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Neuroprotection in the Injured Spinal Cord

*Novel Strategies using Immunomodulation, Stem
cell Transplantation and Hyaluronic acid Hydrogel
carriers*

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Abstract

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The overall aim of this thesis was to establish strategies to minimize secondary damage to the injured spinal cord. Secondary damage that follows spinal cord injury (SCI) involves inflammatory and excitotoxic pathways. Regulation of these pathways using immunomodulatory and neuroprotective substances potentially protects the injured spinal cord from further damage. We also developed and studied resorbable biomaterials to be used as carriers for potential neuroprotectants to the injured spinal cord.

We used transversal spinal cord slice cultures (SCSCs) derived from postnatal mice as a model. SCSCs were maintained on different biomaterials and were studied after treatment with immunomodulatory and/or neurotrophic factors. They were further excitotoxically injured and subsequently treated with interleukin-1 receptor antagonist (IL1RA) or by neural crest stem cell (NCSC)-transplantation.

The results show that biocompatible and resorbable hydrogels based on hyaluronic acid (HA) preserved neurons in SCSCs to a much higher extent than a conventional collagen-based biomaterial or standard polyethylene terephthalate (PET) membrane inserts. Glial activation was limited in the cultures maintained on HA-based hydrogel. The anti-inflammatory factor IL1RA protected SCSCs from degenerative mechanisms that occur during *in vitro* incubation, and IL1RA also protected SCSCs from excitotoxic injury induced by N-Methyl-d-Aspartate (NMDA). IL1RA specifically protected neurons that resided in the ventral horn, while other neuronal populations such as dorsal horn neurons and Renshaw cells did not respond to treatment. Finally, transplantation of NCSCs onto excitotoxically injured SCSCs protected from neuronal loss, apoptosis and glial activation, while NCSCs remained undifferentiated.

The results presented in this thesis indicate that carriers based on HA seem to be more suitable than conventional collagen-based biomaterials since they enhance neuronal survival *per se*. The observed neuroprotection is likely due to biomechanical properties of HA. IL1RA protects SCSCs from spontaneous degeneration and from NMDA-induced injury, suggesting that excitotoxic mechanisms can be modulated through anti-inflammatory pathways. Different neuronal populations are affected by IL1RA to various degrees, suggesting that a combination of different neuroprotectants should be used in treatment strategies after SCI. Finally, NCSCs seem to protect SCSCs from excitotoxic injury through paracrine actions, since they remain undifferentiated and do not migrate into the tissue during *in vitro* incubation.

It seems that combinations of neuroprotectants and carrier substances should be considered rather than one single strategy when designing future treatments for SCI. Incorporation of neuroprotectants such as IL1RA combined with stem cells in injectable biocompatible carriers based on HA is the final goal of our group in the treatment of SCI.

Keywords: Hyaluronic Acid-based hydrogel, motoneurons, microglial cells, Interleukin-1 Receptor Antagonist, Renshaw cells, excitotoxicity, neuroinflammation, Neural Crest Stem Cells

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“Somewhere, something incredible is waiting to be known”

Carl Sagan

To my beautiful daughter Sofia

List of Papers

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

- I Schizas, N., Rojas, R., Kootala, S., Andersson, B., Pettersson, J., Hilborn, J., Hailer, N.P. (2013) Hyaluronic Acid-based hydrogel enhances neuronal survival in spinal cord slice cultures from postnatal mice. *Journal of Biomaterials Applications*, 2014 Feb; 28(6):825-36.
- II Schizas, N., Andersson, B., Hilborn, J., Hailer, N.P. (2014) Interleukin-1 Receptor Antagonist promotes survival of Ventral Horn Neurons and suppresses Microglial Activation in Mouse Spinal Cord Slice Cultures. *Journal of Neuroscience Research*, 2014 Nov;92(11):1457-65.
- III Schizas, N., Perry, S., Andersson, B., Wählby C., Kullander, K., Hailer, N.P. (2015) Differential Neuroprotective effects of Interleukin-1 Receptor Antagonist on Spinal Cord Neurons after Excitotoxic injury. *Submitted*
- IV Schizas, N., König, N., Andersson, B., Vasylovska, S., Hoeber, J., Kozlova E.N., Hailer, N.P. (2015) Transplantation of Neural Crest Cells to Spinal Cord Slice Cultures protects from Excitotoxic Neuronal Damage and inhibits Glial Activation. *Submitted*

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Abbreviations

BBB	Blood Brain Barrier
BDNF	Brain Derived Neurotrophic Factor
BMSC	Bone Marrow Stromal Cells
BMP	Bone Morphogenetic Protein
ChAT	Choline Acetyltransferase
Chr α 2	Cholinergic nicotinic receptor subunit α 2
CNS	Central Nervous System
COX	Cyclooxygenase
ECM	Extracellular Matrix
eGFP	Enhanced green fluorescent protein
GABA	Gamma-aminobutyric acid
GAG	Glycosaminoglycan
GDNF	Glial Derived Neurotrophic Factor
GFAP	Glial Fibrillary Acidic Protein
HA	Hyaluronic Acid
IB ₄	Isolectin B ₄
IL	Interleukin
IL1RA	Interleukin-1 Receptor Antagonist
Krox20	Zinc finger protein Krox20
MHC	Major Histocompatibility Complex
MSC	Mesenchymal Stem Cells
NASCIS	National Acute Spinal Cord Injury Studies
NCSC	Neural Crest Stem Cells
NeuN	Neuronal Nuclei
NF-L	Light-molecular-weight neurofilament
NMDA	N-Methyl-d-Aspartate
NO	Nitric Oxide
PET	Polyethylene Terephthalate
SCI	Spinal Cord Injury
SCSC	Spinal Cord Slice Culture
siRNA	Small interference RNA
SOX2	SRY (sex determining region Y)-box 2
TNF- α	Tumour Necrosis Factor- α
TUNEL	TdT-mediated dUTP Nick End Labelling

Introduction

Secondary Damage after Spinal cord injury

Spinal cord injury (SCI) is associated with irreversible damage to neurons and long fiber tracts such as the descending corticospinal tract and the ascending lateral and anterior spinothalamic tracts. These injuries result in permanent para- or tetraplegia accompanied by sensation disorders such as loss of pain sensation, inability to recognize temperature, and sometimes even a complete absence of sensation. An important aspect of the pathophysiology of SCI is that beyond the primary neuronal death that is a result of the initial trauma, a secondary damage process is initiated. Neuroinflammation, hypoxia and excitotoxicity are pathophysiological pathways related to the secondary damage process, all of which lead to further neuronal demise (Rothwell *et al.*, 1997; Rothwell & Luheshi, 2000; Freire, 2012).

Therefore, any intervention that is able to interrupt this cascade of events would possibly protect the injured spinal cord from further damage. Thus, many therapeutic approaches of SCI target the secondary damage cascade, some through immunosuppression and others by blocking parallel pathways such as those mediated by excitotoxicity. Stem cell transplantation was introduced as a treatment approach after SCI aimed at restoring neuronal loss by inducing neural differentiation of stem cells. However, research over the years has shown that stem cells release a large amount of soluble factors that possess neuroprotective properties, such as trophic, anti-inflammatory and anti-apoptotic compounds (Neirinckx *et al.*, 2014). Thus, apart from the initial aim of substitution of lost neurons, stem cells also seem to support injured neurons through the release of neuroprotective factors.

The role of microglial cells, macrophages and astrocytes in SCI

Microglial cells are easily activated immunocompetent cells that reside in the spinal cord in order to perform important functions such as antigen presentation and phagocytosis of debris. Damage to the spinal cord invariably leads in microglial activation, which in turn is associated with microglial proliferation, secretion of pro-inflammatory cytokines and proteases, and migration to sites of neuronal damage (Woodroffe *et al.*, 1991; Fujita *et al.*,

1998; Aldskogius *et al.*, 1999; Hailer *et al.*, 1999; Bellander *et al.*, 2004; Spulber *et al.*, 2009). This microglial activation results in secondary damage to previously intact neurons and axons that survived the initial trauma (Beatie & Bresnahan 2000).

Once activated, microglial cells proliferate and undergo several key morphological and functional changes including the thickening and retraction of cellular processes, expression of MHC class I and class II proteins, expression of co-stimulatory molecules, release of cytotoxic factors such as tumour necrosis factor (TNF)- α and nitric oxide (NO), and secretion of pro-inflammatory cytokines, resulting in phenotypical and functional changes associated with inflammation. Taken together, the activated state of microglial cells represents a transformation to tissue macrophages. Among the cytokines secreted by activated microglia the proinflammatory cytokine interleukin (IL)-1 β is an important mediator of secondary damage (Woodroffe *et al.*, 1991; Hailer, 2008; Spulber *et al.*, 2009). Apart from its neurotoxic action IL-1 β induces the expression of other pro-inflammatory mediators such as TNF- α and cyclooxygenase (COX)-2, thus playing a regulatory role in the concert of proinflammatory cytokines (Gibson *et al.*, 2004; Kaushik *et al.*, 2013).

Astrocytes are normally located within the white and grey matter interspersed between neurons, other glial cells, and capillaries. Beyond their structural supportive role to neurons astrocytes also provide nutritional support and, most notably, possess a regulatory role between neuronal synapses by secretion and absorption of neurotransmitters (Ullian *et al.*, 2001; Ullian *et al.*, 2004; Eroglu & Barres, 2010). Following SCI astrocytes migrate to the site of injury and take part in the formation of a glial scar, a structural border that becomes non-permissive for neurons and sprouting axons (Fawcett, 2006; Cloutier *et al.*, 2013).

In conclusion, different glial populations that have important functions both in the normal and in the injured spinal cord can after SCI be transformed into cells that perpetuate neuronal damage and that inhibit regeneration.

Crosstalk between inflammatory and excitotoxic pathways

In the context of acute neuroinflammation secretion of nitric oxide (NO), other proinflammatory cytokines such as TNF- α , and proteases all contribute to neuronal demise (del Zoppo *et al.*, 2007; Spulber *et al.*, 2009). In parallel, the concentration of excitatory neurotransmitters such as glutamate is increased as a consequence of the primary neuronal injury during SCI. Glutamate induces excitotoxic mechanisms leading in turn to further neuronal

damage. It is suggested that synthesis and actions of NO could represent a link between inflammatory and excitotoxic pathways: NO is synthesized as a result of inflammatory pathway activation (Bal-Price & Brown, 2001; Bal-Price *et al.*, 2002; Brown & Neher, 2010), and apart from its direct neurotoxic action, NO induces neuronal depolarization and release of excitotoxic concentrations of glutamate (Brown, 2007).

The proposition that IL-1 β is directly neurotoxic (see above) has not remained unopposed: Injections of IL-1 β into the brain do not immediately lead to neurodegeneration, however, IL-1 β exacerbates neuronal death that follows traumatic brain injury (Rothwell *et al.*, 1997). The effects of IL-1 β on neurons are not fully understood, but an increased vulnerability of neurons after exposure to IL-1 β has been suggested through activation of glutamate receptors such as N-Methyl-d-Aspartate (NMDA) receptors (Simi *et al.*, 2007).

It is therefore interesting to investigate the potential neuroprotective effect of anti-inflammatory agents within the context of excitotoxic neuronal damage.

