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Cell Therapy for Spinal Cord Injury, Studies of Motor and Sensory Systems

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Dedicated to my wife, parents and sisters

Abstract

Spinal cord injury represents a significant medical concern, affecting thousands of individuals each year. The majority of victims are young males harmed by traffic accidents, violence, and falls. Severe spinal cord injury interupts nerve fiber pathways connecting the brain and the rest of the body. This leads to paralysis, loss of sensation, and reflex function below the level of injury. It also may significantly affect autonomic activity, including respiratory, vascular, bowel, and bladder control. Over time, spinal cord injury victims are also prone to develop other symptoms such as chronic pain, muscle spasms, and infections.

The first part of the present thesis describes functional and structural alterations that follow a contusion injury in adult rats. We were interested in determining whether the pattern of spontaneous sensory and motor recovery in weight-drop injured rats resembles that observed in human spinal cord injury victims. Using behavioral tests and functional magnetic resonance imaging, we found that rats subjected to a moderate spinal cord injury recovered close to normal hindpaw locomotion in the absence of sensory function. Thus, assessment of locomotor function in the rat is not a good indicator of sensory signaling across the injury site. This is in contrast to humans, where sensory function predicts locomotor outcome.

The failure of nerve fiber regeneration following traumatic injury of the spinal cord has been attributed to the insufficient intrinsic growth capacity and/or growth inhibitory properties of the scar tissue. We characterized sprouting of the severed corticospinal tract using in situ hybridization and stereology. Axotomized pyramidal cells upregulated growth-associated molecules and underwent signifcant sprouting rostral to the injury site. However, outgrowing fibers did not regenerate beyond the lesion but remained rostral to the injury site where they formed a massive "central neurinoma".

The second part of the thesis investigates effects of mesenchymal and neural stem cell grafts on outcome following spinal cord injury. Mesenchymal stem cells were harvested from bone marrow of adult rats, expanded in vitro, and grafted into the lesion site 7 days following a weight drop injury. Engrafted MSCs formed bundles that bridged the epicenter of the injury. MSC-aggregates contained host-derived immature astrocytes and provided directional guidance to regrowing nerve fibers. Transplantation of MSCs led to significantly improved recovery of open-field walking.

We also grafted adult neural stem cells into the lesion 7 days following a contusion injury of the spinal cord. The majority of engrafted cells gave rise to astrocytes. Grafting of these cells improved locomotor function; however, it also causes aberrant axonal sprouting of CGRP-positive fibers that were associated with allodynia-like hypersensitivity of forepaws. Transduction of neural stem cells with neurogenin2 prior to transplantation suppressed astrocytic differentiation of engrafted cells and prevented the development of allodynia. Instead, ectopic expression of neurogenin2 allowed for enhanced oligodendroglial differentiation of engrafted cellsand increased amounts of myelin in the injured area. This was correlated to significantly better recovery of skilled hindlimb motor tasks. Importantly, neural stem cells transduced with neurogenin2 also allowed for improved hindlimb sensory function and increased cortical BOLD-signaling in response to hindpaw stimulation.

To conclude, functional MRI and stereology allowed objective quantitative measurents of functional and structural recovery following spinal cord injury. We used these methods to characterize the effects of two types of stem cell engraftment in spinal cord injury models. We also identified a potentially harmful side effect of stem cell grafting and found a remedy. These kinds of analysis of current experimental treatment strategies are needed to select which approaches to translate into clinical trials.

Table of Contents

List of Papers

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

I. Hofstetter CP, Card PJ, Olson L. A spinal cord pathway connecting primary afferents to the segmental sympathetic outflow system. *Experimental Neurology* 2005 (in press).

II. Hofstetter CP, Schweinhardt P, Klason T, Olson L, Spenger C. Numb rats walk – a behavioral and fMRI comparison of mild and moderate spinal cord injury. *European Journal of Neuroscience* 2003; 18(11):3061-3068.

III. Hofstetter CP, Olson L. Stereological quantification of the corticospinal tract following spinal cord injury. *Manuscript.*

IV. Erschbamer M, Hofstetter CP, Olson L. RhoA, RhoB, RhoC, Rac1, Cdc42 and Tc10 mRNA levels in spinal cord, sensory ganglia and corticospinal tract neurons and long-lasting specific changes following spinal cord injury. *Journal of Comparative Neurology* 2005; 484 (2): 224-233.

