs) were the first cells to be transplanted for the purpose of SCI repair. While belonging to the PNS and promoting axonal regeneration following PNS injuries, SCs have a variety of properties that are potentially valuable in the repair of the injured spinal cord. Among these is the ability of SCs to produce different neurotrophic factors, such as BDNF, NGF and GDNF (glial cell line-derived neurotrophic factor). Promising preclinical studies have shown, among other features, improved myelinisation, enhanced axonal regeneration and functional improvement. Only a few clinical trials have been published hitherto (Silva et al., 2014).
Neural stem cells (NSC) may readily be grown in vitro, and are capable of differentiating into neural and glial cells. Apart from the prospect of replacing lost cells, NSCs also exhibit the ability to produce a variety of neurotrophic factors. Among these is BDNF, potentially providing trophic support to the injured region. Much like with the transplantation of other cell types, the challenge with NSC transplantation has been improving the survival of cells, and controlling their differentiation and migration (Silva et al., 2014).

Mesenchymal stem cells (MSC) are present in many tissues such as the bone marrow and adipose tissue. They are plentiful, simple to obtain, and are considered safe and easy to transplant (Fehlings and Vawda, 2011). Depending on the tissue of origin the MSCs may exhibit different properties. Among other beneficial features, they are capable of producing anti-inflammatory cytokines and neurotrophic factors. Previous clinical trials have unfortunately failed to prove any significant functional improvement of MSC treatment after SCI (Silva et al., 2014).

Olfactory ensheathing cells (OEC) possess the unique ability to regenerate from the periphery and gain access to the CNS. As such, they are of great interest as mediators of structural and functional improvement. While the exact actions of OECs are still uncertain, preclinical studies have shown beneficial effects on axonal regeneration and decrease in the expression of CSPGs in the trauma zone. Clinical trials have proven the safety of transplantation (Silva et al., 2014). A much-publicized case of OEC transplantation in a patient with traumatic SCI has shown anatomical and functional improvements (Tabakow et al., 2014). The study is however based on one patient, with certain objections having been raised regarding the representativity of the patient and the therapeutic and evaluational procedures (Guest and Dietrich, 2015). More research in the field of OEC transplantation is therefore warranted.

2.4.3 Pharmacological therapy

An abundance of pharmacologically very diverse substances have been investigated for the treatment of spinal cord injury. In essence, all of the studied compounds combat one or more of the basic pathophysiological components of the secondary injury. This brief overview will comprise far from all tested substances, instead focusing on the clinically promising ones. Antioxidant therapies will be separately addressed.

Methyl prednisolone (MP) is clinically the most thoroughly studied substance for treatment of acute SCI. MP has been evaluated in three large national studies termed NASCIS I-III performed in 1984, 1990 and 1997. The two latter
have attracted the greatest interest from clinical practitioners. All three were multicentre, randomized, prospective and double-blinded studies. The trials evaluated the effects of treatment on motor, sensory and functional recovery, as well as the safety aspects of MP. Initial evaluation of the results proved disappointing, identifying no improvement in recovery. Post hoc analyses of NASCIS II & III, on the contrary, revealed improved functional outcome when treatment commenced within 8 h post trauma. However, treatment also carried a greater risk of infection, primarily wound infection, pneumonia and severe sepsis. Due to doubts in regard to efficiency and the potential risks of infection, the American Association of Neurological Surgeons (AANS) issued a recommendation in 2013 advising clinical practitioners not to use MP for the treatment of SCI. Currently, different standards are applied in different centres, with some choosing MP for the treatment of selected cases (Bydon et al., 2014, Silva et al., 2014, Witwi and Fehlings, 2015). Being well studied and showing some therapeutic promises, the molecular basis of the beneficial effects of MP treatment is of great interest for possible future development of SCI treatment. The main effect of MP in SCI is interestingly not mediated through the corticosteroid receptor, but rather by the antioxidant properties of the molecule. Among other actions, MP combats lipid peroxidation by acting as a scavenger of free radicals and ameliorating neuroinflammation (Diaz-Ruiz et al., 2000, Silva et al., 2014).

Riluzole is currently the only neuroprotective drug approved by the US Food and Drug Administration, with ALS being the sole indication for treatment. Riluzole acts as a sodium-channel blocker, impeding the uncontrolled and detrimental influx of sodium into cells in the zone of injury. After showing promising results on motor recovery after SCI in a phase I/II, a phase II/III trial is now ongoing (Witwi and Fehlings, 2015, Ahuja et al., 2016).

Magnesium compounds have evoked interest due to their ability to block the NMDA receptor, thus acting to prevent damaged induced by glutamate excitotoxicity. Treatment promotes tissue sparing and cell survival, and combats LP. Phase I and II trials are on-going (Silva et al., 2014, Ahuja et al., 2016).

NSAIDs are, due to their anti-inflammatory properties, a theoretically appealing therapeutic option. However, in preclinical trials the results have been mixed, with at least one of the compounds aggravating the LP reaction. Currently, ibuprofen is the main NSAID considered to hold promise as an anti-inflammatory agent following SCI (Silva et al., 2014).
Minocycline is an antibiotic with anti-inflammatory and neuroinflammation-attenuating properties, improving the sparing of spinal cord parenchyma and strongly decreasing the microglial response after injury. A phase II trial showed promising results in regard to functional improvement, prompting an on-going phase III trial (Ahuja et al., 2016).

Neurotrophic factors of different types have for obvious reasons been under investigation. The effects of BDNF treatment have been extensively studied by our group in preclinical models of CNS and PNS injury. Treatment with intrathecally administered BDNF after SCI salvages motoneurons in general (Novikova et al., 1996) as well as motoneurons belonging to descending rubrospinal motor pathways in rats (Novikova et al., 2000). The neurotrophic factor neurotrophin-3 (NT-3) has been shown to exert similar effects (Novikova et al., 2000, Silva et al., 2014). Various other neurotrophic substances have been investigated preclinically, with more or less promising results (Harvey et al., 2015).

Pharmacological manipulation of the astroglial scar is an appealing approach, possibly holding the promise of regeneration through the injury zone. All things considered, the scar appears to be a crucial challenge to overcome if SCI is to be successfully treated. Chondroitinase ABC (ChABC) is an enzyme capable of degrading certain CSPGs, thereby removing some of the chemical inhibitors of axonal regeneration. Preclinical studies have shown shrinkage of the posttraumatic cavity and of the scar, as well as motor improvement after therapy. No clinical trials have yet been initiated, since a humanized form is still to be developed (Silva et al., 2014, Ahuja et al., 2016). Blocking of the growth inhibiting protein “Nogo” has been proposed as a possible option for therapy. Several antibodies have been evaluated, among others the anti-Nogo antibody. Anti-Nogo has been shown to promote axonal growth and functional recovery in preclinical trials, without causing adverse effects usually associated with uncontrolled sprouting (Silver et al., 2014). The results of an on-going clinical trial are still awaiting publication (Silva et al., 2014).

In conclusion, the outstanding pathophysiological complexity of the SCI demands therapeutic combating of a number of key factors. It is unlikely that one single substance or approach will be sufficient in successfully addressing the whole spectrum of pathological problems. Therefore one can speculate that a combinational treatment, involving pharmacological, cellular and surgical components, will be needed to restore motor, sensory and autonomic function following SCI.
2.4.4 Antioxidants

The term “antioxidant” is defined as a substance that inhibits oxidation reactions caused by oxygen or peroxides (i.e. oxidants). Being dependent on oxidative metabolism for energy production, mammalian cells constantly combat ROS. The ROS however, once believed to be of detrimental effect only, have lately been shown to play a role in a number of important physiological processes, among others acting as signalling molecules enabling cells to react and adapt to changes in their environment (Rhee, 2006, Wang and Michaelis, 2010). Therefore, in order to maintain optimal levels of oxidants, the mammalian organism utilizes both endogenously and exogenously synthetized antioxidants. Examples of important endogenously produced antioxidants are melatonin, vitamin E, glutathione and acetyl-L-carnitine (ALC). Vitamin C is an example of an antioxidant that, while essential for survival, cannot be produced by primates and humans, and must be supplemented through the diet (Smirnoff, 2001).

With oxidative stress playing a most central role in the pathophysiology of the SCI, a therapeutic agent capable of effectively manipulating the production of ROS may be highly valuable. To date, the two only substances proven in clinical trials to act as neuroprotectants in the acute treatment of spinal cord injury do so by acting as antioxidants (Hall, 2011). The best-studied antioxidant therapy after traumatic SCI is methylprednisolone. As discussed earlier, it is the only pharmacological approach that has been studied on a larger scale, yielding positive results. However, a great number of other antioxidants are being studied as potential therapeutic agents after traumatic SCI. Some of these have shown particularly promising results in preclinical trials.

Lazaroids (21-aminosteroids) are chemically similar to methylprednisolone, although they do not act as glucocorticoid receptor agonists. As such, lazaroids lack the adverse effects associated with MP, while retaining and in some cases even exerting stronger antioxidative actions. Two promising compounds currently under investigation are U-74006F and U-83836E. Both are very potent ameliorators of LP. Additionally, they manipulate excitotoxicity, mitochondrial dysfunction and the calcium levels in a favourable way. U-74006F has been shown to improve functional outcome after SCI in preclinical trials (Hall, 2011, Jia et al., 2012).

Melatonin (N-acetyl-5-metoxitryptamine) is a hormone regulating the circadian rhythm. The drug has few adverse effects and readily passes the BBB (Samantaray et al., 2009). It is also a direct antioxidant, scavenging the peroxyl radicals and reacting with peroxynitrate, thereby combating the
effects of LP. As such, melatonin has been studied in the treatment of SCI. It has been shown to ameliorate LP, save motoneurons and improve motor function (Hall, 2011).

*Vitamin E* is an endogenous antioxidant, acting as a direct scavenger of LP-generated ROS. It has shown promising results in preclinical trials in regard to direct scavenging of ROS and protection of the spinal cord after injury (Hall, 2011, Jia et al., 2012).

Although no antioxidants are currently approved for the treatment of SCI, their principal actions combined with current preclinical results warrant further investigation. Many clinical trials have successfully addressed the potential benefits of antioxidant treatment following a number of neurological diseases. Therefore, clinical trials of antioxidant treatment would be of great interest.

### 2.4.5 Acetyl-L-carnitine

Acetyl-L-carnitine (ALC) is an ester of the trimethylated aminoacid L-carnitine. ALC is abundant in the mammalian organism, with the homeostasis of the carnitines being maintained through endogenous production, dietary absorption and controlled elimination. The absorption of ALC takes place in the small bowel (Rebouche and Seim, 1998), while the production mainly occurs in the liver, kidneys, and brain (Rebouche, 2004). Both intravenous and oral administration of ALC lead to a rapid increase in ALC concentration in the blood plasma and cerebrospinal fluid (CSF), with the peptide readily crossing the BBB (Parnetti et al., 1992). The antioxidant is filtered through the kidneys into the urine, with over 90% being reabsorbed (Heard, 2008).

On a cellular level, ALC is an important component of the inner mitochondrial membrane. As such, it is primarily and intimately involved in several aspects of cellular bioenergetics. The functions of ALC in the mitochondria are as important as they are diverse. The peptide, containing an acetyl group, can act as a donor of acetyl to CoA when acetyl is scarce. In the absence of pyruvate, ALC will contribute to continued production of acetyl-CoA, that in turn can enter the citric acid cycle and contribute to increased production of ATP (McEwen et al., 2011). This is particularly important in traumatic SCI, cellular bioenergetics will be greatly compromised. It has been shown that ALC treatment of mitochondria *in vitro* in the absence of pyruvate can restore ATP levels to normal, thus completely replacing pyruvate (Patel et al., 2010). ALC treatment in an *in vivo* rat model of SCI maintained mitochondrial respiration, ATP-levels, and the activities of several metabolically important enzymes, among others NADH dehydrogenase (Patel
et al., 2012). The amino acid carnitine itself also plays a role in the metabolism of fatty acids, mainly by transporting acetyl moieties from the fatty acids to the mitochondria for production of ATP in the citric acid cycle. Subsequently, administration of ALC may improve the metabolism of fatty acids in a state of energy depletion (Hagen et al., 1998).