Neuroprotective substances

Steroids

Hydrocortisone, aldosterone and dexamethasone have all been shown to suppress the number of microglial cells after injury to the central nervous system (CNS) (Vijayan & Cotman, 1987). They also were found to inhibit activation of isolated microglial cells and were associated with rapid electrophysiological alterations in neurons (Ganter *et al.*, 1992). After experimental SCI, methylprednisolone was associated with increased neurofilament preservation (Braughler & Hall, 1984) and improved tissue and axon preservation (Oudega *et al.*, 1999). Experimental approaches indicated that steroids inhibit the secretion of both proinflammatory cytokines such as IL-6 and TNF- α and the neurotoxic agent NO (Chao *et al.*, 1992; Tanaka *et al.*, 1997; Chang & Liu, 2000; Drew & Chavis, 2000; Jacobsson *et al.*, 2006). The exact mechanism of action has not been completely understood even though the classical concept suggests that steroid action involves their intracellular binding to cytosolic receptors which subsequently modulates the nuclear transcription of various genes (Schumacher, 1990).

The application of large quantities of methylprednisolone after SCI was introduced into clinical practice during the 1980s following the National Acute Spinal Cord Injury Studies (NASCIS). Steroid treatment has since then been the only widely established pharmacological therapy after traumatic brain or spinal cord injury (Bracken *et al.*, 1998). However, subsequent clinical studies have reported very limited functional recovery after high-dose

methylprednisolone treatment. This limited efficacy combined with deleterious side effects after administration of high doses of corticosteroids (Hurlbert, 2006) has led many researchers to question the use of steroid treatment after neurotrauma. Nine clinical trials on the use of steroids after SCI failed to demonstrate clinically significant functional improvement (Hurlbert, 2006). Additionally, the results of a randomised placebo-controlled study of about 10,000 patients with brain injury (Glasgow Coma Scale 14 or less) demonstrated that the risk of death or severe disability six months post-injury was significantly higher in the steroid treatment group. Therefore it is suggested that steroids after head injury should not be used routinely (Edwards *et al.*, 2005). Until now, there is no proof of significant functional recovery following pharmacological intervention with steroids after neurotrauma.

It is difficult for corticosteroids such as methylprednisolone, cortisone and dexamethasone with a molecular weight around 360 kDa to cross the blood brain barrier (BBB), even when administered in high doses. Microdialysis in samples from pigs that received methylprednisolone intravenously following SCI showed that the levels of methylprednisolone detected in the plasma were 30 times larger than the concentrations detected in the spinal cord (Bernards & Akers, 2006). Methylprednisolone was loaded in nanoparticles and delivered intracellularly to glial cells to bypass the BBB, leading to improved locomotor function after lateral hemisection (Cerqueira *et al.*, 2013).

Taken together, the above-mentioned findings indicate that steroids—and potentially other immunomodulatory agents—do have the capacity to inhibit glial activation and neuroinflammation, but that systemic administration is problematic and may even be counterproductive. It therefore seems logical to propose continued investigation of immunomodulatory compounds for the treatment of SCI, but by using locally controlled release. Other microglial inhibitors combined with other methods of administration, such as biocompatible drug carriers, could thus be a promising approach in order to achieve immunosuppression in the injured spinal cord.

Interleukin-1 Receptor Antagonist (IL1RA)

IL1RA is a polypeptide with a molecular weight of 17.3 kDa that belongs to the IL-1 family and inhibits the activities of IL-1 α and IL-1 β . Its mechanism of action is the binding and subsequent inhibition of the IL-1 receptor on the cell membrane (Dinarello, 1994).

The potential of IL1RA to influence glial activation and neuronal survival was investigated in many different experimental settings, among others in organotypic hippocampal slice cultures. Application of IL-1 β on NMDA-lesioned organotypic hippocampal slice cultures exacerbated neuronal cell death and further enhanced microglial cell numbers. Treatment of NMDA-

lesioned cultures with IL1RA significantly attenuated NMDA-induced neuronal damage and reduced the number of microglial cells, whereas application of IL1RA in unlesioned organotypic hippocampal slice cultures did not induce significant changes in either cell population. These findings indicate that IL1RA, even when applied for only 4 hours, reduces neuronal cell death and the number of microglial cells after excitotoxic damage (Hailer *et al.*, 2005). Similar effects of IL1RA treatment on microglial cells were observed even when IL1RA was applied as late as 36 hours post-injury, and IL1RA exhibited neuroprotective effects in this *in vitro* model of excitotoxic injury when delivered 16 hours after the injury (Vogt *et al.*, 2008).

The above *in vitro* findings are corroborated by *in vivo* experiments on SCI, indicating that continuous administration of IL1RA to the site of injury results in the reduction of contusion-induced apoptosis (Nesic *et al.*, 2001). After spinal cord contusion local application of IL1RA using a biocompatible gelatine sponge enhances neuronal survival and locomotor function (Zong *et al.*, 2012), and IL1RA is associated with down-regulation of IL-1 β and improved locomotor function after glutamate-induced SCI (Liu *et al.*, 2008).

The effects of IL1RA have also been investigated in *in vivo* models of cerebral ischemia or haemorrhage, further supporting the notion that IL1RA possesses neuroprotective properties (Loddick *et al.*, 1997). Following subarachnoid haemorrhage IL1RA blocks the haem-induced IL-1 β inflammatory pathway and thus reduces the BBB-breakdown (Greenhalgh *et al.*, 2012).

Stem cells as a treatment strategy after SCI

Mesenchymal Stem Cells and Bone Marrow Stromal cells

Mesenchymal stem cells (MSCs) are multipotent cells, which were first isolated from the adult bone stroma during the 1970s (Friedenstein *et al.*, 1974; Friedenstein, 1980). These cells give rise to other cell types that are derived from mesodermal tissue, such as adipocytes, chondrocytes and osteocytes (Pittenger *et al.*, 1999; Bianco *et al.*, 2001). Over the years, MSCs were found to be present in different tissues in adults such as dental and adipose tissue, muscle, synovia and tendon. In embryos MSCs are commonly found in perinatal tissues such as the umbilical cord and Wharton's jelly (Neirincx *et al.*, 2014). Bone Marrow Stromal Cells (BMSCs) are MSCs isolated from bone marrow stroma and they are considered potential candidates for treatment strategies after SCI mainly due to their ability to anorthodox differentiation to neural-like cells (Sanchez-Ramos *et al.*, 2000; Wislet-Gendebien *et al.*, 2005; Morikawa *et al.*, 2009). The ability of BMSCs in neural differentiation *in vivo* remains a matter of debate (Prockop & Oh, 2012), however BMSCs possess neurotrophic, anti-excitotoxic, anti-inflammatory and anti-

apoptotic properties (Bartholomew *et al.*, 2002; Aggarwal & Pittenger, 2005; Crigler *et al.*, 2006; Dasari *et al.*, 2007; Voulgari-Kokota *et al.*, 2012).

Neural Crest Stem Cells

Neural Crest Stem Cells are a different population of stem cell that arise from neural tissue, specifically the neural tube during the early stages of embryonal development of the nervous system. NCSCs migrate to peripheral organs in order to develop into peripheral neurons and glia as well as into other cell types (Huang & Saint-Jeannet, 2004). When NCSCs are co-cultured with pancreatic beta cells they induce proliferation of the cells in pancreatic islets (Grouwels *et al.*, 2012) and protect against cytokine-induced cell death (Ngamjariyawat *et al.*, 2012; Ngamjariyawat *et al.*, 2013), indicating that NCSCs possess anti-inflammatory properties. Little is known about the potential neuroprotective effects of NCSCs following SCI. Approaches using NCSCs after SCI could be interesting to study given that NCSCs arise from neural tissue and would on the one hand more easily differentiate into neurons and on the other hand secrete mediators that are target-specific to neurons.

Biomaterials for drug and/or stem cell delivery

Systemic administration of steroids after SCI has been associated with severe side effects, underlining the need for development of locally controlled release of potentially neuroprotective compounds. In pathophysiologies involving the CNS controlled release at injury sites is even more essential due to the fact that the pharmacological agent must cross the BBB.

The extracellular matrix (ECM) is a part of the microenvironment that surrounds cells, it supplies the signals for interactions between cells, and it provides functional and biochemical support. Therefore, many biomaterials intended as drug carriers are based on elements derived from the ECM (Hilborn, 2011). In the majority of the human tissues the ECM is composed of fibrous proteins, such as collagen, elastin, and glycosaminoglycans (GAGs). However, the basic component of the ECM in the CNS is hyaluronic acid (HA) and not fibrous proteins. The latter are limited and mainly localized to the meninges, blood vessels and fiber tracts (Delpech & Delpech, 1984; Liesi, 1984).

Experiments on mixed cultures of cortical neurons and astrocytes that were maintained on flexible biomaterials showed that these cells respond to matrix rigidity, suggesting that they are not only subjected to chemical but also to mechanical signals (Georges *et al.*, 2006). Slices derived from rat CNS tissue and dorsal root ganglia have been successfully cultured in collagen-based biomaterials, and axonal sprouting occurs under such conditions

(Koyama *et al.*, 2004; Willits & Skornia, 2004; Allodi *et al.*, 2011). However, the use of collagen-based biomaterials as drug carriers for CNS repair has been debated due to collagen's adhesive properties and due to the potential interaction of collagen with integrins (Gupta *et al.*, 2006; Khaing & Schmidt, 2012).

Hyaluronic acid (HA) has been used as the base substance for injectable biomaterials intended for the delivery of molecules such as DNA plasmids (Varghese *et al.*, 2009; Varghese *et al.*, 2010). Delivery of bone morphogenetic protein (BMP)-2 was performed using HA-based biomaterials in order to facilitate bone formation (Bergman *et al.*, 2009; Docherty-Skoggh *et al.*, 2010). Concerning the spinal cord, a biomaterial based on HA and integrated with the nogo-66 receptor antibody was injected after experimental SCI, leading to improved neuronal outgrowth and locomotor function (Wei *et al.*, 2010).

The biodegradation of various biomaterials based on HA has been investigated in order to develop suitable biocompatible carriers. Cross-linked HA-based biomaterials are more stable compared to non cross-linked HA gels. The half-life of non cross-linked HA after injection into the body does not exceed 24 hours as it is rapidly degraded by hyaluronidases, internalization of HA by CD44 cell-surface receptors or by reactive oxygen species (Schanté *et al.*, 2011). The biodegradation of cross-linked HA-based hydrogels is basically achieved by hyaluronidases due to the chemical stability of the cross-linking bond (Varghese *et al.*, 2009; Collins & Birkinshaw, 2013) and it involves a considerably longer procedure than in non cross-linked HA. Moreover, the biodegradation rate of different HA-based hydrogels appears to decrease as the molecular weight of the cross-linking molecule decreases (Jeon *et al.*, 2007).

The biomechanical properties of various cross-linked HA-based hydrogels seem to be dependent on the type of the molecular weight of the polymer chain, the type of the cross-linking reaction, the cross-linking density and the corresponding mesh size (Jeon *et al.*, 2007; Collins & Birkinshaw, 2013). Several cross-linking molecules have been described such as hydrazone (Varghese *et al.*, 2009) or poly(ethylene glycol)-diepoxide (Segura *et al.*, 2005; Noh *et al.*, 2006) and others. The porosity of a scaffold is also considered a factor that affects its biomechanical performance (Collins & Birkinshaw, 2013). It has previously been shown that within scaffolds before and after crosslinking, the porous size was retained after the crosslinking reaction (Collins & Birkinshaw, 2011). The pore size in these scaffolds varied between 40-230 μm in diameter (Collins & Birkinshaw, 2011).