V. Hofstetter CP, Schwarz EJ, Hess D, Widenfalk J, El Manira A, Prockop DJ, Olson L. Marrow stromal cells form guiding strand in the injured spinal cord and promote recovery. *Proceedings of the National Academy of Science of the United States of America* 2002; 99(4):2199-2204.

VI. Hofstetter CP, Holmström N, Lilja J, Schweinhardt P, Hao JX, Spenger C, Wiesenfeld-Hallin Z, Kurpad SN, Frisen J, Olson L. Allodynia limits usefulness of intraspinal neural stem cell grafts; directed differentiation improves outcome. *Nature Neuroscience* 2005; 8(13): 236-253.

Introduction

The spinal cord extends from foramen magnum to the lower border of the first lumbar vertebra. It is the only part of the adult nervous system that fully preserves the primitive segmental arrangement of the embryonic neural tube. From each of the segments a pair of spinal nerves leaves the bony vertebral canal and reaches the periphery. The spinal cord conducts and modulates impulses back and forth between the brain and the body. Ascending sensory pathways transfer sensory information from skin, muscles, joints and organs to the brain. Descending motor pathways control voluntary movements and reflex functions of limbs and the trunk. Other descending pathways modulate the output of the autonomic system which controls homeostasis as well as bowel, bladder and sexual function.

Fractures or dislocation of vertebrae, secondary e.g. to traffic accidents, falls and violence, can cause compression of the spinal cord. Traumatic spinal cord injury severing descending and ascending fiber tracts leads to loss of motor and sensory function caudal to the level of injury. Disruption of fibers that control the autonomic nervous system leads to impairment of vascular, exocrine and endocrine gland, bowel, bladder and sexual function. Loss of supraspinal control of the sympathetic nervous system may lead to orthostatic hypotension, reflex bradycardia and even cardiac arrest. In addition, a majority of spinal cord injury victims develops chronic complications such as urinary tract infections, decubitus ulcers, urolithiasis, chronic pain conditions (Figure 1, Levi et al., 1995a; Christensen and Hulsebosch, 1997), and subsequently depression (Cairns et al., 1996).

Currently, the standard clinical treatment includes surgical stabilization of the vertebral column and application of high doses of steroids. Surgical stabilization prevents posttraumatic instability of the vertebral column and further progression of neurological deficits (Malcolm et al., 1981). Early administration of steroids has been suggested to decrease the amount of tissue damage by lowering the amount of free radicals at the injury site (Bracken et al., 1990, 1998; Bracken, 2001). The effects of this treatment regimen, however, are modest at best (Coleman et al., 2000) and there is a therefore great need for novel treatment strategies that could significantly protect and/or restore function following spinal cord injury.

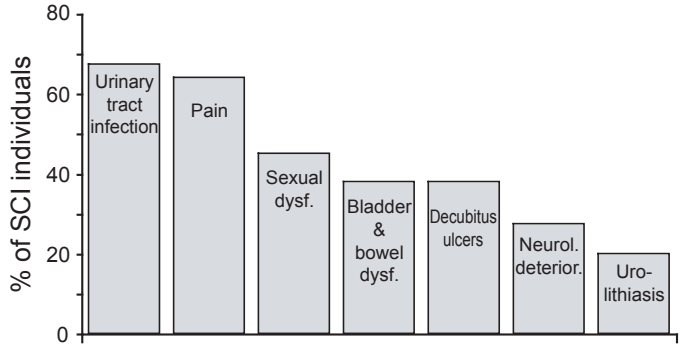

Figure 1. The most common medical problems encountered by spinal cord injured individuals. Data summarized from (Levi at al., 1995a).

Epidemiology

Worldwide 90 million people suffer from spinal cord injuries of varying severity (Jackson et al., 2004). The annual incidence of spinal cord injury is 3–5 per 100 000 in the USA and 1.5–2 per 100,000 in Sweden. This results in approximately 11m000 new cases of spinal cord injury in the USA and 150 in Sweden per year (Levi et al., 1995b; Anson and Shepherd, 1996; Frankel et al., 1998; Hoque et al., 1999; Surkin et al., 2000; van Asbeck et al., 2000). Spinal cord injury victims are predominantly male (80%) and more than half of them are injured between the ages of 16 and 30. In the USA, the most frequent causes for spinal cord injury are motor vehicle accidents, followed by violence, falls and sports injuries (Surkin et al., 2000). In Europe, violence accounts for fewer injuries.