Furthermore, apart from boosting the energy production of the cell, ALC also facilitates the production of the organism’s own antioxidant glutathione. ALC does so by donating one carbon from the acetyl moiety (Aureli et al., 1999, McEwen et al., 2011). Thus, ALC will promote the synthesis of one of the most important reducing agents in the human organism, readily capable of scavenging ROS.

A plethora of preclinical studies showing the beneficial effects of ALC treatment is available. Our group has previously investigated the anti-apoptotic and pro-regenerative properties of ALC in relation to PNI. We have shown that administration of ALC after PNI abolishes sensory neural death, stimulates nerve regeneration, and contributes to reinnervation of the target muscle (Hart et al., 2002, McKay Hart et al., 2002, Wilson et al., 2010).

In regard to SCI, the bioenergetic and antioxidative features of the peptide theoretically make ALC an interesting therapeutic agent in the highly metabolically sensitive and oxidative stress-prone injured spinal cord. As mentioned previously, Patel et al. have demonstrated that treatment maintains the mitochondrial function 24 h following injury, and improves the function of several important metabolic enzymes in rats. The same study showed that administration of ALC twice daily for 7 days after contusion injury to the spinal cord significantly spared the grey matter (Patel et al., 2010). In a follow-up study, the same group confirmed the previous effects on mitochondrial function. Additionally, they found that ALC-treated rats showed significantly decreased volumes of the tissue injured in and around the immediate trauma zone than placebo treated subjects. Furthermore, the sparing of both grey and white matter rostral and caudal to the trauma epicentre was noted, as well as improved locomotive function (Patel et al., 2012). Studies of ALC’s effects on mitochondrial function after SCI have shown a decrease in mitochondrial damage after treatment. ALC treatment also reverses the downregulation of the anti-apoptotic protein Bcl-2 and upregulation of BAX, thereby combating apoptosis (Zhang et al., 2015). The ALC-mediated improvement of motoneuron and sensory neuron survival in the injury zone penumbra, as well as improved motor function, has recently been confirmed (Ewan and Hagg, 2016).
Despite yet not being an approved drug for treatment of CNS disease and injury, the evidence of the beneficial effects of ALC treatment are accumulating. The drug has been used in human studies for a wide variety of degenerative CNS diseases and CNS and PNS injuries. Promising studies have shown positive effects in the treatment of ALS (Beghi et al., 2013), neuropathy (Li et al., 2015), Mb Alzheimer (Palacios et al., 2011), stroke (Zhang et al., 2012b), traumatic brain injury (TBI) (Scafidi et al., 2010), BPI (Zhang et al., 2005) and peripheral nerve injury (PNI).

ALC is generally considered a safe drug, with few adverse effects even at higher dosages. In a human long-term study, pure ALC was administered in dosages up to 1 gram per day for 1 year, with no adverse effects being recorded (Thal et al., 2000). Human studies performed with dosages exceeding 2 grams per day showed good tolerance of the drug (Li et al., 2015). The main reported adverse effects are nausea and vomiting (De Grandis, 1998).

In summary, ALC treatment following SCI ameliorates cell death and tissue destruction, and improves functional outcome in preclinical trials. As such, the compound is of great clinical interest.

2.4.6 N-acetyl-cysteine

N-acetyl-cysteine (NAC) is a thiol and a derivative of the amino acid cysteine. NAC can be synthetized by the organism or administered as a drug orally, intravenously or, in experimental preclinical models, intrathecaly. When ingested orally, NAC is absorbed in the stomach and small bowel, with a bioavailability of 6 to 10% due to first-pass metabolism (Holdiness, 1991). Bound to plasma proteins, NAC is transported to the liver via the portal system, where it is virtually entirely converted to the amino acid cysteine (Cotgreave, 1997, Noszal et al., 2000). The synthetized cysteine then freely passes over biological membranes. Inside the cells, cysteine is the most prominent precursor of glutathione (Atkuri et al., 2007).

From a chemical point of view NAC acts as a direct antioxidant, and readily reduces various radicals (Elbini Dhouib et al., 2016). Furthermore, and perhaps most importantly, NAC is a precursor to glutathione and as such a potential booster of the glutathione levels in the organism. In regard to the central role of glutathione in the defense against free radicals, the contribution of NAC may be very valuable in combating oxidative stress. It has indeed been shown that the loss of glutathione observed in a state of oxidative stress can be reverted by the administration of NAC (Atkuri et al., 2007). Moreover, studies of NAC treatment of various diseases, in which inflammation plays a key role, show that the compound decreases the release
of several inflammatory cytokines. It has for instance been shown that NAC treatment after cerebral ischemia in a rat model showed decrease of the inflammatory cytokines IL-1β and TNFα (Khan et al., 2004).

Our group has previously investigated the effects of NAC treatment following several types of nerve injuries in preclinical animal models. We have shown improved mitochondrial preservation and increased survival of sensory neurons in the DRG after axotomy (Hart et al., 2004, West et al., 2007, Welin et al., 2009). NAC treatment after axotomy has been shown to increase the expression of the anti-apoptotic signaling molecule Bcl-2, and decrease the expression of the pro-apoptotic BAX protein and caspase-3 (Reid et al., 2009). The neuroprotective effects of NAC on motoneurons have also been confirmed, showing significantly increased survival of neurons following ventral root avulsion (Zhang et al., 2005).

In regard to NAC therapy after SCI, new preclinical studies are steadily emerging indicating beneficial effects. It has recently been shown that treatment in rats improves mitochondrial respiration, ameliorates LP, decreases the production of inflammatory cytokines such as IL-6 and TNF-α, decreases leukocyte infiltration, combats apoptotic cell death and decreases demyelination. Furthermore, NAC treatment after SCI is associated with increased tissue sparing and improved long-term motor function (Cavus et al., 2014, Patel et al., 2014, Guo et al., 2015, Gurcay et al., 2016).

NAC is currently clinically approved and abundantly used for several indications. Most prominently it is used as an antidote in acetaminophen intoxication. NAC acts by boosting the liver glutathione levels, glutathione being essential in the conjugation of certain acetaminophen metabolites (Heard, 2008). Furthermore, NAC is used as a mucolytic agent and as a nephroprotectant to prevent contrast-induced injury to the kidneys (Ellis and Cohen, 2009).

In regard to clinical trials, much of the research revolves around potential benefits in the treatment of affective disorders and PNS/CNS injuries. It has been proven to have positive effects in the treatment of bipolar disease (Rosenblat et al., 2016), schizophrenia and compulsive disorders (Berk et al., 2013, Deepmala et al., 2015), among others. Furthermore, NAC has been used both in experimental animals and patients for the treatment of TBI, showing promising results (Hoffer et al., 2013, Eakin et al., 2014).

NAC is considered to have a benign profile of adverse effects. Much like in the case of ALC, the compound is well tolerated even at higher doses and with prolonged treatment. A study in which NAC was administered orally for 4
weeks at a maximum dosage of 3600 mg/day showed no difference in adverse effects compared to placebo (Mardikian et al., 2007). When treating acetaminophen overdose, a total of 21 grams is normally given intravenously in the first 24 h (Selvan et al., 2007). The most common adverse effect with oral administration is gastrointestinal symptoms, while i.v. administration carries a small risk of anaphylactic reactions (Mant et al., 1984, Grandjean et al., 2000).

In conclusion, NAC has been shown to combat several of the key factors of SCI secondary injury, improving functional recovery. The beneficial effects of treatment, in combination with the benign profile of adverse effects, make NAC an interesting possible agent for the treatment of traumatic SCI in humans.
3. Pathophysiology of peripheral nerve injury (PNI)

3.1 Nerve injury types

The severity of a peripheral nerve injury (PNI), and the prospect of functional recovery, is largely dependent on the type and level of injury. According to the generally accepted Seddon’s classification scheme of nerve injuries, peripheral nerve injuries may belong to one of three principal types (Seddon, 1942).

*Neurapraxia* refers to an injury with a transient disruption of nerve conductivity, without disruption of axon continuity, and with preservation of the supportive structures. This injury type is most commonly caused by pressure, and is considered the mildest form of PNI. Neurapraxia is associated with spontaneous restoration of function.

*Axonotmesis* occurs when nerve fibers are damaged, while the supporting perineurium and epineurium are preserved. Among other pathophysiological events, axonotmesis typically induces Wallerian degeneration (see below).

*Neurotmesis* refers to an injury in which the nerve is transected, causing complete detachment from the target organ. While the proximal nerve stump has a potential for regeneration, the reinnervation of the target organ will not occur spontaneously. Surgical nerve repair is necessary for the restoration of function (Lundborg, 2000, Wiberg and Terenghi, 2003). Neurotmesis represents the most severe form of PNI.

3.2 The general pathophysiology of PNI

While neurapraxia is caused by a disturbance in nerve conductivity without significant nerve damage, both axonotmesis and neurotmesis are associated with disturbed nerve integrity. As such, these two types of nerve injury spark a set of well-studied pathophysiological events resulting in nerve degeneration. The events occur in the distal nerve stump, the proximal nerve stump, and the associated spinal cord segments.

The *distal nerve stump* will start undergoing Wallerian degeneration approximately 24 hours after injury, with the process lasting for a total of 5-8 weeks. Wallerian degeneration is associated with the breakdown of the axonal skeleton and membrane, after which the myelin sheath is degraded. In the cases when the target organ is not reinnervated, activated macrophages will clear the axon and myelin debris. The Schwann cells also react to denervation by aligning into a tubular form called the bands of Büngner, intended to help
guide regenerating axons to their target. The purpose of Wallerian degeneration is to optimize the regenerative properties of the distal nerve stump (Stoll and Muller, 1999). In contrast, the proximal nerve stump normally undergoes modest changes that do not extend beyond the most distal node of Ranvier.

The severity of the reaction of motoneurons and glial cells in the spinal cord following neurotmesis is dependent on the age of the subject, the distance from the point of injury to the spinal cord, and whether the nerve was severed or avulsed from the spinal cord. Animal studies have shown that axotomy in newborn animals virtually obliterates the corresponding motoneuron pool, while the same injury causes no motoneuron death in adult subjects (Vejsada et al., 1998, Welin et al., 2008). Axotomy will however always induce changes such as atrophy of motoneurons and loss of dendrites and axons (Carlstedt and Cullheim, 2000). Injuries occurring close to the spinal cord have more severe implications, with injuries proximal to the dorsal root ganglion (DRG), i.e. preganglionic nerve injuries, causing greater changes than those occurring distal to the DRG (i.e. postganglionic nerve injuries) (Novikov et al., 2000, Ma et al., 2001, Jivan et al., 2006).

### 3.3 Brachial plexus injury

Brachial plexus injury (BPI) is a type of peripheral nerve injury afflicting the network of nerves responsible for the motor and sensory functions of the upper limb. Principally, the BPI may be a complication to childbirth or induced by trauma, in the latter case most commonly caused by traffic accidents (Kaiser et al., 2012, Ouzounian, 2014). Anatomically, the brachial plexus is formed by the anterior rami of cervical nerves 5-8 and thoracic nerve 1 (C5-Th1). The cervical roots are devoid of meningeal coating and lack supporting connective tissue, with the nerve being freely movable in the intervertebral foramen. As such, the cervical root entry zone is the weakest point of the nerves, and therefore prone to avulsion (Sakellariou et al., 2014).

Injuries to the brachial plexus are either categorized as being preganglionic or postganglionic. The preganglionic avulsion injury is considered to be the most severe type, and is associated with a poor prognosis.