One of the challenges concerning cross-linked biomaterials that are formed *in situ* is that the side products of the crosslinking reaction should not be toxic to the surrounding tissue. Therefore, the interaction between the tissue and the biomaterial must be carefully investigated.

Spinal Cord Slice Cultures (SCSCs)

SCSCs are a useful *in vitro* model for the study of SCI. SCSCs enable the screening of several neuroprotective substances at different concentrations (Krassioukov *et al.*, 2002; Stavridis *et al.*, 2005). Furthermore, SCSCs can be easily modified and used for the study of axonal regeneration (Bonnici & Kapfhammer, 2008; Stavridis *et al.*, 2009; Allodi *et al.*, 2011). A majority of experiments have been carried out on tissue derived from postnatal rats, while reports on well-preserved SCSCs derived from postnatal mice are lacking.

The term “organotypic culture” implies an interaction between different types of cells derived from a complex tissue or organ (Freshney 2005). Organotypic hippocampal slice cultures, longitudinally sectioned SCSCs, and even organotypic skin cultures have been described (Bellander *et al.*, 2004; Stavridis *et al.*, 2005; Bonnici & Kapfhammer, 2008; Vogt *et al.*, 2008; Oh *et al.*, 2013). The cultures used in our studies could be considered “organotypic” in the sense that they preserved their basic cytoarchitecture, a prerequisite for interactions between different types of neurons and glial cells. However, a major difference between organotypic slice cultures and *in vivo* experiments lies in the loss of tissue perfusion by blood during *in vitro* experiments. The continuous blood flow *in vivo* supplies the target organ, in our case the spinal cord, with factors in a dynamic and not in a static way. Moreover, during experimental SCI *in vivo*, the spinal cord is potentially subjected to signals coming from cells or organs outside the CNS. In the context of SCSCs a very important aspect is the disruption of BBB that occurs during the preparation of SCSCs. It should be taken into consideration that the concentrations of substances used in experiments involving SCSCs represent the amount that should come across the BBB during *in vivo* experiments or clinical trials.

Thus, our SCSCs can be considered organotypic in terms of neuronal and glial arrangement. The microarchitecture is preserved after several days and even weeks of *in vitro* incubation. However, our cultures cannot be considered organotypic in terms of fiber tract preservation since these structures are axotomized during culture preparation. Therefore, our cultures can be considered a reliable model for studying neuronal preservation after experimental SCI, however, it is not a good model to study the preservation of long ascending and descending fiber tracts.

In our studies, we used SCSCs derived from postnatal mice since this choice of species enables the use of transgenic animals. We used transgenic mice in order to visualize specific cell types described in detail in the materials and methods session. Transgenic mice deficient for IL-1 have been used for the study of neurodegenerative disorders such as Parkinson’s disease (Tanaka *et al.*, 2013). Transgenic mice that overexpress IL1RA were used in order to study the role of IL-1 in the developing brain (Spulber *et al.*, 2011),

and IL1RA knockout mice have been used for the study of other pathological conditions such as *Helicobacter pylori*-induced gastric inflammation, intervertebral disk degeneration and arthritis (Cao *et al.*, 2012; Huang *et al.*, 2013; Phillips *et al.*, 2013).

IL-1 and IL1RA knockout or overexpressing mice could be used in future experiments in order to examine and further understand the role of the above-mentioned cytokines in the context of SCI.

Aims of the thesis

1. To establish an *in vitro* model for the study of SCI using cultures from spinal cord tissue. To examine whether different biomaterials that are intended to be used as carriers of neuroprotectants or stem cells, such as HA-based hydrogels, have protective effects on SCSCs when compared to standard culture substrates such as collagen gel or PET culture inserts.
2. To treat SCSCs that undergo spontaneous degenerative changes during *in vitro* incubation with the immunomodulatory substance IL1RA, in combination with or without neurotrophic support, with the aim of improving neuronal survival.
3. To use IL1RA in order to treat SCSCs subjected to excitotoxic injury with NMDA. Furthermore, to study the potential neuroprotective effect of IL1RA on different neuronal populations within these injured cultures.
4. To transplant NCSCs onto SCSCs subjected to excitotoxic injury with NMDA and to examine whether NCSCs protect SCSCs from excitotoxicity.

Hypotheses

1. SCSCs cultured on a biomaterial based on HA are superior in terms of neuronal survival and glial activation compared to SCSCs maintained on a collagen based biomaterial or PET inserts.
2. Application of IL1RA (in the presence or absence of neurotrophic support with neurotrophin [NT]-3) protects SCSCs from degeneration during *in vitro* incubation by preserving the number of neurons and by suppressing glial activation.
3. IL1RA protects SCSCs from excitotoxic damage by counteracting the neuronal loss observed after application of NMDA, by protecting neurons and/or glial cells from apoptosis, and by suppressing glial activation. Neuronal subpopulations such as ventral horn NeuN-positive neurons, dorsal horn NeuN-positive neurons and Renshaw cells differ in their response to immunomodulatory treatment.
4. Transplantation of NCSCs onto SCSCs subjected to excitotoxic damage counteracts neuronal loss, protects from apoptosis and suppresses glial activation.

Materials and Methods

The model — Spinal Cord Slice Cultures

All experiments were conducted after approval by the local ethics committee (Uppsala Ethical Committee on Animal Welfare, approval numbers C5/9 and C 346/11). Postnatal mice were euthanatized by decapitation and the skin above the lower back was surgically removed to expose the lumbar and sacral region. The spine was detached from the sacrum and a 23-gauge cannula was caudally inserted 2-3 mm into the spinal canal. Ice-cold preparation medium was injected through the cannula and the spinal cord was flushed out through the cervical spine. Using a tissue chopper, 500 μm slices were obtained and immediately transferred to Petri dishes containing ice-cold preparation medium. More details on the method of slice culture preparation can be found in paper I.

Types of media

Three types of media were used during our experiments and they are presented in the table below:

Table 1. *Types of media*

Preparation medium	Culture medium	NCSC differentiation medium
100% Minimal Essential Medium (D-MEM; SVA Uppsala Sweden)	50% D-MEM (SVA)	D-MEM F12 supplemented with
10 $\mu\text{g}/\text{mL}$ glutamine (Sigma, Stockholm, Sweden)	25% Hank's balanced salt solution (HBSS, Gibco, Stockholm, Sweden)	1.2% non-essential amino acids (NEAA 100x; Invitrogen, Stockholm, Sweden)
	25% Normal Horse Serum (NHS, Gibco)	1% N-2 supplement (100x; Invitrogen)
	20 $\mu\text{g}/\text{mL}$ glutamine (Sigma)	1% B-27 supplement (50x; Invitrogen)
	1 $\mu\text{g}/\text{mL}$ insulin (Sigma)	
	0.8 $\mu\text{g}/\text{mL}$ vitamin C (Sigma)	
	2.4 mg/mL glucose (Sigma)	
	0.1 mg/mL streptomycin (SVA)	
	100 U/mL penicillin (SVA)	

Mice

The majority of the experiments were conducted using SCSCs derived from wild-type C57/B16 mice. Transgenic *Chrna2-Cre/R26^{Tom}* mice were used to generate the SCSCs used for the study of Renshaw cells, a subpopulation of ventral horn interneurons. The cholinergic nicotinic receptor subunit alpha 2 (*Chrna2*) was marked with dTomato fluorescent protein (Leao *et al.*, 2012). *Chrna2* is considered a marker of Renshaw cells when it involves small to medium sized neurons in the ventral horn. Heterozygous transgenic C57/B16 mice with enhanced green fluorescent protein (eGFP) or DsRed were used in order to generate autofluorescent NCSCs.

Preparation and maintenance of hydrogels

Cross-linked HA-based hydrogel

Two components of HA, a hyaluronic aldehyde and a hydrazide-modified HA, were mixed in order to form a cross-linked HA-based hydrogel. First, HA was transformed to hyaluronic aldehyde (Figure 1A). Subsequently, HA was functionalized to yield carbazate groups to form the hydrazide modified HA (Figure 1B). Mixing the two components led to the formation of a hydrazone cross-linked HA hydrogel within 1 minute (Figure 1C).

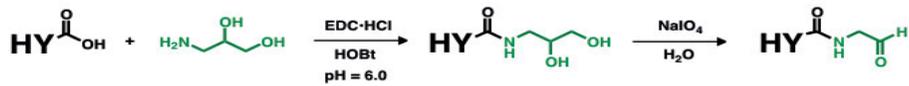


Figure 1A. Formation of Hyaluronic aldehyde (HY: Hyaluronic acid)

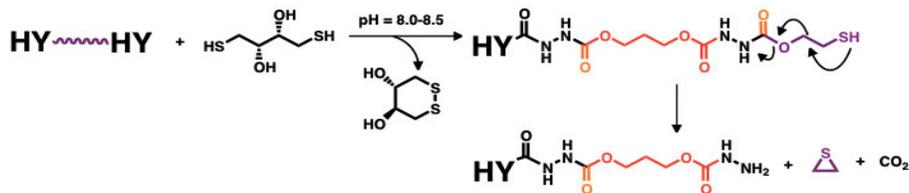


Figure 1B. Formation of hydrazide modified HA

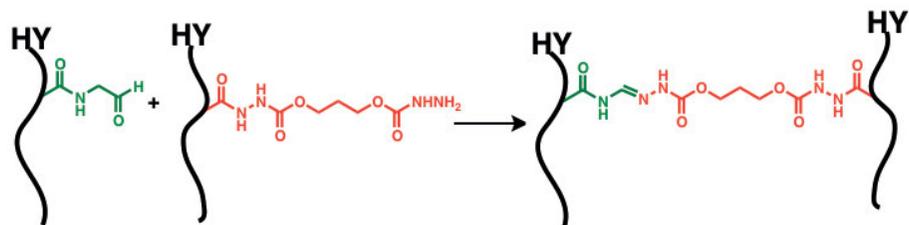


Figure 1C. Formation of hydrazone cross-linked HA

Non-cross-linked HA-based hydrogel

Healon 5® is a patent-protected, non cross-linked HA-based hydrogel obtained from Abbott (Uppsala, Sweden). The HA in Healon 5® is extracted from rooster combs and is dissolved in a physiological buffer solution at a concentration of 23 mg/mL. It has an osmolarity and pH similar to the aqueous humour of the eye, and the product is originally intended for use during cataract surgery in the human eye.

Collagen gel

Rat-tail collagen (CN: 354236, BD Biosciences Uppsala, Sweden) at a concentration of 3.68 mg/mL was mixed with sodium bicarbonate and Minimal Essential Medium and incubated for 2 hours at 37°C in order to allow gel formation.

Preparation of NCSCs

Primary cultures of boundary cap derived NCSCs were obtained from dorsal root ganglia (DRG) dissected from embryos of transgenic mice ubiquitously expressing eGFP or DsRed. The method is described in detail in paper IV. NCSCs formed neurospheres after a two-week period in culture, at which point they were ready for use.