Due to improved emergency medical techniques, immediate survival following spinal cord injury has improved over the past 50 years. Presently, the majority (90%) of spinal cord injury victims survive and live near-normal life spans. The costs associated with the medical treatment of a single spinal cord injury victim can reach as high as US$1.35 million depending on the severity of the injury and the age when the individual was injured. In 1992, for example, the direct costs for medical care related to spinal cord injury exceeded US$7 billion per year in the USA.

Improved neurointensive care and introduction of high-dose methylprednisolone treatment in the acute phase following injury have increased the proportion of incomplete injuries. Thus, while complete spinal cord injuries used to outnumber incomplete injuries by 3:2, this ration is now inverted (Levi et al., 1995a; Bracken et al., 1997). However, recovery is still limited and the consequences of spinal cord injury are often functionally devastating.

Pathopysiology

Traumatic injury leads to immediate and irreversible primary tissue damage (Allen, 1911). Experimental studies demonstrate that primary gross tissue damage is remarkably limited. Typically, there is hemorrhage in the central gray matter whereas the surrounding white matter appears to be morphologically intact within the first hours following injury (Balentine, 1978; Noble and Wrathall, 1989; Rosenberg and Wrathall, 1997). In the acute phase, mechanical disruption of microvasculature causes vasospasm and thrombosis at the injury site (Tator and Fehlings, 1991). This leads to a substantially reduced delivery of oxygen to the tissue over several hours following injury (Ducker et al., 1978; Stokes and Garwood, 1982) and subsequent ischemic necrosis (Kobrine, 1975; Sandler and Tator, 1976). Metabolic disturbances lead to failure of normal neuronal function and a stage of spinal chock (Bach-y-Rita and Illis, 1993).

Minutes following injury, secondary damage of initially spared tissue is initiated by excessive production of free radicals, release of excitatory neurotransmitters and inflammatory reactions. Direct mechanical stimulation or mobilization of calcium increases phospholipase activity leading via the cycloocygenase pathway to the formation of pro-inflammatory prostanoid and free radicals that can cause oxidation of fatty acids in cellular membranes (lipid peroxidation) (Braughler and Hall, 1989). Oxidative stress disables key respiratory chain enzymes, alters DNA-associated proteins and inhibits sodium-potassium ATPase, together inducing metabolic collapse and subsequent necrotic or apoptotic cell death (Cuzzocrea et al., 2001).

Excessive release of the excitatory neurotransmitter glutamate can reach toxic levels as early as 15 minutes following injury, contributing to secondary tissue damage (Panter et al., 1990; Liu et al., 1991). Activation of NMDA receptors leads to intracellular accumulation of calcium, which in turn triggers processes that can lethally alter cellular metabolism (Choi, 1987, 1988). These processes

lead via activation of calpains, phospholipase A2 and lipoxygenases as well as via the generation of free radicals and dysregulation of mitochondrial oxidative phosphorylation to apoptotic cell death. Excessive glutamate also causes sodium influx via NMDA and AMPA/kainate receptors and hence cellular edema. Glutamate exitotoxicity appears to be further aggravated by the presence of TNF-∝ (Hermann et al., 2001). Ischemia, oxidative stress and excitotoxicity lead to secondary cell loss at the injury site by necrosis and apoptosis. The latter is especially interesting from a therapeutic standpoint. Apoptosis is ATP dependent and requires the production of new proteins in order to be carried out. It involves intracellular pathways which eventually activate caspases (Nicholson and Thornberry, 1997) that could be targets for pharmacological intervention aimed at delaying the process until it is no longer triggered by the environment.

Traumatic spinal cord injury elicits a strong inflammatory response with recruitment of inflammatory cells that secrete lytic enzymes and cytokines. The role of inflammation for outcome following spinal cord injury can be considered as a "dual-edged sword" since both deleterious and neuroprotective effects of the inflammatory response have been described. Increased expression of endothelial CAMs recruits leukocytes to the injury site (McTigue et al., 1998; Schnell et al., 1999). Invading macrophages produce pro-inflammatory cytokines such as TNFα, IL-1 and IL-6 and activate cyclooxygenases. Low concentrations of TNFα, IL-1 and IL-6 have been suggested to be neuroprotective (Cheng et al., 1994; Barger et al., 1995; Murphy et al., 2000). TNFα prevents glutamate induced cell death and suppresses the accumulation of free radicals (Cheng et al., 1994; Barger et al., 1995). Low concentrations of IL-1 and IL-6 have been suggested to induce production of glial-derived neurotrophic growth factor (Ho and Blum, 1997) and brain-derived growth factor (Murphy et al., 2000), respectively. On the other hand, high concentrations of TNFα, IL-1 and IL-6 can activate transcription factors such as AP-1 or NF-κB which mediate cell death (Li et al., 2001; Bareyre and Schwab, 2003).