Avulsion injury occurs when the nerve root is pulled out from its entry zone into the spinal cord. The avulsion injury, when afflicting the ventral root, is the most severe and direct injury that can be inflicted on a motoneuron. As such, ventral root avulsion will cause the most dramatic morphological changes in the spinal cord. Avulsion induces a significant death of motoneurons observable already 1 week after injury, with the majority of loss
occurring after the second and third week (Koliatsos et al., 1994, Hoang et al., 2003). The motoneurons that survive will undergo atrophy, causing 50% degeneration 4 weeks after injury (Novikov et al., 2000), and a total of 90% loss of affected motoneurons in long-term studies (Carlstedt and Cullheim, 2000). The extensive apoptotic death of motoneurons is known to be paralleled by the apoptotic death of interneurons (Oliveira et al., 2002). It is well known from studies of dorsal root avulsion (DRA) that the loss and atrophy of cells in turn will induce a general loss of dendrites, axons and synapses belonging to the cells (Chew et al., 2011). Avulsion also induces a glial response, with the influx of microglial and astroglial cells (Koliatsos et al., 1994, Ohlsson et al., 2006).

Peripheral axotomy, in contrast, is considered the least severe of the postganglionic nerve injuries. As previously mentioned, peripheral axotomy of a nerve in adult subjects does not cause the death of motoneurons (Carlstedt and Cullheim, 2000). Even a very proximal transection of an axon, for example transection of the ventral branch of the seventh cervical spinal nerve (C7), will only cause a loss of 15% of motoneurons after 4 weeks (Jivan et al., 2006). Correspondingly, the effects of injury on interneurons, dendrites, synapses and axons are less pronounced after axotomy than after avulsion injuries, as has been shown previously when studying dorsal root rhizotomy (DRR) (Chew et al., 2011).

From a clinical point of view, BPI caused by the mere extension of the nerve (i.e. neurapraxia) will result in a transient loss of function, with spontaneous recovery of function and no need for surgical intervention. In contrast, a BPI caused by the loss of nerve continuity, if left untreated, will not spontaneously reinnervate the target organ. Both the pre- and postganglionic injury will require surgical restoration of nerve continuity if improvement of function is to be expected. The surgical approach however differs greatly depending on injury type. The loss of nerve continuity in a postganglionic injury can be restored using autologous nerve grafts harvested from the patient’s leg, and may contribute to an improved function. Experimental studies indicate that an early repair of the nerve is associated with increased survival of motoneurons and improved regeneration (Ma et al., 2003, Jivan et al., 2006). Clinical results show that early surgical intervention is associated with a better functional outcome than delayed nerve repair, indicating that BPI should be surgically treated within 2 months for optimal outcome (Jivan et al., 2009). In contrast, the preganglionic avulsion injury cannot be repaired using a nerve graft. Instead, in order to reinnervate the target organ, the transfer of a nerve, for instance the intercostal nerves or the phrenic nerve, can be considered (Bhandari and Maurya, 2014, Sun et al., 2014).
3.3.1 Diagnostics of BPI

The appropriate choice of treatment and its timing is dependent on the availability of good diagnostic tools. Ideally, a diagnostic tool, or set of tools, capable of early and reliable establishment of injury level - i.e. determination whether the injury is pre- or postganglionic - would be of great clinical value. A number of different modalities are currently in clinical use, and modern diagnostics is based on a combination of clinical tests, electrodiagnostics, plain X-rays and different CT and MRI protocols (Sakellariou et al., 2014). Despite advances in radiological diagnostics, no method alone is sufficiently reliable for the determination of injury level. This is especially true for the time early after injury. In certain cases, surgical exploration is required for establishment of diagnosis. Therefore, the need for improved diagnostics of BPI persists.
Specific aims

The overall goal of this thesis was to investigate the role of the antioxidants acetyl-L-carnitine (ALC) and N-acetyl-cysteine (NAC) in the treatment of spinal cord injury. Moreover, a novel approach for the detection of retrograde degeneration in the spinal cord using magnetic resonance imaging (MRI) was developed.

The specific aims were:

- To study the neuroprotective effects of intrathecal ALC and NAC treatment after SCI on tibial motoneurons and glial cells following lumbar spinal cord injury (paper I).

- To investigate the effects of NAC and ALC treatment on descending rubrospinal neurons, secondary degeneration in the trauma zone, and neuroinflammation following cervical spinal cord injury (paper II).

- To elucidate the mechanisms underlying modulation of the microglial response by ALC in vitro (paper III).

- To develop and evaluate a novel non-invasive magnetic resonance imaging (MRI)-based protocol for objective quantification of neuronal degeneration in the spinal cord after ventral root avulsion and peripheral nerve injury (paper IV)
Materials and methods

4. Operative & radiological procedures

4.1 Experimental animals & anaesthetic procedures (papers I, II & IV)

All experiments were performed on adult female Sprague-Dawley rats (10-12 weeks). The experimental procedures and animal care were carried out in accordance with Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes, and approved by the Northern Swedish Committee for Ethics in Animal Experiments. All surgical procedures were performed under general anaesthesia, using a mixture of ketamine (Ketalar, Parke-Davis, Pfizer, New York, NY, USA; 100 mg/kg i.v.) and xylazine (Rompun, Bayer, Leverkusen, Germany; 10 mg/kg i.v.). Following surgery, the rats were given the analgesic Finadyne (Schering- Plough, Denmark; 2.5 mg/kg, s.c.), normal saline (4 ml, s.c.), and the antibiotic benzylpenicillin (Boehringer Ingelheim, Ingelheim am Rhein, Germany; 60 mg, i.m.). Each animal was housed alone in a cage after surgery, and was exposed to 12-h light/dark cycles, with ad libitum access to food and water. In experiments involving in vivo MRI scanning, the rats were anaesthetized using isoflurane (Attane vet®, 1000 mg/g, Oiramal Healthcare, UK), administered through a breathing mask. Respiration and temperature were monitored using a respiration pillow and a rectal probe, respectively (SA Instruments Inc., Stony Brook, USA).

4.2 Experimental models of SCI (papers I & II)

Following either cervical or lumbar laminectomy, the C3 (paper II) or L5 (paper I) spinal cord segment was identified, and the dorsal root entry zone penetrated using a 23 G needle. One blade of a pair of Vannas spring scissors (Fine Science Tools GmbH, Germany) was inserted in the wound, after which spinal cord hemisection was performed using the second blade. Care was taken not to damage the ventral and dorsal roots. The spinal cord was covered with stretched parafilm and a piece of Spongostan®, and the muscle and skin were closed in layers.
4.3 Experimental models of BPI (paper IV)

The experimental animals subjected to preganglionic nerve injury underwent L4 and L5 ventral root avulsion. Following lumbar laminectomy, the L4 and L5 ventral roots were identified. After transection of the roots was performed using a pair of Vannas spring scissors, after which the proximal stump of each transected nerve ventral root was grasped using a pair of jeweller’s forceps. The roots were carefully and slowly pulled in the caudal direction, tangentially to the spinal cord, until the root was ruptured and came out in its entire length. Post mortem, the spinal cord was inspected and both L4 and L5 ventral roots were confirmed to be avulsed from their respective segments.

The experimental animals subjected to postganglionic nerve injury underwent a unilateral left side transection of the sciatic nerve at the upper border of the quadratus femoris muscle. The proximal and the distal stumps were covered with a custom-made blind-ending polyethylene cap, sutured to the epineurium using Ethilon™ 9-0 sutures. This was done to prevent spontaneous regeneration of the nerve.

4.4 Retrograde neuronal labelling using Fast Blue (papers I & II)

In the experiments investigating survival of specific motoneuron pools, retrograde labelling using the non-toxic fluorescent tracer Fast Blue (FB) (EMS-Chemie GmbH, Germany) was performed one week prior to L5 hemisection. Labelling of the tibial neuronal pool in the L4-L5 spinal cord segments (paper I) was performed by transection of the tibial nerve at the level of the popliteal fossa. The proximal nerve stump was placed in a polyethylene tube containing a 2% aqueous solution of the tracer (Novikova et al., 1997b). The tube was sealed using a mixture of Vaseline and silicone grease, preventing leakage of FB. After 2 h of immersion the tube was removed, the nerve stump gently rinsed in normal saline, and the muscle and skin sutured in layers.

Labelling of rubrospinal neurons (paper II) was performed with the help of a small pellet prepared from 1-2 μL of an aqueous solution of FB. The pellet was placed in the lesion cavity caused by the C3 hemisection, and the wound was closed as described in “4.2 Experimental models of spinal cord injury (papers I & II)”. 
4.5 Antioxidant treatment (papers I-II)

The treatment with either N-acetyl-cysteine (200 mg/ml in normal saline; BioPhausia) or acetyl-L-carnitine hydrochloride (75 mg/ml in normal saline; Sigma-Aldrich) commenced immediately after the infliction of hemisection. The appropriate dosages were derived from previous studies (Hart et al., 2002, Zhang et al., 2005, Welin et al., 2009). It has previously been confirmed that vehicle treatment with PBS or saline does not affect the survival of motoneurons following SCI (Novikov et al., 1997, Novikova et al., 1997a, Zhang et al., 2005). The drugs were administered using an Alzet osmotic minipump (Alza Corp., Palo Alto, CA), filled with either ALC or NAC. The pump was implanted subcutaneously in the neck of the rat. Following partial L6 laminectomy, a polyethylene catether (Intermedic PE-60) was implanted in the lumbar subarachnoidal space. The implantation site was covered with Spongostan® and the catheter was fixed to the Si bone using Histoacryl® and to the back muscles with the help of multiple sutures. The proximal end of the catheter was connected to the pump, after which treatment immediately commenced. The speed of infusion corresponded to the dosages of 2.4 mg/day of NAC and 0.9 mg/day of ALC, and continued for 14 days until the pump was replaced with a fresh full pump containing the same solution. The animals subjected to 8 weeks of antioxidant treatment underwent a total of three pump changes.

4.6 MRI image acquisition (paper IV)

All scans were performed on a 9.4 T Bruker BioSpec 94/20 USR system, connected to a mouse heart array coil combined with a 87 mm QUAD resonator coil and running ParaVision® software (Bruker BioSpin Group, Bruker Corporations, Germany). The experimental animals were anaesthetized using isoflurane, as described in “4.1 Experimental animals & anaesthetic procedures (papers I, II & IV)”. In order to establish the position of the animal in the scanner and to identify anatomical landmarks relevant for the planning of the subsequent scans, a series of orientational pilot scans were performed. A coronal scan of the thoracic spine was utilized to establish the position of the 13th rib, with the caudal tip of the first caudally situated DRG, DRG 13, known to correspond to the L4 spinal ventral root entry zone. The obtained images were then used to position a total of 8 image slabs oriented transversely, covering the L4 and L5 segments. Account was taken to the curved shape of the spinal cord, with all the slabs being carefully placed at 90° in relation to the scanned segment.
The data was acquired using a T2-weighted TurboRARE sequence (TR 2000.0 ms, TE 11.0 ms, field of view (FOV) 20x20 mm, matrix 256x256, slice thickness 2 mm, 4 averages). The scans were gated for respiration, with an approximate acquisition time of 35 minutes.

5. Tissue processing and cell culture

5.1 Tissue harvest (papers I, II, & IV)

Following the conclusion of experiments, the animals were deeply anaesthetized with an intraperitoneal injection of sodium pentobarbital (240 mg/kg, Apoteksbolaget, Sweden).

In animals intended for Western blotting (WB), harvest of either the L4-L5 segments rostral to the injury (paper I), or tissue rostral and caudal of the C2 injury site (paper II), was performed. The spinal cord tissue was divided in the sagittal plane, and in the case of the latter study (paper II), the spinal cord was additionally divided transversely, thus separating tissue rostral and caudal from the injury site. Immediately afterwards, the tissue samples were frozen in liquid nitrogen.

Animals intended for cell counts and immunohistochemical analysis (papers I, II & IV) underwent transcardial perfusion with Tyrode’s solution, followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4). Following perfusion, the L4-L6 (paper I), C1-C3 (paper II) and L4-L5 spinal cord segments were harvested, post fixed in the same PFA solution, cryoprotected in 10% and 20% sucrose solution for 2-3 days, and finally frozen in liquid isopentane. Tissue intended for counts of FB-labelled motoneurons (paper I) and rubrospinal neurons (paper II) was cut in 50-µm-thick serial horizontal sections on a vibratome (Leica Instruments, Wetzlar, Germany), mounted on gelatine coated glass slides, air dried, briefly immersed in xylene and covered slipped in dibutyl phthalate xylene (DPX). Tissue intended for immunohistochemical analysis was cut in 16-µm-thick serial sections using a cryomicrotome (Leica Instruments, Wetzlar, Germany), thaw-mounted onto SuperFrost Plus slides (Thermo Fisher Scientific, USA), dried overnight at room temperature, and stored at -85 °C before further processing.