NMDA-induced excitotoxic injury

After a three-day *in vitro* incubation on Healon 5®, SCSCs were subjected to NMDA-induced excitotoxic injury. SCSCs were exposed to culture medium containing NMDA (50 µmol/mL) for four hours.

Immunohistochemistry (IHC)

The method of IHC is based on the ability of a primary antibody to bind to an epitope on or inside a cell. A detectable substance, for example a fluorophore, is in turn connected to the primary or a secondary antibody. Goat anti-rabbit secondary antibody was used (Vector Laboratories Järfälla, Sweden). The primary antibodies and lectin used in our experiments are listed in table 1.

For immunohistochemistry we used both unsectioned cultures (200-300 µm) and cryostat sections of cultures (14 µm). The basic difference between the two methods was that unsectioned cultures were incubated for longer periods in the presence of the primary antibody and the other reagents compared to thin cryosections in order to penetrate to the full thickness of the

culture. They subsequently required longer washing periods. Various primary antibodies differ concerning their permeation rate into a tissue. The consequence was that ChAT primary antibodies and TUNEL labeling did not yield satisfactory staining results when used in unsectioned cultures, and therefore cryosections were stained in these staining protocols.

Table 1. *Primary antibodies and lectin*

Primary Antibody or Lectin	Company/Catalogue number (CN)	Dilution	Application	Marker
NeuN	Millipore/ABN78	1:500	Cultures	Neurons
ChAT	Novus/NB110-89724	1:2500	Sections	Motorneurons
GFAP	Abcam/AB7260	1:2000	Sections	Astrocytes
NF-L	Millipore/AB9568	1:500	Cultures	Axons
IB ₄	Sigma-Aldrich/L2895	1:20	C/S	Microglial cells
MacII	BioSite/ CL8942F	1:400	Cultures	Activ. mic. Cells
SOX2	SantaCruz/sc-17320	1:100	Sections	Immature NCSC
Krox20	BioSite/LS-B3577	1:50	Sections	Immature NCSC

TdT-mediated dUTP Nick End Labelling (TUNEL)

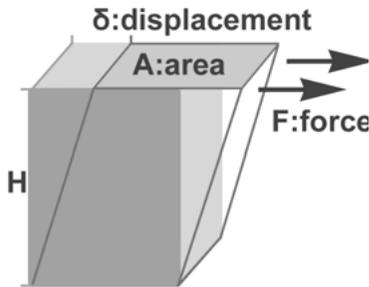
Chromatin cleavage or DNA fragmentation is considered the most characteristic biochemical feature of apoptosis, or programmed cell-death (Wyllie *et al.*, 1984; Martz & Howell, 1989). Apoptosis is furthermore accompanied by morphological changes in the nucleus identified by electron microscopy. These changes include nuclear chromatin condensation, appearance of pedunculated protuberances on the nuclear surface and compactness of cytoplasmic organelles (Wyllie *et al.*, 1984). However, identification of apoptotic cells using conventional light microscopy is difficult.

We used terminal deoxynucleotidyl transferase (TdT) in order to label DNA strand breaks in the nucleus, based on a previously described method (Gavrieli *et al.*, 1992). TUNEL enzyme and TUNEL label (enzyme CN: 11767305001, label CN: 11767291910, Roche, Stockholm, Sweden) were mixed according to the manufacturer's protocol in order to create a TUNEL-fluorescein complex solution that was applied on culture sections. The method is further described in papers III and IV.

Rheology

Rheology is the study of material flow. It is mainly applied to liquids, but also to materials with plastic flow such as hydrogels. Plastic flow is the capacity of a material to behave as a fluid when the applied stress reaches a critical value. The material is placed on a rheometer and subjected to oscilla-

tory stress in order to determine a linear viscoelastic region. The elastic modulus G' is calculated based on the deformation of the material as shown in figure 2.



$$E_{\text{modulus}} (G') = \text{Stress/Strain}$$

$$\text{Stress} = F/A$$

$$\text{Strain} = \delta/H$$

Figure 2. Rheology

ELISA

Enzyme-linked Immunosorbent Assay (ELISA) is a method that allows the detection of proteins in a solution. The protein is immobilized on a pre-coated polystyrene plate and a primary antibody conjugated to a detection enzyme that reacts with the protein is added. The measurement is performed by recognition of the detection enzyme with a procedure called spectrophotometry. During that procedure, transmitted light absorbance is measured at defined wavelengths by a spectrophotometer.

Light absorbance reflecting standard concentrations of the protein is primarily measured and a standard curve that is a function of protein concentration is generated. Each value of absorption thus corresponds to a value of protein concentration.

Confocal laser scanning microscopy

In our studies the excitation of fluorophores was performed with a laser beam (wavelength 488 nm for green fluorophores, 543 nm for red fluorophores, and 405 nm for DAPI). Emitted light was detected between 505-550 nm for green fluorophores, from 560 nm and higher for red fluorophores, and between 420-460 nm for DAPI. All fluorophores used in our studies along with their excitation/emission spectra are presented in table 3.

During confocal laser microscopy, light emitted from the specimen is passed through a pinhole before it is collected by a photodetector. Therefore, by adjusting the size of the pinhole, features that fall outside the pinhole (in this case features out of the plane of focus) are not registered. Thus, an im-

age of an optical section is produced (Figure 3). We used a magnification of 200 and a pinhole that produced an optical section of 1.2 μm .

Table 2. *Fluorophores used in our studies with excitation/emission spectra*

Fluorophore	Color	Excitation	Emission
DyLight 488	Green	493 nm	518 nm
DyLight 549	Red	556 nm	571 nm
Enhanced green fluorescent protein (eGFP)	Green	488 nm	509 nm
FITC	Green	492 nm	520 nm
tdTomato	Red	554 nm	581 nm
DsRed	Red	554 nm	586 nm
DAPI	Cyan	358 nm	461 nm

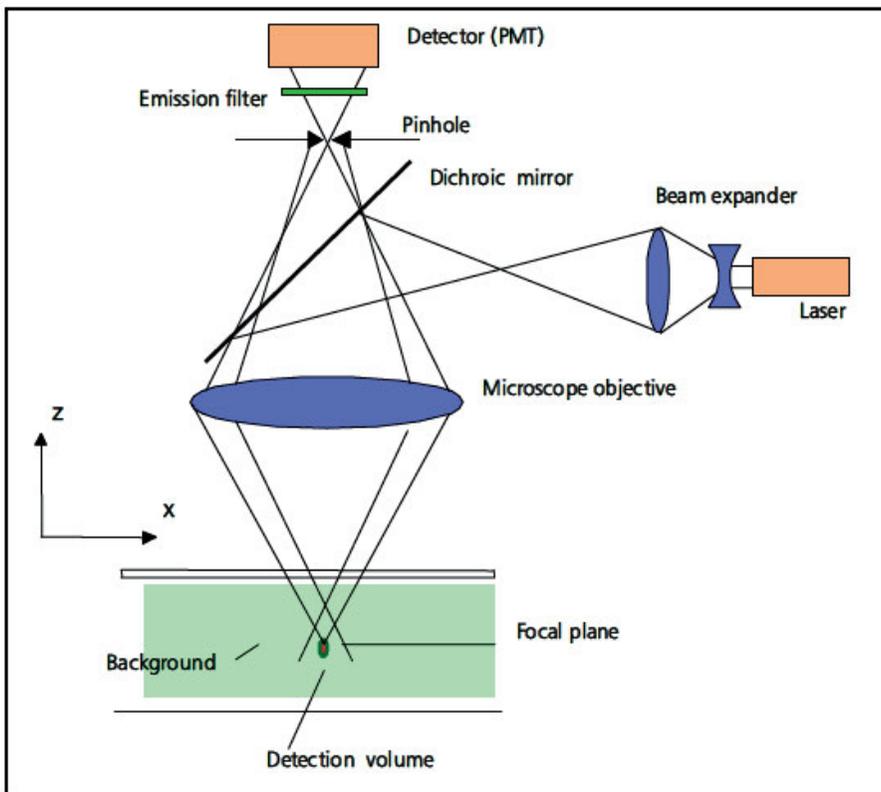


Figure 3. Simplified sketch of a confocal microscope. Features that fall outside the detection pinhole (i.e., features out of the plane of focus that is indicated by the red point) are not registered. Reprinted from the manual “Confocal Laser Microscopy Principles” with the kind approval of Karl Zeiss AB.

Image analysis

Papers I and II

In papers I and II NeuN- and ChAT-positive neurons showing distinguishable cytoplasmic staining were manually counted using the Image-J software. GFAP-positive astrocytes and microglial cells marked with IB₄ were manually counted using the same software. Neurons were counted separately within the ventral and dorsal horns, and astrocytes and microglial cells were counted separately within the white and grey matter.

In paper II activated and resting microglial cells marked with IB₄ were counted using an automated method based on color intensity and circularity index (Heppner *et al.*, 1998).

Papers III and IV

An automated method of quantification was developed in collaboration with the Centre of Image Analysis at Uppsala University in order to identify and count NeuN-positive neurons, MacII-positive microglial cells and objects labeled by TUNEL staining. The method is described in detail in paper III (see supporting information in paper III).

The major problems that we had to solve concerning automated image analysis were: 1. The presence of a high background signal, partly due to background staining intensity, and partly due to the inability of the washing buffer to penetrate into an unsectioned SCSC to the same extent as in a section. The sample was actually a thick SCSC of 200-300 μm and not a slice of 14 μm . 2. Neurons that overlapped with each other created clusters and increased the staining intensity in some parts of an image. In consequence, the percentage of positive structures (neurons) that covered an image varied substantially between images.

These problems were solved by first using a filter in order to smoothen the image and to reduce signal noise by eliminating pixel artefacts. Subsequently the high background signal was reduced by enhancing features in each image using a special function called “enhance or suppress features” based on the typical size of neurons. Finally, positive neurons were identified using Otsu thresholding, a method that reduces a greylevel image to a black and white binary image, thus creating a “foreground” (neurons) and a “background” (Otsu, 1979). By using “CellProfiler” software we had the opportunity to split the image into either foreground and background or foreground, mid-level, and background. Otsu thresholding is considered a reliable approach especially when the percentage of the foreground of an image varies from image to image. A validation study comparing manual counting and automated analysis using CellProfiler was performed (see Results section).

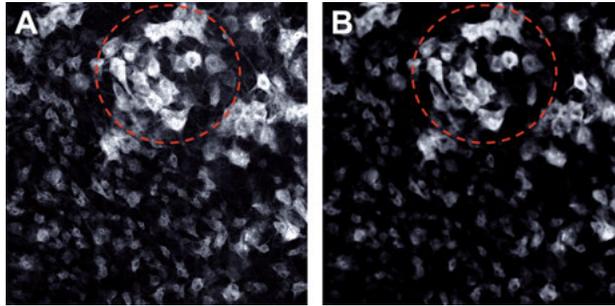


Figure 4(A-B). Micrographs through the ventral horn of SCSC captured using confocal laser microscopy and analyzed with the CellProfiler software. Before smoothing and enhancement (A) the presence of high background and artefacts were pronounced especially in the area inside the dashed circle. After smoothing and enhancement (B) the neurons were considerably more distinguishable than in image A.