Oligodendrocytes appear particularly vulnerable to apoptotic cell death. Different mechanisms account for the loss of oligodendrocytes in various locations in relation to the injury center. At the injury center oligodendrocytes die due to cellular disruption and necrosis. In regions surrounding the lesion, secondary degeneration of oligodendrocytes is evident 6 hours post injury (Crowe et al., 1997). Extracellular excitatory amino acids have been suggested to induce apoptosis via AMPA/kainate receptors that are expressed by oligodendrocytes (Gallo and Russell, 1995; Bernstein et al., 1996). This is supported by the observation that microinjection of an AMPA/kainate receptor antagonist reduces loss of oligodendrocytes and myelin pathology (Rosenberg et al., 1999). Whereas oligodendrocytes undergo apoptosis, intact axons, astrocytes and blood vessels remain in the spared white matter surrounding the lesion (Li et al., 1999). In areas of Wallerian degeneration remote to the injury center oligodendrocytes undergo apoptosis for weeks following injury. This might be due to withdrawal of trophic factors after axonal loss (Barres et al., 1993). Since oligodendrocytes myelinate multiple axons, loss of these cells could also lead to demyelination of spared axons in regions containing both dying and surviving axons.

Demyelination impairs conduction properties of spared axons (Waxman, 1989). Some degree of spontaneous remyelination is seen and may be the result of invading Schwann cells (Blight and Young, 1989) and/or oligodendrocyte precursor cells (Gensert and Goldman, 1997). This remyelination is often abnormal, with some areas remaining partially demyelinated and other areas possessing thin myelin sheaths (Griffiths and McCulloch, 1983). Consequently, incomplete spinal cord injury can be associated with impaired axon conduction properties (Nashmi and Fehlings, 2001).

Experimental spinal cord injury treatment strategies

Pharmacological therapy

Administration of high doses of methylprednisolone is currently standard clinical practice for acute spinal cord injury. The mechanisms by which corticosteroids promote neuroprotection are proposed to include the scavenge of free radicals (Hall and Braughler, 1982; Hall, 1992) and down-regulation of inflammation-related genes such as pre-pro complement C, MHC class I and thrombin (Almon et al., 2002). Beneficial effects of methylprednisolone on functional recovery have been demonstrated in randomized, prospective, double-blind, multicenter studies NASCIS II (Bracken et al., 1990) and NASCIS III (Bracken et al., 1997). However, these clinical studies have been criticized because of design and statistical analysis problems (Coleman et al., 2000). Moreover, methylprednisolone can cause significant side effects such as sepsis and pneumonia.

Blocking of excitotoxic receptors is another strategy to diminish secondary tissue damage. Beneficial treatment effects have been reported following blocking of NMDA (Faden and Simon, 1988; Kochhar et al., 1988; Feldblum et al., 2000; Li and Tator, 2000) or AMPA (Wrathall et al., 1996; Mu et al., 2002) receptors. At the University of Montpellier a clinical trial investigated the effect of NMDA-receptor blocker application (gacyclidine) within 3 h of injury. Overall, treatment did not result in statistically significant improvement of function, however there was a trend suggesting that patients with the highest dose improved (Steeves et al., 2004).

Immunomodulatory treatments

Inflammatory processes appear to mediate beneficial as well as detrimental effects on the outcome following spinal cord injury. Blocking TNFα either with a neutralizing antibody (Lavine et al., 1998) or by IL-10 (Bethea et al., 1999) decreased the lesion size and resulted in significant improvement in function. Infusion of an IL-1 receptor antagonist diminished injury-induced apoptosis (Nesic et al., 2001). Blocking of the IL-6 receptor following spinal cord injury reduced astrogliosis and connective tissue scar formation and allowed for improved functional recovery (Okada et al., 2004).