5.2 Western blotting (papers I, II & III)

The spinal cord tissue (papers I & II) was homogenized using buffer (pH 6.9) containing 5 mM Ethylene glycol tetraacetic acid (EGTA), 100 mM Piperazine-N,N’-bis(2-ethanesulfonic acid) (PIPES), 5 mM MgCl₂, 20% (v/v) glycerol,
0.5% (v/v) Triton X-100 and protease inhibitor cocktail (Sigma–Aldrich). Sample protein levels were quantified using a detergent compatible protein assay (Bio-Rad, Hercules, CA, USA). The samples to create the lysates were either run individually (paper I) or pooled together from 4-5 animals (paper II). The samples were denatured at 95 °C and transferred to a sodium dodecyl sulphate-polyacrylamide gel. A total of 10 µg (paper I) or 20 µg (paper II) of protein was used for each sample. Following the transfer of protein to a nitrocellulose membrane, the blots were blocked with 5% (w/v) non-fat milk or 1% bovine serum albumin (BSA) (w/v) in Tris buffer saline with Tween (TBST). After blocking, the blots were incubated with mouse anti-GFAP antibody (1:500; NeoMarkers, USA), mouse anti-OX42 antibody (1:200; Santa Cruz Biotechnology Inc, Dallas, TX, USA), mouse anti-ED1 antibody (1:300; Abcam, Cambridge, UK), rabbit anti-caspase 3 antibody (1:1000; Cell Signalling Technology, Danvers, MA, USA), rabbit anti-BAX antibody (1:200; Santa Cruz Biotechnology Inc, USA), and the loading control rabbit anti-beta tubulin (1:5000; Abcam, Cambridge, UK) at 4 °C overnight. After incubation the blots were washed in TBST 6x5 min and incubated with either mouse or rabbit IgG HRP-conjugated secondary antibody (Cell Signalling Technology, Danvers, MA, USA) for 1 h. Following a series of 6 x 5-min washes in TBST, the blots were exposed to ECL reagent (GE Healthcare, Little Chalfont, UK) for 1 min and developed onto Kodak XPS films. The blots were re-probed with anti-actin (1:5000; Millipore, Sweden) as a loading control (paper I). After development, the films were scanned using an Epson photo scanner.

For the in vitro experiments (paper III), cells were seeded in 6-well plates at a density of 100,000 cells per well. After attachment to the plastic surfaces the cells were serum starved overnight. After serum starvation, the cells were either treated with fresh serum-free medium or serum-free medium containing ALC (pH adjusted to pH 7.6). At different time points after exposure to ALC, the cultures were washed in PBS, detached from the plastic with a cell scraper, and suspended in lysis buffer containing 5 mM EGTA, 100 mM PIPES, 5 mM MgCl₂, 20% (v/v) glycerol, 0.5% (v/v) Triton X-100, protease inhibitor cocktail and phosphatase inhibitor cocktail (Sigma-Aldrich). Sample protein concentrations were quantified with the DC protein assay (Bio-Rad, Sweden). For each sample, 30µg of protein was denatured at 95°C, and loaded onto gels made from 10% Tris–HCl polyacrylamide for SDS-PAGE. Protein transfer to a nitrocellulose membrane was performed, after which the blots were blocked with 5% (w/v) bovine serum albumin in Tris-buffered saline with Tween-20. The blots were then incubated with either anti-Phospho-PI3 Kinase p85 (Tyr458)/p55 (Tyr199) antibody (Cell Signalling Technology, 1:1000 dilution) or anti-Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody (Cell Signalling Technology, 1:1000 dilution) overnight at 4°C. After washings, membranes were incubated with anti-rabbit HRP-conjugated secondary
antibodies (Cell Signalling, 1:2000 dilution). Chemiluminescence signals were initiated by addition of ECL substrates (GE Healthcare, Sweden) and images captured using an Odyssey Imaging System (LI-COR Biosciences GmbH, Germany).

5.3 Cell culture (paper III)

Culture of the human microglial cell line (HMC3) was performed in growth medium consisting of Minimum Essential Medium α (MEM-α) supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin (all from Invitrogen) on tissue culture flasks (Nunc Brand). At passages 3-6, cells were passaged to new culture flasks by using trypsin/EDTA and seeded at a density of 1,000 cells/cm². The cell cultures were either untreated or treated with 25-30 mM ALC diluted in growth medium, which was pH-adjusted to pH 7.6. At 3, 6 and 9 days following plating, the cells were again trypsinized and counted using a haemocytometer.

5.4 qRT-PCR (paper III)

Total RNA isolated using a Qiagen RNeasy kit was converted into complementary DNA (cDNA) using the iScript™ cDNA synthesis kit (Bio-Rad) and the polymerase chain reaction (PCR) was performed using SsoFast™ EvaGreen supermix (Bio-Rad) in a CFX96 Optical Cycler followed by analysis using the CFX96 manager software (Bio-Rad). The reactions were optimised and processed according to the manufacturer with initial denaturation/DNAPolymerase activation at 95°C for 30s followed by PCR: 95°C for 5s, variable annealing temperature (as below) for 5s, and 65°C for 5s repeated for 40 cycles. Data were calculated as relative expressions according to the ΔΔC(t) principle. Primers were manufactured by Sigma, UK: bdnf forward primer 5'-AGAGGCTTGACATCGTGCTG-3’, reverse primer 5'-CAAAGGCACCTGACTGAGTC-3’ with annealing at 64.4°C; gdnf forward primer 5'-CACCAGATAACAAATGGGTGC-3’, reverse primer 5'-CGACAGGTTCATCGAAGAGGC-3’ with annealing at 65.8°C; nfg forward primer 5'-ATACAGCGGGAACACACTCA-3’, reverse primer 5'-GTCCACAGTAATGTGGCGGGTC-3’ with annealing at 65.1°C; il-6 forward primer 5'-AGAGACTTGTGAGTGGAAA-3’, reverse primer 5'-CAGGGGTGGTTATTGCATCT-3’ with annealing at 63.8°C and the reference gene gapdh forward primer 5'-GAAGGTGAGTGGAGTGGAGT-3’ and 5'-CAAGCCTTCCCCGTCTCACG-3’ with annealing at 59°C.
5.5 Enzyme linked immunosorbent assay (ELISA) (paper III)

Cells were seeded at a density of 100.000 cells/300µl in 24-well plates and maintained for 3 days in control growth medium or medium supplemented with 25 mM ALC (pH adjusted to pH 7.6). Thereafter, ELISAs were performed in order to investigate the presence of BDNF and GDNF proteins (R&D Systems, USA).

The cells intended for the multi-analyte ELISA array were seeded in T75 flasks (Thermo Fisher Scientific, Roskilde, Denmark) at a density of 10.000.000 cells/10 ml and maintained for 2 days in control growth medium or medium supplemented with pH adjusted 30 mM ALC. The conditioned medium was used in a multi-analyte ELISAs to investigate the effects of ALC treatment on various inflammation-associated cytokines and chemokines (Qiagen, Sweden). The ELISAs were performed in accordance with the instructions provided by the manufacturer.

5.6 Immunohistochemical stainings (papers I, II & IV)

Serial tissue sections were processed for the demonstration of neuron markers and glial cells. After blocking with normal serum, the following primary antibodies were applied: mouse anti-neuronal-nuclei antibody (NeuN; 1:200, Chemicon) (paper IV), mouse anti-microtubule-associated protein-2 (MAP2; 1:100, Chemicon) (paper I), mouse anti-microtubule-associated protein-2 (MAP2; 1:200, Chemicon) (paper IV), rabbit anti-synaptophysin (SYN; 1:500, Dako) (papers I & IV), rabbit anti-glial fibrillary acidic protein (GFAP; 1:1000, Dako) (papers I & IV), monoclonal antibodies reacting with C3bi complement receptors (OX42; 1:200-1:250, Serotec) (papers I, II & IV), a cocktail of monoclonal antibodies reacting with 68 kDa, 160 kDa and 200 kDa neurofilament proteins (NF; 1:200; Zymed Laboratories) (paper I), rabbit anti-serotonin antibodies (5-HT;1:500, Sigma–Aldrich) (paper IV). Following rinsing in PBS, secondary goat anti-mouse and goat anti-rabbit antibodies Alexa Fluor® 488 and Alexa Fluor® 568 (1:300; Molecular Probes, Invitrogen) were applied for 1 h in darkness and at room temperature. All the slides were cover slipped with ProLong mounting media containing 4’,6-diamidino-2-phenylindole (DAPI) (Invitrogen, Carlslsbad, CA, USA). The staining specificity was tested by omission of primary antibodies.
6. Analytical procedures

6.1 Counts of Fast Blue labelled motoneurons (papers I-II)

Identification of FB labelled tibial motoneurons was carried out using a Leitz Aristoplan fluorescent microscope equipped with UV filter block A (Leica, Germany) with the excitation wavelength range between 340 & 380 nanometers and an emission wavelength of 430 nanometers (paper I). The tracer is found in the cell bodies and proximal dendrites, but does not stain the nuclei (see paper I, Figure 1A, insertion). In sections exhibiting very strong cytoplasmic labelling causing the masking of unstained nuclei, the particular sections were left to fade for 2-3 min, after which they were re-examined. The motoneurons were counted manually at 250x final magnification in every section, yielding a total number of labelled profiles. The labelled profiles were counted within a 2 mm distance both rostral and caudal of the middle of the hemisection cavity. This was done to establish whether a difference in reaction could be seen depending on which side of the lesion site the motoneurons are found. No correction was done for split nuclei due to the nuclear diameters being small compared to the slice thickness. Our group has previously shown the validity of this method of assessing retrograde neural cell death. We have shown that it yields counts similar to those obtained using reconstructions from serial sections (Novikov et al., 1997) and the physical dissector (Ma et al., 2001).

FB labelled rubrospinal neurons (paper II) were identified in the brainstem at a final magnification of 250x. As when counting labelled tibial motoneurons, the counts were not corrected for split nuclei.

6.2 Immunohistochemical analysis (papers I, II & IV)

The immunostained preparations were examined using a Leitz Aristoplan fluorescent microscope (paper I) or a Nikon Eclipse 80i microscope (paper II & IV) (Nikon Instruments Europe B.V.). Images of the immunostained sections were captured using either a Nikon DXM1200 digital camera (paper I) or a Nikon DS-U2 digital camera (paper II & IV).

Changes in the area occupied by NeuN positive neurons in the ventral horn (paper IV), presence of OX42 positive microglial cells (paper I, II & IV), PanNF positive nerve fibers, MAP2 positive dendritic branches, SYN positive
synapses and GFAP positive astroglial cells (papers I, II & IV) was studied in the ventral horn of the L4 & L5 segments (papers I & IV) or the ventral horn of the C2 segment (paper II). The sprouting of 5-HT positive axons was studied directly by the lesion site (paper II). When assessing the presence of NeuN-positive neurons, the images were captured at 4x final magnification, capturing the whole ventral horn (paper IV). When assessing all other markers, 40x final magnification was used (papers I, II & IV). Pictures depicted the spinal cord segments affected by PNI (paper IV), the immediate vicinity of the SCI lesion site (paper II) or the area 800-1600 µm rostral to the SCI lesion (paper I). Damaged sections were completely excluded, while artifacts and large blood vessels were excluded when outlining the region of interest (ROI). The area occupied by the immunostained profiles was calculated using Image-Pro Plus software (Media Cybernetics, Inc., USA), yielding the percentual area occupied by immunostaining. A standardized protocol was employed, with manual outlining of the ROI (papers I, II & IV).