Statistical analysis

Data were described using mean values \pm the standard error of the mean (SEM). Analysis of variance (ANOVA) was used for the analysis of differences between multiple groups. Model assumptions (sphericity and normality) were tested with Levene's test concerning sphericity and the Kolmogorov-Smirnov test concerning normality. When variances in different groups were equal and when data were normally distributed, analysis was performed using a one-way ANOVA and planned contrasts. Dunnett's test was used for post-hoc comparisons, assuming that the experimental groups would be superior to the respective negative control. When the assumption of equal variances between groups was violated, robust tests (Welch and Brown-Forsythe) were used. Data that were not normally distributed were analysed by the Kruskal-Wallis-test followed by multiple Mann-Whitney U tests. The level of statistical significance (p) was set at 0.05.

Results and Discussion

Paper I

The major finding of paper I was the significantly improved neuronal, especially motoneuronal, survival in SCSCs maintained on a cross-linked HA-based hydrogel compared to cultures maintained on PET inserts or collagen gel (Number of neurons per slide: HA-based hydrogel: 90.4 ± 9.4 , collagen gel: 34.3 ± 3.5 , PET inserts: 38.6 ± 3.2). Soluble HA failed to improve neuronal survival of SCSCs. Furthermore, microglial activation was limited in the grey matter of cultures maintained on HA-based hydrogel compared to cultures maintained on PET inserts. The elastic modulus of HA-based hydrogel was considerably higher than that of collagen gel.

The findings in paper I indicate that the mechanical rather than chemical properties of the cross-linked HA-based hydrogel contribute considerably to neuroprotection inside cultured spinal cord tissue. This makes HA-based hydrogel an interesting candidate for further studies investigating its suitability as a drug carrier. The elastic modulus of the cross-linked HA-based hydrogel was approximately 1,200 Pa, which is within the range of the mammalian neural tissue that lies between 500 and 1,000 Pa (Leipzig & Shoichet, 2009). In contrast, collagen gel was considerably softer than the above-mentioned HA-based hydrogel with an elastic modulus around 25 Pa, while PET membranes were considerably harder with a typical elastic modulus of four G-Pa.

Even though SCSCs and tissue from dorsal root ganglion have been successfully cultured on collagen-based biomaterials (Koyama *et al.*, 2004; Willits & Skornia, 2004; Allodi *et al.*, 2011), the use of collagen-based biomaterials as drug carriers has been debated due to the adhesive properties and potential interaction with integrins of collagen (Gupta *et al.*, 2006; Khaing & Schmidt, 2012). However, modification of HA-based hydrogel with other fibrous proteins such as fibronectin can increase its adhesion to the surrounding cells and tissue (Kisiel *et al.*, 2013b). Whether the alteration of the adhesive properties of HA-based hydrogel will affect neuronal survival and axonal sprouting is a matter for future investigation.

HA is the basic component of the ECM surrounding neurons (Delpech & Delpech, 1984). HA has a molecular weight that varies between 100 and 8,000 kDa and can easily be modified and transformed into many physical forms such as soft or stiff hydrogels and viscoelastic solutions (Burdick &

Prestwich, 2011). Hydrogels based on HA have been injected into brain and spinal cord defects without causing further neuronal damage, sometimes even resulting in neuroprotective effects: Specifically, a hydrogel blend of 2% HA and 7% methylcellulose was injected intrathecally in rats subjected to SCI and this was associated with improved locomotor function and decreased inflammation compared to controls (Gupta *et al.*, 2006; Austin *et al.*, 2012). In another experiment, HA-based hydrogel was injected into cortical defects in rats and prevented glial scarring (Hou *et al.*, 2005). Furthermore, when the hydrogel was modified with laminin, it promoted axonal outgrowth (Hou *et al.*, 2005). In fact, it has previously been shown that adding high molecular weight HA to astrocyte cultures it prevents astrocyte activation and subsequent glial scarring (Struve *et al.*, 2005).

The ability of a biomaterial carrier to release the integrated substance into the surrounding tissue is critical in order for the biomaterial to be considered as a suitable drug carrier. In cross-linked hydrogels based on HA, the diffusion of small water-soluble molecules occurs rapidly whereas hydrophobic molecules are more slowly released into the tissue within a period of days or weeks (Prestwich, 2011). However, large molecules and particles larger than 40 kDa are not so easily released into the tissue and the biomaterial has to be dissolved by hyaluronidases in order to allow their dissemination (Prestwich, 2011). Therefore, IL1RA with a molecular weight of 17.3 kDa would diffuse rapidly into the surrounding tissue. The neuroprotective molecule anti-NogoA has been integrated into nanoparticles in a HA-based hydrogel showing sustained *in vitro* release (Stanwick *et al.*, 2012). The same hydrogel has been used for the delivery of neural stem cells to the injured spinal cord (Mothe *et al.*, 2013).

Paper II

The SCSCs in paper II were maintained on collagen gel, a substrate that in paper I was associated with a high extent of neuronal loss and culture degeneration. The major finding in paper II was the improved neuronal survival in the ventral horn of SCSCs maintained in the presence of IL1RA compared to untreated cultures. This was independent of whether cultures were supported by the trophic factor NT-3 or not. SCSCs maintained under control conditions (i.e. untreated cultures) degenerated substantially after eight days *in vitro*, however a combination of IL1RA and trophic support using NT-3 preserved the number of neurons at a level comparable to that observed directly after culture preparation (Number of neurons per slide: Untreated cultures: 18.2 ± 2.4 , IL1RA alone: 28.3 ± 3.0 , NT-3 alone: 38.4 ± 7.2 , IL1RA and NT-3: 46.4 ± 9.4). Apart from the use of IL1RA and NT-3, the major difference of the experimental setting described in paper II compared to the setting described in paper I was the different substrate. Cultures maintained on a

collagen gel showed early signs of degeneration while SCSCs maintained on a cross-linked HA based hydrogel described in paper I were well preserved. The absolute number of neurons in the ventral horn of untreated cultures after four days *in vitro* in paper II (30.8 ± 3.3) was at the same level as the number of neurons observed in the collagen gel group presented in paper I (34.3 ± 3.5).

Concentrations of IL-1 β measured by ELISA peaked after six hours in all groups, except for the groups with the highest degree of neuronal preservation (i.e. cultures treated with a combination of IL1RA and NT-3).

The microglial activation was limited after eight days *in vitro* in cultures treated with a combination of IL1RA and NT-3, or NT-3 alone, or IL1RA alone, when compared to untreated cultures. The inhibition of microglial activation was observed both in the white and grey matter of cultures.

The findings in paper II make IL1RA an interesting candidate for further investigations concerning its neuroprotective properties. Liberation of IL-1 β from freshly prepared SCSCs follows a pattern of an early IL-1 β peak during the first six hours followed by a decline, previously described *in vivo* (Bartholdi & Schwab, 1997). It is reasonable to assume that the source of IL-1 β is activated microglial cells responding to substantial neuronal injury that occurs during culture preparation. Treatment of organotypic hippocampal slice cultures with similar concentrations of IL1RA was associated with an inhibition of microglial activation even after delayed application following excitotoxic neuronal damage with NMDA and IL-1 (Hailer *et al.*, 2005; Vogt *et al.*, 2008). Following experimental SCI, application of higher concentrations of IL1RA was associated with morphological and functional recovery. Specifically, continuous administration of IL1RA at a concentration of 750 ng/mL using a mini-pump was associated with a reduction of contusion-induced apoptosis (Nesic *et al.*, 2001). In addition, a considerably higher concentration of IL1RA (2 mg/mL) was used for one single local administration after experimental SCI (Zong *et al.*, 2012). It is reasonable to assume that higher concentrations of IL1RA are needed in order to reach a therapeutic effect in *in vivo* experiments, especially when only one single dose is administered.

Whether neuroprotection brought about by IL1RA is a result of the observed suppressed microglial activation or if IL1RA has direct effects on neurons is still a matter of speculation. Inhibition of microglial activation could result in decreased secretion of neurotoxic agents, thereby indirectly reducing neurotoxicity. However, direct neuroprotective actions of IL1RA on neurons could also be considered a possibility since microglial activation and neuronal demise are exacerbated after application of IL-1 following excitotoxic neuronal damage (Hailer *et al.*, 2005; Vogt *et al.*, 2008). Although the action of IL-1 on neurons is not fully understood, experimental evidence supports the idea of increased neuronal vulnerability to excitotoxicity through an activation of NMDA receptors under the influence of IL-1

(Simi *et al.*, 2007). Toxin B of *clostridium difficile* has an excitatory effect on enteric neurons that is mediated through an IL-1 pathway, and this could be a potential target for inhibition by IL1RA (Neunlist *et al.*, 2003). Finally, the excitatory effect of IL-1 on cerebellar Purkinje cells is absent in IL-1 receptor knockout mice (Motoki *et al.*, 2009). However, administration of IL-1 on primary cultures of cortical neurons attenuates rather than exacerbates the excitatory effects of NMDA (Strijbos & Rothwell, 1995). Based on these partially contradictory findings, it seems possible that IL1RA-induced neuroprotection is a result of decreased microglial activation and indirect neuroprotective effects, but direct neuronal effects of IL1RA cannot be excluded. Thus, more studies such as neurophysiological studies under the influence of IL-1/IL1RA or application of IL-1/IL1RA on mixed cultures of neurons and microglial cells need to be performed in order to elucidate the mechanisms of action of IL-1 on neurons.

Evidence of a crosstalk between neuroinflammation and excitotoxicity provided the basis of paper III in order to investigate the effect of IL1RA in cultures subjected to excitotoxic injury. The concentration of IL1RA in the experiments described in paper II was 100 ng/mL and was chosen based on previous experiments (Hailer *et al.*, 2005; Vogt *et al.*, 2008). However, when experiments on excitotoxically injured SCSCs were planned, a wider range of IL1RA concentrations was investigated, since it was unclear whether neurons and glial cells would respond the same way to the initial concentration of IL1RA (100 ng/mL) after excitotoxic injury.

Paper III

A non cross-linked HA-based hydrogel, Healon 5®, was used as a cultures substrate in paper III in order to ensure improved neuronal survival and thereby enable experiments using NMDA-induced excitotoxic injury. Excitotoxic injury with NMDA dramatically reduced the number of neurons counted within the ventral horn (no NMDA: 115.3 ± 11.8 , NMDA: 67.9 ± 6 , NMDA+IL1RA: 98.1 ± 11), while the neuronal population in the dorsal horn was not particularly affected either by excitotoxic damage or by IL1RA treatment. Due to the different substrates used in paper I (cross-linked HA based hydrogel) and paper II (collagen gel) direct comparisons between the absolute numbers of neurons between papers I, II and III were not feasible.

A possible explanation of the resistance of dorsal horn neurons to excitotoxic mechanisms might be their expression of calbindin, a protein that regulates the concentration of intracellular Ca^{2+} and is considered to be an intrinsic neuroprotective factor (Ren & Ruda, 1994; Sun *et al.*, 2011; Yuan *et al.*, 2013). The number of Renshaw cells, a subpopulation of ventral horn interneurons, was vastly reduced after NMDA excitotoxicity. The above subpopulation of Renshaw cells was even more vulnerable to degenerative

mechanisms that occur during *in vitro* incubation than other NeuN-positive ventral horn neurons.