Although phagocytic macrophages have been traditionally implicated in secondary destruction of neural tissue following traumatic injury (Blight, 1985; Blight, 1992), it has been suggested that the macrophage recruitment following spinal cord injury is in fact not sufficient as compared with peripheral nerve injury. In the latter, early and robust macrophage invasion contributes to the removal of myelin debris, which impedes axonal regrowth, and stimulates the proliferation of Schwann cells. Implantation of activated macrophages into a completely transected spinal cord of adult rats resulted in partial recovery of motor function (Rapalino et al., 1998). Conversely, however, there is evidence suggesting that depletion of macrophages may result in preservation of myelinated axons and improved functional outcome following injury (Popovich et al., 1999). In Israel, a phase I clinical trial demonstrated the safety of autologous macrophage transplantation into the damaged spinal cord within 2 weeks of injury. Since this study found modest improvements in some patients a phase II clinical trial has now been launched.

Neurotrophic factors

Neurotrophic factors have been demonstrated to promote cell survival, dendritic modeling and axonal regeneration (Thoenen, 1995). The up-regulation of intrinsic neurotrophic factors following spinal cord injury is limited (Thoenen, 1995; Widenfalk et al., 2001). Different approaches have been developed to deliver neurotrophins to the lesion site. Robust long-term release of neurotrophic

factors has been reported following implantation of genetically modified fibroblasts (Tuszynski et al., 1996; Grill et al., 1997). Transplantation of cells that serve as a source of growth factors circumvents the need for genetic engineering of cells prior to transplantation. Beneficial treatment effects have been reported following transplantation of neurotrophic factor producing cells such as dental pulp cells (Nosrat et al., 2001), olfactory ensheathing cells (Lipson et al., 2003), marrow stromal cells (Chopp et al., 2000; Wu et al., 2003; Ankeny et al., 2004; Ohta et al., 2004; Zurita and Vaquero, 2004; Kamada et al., 2005; Neuhuber et al., 2005), neural stem cells (Ogawa et al., 2002) and embryonic stem cells (McDonald et al., 1999).

Blocking of growth inhibiting factors

The failure of spontaneous axonal regeneration has been attributed to the elevated expression of several growth inhibitory molecules such as Nogo (Caroni and Schwab, 1988; Bregman et al., 1995), myelin associated glycoprotein (McKerracher et al., 1994) and oligodendroycyte-myelin glycoprotein (Wang et al., 2002). Regenerating axons may also face increased deposition of extracellular matrix molecules such as chondroitin sulfate proteoglycans (Davies et al., 1999; Lemons et al., 1999; Jones et al., 2002) that make the scar tissue non-permissive for the regrowing nerve fibers (Silver and Miller, 2004). Treatment of animals subjected to a hemitransection of the spinal cord with IN-1, a monoclonal antibody directed against Nogo, improved axonal regeneration (Bregman et al., 1995). Increased axonal regeneration has also been reported following inhibition of chondroitin sulfate synthesis (Grimpe and Silver, 2004) or following digestion of its side chains by chondroitinase ABC (Bradbury et al., 2002).

Cell transplantation

Cell transplantation strategies aim at improving functional outcome following spinal cord injury by providing a scaffold for axonal regeneration and/or replacing lost neural tissue.

Marrow stromal cells have been suggested to provide support for directed axonal regeneration and have shown to improve functional recovery following experimental spinal cord injury (Chopp et al., 2000; Wu et al., 2003; Ankeny et al., 2004; Ohta et al., 2004; Zurita and Vaquero, 2004; Kamada et al., 2005; Neuhuber et al., 2005). However, the contribution of enhanced nerve fiber outgrowth to functional benefits is not clear. Rather it appears that MSCs mediate beneficial effects via release of growth factors (Ankeny et al., 2004) and ensheathment of spared nerve fibers (Akiyama et al., 2002a, b; Inoue et al., 2003).

Repair of lost myelin with peripheral type myelin has also been observed following transplantation of Schwann cells (Blakemore and Crang, 1985; Duncan et al., 1988; Bachelin et al., 2005). Grafting of both rat and human Schwann cells into demyelinated lesions improves conduction velocity and the ability to follow high frequency stimuli (Honmou et al., 1996; Kohama et al., 2001). Upon transplantation into a contusion injury Schwann cells myelinate nerve fibers and improve hindlimb locomotor function (Takami et al., 2002).

Transplantation of olfactory ensheathing cells has been shown to enhance axonal regeneration (Ramon-Cueto et al., 1998) and to promote recovery of motor function following a transection injury of the spinal cord (Ramon-Cueto et al., 2000). However, their ability to migrate and to guide regrowing axons appears to be limited by scar tissue (Lee et al., 2004a). This might explain controversial results regarding their effectiveness in traumatic contusion injuries (Takami et al., 2002; Lipson et al., 2003; Plant et al., 2003). In addition to promote axonal regeneration, olfactory ensheathing cells also form peripheral myelin upon grafting into demyelinated CNS lesion.