The images of the NeuN-positive ventral horn neuron pool (paper IV) were assessed using the same software and the identical standardized protocol that was employed when assessing the MRI images (see “6.5 MRI image analysis (paper IV)”). The identical anatomical landmarks were used to outline the ventral horn in the histological preparations. The ratio between the area of the injured and healthy side was calculated correspondingly.

### 6.3 Western blot analysis (papers I, II & III)

For paper I, the scanned films were analysed using Scion Image (Scion Corporation, Maryland, USA) in order to perform peak area integration, determining the area of each band in pixel units. The optical density was expressed as a ratio of the corresponding signal for β-actin. For paper II, a qualitative interpretation of the subsequent images was performed. For paper III, qualitative assessments of the WB was made.

### 6.4 Image processing (papers I-IV)

For the preparation of figures for publication, the captured images were resized, grouped in a single canvas and labelled using the Adobe Photoshop CS4 software (Adobe Systems; San Jose, CA, USA). Adjustment of contrast and brightness were performed in order to optimize image clarity.
6.5 MRI image analysis (paper IV)

The images obtained were exported to the Digital Imaging and Communications in Medicine (DICOM) format from ParaVision®, converted to high quality TIFF format pictures and assessed using Image-Pro Plus software (Media Cybernetics, Inc., USA). Initially, a line was drawn running through the anterior spinal artery, the central canal of the spinal cord, and through the posterior spinal vein, dividing the spinal cord in a right and left side. Another line was then drawn, running in a lateral direction, from the central canal, positioned at a 90° angle to the first line. These two lines served as references, produced the antero-posterior and medial-lateral border of the left and right ventral horn. Utilizing the lines, the ventral horns on both sides were outlined, and the area on each side was measured (Fig. 4). The ratio between the area of the injured and uninjured side was subsequently calculated.

Fig. 4 The yellow line is drawn through the anterior spinal artery, the central canal and posterior spinal vein, creating the left-right border. Another line, running at a 90° angle to the yellow line and starting from the central canal, comprises the anterior-posterior border. Measurements are performed in the same manner in histological images (left) and MRI images (right).

6.6 Statistical analysis

One-way analysis of variance (ANOVA), followed by a post hoc Newman-Keuls Multiple Comparison Test was used to determine statistical differences between the experimental groups in paper I and II. In paper III and IV, the statistical differences between groups were established using the paired and unpaired t-test, respectively. Significance was set as *p<0.05, **p<0.01, ***p<0.001. All statistical analyses were performed using the statistical software Prism® (GraphPad Software, Inc; San Diego, CA, USA).
Results

7. Effects of antioxidant treatment after SCI

7.1 Survival of spinal motoneurons (paper I)

The effects of NAC and ALC treatment on neuronal survival were evaluated by pre-labelling of tibial motoneurons using the fluorescent dye FB. The effects of antioxidant treatment (4 weeks) on the survival of motoneurons in the vicinity of the trauma zone were investigated. In congruence with previous findings, all FB-labelled tibial motoneurons were found in the L4–L6 spinal cord segments (Swett et al., 1986, Novikova et al., 1996), forming a 5.3±0.3 mm long cell column. In uninjured control animals, at 1 week after the application of tracer to the cut peripheral nerve, the tibial motoneuron pool contained 1656±23 labelled cell bodies with clearly visible primary and secondary dendrites (paper I Fig. 1A). Hemisection of the spinal cord at L5 lumbar level induced significant death of motoneurons. After 4 weeks only 62% of the labelled motoneurons remained (paper I Fig. 1B and Fig. 2A). The counting of labeled neurons within a 2 mm distance rostral and caudal to the lesion site showed an increase in the rate of degeneration in motoneurons rostral to the injury zone (rostral: 233±33 motoneurons; caudal: 358±32 motoneurons; mean±SEM; p<0.05). Furthermore, surviving motoneurons lost FB labelling in their dendritic branches, and were surrounded by numerous small FB-positive cells, probably representing activated microglial cells (paper I Fig. 1B). Treatment with NAC resulted in 78% survival (paper I Fig. 2A) of the tibial motoneurons. Labelling of primary dendrites was partially preserved, and the number of microglia-like cells labelled with FB was decreased (paper I Fig. 1C). Treatment with ALC similarly induced an increased survival of tibial motoneurons (80% survival, paper I Fig. 1D and Fig. 2A). The preservation of FB labelling in primary and secondary dendrites was not equality pronounced.

7.2 Survival of rubrospinal neurons (paper II)

The survival of rubrospinal neurons was evaluated by pre-labelling with FB. Long-term (8 weeks) effects on the survival of rubrospinal neurons projecting caudal to the spinal cord injury zone were investigated. In the control group of uninjured animals 1 week after Fast Blue labelling, the rubrospinal neuronal pool contained 3533±171 labelled cells mean±S.E.M., paper II Fig. 2A, F). Eight weeks after SCI, 61% of labelled rubrospinal neurons remained (paper II Fig. 2B, F). Additionally, plenty of small FB-positive profiles, resembling microglial cells, were observed in the red nucleus (paper II Fig. 2E). ALC treatment
significantly ameliorated cell death, resulting in an 88% neuron survival (P<0.01; Fig. 2D, F). The effect of NAC therapy was slightly weaker with the survival of 80% of the neurons (P<0.05; Fig. 2C, F). Both NAC and ALC treatment improved the preservation of Fast Blue labelling in the proximal dendritic branches (paper II Fig. 2C, D).

7.3 Expression of apoptotic markers (paper II)

The short-term effects (2 weeks) of antioxidant treatment on the expression of apoptotic markers in the spinal cord lesion site after C3 hemisection were studied. Tissue harvested from the cervical spinal cord level was analyzed for the expression of the apoptotic markers BAX and caspase 3, using WB. The samples representing each group (CONT, SCI, NAC, and ALC) were made from pooled samples created from tissue that was harvested from four to five animals. Antioxidant therapy decreased the expression of the pro-apoptotic protein BAX (paper II Fig. 1). Both antioxidants also decreased the levels of caspase 3 in the tissue (paper II Fig. 1). Treatment did however not seem to exert an equally strong effect on the expression of caspase 3 as it did on the expression of BAX.

7.4 Axonal and dendritic degeneration and axonal sprouting (paper I & II)

The effects of antioxidant treatment on the degeneration of MAP2-positive dendrites in the vicinity of the trauma zone was studied 4 weeks following L5 hemisection (paper I). Preparations immunostained for MAP2 and synaptophysin in the L4–L5 spinal segments were quantified. In control animals approximately 36% and 9% of the ventral horn neuropil was occupied by dendritic branches and synaptic boutons, respectively (paper I Fig. 2B,C and Fig. 3 A–C). Presynaptic synaptophysin labelling was found around neuronal cell bodies and proximal dendrites (Paper I Fig. 3C), while the ventral horn neuropil displayed a more diffuse staining pattern. Spinal cord injury decreased MAP2 and synaptophysin immunostaining by 39% and 47%, respectively (P<0.05; paper I Fig. 2B,C and Fig. 3D,E). Furthermore, a reduction of synaptophysin labelling surrounding motoneuron cell bodies and proximal dendrites was observed (paper I Fig. 3F). NAC therapy improved MAP2 and synaptophysin immunoreactivity to 89% and 107% of control values, respectively (P<0.05; paper I Fig. 2B,C and Fig. 3G,H). Treatment with ALC showed similar effects on dendritic branches and synaptic boutons, with return to 76% and 111% of control MAP2 and synaptophysin staining, respectively (P<0.05; paper I Fig. 2B,C and Fig. 3J,K). Treatment did not to
recover the pattern of synaptophysin labelling around cell bodies typically found in uninjured subjects (paper I Fig. 3I,L).

The effect of antioxidant treatment on the sprouting of PanNF-positive axons close to the trauma zone 4 weeks after spinal cord hemisection was evaluated (paper I). In control animals, the area of the ventral horn occupied by PanNF-positive axons was approximately 14% (paper I Fig. 2D and Fig. 4A). Spinal cord hemisection decreased PanNF immunostaining by a third (P<0.05; paper I Fig. 2D and Fig. 4D). NAC therapy restored pan-neurofilament immunostaining to normal values (104%, P<0.05; Fig. 2D and Fig. 4G), whereas ALC was somewhat less effective (86% recovery; P<0.05; paper I Fig. 2D and Fig. 4J).

The sprouting of 5HT-positive raphaespinal axons in the immediate vicinity of the trauma zone 8 weeks following C3 hemisection was also investigated (paper II). In untreated subjects only a few 5HT-positive axons grew from the rostral spinal cord into the trauma zone after hemisection (paper II Fig. 3A, B). NAC and ALC treatment induced the arborization of numerous raphaespinal axons in the trauma zone. The axons did however not enter the distal part of the spinal cord (paper II Fig. 3C–F). The density of raphaespinal terminals in the ventral horn of the C2 segment rostral to the lesion site was assessed. Quantification showed that SCI induced a significant, twofold increase of the area occupied by raphaespinal terminals (P<0.05; paper II Fig. 4I, A, B). Treatment with NAC and ALC further stimulated the sprouting of axons rostral to the lesion site, and resulted in a 4.5- and fivefold increase of the area occupied by axonal terminals, respectively (P<0.001; paper II Fig. 4I, C, D).

7.5 Effects of antioxidants on glial cell reactions (paper I & II)

The short-term results (2 weeks) of antioxidant therapy on the expression of neuroinflammation-associated markers found in the spinal cord lesion site after C3 hemisection were studied (paper II). WBs showed an ameliorating effect of NAC and ALC treatment on the neuroinflammatory reaction. A decreased expression of both ED1 (a marker of activated microglia/macrophages) and OX42 was found in animals undergoing antioxidant treatment, compared to untreated animals (paper II Fig. 1).

WB analyses of L4-L5 spinal cord segments rostral to the lesion site 4 weeks after L5 hemisection were studied in regard to the mentioned neuroinflammatory markers (paper I). Expression of ED1 and OX42 were
barely detectable in tissue from uninjured control animals, while they were up-regulated following spinal cord injury (P<0.001; paper I Fig. 5). Antioxidant treatment significantly down-regulated the expression levels of ED1 (P<0.001) and OX42 (P<0.05 for NAC and P<0.01 for ALC). The expression of OX42 was also investigated in histological preparations of the L4-L5 segments rostral to the lesion site 4 weeks after injury (paper I). In control rats, the area of the ventral horn occupied by OX42-positive microglial cells was approximately 4% (paper I Fig. 2F and Fig. 4C). Spinal cord hemisection increased the OX42 immunoreactivity dramatically in the L4–L5 ventral horn neuropil rostral to the injury site (paper I Fig. 2F and Fig. 4F; P<0.05; 576%). Both NAC and ALC treatment significantly decreased the OX42 immunoreactivity in the ventral horn (P<0.05; paper I Fig. 2F and Fig. 4I, L).

A long-term investigation (8 weeks) of the neuroinflammatory modulating properties of treatment after spinal cord injury was performed in animals undergoing C3 hemisection (paper II). Investigation of immunohistological preparations of tissue close to the trauma zone revealed that hemisection caused a dramatic, almost 40-fold increase in the area occupied by OX42-positive microglia in the ventral horn (P<0.001; paper II Fig. 4J, E, F). Treatment with NAC and ALC attenuated the hemisection-induced microglial response. Both antioxidants decreased the OX42-immunoreactivity by approximately 70% (P<0.001; paper II Fig. 4J, G, H).

The possible effects of antioxidant treatment on the astroglial component of the scar 4 weeks after L5 hemisection were investigated using WB (paper I). Spinal cord hemisection resulted in an up-regulation of GFAP compared to tissue from corresponding segments in uninjured animals (P<0.01; paper I Fig. 5). Antioxidant treatment did not influence the protein levels (P>0.05; paper I Fig. 5). Further investigation of the immunoreactivity for GFAP-positive astroglial cells in the L4-L5 segments rostral to the lesion site was performed. In uninjured control rats roughly 7% of the ventral horn neuropil was occupied by GFAP-positive astroglia (paper I Fig. 2E and Fig. 4B). After hemisection, a dramatic increase in the presence of GFAP-positive astroglia was found in the ventral horn (P<0.05; paper I Fig. 2E and Fig. 4E). Antioxidant treatment did not affect the immunoreactivity of GFAP-positive astroglial cells in the ventral horn neuropil (paper I Fig. 2E and Fig. 4H, K).
8. Effects of ALC treatment on cultured human microglia (paper III)

8.1 The effect of ALC on the proliferation of HMC3 cells

The HMC3 cells (paper III Fig. 1A) were plated either without (control) or with the addition of 25 mM of pH-adjusted ALC (+ALC) to the cell cultures. No difference in the rate of cell proliferation was observed between the groups at 3, 6 and 9 days after the plating of cells (p>0.05; paper III Fig. 1B).