Treatment with IL1RA protected SCSCs from excitotoxically induced neuronal loss and prevented post-injury micro- and astroglial activation, an observation that is in line with previous studies describing neuroprotective effects of IL1RA (Rothwell & Luheshi, 2000; Hailer *et al.*, 2005; Vogt *et al.*, 2008). In part, neuronal preservation was attributed to the properties of the biomaterial Healon 5®, therefore direct comparisons between SCSCs described in paper II and those described in paper III were not performed. SCSCs maintained on collagen gel as described in paper II degenerated substantially after only a four-day *in vitro* incubation.

Renshaw cells were more vulnerable during *in vitro* incubation and were not rescued by IL1RA. Moreover, Renshaw cells appeared to be more sensitive to excitotoxicity than ventral horn neurons, and they did also not respond to IL1RA treatment after NMDA-induced injury. Renshaw cells are inhibitory interneurons that reside in the spinal cord. Among the eleven described classes of interneurons (Tanabe & Jessell, 1996) they represent the only class that receives afferents directly from motoneurons and they mediate recurrent inhibition to them. This is performed through production and co-release of gamma-aminobutyric acid (GABA) and glycine (Schneider & Fyffe, 1992; McIntire *et al.*, 1997; Sagne *et al.*, 1997; Chang & Martin, 2009). It is unknown how Renshaw cells are affected by an alteration of the afferent inflow from motoneurons when the latter are excitotoxically damaged. If we presume that Renshaw cells are resistant to excitotoxic mechanisms due to their expression of calbindin, it is reasonable to assume that potential loss of or alterations to motoneuron-derived signaling could affect the survival of Renshaw cells. In contrast to the latter proposition for Renshaw-cell degeneration, ventral horn interneurons (including Renshaw cells) seem to survive a massive motoneuron loss *in vivo* (Lim *et al.*, 2000), however, it is unclear how Renshaw cells behave when their target motoneurons are subjected to excitotoxic injury.

The rescue of NeuN-positive ventral horn neurons by IL1RA after NMDA-induced excitotoxic damage may represent actions through anti-inflammatory pathways. This suggests that the pathophysiological conditions of excitotoxicity and inflammation are linked. A crosstalk between these pathways has been described both in acute and chronic disorders mentioned in more detail in paper III (Centonze *et al.*, 2009; Rossi *et al.*, 2012).

Since both the presence of HA-based hydrogels (both cross-linked HA described in paper I and non cross-linked Healon 5®) and IL1RA were associated with favorable effects on neurons, the integration of HA-based hydrogels with potential neuroprotective substances such as IL1RA seems to be the next logical step in the context of controlled drug release after SCI. IL1RA has a molecular weight of 17.3 kDa and it should be expected that it would easily diffuse from a HA hydrogel into the surrounding tissue. How-

ever, the *in vitro* release of the substance should be studied in order to determine the kinetics of this process. Incorporation of BMP-2 into the cross-linked HA-based hydrogel described in paper I has been achieved by dissolving the substance in one of the components of the hydrogel (Bergman *et al.*, 2009).

Paper IV

The major finding of paper IV was the substantial neuroprotection induced by NCSCs transplanted onto SCSCs that were previously subjected to excitotoxic injury. Transplantation of NCSCs onto such lesioned cultures was associated with prevention of neuronal loss, protection from apoptosis, and inhibition of micro- and astroglial activation.

The majority of transplanted NCSCs showed very few signs of either neural or glial differentiation after a six-day *in vitro* incubation on the surface of SCSCs, while markers of undifferentiated NCSCs—such as SOX2 and Krox20—were abundantly expressed. It is thus plausible that the favorable effect of NCSCs on SCSCs is mediated by soluble neuroprotective factors released from NCSCs. Soluble factors such as brain derived neurotrophic factor (BDNF), glial derived neurotrophic factor (GDNF) and NT-3 that could positively affect SCSCs have been identified in a recent study of coating pancreatic islets with NCSCs (Lau *et al.*, 2015). The fact that NCSCs transplanted onto SCSCs seem to be arrested in an undifferentiated state is interesting. This observation is consistent with the fact that stem cells are involved in paracrine actions and regulate their environment by releasing a large amount of soluble factors (Baraniak & McDevitt, 2010). It is expected that NCSCs would gradually lose their capacity of growth factor release as they differentiate into other cell types such as neurons and glia, although relevant literature on the comparison between differentiated and undifferentiated NCSCs is lacking. In fact, the release of angiogenetic factors such as hepatocyte growth factor (HGF) by human adipose-derived stem cells is higher when the latter are in an undifferentiated state rather than when they start to differentiate to other cell types (Kilroy *et al.*, 2007; Baer *et al.*, 2010).

It would be interesting to investigate whether NCSCs show signs of differentiation when they are transplanted onto SCSCs and maintained in the presence of NCSC differentiation medium. An experimental setting was prepared for that purpose by incubating SCSCs in the presence of NCSC differentiation medium. However, the effect of NCSC differentiation medium on SCSCs was detrimental, reducing the number of neurons to nearly zero after only a four-day *in vitro* incubation, presumably due to the absence of serum in the NCSC differentiation medium. Considerable amounts of normal horse serum (approximately 25% of the volume of regular culture

medium) seem to be essential for the survival of neurons in SCSCs. On the other hand, large amounts of normal horse serum seem to prevent differentiation of glial precursor cells in culture (Fedoroff & Hall, 1979), and the observed arrest of the transplanted NCSCs in an undifferentiated state may have been due to the high concentration of serum required to maintain our slice cultures.

In order to establish a type of medium that is suitable for both SCSC incubation and NCSC differentiation, on-going research continues in our laboratories by using different types of media in order to further characterize and study the behaviour of NCSCs transplanted onto SCSCs.

Findings not presented in papers I-IV

Histological evaluation of SCSCs

In addition to the manual and automated quantitative methods the integrity of SCSCs was histologically evaluated using conventional staining with hematoxyline and eosin (H&E). We found that fixation of SCSCs using a method described in a previous study (Stavridis *et al.*, 2005) with some modifications was associated with improved tissue preservation compared to the fixation method previously used in our laboratory and described in paper I. Both fixation methods are presented in table 3.

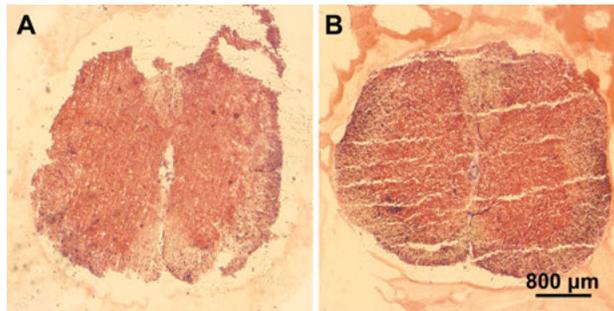


Figure 5. SCSCs were obtained and maintained on a collagen gel in the presence of culture medium for four days. The cultures were subsequently divided into two groups and fixed using a method described in a previous study (Stavridis *et al.*, 2005) with some modifications (A) or a method described in paper I (B). Cultures were evaluated by two independent blinded observers. SCSCs fixed with the first method (A) showed improved tissue quality and were more resistant to sectioning artefacts than cultures fixed with the second method (B).

Tissue sectioning was generally associated with the presence of artefacts and fragmentation of sections. However, tissue sectioning was used at the beginning of our project particularly to histologically evaluate the preservation of cultures and in order to confirm that the central parts of SCSCs were

viable. Parts of the above experiment are presented in paper I where SCSCs were immunohistochemically stained for ChAT after tissue sectioning.

In order to minimize the occurrence of artefacts and fragmentation, unsectioned SCSCs were used in the majority of the experiments. Cryosections of SCSCs were used in order to minimize high intensity background in paper I, specifically when using the markers ChAT, GFAP and IB₄. In fact, during the pilot phase of the experiments the background staining intensity in unsectioned cultures was considerable. Concerning GFAP and IB₄, the problem of background staining intensity was solved by further developing and refining our method of staining unsectioned cultures. However, we were not able to solve that issue in the ChAT staining protocols, probably due to the fact that the ChAT primary antibody has inferior permeation into the tissue when compared to GFAP primary antibodies or IB₄.

Furthermore, cryosections of SCSCs were used in papers III and IV for TUNEL-NeuN-DAPI triple staining since adequate TUNEL staining could not be achieved in unsectioned cultures.

When unsectioned SCSCs were stained by IHC no major differences were noted between the two fixation methods regarding tissue quality. The fixation method initially developed by Stavridis et al. (2005) and modified in our lab was chiefly used when cryosections of SCSCs were studied, except in paper I.

Table 3. *Brief description of the two fixation methods*

Initially used in our lab (paper I)		Developed by Stavridis et al (2005) and modified in our lab	
<i>Solutions</i>	<i>Timing</i>	<i>Solutions</i>	<i>Timing</i>
Zamboni's fixative: 4% paraformaldehyde (PFA) in 0.2 M Sorensen phosphate buffer (SPB) containing 0.2% picric acid	24 hours	4% PFA, 0.1% glutaraldehyde and 15% picric acid in 0.2 M SPB	15 minutes
20% sucrose solution (in SPB)	Until the yellow colour disappears	4% PFA and 15% picric acid in 0.2 M SPB 30% sucrose solution (in SPB)	2 hours Until the yellow colour disappears

SCSCs degenerate substantially when maintained on a rough HA-based hydrogel

In order to examine whether the surface of the substrate was important for the survival of SCSCs we used a patent-protected cross-linked HA-based hydrogel (Durolane®). Durolane® is commercially available (Q-Med, Uppsala, Sweden) and it is used as an injectable substance for the treatment of osteoarthritis.

The HA in Durolane® is produced by bacterial fermentation and it is highly purified. One of the main differences between Durolane® and the cross-linked HA-based hydrogel used in our studies is that the cross-linking reaction in Durolane® is not performed *in situ* as in the above-mentioned HA-based hydrogel.

After the cross-linking reaction, Durolane® is crushed to create gel particles of a diameter of approximately 2-3 mm and it is then stored in syringes for further use. Therefore, when Durolane® is placed on PET inserts it creates a rough surface of biomaterial that stands in stark contrast to the cross-linked HA-based hydrogel described in paper I. The latter forms a smooth surface since the cross-linking reaction is performed *in situ* inside the PET inserts.

SCSCs maintained on Durolane® for four days were completely disrupted and the microarchitecture of the tissue was entirely lost, suggesting that a smooth surface of biomaterial was essential for the survival of SCSCs.

Degeneration of SCSCs maintained on collagen gel

Cultures maintained on collagen gel in the presence of culture medium that did not contain IL1RA or trophic support degenerated substantially during *in vitro* incubation (as described in paper II). SCSCs maintained on collagen gel were excitotoxicity damaged using NMDA after a three-day *in vitro* incubation. Comparisons between SCSCs exposed to NMDA and uninjured cultures were performed at different time points with respect to neuronal survival (Figure 5).

In contrast to SCSCs maintained on Healon 5® (described in paper III), SCSCs maintained on collagen gel degenerated to such an extent after a three-day *in vitro* incubation that NMDA-induced injury was not able to further reduce the number of neurons in the ventral horn. We therefore assumed that the choice of biomaterial was a critical factor in order to maintain SCSCs in a satisfactory condition for further experiments.

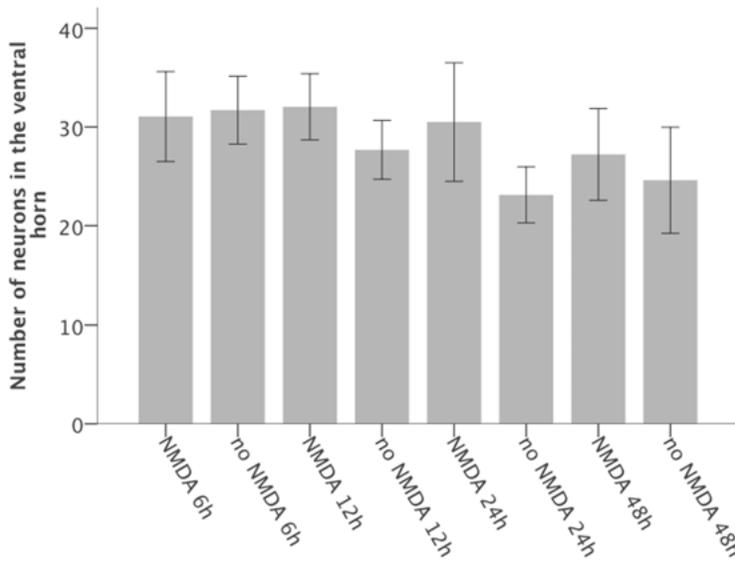


Figure 6. Number of neurons in the ventral horn of SCSCs maintained on collagen gel and exposed to NMDA three days after culture preparation (NMDA) or remained uninjured (no NMDA). SCSCs were exposed to NMDA for four hours and fixed after six, twelve, 24 and 48 hours after NMDA application. No statistically significant differences in the number of neurons were noted between SCSCs exposed to NMDA compared to uninjured cultures at any time point. Error bars denote SEM.

Degeneration of Renshaw cells in uninjured cultures

SCSCs derived from transgenic *Chrna2-Cre/R26^{Tom}* were initially maintained *in vitro* under control conditions for three days prior to NMDA-induced injury. Analysis at that time point revealed a dramatic degeneration of Renshaw cells, with their number not exceeding 2 or 3 cells per culture. Thus, in order to enable valid conclusions on the effects of excitotoxicity and IL1RA treatment on the numbers of Renshaw cells we used a narrower time frame between culture preparation and application of NMDA. SCSCs were prepared and immediately placed on Healon 5® that was pre-incubated with culture media either containing NMDA or not. The experimental setting is described in detail in paper III.

Validation of automated image analysis

The automated image analysis was validated by examining the correlation between automated and manual counts (Pearson's correlation analysis). For that purpose, 27 randomly chosen images were initially analyzed manually using the Image-J software and subsequently re-analyzed automatically us-

ing the CellProfiler software. The results indicated that the manual and automated methods of quantification correlated substantially with each other ($r^2=0.72$, $p<0.001$, Figure 7).

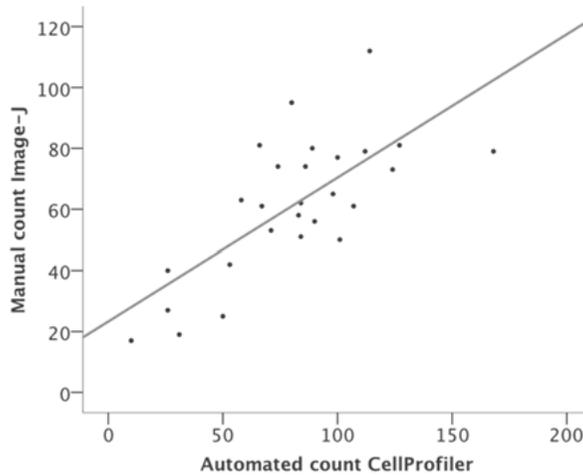


Figure 7. Graph showing the correlation between the manual and the automated methods of quantification.

Ongoing research in the field

Development of novel biomaterials

Since the beginning of this research project many new developments have been undertaken regarding the use of resorbable biomaterials. The HA-based hydrogel used in paper I has been modified with either collagen, GAGs or fibronectin, and it has been used in applications such as the delivery of bone morphogenetic protein (BMP)-2 and the study of neuronal differentiation in neural stem/progenitor cell cultures (Brannvall *et al.*, 2007; Kisiel *et al.*, 2012; Kisiel *et al.*, 2013a; Kisiel *et al.*, 2013b). It would be interesting to study the potential of migration of NCSCs on a collagen-integrated HA-based hydrogel since collagen possesses adhesive properties and could therefore provide a suitable substrate for migration of NCSCs.

Another potential application for the above biomaterial could be the study of spontaneous axonal sprouting from SCSCs. We have observed axonal sprouting from SCSCs in paper II. Axonal sprouting *in vitro* from tissues such as dorsal root ganglia and sensorimotor cortex has also been reported in earlier studies (Willits & Skornia, 2004; Stavridis *et al.*, 2009; Boato *et al.*, 2011). In pilot experiments we observed a difference between SCSCs maintained on collagen gel or Healon 5® regarding the density and length of sprouting axons. SCSCs maintained on Healon 5® had more numerous and

longer axons compared to SCSCs maintained on collagen gel (Figure 8). The model is still under refinement and future experiments in the field are planned.

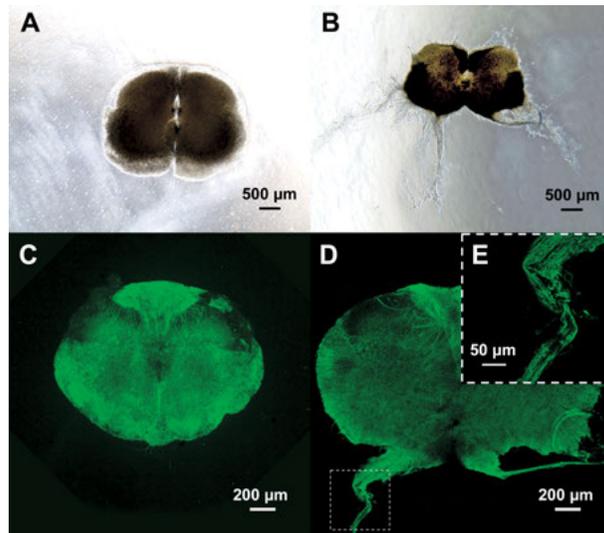


Figure 8. SCSCs maintained on collagen (A, C) and Healon 5® (B, D) for eight days. The cultures were first photographed with a camera attached to an inverted light microscope (A, B) and subsequently immunohistochemically stained against NF-L (C, D). The axon-like structures observed in B are positive for NF-L as shown in D and in a higher magnification in E. These axons, however, lose their initial morphology observed *in situ* (B and D) due to the fact that the biomaterial that supports them is dissolved during the staining procedure.

Small interfering RNA (siRNA)

Small interfering RNA is a class of double-stranded RNA molecules first described in 1998 (Fire *et al.*, 1998) and led to a Nobel Prize award in 2006 to Andrew Fire and Craig Mello. The most notable function of siRNA is the interference and silencing of specifically targeted genes, thus regulating protein expression. It has been argued that RNA interference could represent the genome's immune system (Plasterk, 2002) and since the first report in 1998, siRNA has been used as a type of gene therapy, especially concerning approaches for cancer treatment (Fujita *et al.*, 2015; Jovanovic *et al.*, 2015).

Collaborators in the biomaterial group have developed a type of siRNA with a specific oligonucleotide extension allowing cell penetration and endosomal escape. This type of siRNA is easily delivered *in vitro* to various cell types such as osteoblasts and astrocytes without involving any kind of carrier (unpublished data). Experiments using this modified siRNA have been undertaken on our SCSCs. Preliminary results indicate siRNA uptake by neu-

rons inside SCSCs. In addition to the imaging approach based on confocal laser scanning microscopy we are presently developing a method of cell image analysis called “image streaming” that will hopefully help us investigate siRNA uptake by neurons inside SCSCs. Future strategies include RNA interference leading to silencing of genes that regulate the expression of various factors involved in the secondary damage cascade after SCI.

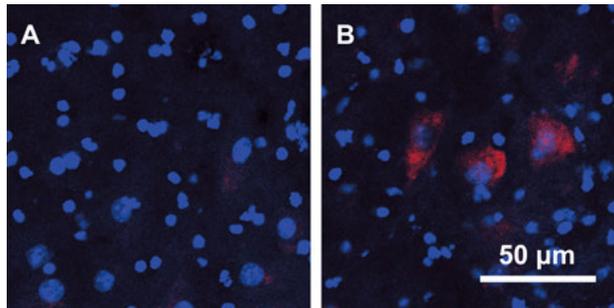


Figure 9. Micrographs of sections through the ventral horns of SCSCs maintained for four days *in vitro* in the presence of culture medium alone (A) and in the presence of culture medium containing siRNA. siRNA was conjugated with Cy-3 (red). Sections were counterstained with DAPI (blue). Red cellular structures in B show a neuronal phenotype, not present in A, suggesting siRNA uptake by neurons.

Concluding remarks and future perspectives

SCI is a devastating condition resulting in permanent para- or tetraplegia, neuropathic pain, dysesthesia and other sensation disorders. The incurability of SCI is mainly due to processes of secondary damage that aggravate the initial degree of tissue damage, and limited axonal regrowth after injury (Joosten *et al.*, 1995; Bregman *et al.*, 1997; Adamchik *et al.*, 2000). Our overall aim was to protect neurons that survived the acute phase of SCI from mechanisms that occur during the process of secondary damage.

This thesis has mainly focused on attempts at achieving neuroprotection during the secondary injury process by means of immunomodulation, particularly IL1RA. Transplantation of NCSCs was also studied as an approach to counteract excitotoxic neuronal damage. NCSCs seem to exert neuroprotective actions through the release of soluble factors. Based on the results of this thesis it seems that IL1RA is neuroprotective, both to neurons that undergo degenerative changes and to those that are subjected to excitotoxic damage.

Moreover, we have investigated the potential of various HA preparations to be used as carriers of neuroprotectants and/or cells. The role of HA-based biomaterials after SCI seems to be essential not only in order to ensure local controlled release of substances to the injury site. The results presented in

this thesis indicate that HA-based hydrogels—both cross-linked and not cross-linked gels—possess neuroprotective properties per se. The incorporation of neuroprotective substances and/or stem cells within biocompatible biomaterials is the final goal of these experiments, a goal that hopefully has come a bit closer. This would enable the development of injectable integrated biomaterials to be delivered at the time of decompressing and stabilizing surgery that is routinely after SCI.

Erratum

Regarding the fixation of cultures in the papers III and IV, I have by mistake referred to a study that is not consistent with the methodology used. Specifically, in papers III and IV it is mentioned that the fixation is performed according to a method described in paper I, which is not actually true. The fixation described in paper III and IV was actually performed according to a method described previously (Stavridis *et al.*, 2005) with some modifications. Since papers III and IV are still under review, these mistakes will be corrected before final publication.