8.2 Effects of ALC on the production of neurotrophic factors by HMC3 cells

The effects of ALC addition to microglial transcription of the neurotrophic factors BDNF, GDNF, NGF and IL-6 was studied using qRT-PCR. Compared to control cells (control), cells treated with ALC (+ALC) showed a significantly increased transcription of mRNA for BDNF (p<0.01; paper III Fig 2A), GDNF (p<0.001; paper III Fig. 2B), NGF (p<0.01; paper III Fig. 2C) and IL-6 (p<0.01; paper III Fig. 2D). The increase in the transcription of mRNA for IL-6 after stimulation with ALC was particularly strong, exhibiting a 33-fold increase compared to control cells.

The secretion of the neurotrophic factors by HMC3 cells after ALC stimulation was investigated using ELISA. The analysis revealed a 2.5-fold increase in BDNF secretion after addition of ALC, compared to the control group (p<0.05; paper III Fig. 3A). The secretion of GDNF was not significantly changed in the treated group, compared to the control group (p>0.05; paper III Fig. 3B). A multi-analyte ELISA was performed in order to investigate the effects of ALC treatment on the microglial secretion of the most common inflammation-associated cytokines, including IL-6. The analysis showed that ALC treatment strongly increased the secretion of IL-6 compared to the control group (paper III Fig. 3C). No effects of ALC treatment on the secretion of the other investigated cytokines was observed. Most notably, no effect of treatment was seen on the secretion of IL-1β and TNF-α, the two cytokines normally secreted together with IL-6 as part of the typical inflammatory response (paper III Fig 3C).
8.3 The effect of ALC on phosphorylation of phosphoinositide 3-kinase (PI3K)

The possible activation of PI3K induced by ALC was investigated. The addition of ALC to cell cultures of human microglial cells induced a rapid increase in the phosphorylation of PI3K. The increase was observable after 10 and 20 minutes, returning to basal levels by 24 hours. There was no effect of ALC on another signaling pathway, involving MAPK (paper III Fig. 4).
9. Retrograde degeneration after PNI (paper IV)

9.1 Axonal terminals and dendrites

The effects of axotomy and ventral root avulsion on dendrites, synaptic boutons, and axons belonging to the neurons of the ventral horn were investigated. This was performed by immunohistochemical staining with MAP2, SYN, and PanNF, respectively.

Following axotomy, a decrease was observed in the area occupied by MAP2 positive dendrites on the injured side compared to the non-injured side (p<0.01). On the uninjured side, MAP2 antibody staining occupied about 19% of the ventral horn, while the staining occupied approximately 15% of the ventral horn area on the injured side (paper IV Fig. 2A). In contrast, following ventral root avulsion, a significant and strong decrease in the presence of MAP2-positive dendrites on the injured side compared to the uninjured side was observed in the ventral horn (p<0.001; paper IV Fig. 2A). MAP2 staining occupied 23% of the ventral horn of the uninjured side, while MAP2 occupied 5% of the area on the side of injury, indicating close to a five-fold decrease in dendrites after avulsion. The density of synapses after ventral root avulsion was studied.

The possible changes in the density of synapses after nerve injury were studied by staining for SYN. Ventral root avulsion caused a significant decrease in the density of SYN-positive synapses in the ventral horn of the injured side compared to the non-injured side (p<0.001; paper IV Fig. 2B). The staining occupied 3% of the ventral horn area of the former, and 7% of the latter (paper IV Fig. 2B). Following axotomy, no significant difference in the density of synaptophysin positive synapses was observed (p>0.05; paper IV Fig 2B).

Changes in the density of axons were investigated by analysis of the density of PanNF-positive axons in the ventral horn. Axotomy induced no statistically significant difference in the density of PanNF-positive axons when comparing the axotomised and non-injured side (p>0.05; paper IV Fig 2C). In animals that had undergone ventral root avulsion, a significant decrease of PanNF-positive axons was found on the injured side compared to the uninjured side (p<0.001; paper IV Fig. 2C), with the staining covering about 1% of the ventral horn of the former and approximately 6% of the latter (p<0.001; paper IV Fig. 2C).
A comparison was also made between the ventral horn on the uninjured side after ventral root avulsion and the uninjured side after axotomy. Ventral root avulsion caused an increased dendrite (p<0.05; paper IV Fig. 2A) and axon (p<0.05; paper IV Fig. 2C) density, compared to axotomy. The finding indicates that the more severe ventral root avulsion injury causes the onset of a sprouting reaction, which was not observed after the less severe axotomy injury.

9.2 Glial cell reactions

The effects of axotomy and ventral root avulsion on the density of microglial and astroglial cells in the ventral horn were studied by staining for OX42 and GFAP, respectively. We found that axotomy induced an almost four-fold increase in the presence of OX42-positive microglia in the ventral horn of the injured side compared to the non-injured side (p<0.01; paper IV Fig. 3A). Ventral root avulsion caused a significant (p<0.001; paper IV Fig 3A), 10-fold, increase in the presence of OX42-positive microglia in the ventral horn on the side of avulsion, compared to the uninjured side.

Study of the astroglia reaction after axotomy showed a significant increase in the presence of GFAP-positive astroglial cells on the injured side compared to the uninjured side (p<0.001; paper IV Fig. 3B). Astroglial cells covered about 2% of the ventral horn of the uninjured side compared to roughly 3% on the injured side (paper IV Fig 3B). Ventral root avulsion induced a strong and statistically significant (p<0.001; paper IV Fig. 3B) increase in the density of GFAP positive astroglial cells in the ventral horn of the injured side, compared to the non-injured side. The density in the injured ventral horn was about 7%, compared with 2% for the uninjured side (paper IV Fig 3B).

9.3 The ventral horn area on histological preparations

The neuronal pool of the ventral horn was assessed by measurement of the area containing NeuN-positive neurons. A relative ratio of shrinkage was calculated for every animal. This was done in the same way as when calculating the ventral horn size in MRI images of the corresponding spinal cord segments (see "6.5 MRI image analysis (paper IV)"). In rats subjected to sciatic axotomy, no statistically significant shrinkage in the size of the neuron pool was detected 4 weeks after injury (p>0.05; paper IV Fig 1B & 1E). Subjects undergoing ventral root avulsion exhibited a shrinkage of roughly 44% on the injured side compared to the non-injured side (paper IV Fig 1C & 1E). Calculation of the size ratio of the neuron pool showed a strong statistical
difference between animals that underwent axotomy, compared to animals that underwent ventral root avulsion (p<0.001; paper IV Fig. 1E), with the latter exhibiting a much smaller area ratio.

9.4 The ventral horn area on MRI

Sciatic nerve axotomy caused no statistically significant difference in the ventral horn size when comparing the injured side with the contralateral side after 4 weeks (paper IV Fig. 1D). In rats subjected to ventral root avulsion, a significant difference was found between the injured and the non-injured side (p<0.001), with about 34% shrinkage of the ventral horn area on the injured side compared to the uninjured side (paper IV Fig. 1D). The ventral horn area ratio was statistically different when comparing subjects undergoing axotomy with those subjected to avulsion (p<0.001; paper IV Fig. 1 D). This indicates that the two different types of injuries can be distinguished from each other using MRI.

9.5 Comparison of histological findings and MRI measurements

Measurements of the size of the ventral horn area occupied by neurons in histological preparations was performed in an identical matter as measurements of the ventral horn grey matter on MRI images. This enabled comparison of the injured/uninjured area ratio yielded by the two methods after both axotomy and ventral root avulsion. Following axotomy, no significant difference in ratio was found between the two methods (p>0.05; paper IV Fig. 3C). Comparing the area ratio following ventral root avulsion, we found a significant difference (p<0.01; paper IV Fig. 3D), with the ratio calculated from MRI image measurements being higher than calculations based on images of histological preparations.
Discussion

10. Antioxidant treatment following SCI

10.1 Neuroprotective effects of antioxidant treatment after SCI (papers I & II)

SCI causes the loss of spinal motoneurons and glial cells close to the trauma zone (Dusart and Schwab, 1994, Grossman et al., 2001, Beattie et al., 2002, Zhang et al., 2012a), as well as atrophy and death of ascending and descending neurons projecting above and below the injury site (Kwon et al., 2002, Deumens et al., 2005). A minority of cells will die due to necrosis in the immediate aftermath of the injury. The main loss of neurons and glial cells will occur through apoptosis (Donnelly and Popovich, 2008).

It is well known that the mitochondria are intimately involved in the triggering of apoptosis of neurons and glial cells following SCI (Sullivan et al., 2005, Merenda and Bullock, 2006, McEwen et al., 2011). Oxygen and energy deprivation, ROS formation and oxidative stress are known triggers of mitochondria-mediated apoptosis (Lin and Beal, 2006, Jia et al., 2016). Not surprisingly, various antioxidant treatments combating mitochondrial dysfunction and ROS formation have been shown to exert anti-apoptotic actions following SCI (Jia et al., 2012).

The presented studies show the anti-apoptotic actions of the antioxidants NAC and ALC on neurons following SCI. Treatment contributed both to the survival of spinal motoneurons and motoneurons belonging to the descending motor pathways projecting caudal to the injured segments. Moreover, the anti-apoptotic effects were observable both at an early (2 weeks) and a later time point (8 weeks).

Both ALC and NAC exert much of their biochemical actions by combating detrimental mitochondrial processes. ALC has the ability to ameliorate energy deprivation by acting as an energy substrate, boost ATP production and stabilize the mitochondria after SCI (Patel et al., 2010, McEwen et al., 2011, Patel et al., 2012). Furthermore, ALC increases the levels of glutathione, improving ROS defense (Aureli et al., 1999, McEwen et al., 2011). NAC exerts similar effects, acting as a direct antioxidant as well as boosting glutathione levels (Atkuri et al., 2007, Elbini Dhouib et al., 2016). In relation to SCI-induced apoptosis in the spinal cord, it is well known that decreasing levels of
ATP and glutathione are paralleled by an increase of apoptotic proteins, such as caspase 3 (Jia et al., 2016). Indeed, various ROS such as $O_2^{-}$ and $H_2O_2$, are important initiators of the apoptotic cascade inducing the transcription of, among other apoptotic proteins, the caspases (Mates and Sanchez-Jimenez, 2000, Haddad, 2004).

Investigation of potential mechanisms underlying apoptotic cell death after SCI revealed that ALC and NAC treatment alter the balance between the anti-apoptotic protein Bcl-2 and the pro-apoptotic BAX protein. The two proteins act antagonistically, mainly through the apoptosis-mediating caspase 3 (Adams and Cory, 1998, Rudel, 1999). An increase in BAX will increase the permeability of the mitochondrial membrane by formation of pores. Subsequently, cytochrome C and ROS are released from the mitochondria, and activate different caspases (Benn and Woolf, 2004), particularly acting on caspase 3 (Putcha and Johnson, 2004). Caspase 3 is crucial for the initiation of the apoptotic cascade in the nervous system (Springer et al., 2001, Snigdha et al., 2012) as well as elsewhere in the organism (Porter and Janicke, 1999), being capable of triggering extensive damage and degeneration. Oxidative stress and mitochondrial dysfunction have been shown to be tightly linked to the activation of BAX and caspase 3, as well as other executors of apoptosis (Hagberg, 2004, Lin et al., 2012). Indeed, various aspects of the secondary degeneration after SCI – LP, increased calcium levels and iron-catalysed oxidant production – all promote the release of pro-apoptotic proteins from mitochondria (Fiskum et al., 2003, Belizario et al., 2007).