Sammanfattning på svenska

Frakturer i kotpelaren kan ge upphov till skador på ryggmärgen som kan leda till förlamning och känselbortfall. Ryggmärgen skadas omedelbart i samband med traumat men ett stort antal nervceller och nervbanor i den komprimerade ryggmärgen skadas senare sekundärt genom bl.a. inflammatoriska och excitotoxiska mekanismer, en process som kallas sekundärskada. Det övergripande syftet med avhandlingen var att utveckla strategier för att minimera sekundärskadan i den komprimerade ryggmärgen. Genom att blockera inflammatoriska och excitotoxiska mekanismer skulle sekundärskadan kunna reduceras. Vi utvecklade och studerade resorberbara biomaterial som skulle kunna användas som bärare för neuroprotektiva substanser och/eller stamceller.

Vi använde som modell ”slice”-kulturer av ryggmärg tagna från nyfödda möss, alltså tunna transversella skivor från ryggmärgen. Slicekulturerna odlades på olika material och studerades efter behandling med immunomodulerande och/eller neurotrofiska faktorer. I andra experiment skadades kulturerna excitotoxiskt för att sedan behandlas med interleukin-1 receptorantagonist (IL1RA) eller genom transplantation av stamceller (neural crest stem cells [NCSC]).

Resultaten visar att biokompatibla och resorberbara biomaterial baserade på hyaluronsyra (HA) bevarade antalet neuroner i slicekulturerna i en mycket högre grad än konventionella substrat såsom kollagenbaserade biomaterial eller polyetentereftalat (PET)-membran. Samtidigt hämmades aktiveringen av gliaceller i de kulturer som odlades på HA-baserade biomaterial. Den antiinflammatoriska faktorn interleukin-1 receptor antagonist (IL1RA) skyddade slicekulturerna från degenerativa förändringar som annars förekommer under *in vitro* inkubation, och skyddade även slicekulturerna från excitotoxisk skada som framkallats genom exponering av kulturerna mot N-metyl-d-aspartat (NMDA). IL1RA skyddade specifikt neuroner lokaliserade i de ventrala hornen, medan andra neuronala populationer såsom neuroner i de dorsala hornen och Renshaw-celler inte svarade på behandling med IL1RA. Transplantation av NCSCs skyddade excitotoxiskt skadade slicekulturer från neuronal förlust, apoptos och glial aktivering. NCSCs migrerade på slicekulturernas yta men inte in i vävnaden och förblev odifferentierade, d.v.s. de utvecklades varken till neuroner eller gliaceller.

De resultat som presenteras i denna avhandling visar att biomaterial baserade på HA per se har neuroprotektiva effekter på ryggmärgsvävnad och de

verkar vara lämpligare än konventionella kollagenbaserade biomaterial. Den observerade neuroprotektionen beror sannolikt på HA-gelens biomekaniska egenskaper. IL1RA skyddar ryggmärgskulturer från degenerativa förändringar och från NMDA-inducerad excitotoxisk skada vilket tyder på att excitotoxiska mekanismer kan moduleras genom anti-inflammatoriska substanser. Olika neuronala populationer påverkas av IL1RA i olika grad vilket tyder på att en kombination av neuroprotektiva substanser bör användas i behandlingsstrategier efter ryggmärgsskada. Slutligen tycks NCSCs kunna skydda ryggmärgsvävnad från excitotoxisk skada genom att utsöndra neuroprotektiva faktorer.

Det förefaller att kombinationer av flera neuroprotektiva ämnen bör övervägas som behandling efter ryggmärgsskada och att HA-baserade gelmatri-ser verkar vara mycket lämpliga som bärare för både neuroprotektiva substanser och stamceller. I förlängningen bör denna strategi kunna leda fram till injicerbara biomaterial laddade med neuroprotektiva substanser såsom IL1RA och/eller stamceller som kan appliceras i samband med dekomprimerande och stabiliserande kirurgi.

Περίληψη στα Ελληνικά

Κατάγματα στη σπονδυλική στήλη ενδέχεται να προκαλέσουν τραυματικές κακώσεις του νωτιαίου μυελού, οι οποίες με τη σειρά τους οδηγούν σε παράλυση. Ο νωτιαίος μυελός εκτός από την πρωτοπαθή βλάβη που θα υποστεί, υπόκειται στις περισσότερες των περιπτώσεων και σε δευτεροπαθή βλάβη η οποία είναι πολλαπλάσια της πρωτοπαθούς κάκωσης. Η δευτεροπαθής βλάβη, ως αποτέλεσμα φλεγμονωδών και νευροδιεγερτικών τοξικών αντιδράσεων, καταστρέφει έναν μεγάλο αριθμό νευρώνων καθώς και ανιόντων και κατιόντων νευρικών οδών που αρχικά είχαν επιβιώσει της πρωτοπαθούς βλάβης.

Ο γενικός στόχος της παρούσας διατριβής είναι να χαραχθούν στρατηγικές για την αντιμετώπιση της δευτεροπαθούς βλάβης που ακολουθεί τον τραυματισμό του νωτιαίου μυελού. Η δευτεροπαθής νευρογενής βλάβη λαμβάνει χώραν διά μέσου φλεγμονωδών και νευροδιεγερτικών τοξικών οδών και η ρύθμιση ή διακοπή αυτών των οδών με τη χρήση αντιφλεγμονωδών και νευροπροστατευτικών ουσιών ενδέχεται να προστατεύσει τον τραυματισμένο νωτιαίο μυελό από περαιτέρω δευτεροπαθή βλάβη. Έχουμε επίσης μελετήσει και αναπτύξει απορροφήσιμα βιοϋλικά μορφής υδρογέλης με σκοπό χρησιμοποιηθούν ως φορείς των προαναφερθέντων αντιφλεγμονωδών και νευροπροστατευτικών ουσιών μετά από πιθανό τραυματισμό του νωτιαίου μυελού.

Χρησιμοποιήσαμε ως πειραματικό μοντέλο καλλιέργειες νωτιαίου μυελού προερχόμενες από ποντίκια. Οι καλλιέργειες επώστηκαν σε διάφορα βιοϋλικά και αξιολογήθηκαν μετά από θεραπεία με τον αντιφλεγμονώδη παράγοντα «αναστολέα του υποδοχέα της ιντερλευκίνης 1» (IL1RA) και/ή νευροτροφικούς παράγοντες. Επιπρόσθετα, οι καλλιέργειες υπέστησαν νευροδιεγερτική τοξική βλάβη χρησιμοποιώντας τον παράγοντα N-Μεθύλ-δ-Ασπαρτικό οξύ (NMDA) και ακολούθως θεραπεία με IL1RA ή θεραπεία με μεταμόσχευση βλαστικών κυττάρων νευρικής ακρολοφίας.

Τα αποτελέσματα έδειξαν ότι μια βιοσυμβατή και απορροφήσιμη υδρογέλη από υαλουρονικό οξύ (ΥΟ), διατήρησε τον αριθμό των νευρώνων σε πολύ μεγαλύτερο βαθμό από ό,τι άλλα συνήθη βιοϋλικά όπως ένα συμβατικό βιοϋλικό με βάση το κολλαγόνο ή από ό,τι πρότυπα ένθετα κυτταροκαλλιέργειας που εμπεριέχουν μεμβράνες από τερεφθαλικό πολυαιθυλένιο. Συγχρόνως η νευρο- και μικρογλοιακή αντίδραση στις καλλιέργειες περιορίστηκε όταν αυτές επώστηκαν στην υδρογέλη από ΥΟ. Ο αντιφλεγμονώδης παράγοντας IL1RA συσχετίστηκε με την προστασία

των καλλιέργειών από εκφυλιστικούς μηχανισμούς που διαδραματίζονται κατά τη διάρκεια της επώασης. Επίσης ο παράγοντας IL1RA διατήρησε τους νευρώνες σε καλλιέργειες που υπέστησαν νευροδιεγερτική τοξική βλάβη με NMDA. Η νευροπροστατευτική ιδιότητα του παράγοντα IL1RA ήταν εμφανέστατη στους νευρώνες του προσθίου κέρατος του νωτιαίου μυελού, ενώ άλλοι πληθυσμοί νευρώνων, όπως οι νευρώνες του οπισθίου κέρατος και οι νευρώνες του Renshaw δεν ανταποκρίθηκαν στη θεραπεία. Τέλος, η μεταμόσχευση βλαστικών κυττάρων νευρικής ακρολοφίας σε καλλιέργειες οι οποίες υπέστησαν νευροδιεγερτική τοξική βλάβη έδειξε να προστατεύει τις καλλιέργειες από μαζική απώλεια νευρώνων, μηχανισμούς απόπτωσης, και νευρο- και μικρογλοιακή αντίδραση. Τα βλαστικά κύτταρα μετανάστευσαν κατά μήκος της επιφάνειας των καλλιέργειών και δεν διείσδησαν διά μέσου αυτών, ενώ παράλληλα παρέμειναν αδιαφοροποίητα.

Τα αποτελέσματα που παρουσιάζονται στην παρούσα διατριβή υποδεικνύουν ότι βιοϋλικά υδρογέλης με βάση το ΥΟ φαίνεται να είναι καταλληλότερα από συμβατικά βιοϋλικά με βάση κολλαγόνο, δεδομένου ότι ενισχύουν επιπλέον την επιβίωση νευρώνων. Η παρατηρούμενη νευροπροστασία είναι πιθανότερο να οφείλεται στις βιο-μηχανικές ιδιότητες της υδρογέλης βασισμένης στο ΥΟ. Ο παράγοντας IL1RA προστατεύει τις καλλιέργειες τόσο από εκφυλιστικές αλλοιώσεις καθώς και από νευροδιεγερτική τοξική βλάβη, υποδηλώνοντας ότι νευροδιεγερτικές τοξικές οδοί μπορούν να διακοπούν ή να τροποποιηθούν διά μέσου αντιφλεγμονωδών οδών. Διαφορετικοί πληθυσμοί νευρώνων απαντούν στη θεραπεία με IL1RA σε διάφορους βαθμούς, γεγονός που υποδηλώνει ότι συνδυασμός διάφορων νευροπροστατευτικών και αντιφλεγμονωδών μέσων θα πρέπει να αξιολογείται σε θεραπευτικές στρατηγικές μετά τον τραυματισμό του νωτιαίου μυελού. Τέλος, τα βλαστικά κύτταρα νευρικής ακρολοφίας φαίνεται να προστατεύουν τις καλλιέργειες από νευροδιεγερτική τοξική βλάβη μέσω παρακρινών δράσεων, δεδομένου ότι δεν μεταναστεύουν διά μέσου των καλλιέργειών και παραμένουν αδιαφοροποίητα κατά τη διάρκεια της επώασης.

Γίνεται πλέον όλο και πιο φανερό ότι στο πλαίσιο της τραυματικής βλάβης του νωτιαίου μυελού θα πρέπει να αξιολογείται ένας συνδυασμός προσεγγίσεων με τη χρήση νευροπροστατευτικών ουσιών αντί μίας απλής στρατηγικής. Η ενσωμάτωση αντιφλεγμονωδών και νευροπροστατευτικών παραγόντων όπως IL1RA ή/και βλαστικών κυττάρων σε ενέσιμα βιοσυμβατά υλικά με βάση το ΥΟ καθώς και η χορήγησή τους κατά τη διάρκεια της αποσυμπιεστικής και σταθεροποιητικής επέμβασης, είναι ο απώτερος στόχος της έρευνάς μας στην αντιμετώπιση των τραυματικών κακώσεων του νωτιαίου μυελού.

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