An alteration of the activity of apoptotic proteins after SCI, with a decrease of BAX and caspase 3, may at least in part explain the improved survival of central neurons observed following ALC & NAC treatment. This is well in line with previous observations that several different antioxidants, among others melatonin and vitamin E, are capable of ameliorating apoptosis by combating oxidative stress (Kowluru and Koppolu, 2002, Onur et al., 2004, Wu et al., 2005). ALC has previously been shown to reduce apoptosis by decreasing both BAX (Onur et al., 2004) and caspase 3 (Pillich et al., 2005, Di Cesare Mannelli et al., 2007, Busquets et al., 2012). NAC has similarly been shown to inhibit apoptosis by decreasing the expression of BAX and caspase 3 in experimental models of oxidative stress and neurotrauma (Reid et al., 2009, Reid et al., 2010, Cort et al., 2012, Kucuksayan et al., 2013).

Apart from the loss of neurons, it is well known that degeneration of spinal motoneurons after SCI also causes the loss of dendrites, synapses and axons (Bernstein et al., 1984, Cummings and Stelzner, 1988, Nacimiento et al., 1995, Grossman et al., 2001). Thus, the subsequent connectivity in the region will diminish after injury. While hemisection of the spinal cord is common in
experimental models of SCI, hemisection is unusual in the clinical setting (Rahimi-Movagha, 2014). For the most part remaining neural pathways can be found that traverse the injury zone. Therefore, improved preservation of dendrites and axons, in combination with possible additional sprouting of axons, may be of great therapeutic and rehabilitational value. Considering the remarkable structural and functional plasticity of the CNS, sprouting through the uninjured part of the spinal cord may contribute to the reconstitution of function after SCI. It is indeed well known that treatment with certain neuroprotective compounds such as BDNF and the antioxidant melatonin may induce sprouting, thus improving motor function after SCI (Mamounas et al., 2000, Jin Y, 2015).

The presented studies reveal the beneficial effects of ALC and NAC treatment on the preservation of dendrites and synapses 4 weeks following SCI. This effect is principally secondary to improved motoneuron survival. Furthermore, following 8 weeks of treatment with antioxidants, it was shown that therapy increased the sprouting of raphaespinal 5-HT-positive axons in the vicinity of the trauma zone. Although no fibers were observed growing across the injury site in the presented experimental model of hemisection, the sprouting fibers could possibly cross through preserved pathways in clinical subjects with incomplete injury. Indeed, ALC treatment in an experimental model of spinal cord contusion injury contributed to the preservation of the locomotor circuitry and subsequent improvement of functional outcome (Patel et al., 2012). Treatment with NAC following experimental contusion injury similarly improved locomotor function (Patel et al., 2014).

The pronounced and diverse effects of ALC and NAC treatment on mitochondrial function and apoptotic pathways may explain the increased survival of motoneurons after SCI. The anti-apoptotic actions of the antioxidants, coupled with a benign profile of side effects, may therefore make NAC and ALC eligible for clinical trials as neuroprotectants after SCI. Furthermore, it could be speculated that the preservation of the neural circuitry and induction of axonal sprouting observed after treatment could contribute to functional improvement in subjects with incomplete SCI.
10.2 ALC & NAC ameliorate neuroinflammation after SCI (papers I & II)

The neuroinflammatory response after traumatic SCI is immense, long-standing, and is a dominating pathophysiological feature of the secondary spinal cord degeneration (Hausmann, 2003, Silva et al., 2014). Neuroinflammation leads to progressive parenchymal destruction beyond the immediate trauma zone, with loss of neurons and glial cells (Bareyre and Schwab, 2003). While the experimental models used in the presented studies principally involve a laceration injury, the clinical scenario of SCI most often involves blunt trauma. Blunt trauma is associated with much greater forces being transmitted to the spinal cord parenchyma, causing more extensive injury and a considerably stronger inflammatory response (Siegenthaler et al., 2007). As such, a clinically useful future treatment for SCI will inevitably have to address the neuroinflammatory response.

The mitochondria are the main source and target of ROS after injury. ROS are in turn also potent triggers of neuroinflammation. The combating of mitochondrial dysfunction, ROS creation and neuroinflammation are therefore intimately interlinked. Indeed, a number of antioxidant treatments targeting mitochondrial dysfunction and ROS formation have been shown to reduce the neuroinflammatory response (McEwen et al., 2011, Palacios et al., 2011, McBean et al., 2016). The diverse, mitochondria-specific and ROS combating properties of ALC and NAC make both antioxidants theoretically well suited for the suppression of neuroinflammation (Khan et al., 2004, Patel et al., 2010, Patel et al., 2012, Patel et al., 2014).

The presented studies show the ability of ALC and NAC to dampen the neuroinflammatory response following cervical and lumbar spinal cord injury. The anti-inflammatory effects of treatment were observed already after 2 weeks, showing a decreased expression of ED1, a marker of activated macrophages and microglia associated with cytotoxic actions (Kullberg et al., 2001). This finding is also well in line with previous observations showing that NAC treatment down-regulates the presence of ED1 already at 24 hours after stroke in mice (Khan et al., 2004). The presented study is the first to show an early effect of ALC therapy on the presence of ED1-positive microglia after SCI. With the destructive properties of neuroinflammation commencing already minutes to hours after injury, an early effect on activated microglia may be of great therapeutic value (Alexander and Popovich, 2009).
Studying the longer-term actions of antioxidants on the presence of OX42 positive microglial cells close to the trauma zone, a sustained effect of treatment was observed. Both antioxidants strongly ameliorated the neuroinflammatory response at 4 and 8 weeks after injury. Neuroinflammation after SCI is considered chronic, and may last indefinitely (Alexander and Popovich, 2009). Therefore, the possibility of dampening neuroinflammation using ALC and NAC may be of clinical value. The sustained anti-inflammatory effects, in combination with the good tolerance and few adverse effects associated with high-dose treatment over longer periods, may make both ALC and NAC suitable candidates for future clinical trials.

10.3 ALC & NAC exert no obvious effect on astroglial scar formation (paper I)

The robust and early onset of scar formation after SCI is one of the crucial pathophysiological aspects preventing regeneration after injury. The complex composition of the scar, in particular the chemical barrier posed by a number of proteins inhibiting axonal growth, has yet to be successfully addressed in human subjects (Kawano et al., 2012, Ahuja and Fehlings, 2016).

Experiments in paper I investigated the effects of treatment on the astroglial component of the scar. Treatment with ALC and NAC did not alter the density of astroglial cells in the vicinity of the trauma zone. It has previously been shown that ALC and NAC improve the survival of cultured astroglial cells (Calabrese et al., 2005, Visalli et al., 2007, Abdul Muneer et al., 2011). It is therefore possible that antioxidant treatment following SCI will not decrease the presence of astroglial cells, due to improved survival. Furthermore, the chemical composition of the scar was not further investigated. Therefore, no conclusions can be drawn in regard to possible alterations of the permeability of the scar. However, since raphaespinal 5-HT-axons were not found to cross the injury site and grow into the distal part of the spinal cord, it is unlikely that treatment significantly altered scar permeability.
11. ALC treatment of microglial cell cultures (paper III)

11.1 ALC stimulates microglial secretion of neurotrophins *in vitro*

The notion that microglia only mediate death of cells and parenchymal destruction after SCI has long been disproved. Indeed, microglia are key players in the regenerative processes following injury, secreting important neurotrophic factors. BDNF, GDNF, NGF and IL-6 are some of the neurotrophic factors produced by microglia, with activation of the cells being known to induce or further increase secretion (Nakajima et al., 2001, Erta et al., 2012, Ferrini and De Koninck, 2013). The pro-regenerative potential of microglia may therefore be an interesting therapeutic target, provided that the secretion of neurotrophins can be successfully manipulated.

The neuroprotective and neurogenesis-stimulating properties of BDNF following after a variety of CNS diseases and injuries are well documented (Kaplan et al., 2010). In the context of SCI, BDNF treatment has been shown to exert antioxidative properties, combat lipid peroxidation and decrease neuroinflammation (Joosten and Houweling, 2004). Treatment has also been demonstrated to improve the survival of local motoneurons, decrease the necrotic zone, and improve axonal regeneration after injury (Novikova et al., 1996, Novikova et al., 2002). The presented finding of ALC-induced increase in microglial secretion of BDNF is novel. It elucidates yet another possible mechanism through which ALC may exert its anti-apoptotic and pro-regenerative actions. Indeed the antioxidant NAC, sharing several biological features with ALC, has been shown to act as an antioxidant by activating the BDNF/TrkB signalling pathway following SCI (Zhou et al., 2009, Berk et al., 2013). The study presented in this thesis adds to a growing body of evidence suggesting that ALC may play a diverse and useful role in the treatment of SCI.

Apart from inducing an increased secretion of BDNF, the addition of ALC also boosted microglial release of IL-6. While the addition of ALC to cultures of peripheral blood cells has previously been shown to induce the production of IL-6 (Kouttab and De Simone, 1993), this study is the first to demonstrate the effects of ALC on microglial IL-6 release. IL-6 is a complex interleukin, capable of mediating a variety of diverse actions. As such, the cytokine can both act as a pro-inflammatory agent, as well as exerting anti-inflammatory and pro-regenerative effects. Secretion of IL-6 with the concomitant secretion...
of IL-1β and TNF-α is the type of neuroinflammatory response typically associated with tissue destructions and other negative aspects of inflammation (Yan et al., 1992, Wang et al., 1994, Xing et al., 1998). In contrast, this study found no increase of IL-1β or TNF-α, showing an isolated and pronounced increase in IL-6. This finding may indicate that ALC stimulation of cells does not cause the release of IL-6 in a pro-inflammatory and potentially destructive manner. Rather, it is possible that the released IL-6 induces beneficial and neurotrophic actions. IL-6 is a known modulator of the levels of other pro-inflammatory cytokines, thereby acting as an anti-inflammatory and neurotrophic agent (Xing et al., 1998, Mukaino et al., 2010, Scheller et al., 2011). Which action the cytokine will exert depends largely on the subsequent pathways it will activate (Scheller et al., 2011). Further research is needed in order to elucidate which pathways the IL-6 released by microglia will activate, and the cytokine’s subsequent actions in vivo.

The effects of ALC treatment on the secretion of NGF and GDNF by unactivated microglia were also investigated. While ALC increased the transcription of the genes for both neurotrophins, no increase in secretion was found. Although a disappointing discovery at first glance, this finding is well in line with what is known about unactivated microglia. It has been previously shown that microglia in culture do not produce NGF if not activated by, for example, lipopolysaccharide (LPS) (Nakajima et al., 2001). Similarly, the secretion of GDNF by microglia is largely dependent on the activation of the cells (Batchelor et al., 1999). It can be speculated that ALC treatment, inducing increased transcription of the genes for both neurotrophins, may further boost the secretion of NGF and GDNF if applied to activated microglia. As such, the antioxidants may possibly contribute to increased neurotrophic support after nerve injury. To corroborate this, further experiments in a representative model of activated microglia is needed.

11.2 ALC rapidly increases the activation of PI3K

Recent studies of the actions of ALC in the CNS have implicated phosphoinositide 3-kinase-associated pathways (PI3K) as one of the targets for the antioxidant. ALC’s activation of the PI3K/AKT signalling pathway has been shown to contribute to the restoration of synaptic transmission after hypoxic brain injury (Kocsis et al., 2016). Furthermore, activation of PI3K-signaling in macrophages has been shown to supress NF-κB, with subsequent decrease of the expression of pro-inflammatory genes (Kok and Saez, 2014). It has also been demonstrated that ALC is capable of activating the PI3K/AKT/BDNF/VEGF pathway in the mouse brain, with subsequent antidepressant-like effects (Wang et al., 2015).
Results in paper III showed a rapid increase in the phosphorylation of PI3K in microglial cell cultures after addition of ALC. It is hard to draw any conclusions in regard to possible beneficial aspects of the observed activation. However, in the light of previous findings of the association between ALC’s activation of PI3K-pathways and positive effects in CNS morbidity, additional experiments further elucidating possible therapeutic implications are of interest.
12. MRI can be used to differentiate pre- & postganglionic injury (paper IV)

The current diagnostic procedures for the establishment of BPI level in clinical practice rely on several modalities. None of these is in itself sufficient for accurate diagnosis in all patients. Despite efforts to improve radiological diagnostics, surgical exploration is still needed in some cases to determine whether the injury is pre- or postganglionic. It is possible that an improvement of diagnostics may be achieved by a novel radiological approach to the problem. Hitherto, the focus of radiologists has mainly been directed towards identifying avulsed nerve roots and secondary signs of avulsion injury. To our knowledge, the possible differences in morphological changes in the spinal cord following the two fundamentally very different nerve injuries, have yet not been investigated.

12.1 MRI of the spinal cord in the experimental model of nerve injury

Ventral root avulsion (VRA) is a devastating injury to the afflicted motoneurons, causing considerable morphological and physiological changes in the spinal cord segments to which the injured nerve projects. Among the most prominent events occurring in the spinal cord after injury are oxidative stress, neuroinflammation, a variety of metabolic changes, and the extensive apoptotic death of motoneurons (Piehl et al., 1999, Chai et al., 2000, Zhang et al., 2005, Wang et al., 2011). VRA causes a rapid and extensive death of motoneurons, with 25% perishing after 1 week, and an approximate loss of 70% of motoneurons by 3 weeks (Li et al., 1995). The death of motoneurons after VRA will be paralleled by the loss of axons, dendrites and synapses. Furthermore, degeneration of motoneurons will also lead to apoptotic death of interneurons, causing a general depletion of the neuron pool in the affected segments (Oliveira et al., 2002).

In contrast to VRA, axotomy of a peripheral nerve will not induce changes of equal severity after 4 weeks. Following peripheral axotomy in adult subjects, no loss of motoneurons will occur (Carlstedt and Cullheim, 2000, Zhang et al., 2005). The changes in the affected segments are generally less pronounced after axotomy than after VRA (Brannstrom and Kellerth, 1998, Ma et al., 2001, Jivan et al., 2006). Furthermore, the neuroinflammatory response is milder after axotomy than after VRA (Yuan et al., 2003).
Experiments in paper IV confirm the previous observations that VRA causes more extensive changes in the spinal cord than axotomy. After 4 weeks, VRA caused a considerable shrinkage of the ventral horn neural pool on the injured side compared to the uninjured side. Meanwhile, no shrinkage of the neuron containing area was observed after axotomy. It is possible that the loss of motoneurons also causes the loss of functionally and anatomically associated interneurons, thus leading to a general loss of neurons in the affected spinal cord segment. Axotomy, on the contrary, causes no loss of motoneurons and subsequently no depletion of associated interneurons at this time point. Measurements of the ventral horn neuron pool size showed that avulsion caused a 44% shrinkage of the neuron pool size on the size of avulsion compared to the uninjured side. Following axotomy, no detectable changes in neuron pool size were found on the side of injury.

Apart from the discrepancy in regard to the size of the neuron pool, other differences in the ventral horn architecture were found following the two injury types. VRA caused an extensive loss of dendrites and axons, while axotomy only caused mild dendritic depletion and no apparent loss of axons. Furthermore, while axotomy did induce an increase in the presence of microglial and astroglial cells in the affected spinal cord segments, the increase was much less pronounced than after VRA.

Applying the same protocol used to measure the ventral horn neuron pool size in histological material, the ventral horn size was measured in images obtained using MRI. Measurements revealed that VRA caused a 34% shrinkage of the ventral horn size on the injured side compared to the uninjured side. The discrepancy in ventral horn size was immediately detectable by the naked eye. Axotomy, on the contrary, induced no detectable shrinkage of the ventral horn on the injured side compared to the uninjured side. Thus, MRI enabled an accurate and non-invasive method of differentiating VRA and axotomy.

While both histological images and images obtained using MRI showed similar results, a discrepancy was noted in the extent of shrinkage as measured by the two methods. Compared to measurements of the histological preparations, MRI measurements tended to somewhat underestimate the shrinkage of the ventral horn after VRA. This can be attributed to the essential difference in what each method measures. Histological measurements of the neuron pool only quantify the area occupied by stained neurons. Measurements of MRI pictures, on the other hand, are based on the ventral horn as it appears on images obtained by a radiological method. The method itself is not specific for the detection of neurons, rather capturing the entire signal emitted by the grey matter. Principally, all cells present in the
ventral horn may contribute to the generation of signal. This includes microglial and astroglial cells, whose numbers increase dramatically after VRA. Consequently, these cells may contribute to a masking of the shrinkage of the ventral horn. Nevertheless, despite the lack of perfect congruence between histology and MRI, the study validates spinal cord MRI as a method of non-invasive in vivo differentiation of pre- and post-ganglionic nerve injury in rats.

12.2 Possible practical applications of spinal cord MRI

The status of spinal CT combined with computed tomographic myelography (CTM) has long been the radiological gold standard for clinical brachial plexus diagnostics. Lately, it has been increasingly challenged by novel MRI-based diagnostic approaches in clinical practice (Doi et al., 2002, Silbermann-Hoffman and Teboul, 2013, Somashekar et al., 2014). However, despite continuous technical and methodological improvements, the MRI protocols used today are still regarded as unreliable for the diagnostic assessment of brachial plexus injury (Sakellariou et al., 2014). Three-dimensional MRI, for example, has been reported to have a mere 74% accuracy when compared to surgical exploration (Bhandari et al., 2009).

An important part of CT/CTM diagnostics of avulsion injury is the identification of pseudomeningocele, which may form after avulsion. Due to haemorrhage obscuring the view, pseudomeningocele can be visualized first 3-4 weeks after injury, when the coagulated blood can be expected to have cleared. The presence of this particular sign is considered to be an indirect indication of avulsion injury. Current MRI protocols, like CT, enable the identification of pseudomeningocele. Moreover, other secondary signs of injury such as neuroma formation and local avulsion-induced edema can be identified using MRI. Apart from the indirect signs of injury, MRI can also visualize the plexus virtually in its entirety, including avulsed nerve roots (Castillo, 2005, Caranci et al., 2013).

Despite considerable technical advances, MRI diagnostics is still plagued by a number of concerns. Relatively large slice thickness is required in order to assure an acceptable signal-to-noise-ratio (SNR). Moreover, regional disturbances caused by respiration, circulation and swallowing also pose a practical obstacle when attempting to obtain images of optimal quality (Sakellariou et al., 2014). In regard to the identification of pseudomeningocele cases of false-positive findings of on MRI do occur, as well as cases in which
avulsion is found upon surgical exploration despite the lack of indicative MRI findings (Bhandari and Maurya, 2014).

Essentially, both the current MRI and CT/CTM approaches are at least in part based on finding indirect evidence of avulsion injury. The results of study IV are the first to demonstrate an MRI-based method of diagnosing root avulsion through direct measurements of the ventral horn grey matter. The method was successfully used in an experimental model of nerve injury, accurately differentiating between pre- and postganglionic injury. The possible implications of these findings may be of both preclinical and clinical interest.

From a preclinical perspective, the possibility of non-invasive in vivo monitoring of changes in the spinal cord ventral horn are of great interest. A validated, MRI-based method of following the possible effects of neuroprotective treatment, and its effects on the survival of motoneurons, may be of great value in preclinical evaluation of neuroprotective substances. The method would enable continuous in vivo monitoring of the neuroprotective effects of therapeutic agents. A powerful neuroprotective agent capable of significant salvage of motoneurons after VRA, such as for example BDNF or GDNF (Li et al., 1995, Novikov et al., 1995), would likely cause a preservation of the ventral horn detectable on MRI. The proposed MRI protocol would also enable repeated measurements in the same subject over time, thus minimizing interindividual variation and the number of research animals needed in the experiments.

From a clinical perspective, an entirely new approach to BPI diagnostics may be of great value. The presented study validates the ability of the method to accurately detect VRA already after 4 weeks. Comparatively, CT/CTM may detect signs of VRA at about the same time point, although this is highly dependent on the presence of pseudomeningocele (Sakellariou et al., 2014). From a therapeutic point of view, “early repair” is considered to have been provided if the operation is performed within 8-12 weeks after injury (Bhandari and Maurya, 2014). Clinical data suggests that operation within 2-3 months is necessary in order to ensure an optimal clinical outcome (Jivan et al., 2009, Aralasmak et al., 2010). In this temporal context the presented MRI approach, if successfully converted to a clinically applicable protocol, may aid diagnostics and enable the choice of appropriate treatment at a suitable time point. Furthermore, post-treatment MRI scanning of the spinal cord may help the clinician to monitor recovery and intervene in the absence of radiological signs of recovery, or signs of further ventral horn degeneration.
In conclusion, MRI of the spinal cord is a novel approach for the differentiation of pre- and postganglionic nerve injury. It offers a non-invasive *in vivo* method of monitoring neural degeneration and neuroprotective treatment in preclinical studies. Hopefully a similar, clinically applicable MRI protocol may be an aid in future diagnostics of BPI.
Conclusions

Paper I & II  Both NAC and ALC act as neuroprotectants and anti-inflammatory agents after SCI. NAC and ALC have an anti-apoptotic effect on local motoneurons as well as descending brainstem neurons projecting caudal to the injury site. The anti-apoptotic and anti-inflammatory effects are detectable both at an early and late time point after injury. Treatment preserves dendrites, axons and synapses belonging to the cells found in the vicinity of the trauma zone. The antioxidants also induce sprouting of local axons. Treatment exerts no apparent effect on scar formation.

Paper III  The addition of ALC to cultures of unactivated human microglial cells stimulates their release of BDNF and IL-6. ALC does not cause the concomitant secretion of IL-1β and TNF-α, indicating that the microglia do not respond in a classically pro-inflammatory manner. ALC causes an increase in the phosphorylation of PI3K in microglial cell cultures, which may be associated with pro-regenerative properties.

Paper IV  Pre- and postganglionic nerve injury cause distinctively different changes in the ventral horn grey matter in histological preparations. These changes can also be detected using MRI of the affected segments.
Future prospects

The studies presented in this thesis show the neuroprotective, anti-inflammatory and sprouting-inducing actions of ALC and NAC treatment after SCI. From a therapeutic point of view, both agents may be of future clinical interest. Good tolerability with few adverse effects even at higher doses and longer treatment times encourages clinical trials. The presented preclinical experiments involved intrathecal administration of the antioxidants. In a clinical scenario, however, NAC is easily administered intravenously, while ALC can be given in preparations for oral use. In the light of possible benefits associated with treatment, there are no real obstacles to a clinical trial of ALC and NAC after SCI. If proven clinically useful, it is tempting to speculate that the antioxidants may be included in a future combinational therapy for SCI, probably coupled with surgical and/or cellular approaches.

The presented MRI protocol for the differentiation of pre- and post-ganglionic nerve injury is unique in its kind. No other MRI approach hitherto has addressed the changes occurring in the spinal cord grey matter after nerve injury. Combined with the accuracy in differentiating the two injury types, this novel approach is intriguing for clinical use. Naturally, this proof-of-principle study does not entirely reflect the clinical scenario of injury and diagnosis. When developing a protocol for clinical examination of changes in the cervical spinal cord after BPI, a first step would be to compare established avulsion injuries with peripheral axotomies. Patients with established, long-standing, and extensive avulsion injuries could comprise the former group. The axotomy group could be composed of patients with upper limb amputations. If proven successful in differentiating the injuries in regard to changes in the spinal cord, changes induced by axonal injuries closer to the spinal cord could be studied in pursuit of a clinically useful protocol.

Hopefully an MRI-based method derived from the presented study could be used as a complement in future MRI diagnostics of BPI. The examination could be performed during the same session as the other MR scans, as it would not require significant repositioning of the patient or change of hardware equipment. Optimally, the contribution of spinal cord MRI would aid the clinical practitioner in assessing the level of injury. This would be particularly valuable in ambiguous cases, where root avulsion is suspected despite lack of secondary signs of injury in MRI images.
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