Whereas there are some differences in the appearance of myelination formed by olfactory ensheathing cells compared to Schwann cells, they have similar beneficial effects on conduction properties (Imaizumi et al., 1998, 2000). Recently, remyelination has also been described following transplantation of neural stem cells (Hammang et al., 1997; Oka et al., 2004).

Bridging strategies

Guiding structures such as peripheral nerve bridges (Aguayo et al., 1981) have been shown to restore a degree of function after complete spinal cord injury (Cheng et al., 1996; Lee et al., 2002; Fraidakis et al., 2004; Lee et al., 2004b; Tsai et al., 2005), and biodegradable polymers (Friedman et al., 2002; Novikova et al., 2003) can also be used to bridge a gap after spinal cord injury. Recently, grafting of olfactory ensheathing cells and delivery of chondroitinase ABC has been demonstrated to make the interfaces between host and bridge more favorable to nerve fiber outgrowth (Fouad et al., 2005).

Aims

- Characterize possible segmental coupling between the sensory and sympathetic system
- Characterize the relationship between spontaneous motor and sensory recovery following spinal cord injury
- Investigate the responses of a downstream signaling component of the Nogo system, Rho, and related genes to spinal cord injury
- Compare effects of mesenchymal and neural stem cells on local pathology and recovery following spinal cord injury
- Identify possible side effects of novel treatment strategies

Materials and Methods

Animals and postoperative care (Paper I – VI)

We used adult female Sprague Dawley rats ((B&K Universal, Sollentuna, Sweden) and Lewis rats (Charles River). Following operations animals received intraperitoneal injections of 0.03 mg/kg buprenorphin (Temgesic®, Schering-Plough, NJ, USA) to relieve postoperative pain. During the first week after spinal cord injury, cages were warmed by heating pads. Bladders were emptied manually three times a day and animals received antibiotics (Borgal®, Hoechst) to prevent urinary tract infections.

Viral tracing (Paper I)

The recombinant Bartha strain 152 of pseudorabies virus (PRV-Bartha, (Billig et al., 2000)), which expresses green fluorescent protein, was used to study the control of the sympathetic outflow to lumbar DRGs. PRV-Bartha was propagated on PK15 cells to a titer of 1.7×10^8 plaque forming units per milliliter (pfu/ml). The virus was aliquoted in 100 µl batches and stored at –80°C. Single aliquots were thawed immediately prior to injection and stored on ice during operation.

Under anesthesia, right lumbar dorsal root ganglia L3 to L6 were exposed by a hemilaminectomy of L3 – L6 and removal of facet joints L3/L4 – L6/S1. Virus suspension was injected by stereotaxic technique into dorsal root ganglia L3 to L6 using a glass pipette with a tip diameter of 80 µm attached to a 10 µl microliter syringe. The viral suspension was infused at 0.5 µl per minute and after complete injection the needle was left in place for 2 minutes. For double tracing experiments 0.05% cholera toxin B subunit (CTB, List laboratories, CA, USA) was added to the viral suspension.

The Bartha-strain is an attenuated strain derived from the virulent Becker strain of pseudorabies virus. The latter spreads both in an anterograde and a retrograde fashion in a chain of synaptically linked neurons. The attenuated Bartha strain spreads in a strictly retrograde fashion (Brideau et al., 2000; Husak et al., 2000; Tomishima and Enquist, 2001; Pickard et al., 2002) due to a deletion in the unique short region which codes for Us9, gE and gI viral membrane proteins. Us9 appears necessary for axonal localization of viral membrane proteins and subsequent transport to the axon terminals of infected neurons (Tomishima and Enquist, 2001). gE and gI accumulate at nerve terminals and facilitate the spread of infection from pre- to postsynaptic neurons (Dingwell et al., 1995; Brideau et al., 2000). Absence of Us9, gE and gI viral membrane proteins renders the virus incapable of anterograde transport in the CNS. Therefore the attenuated Bartha-strain did not

enter the spinal cord via the dorsal roots but instead entered the spinal cord via the sympathetic system in a purely retrograde fashion.

Immunohistochemistry (Paper I, VI, V and VI)

Animals were deeply anesthetized (Pentobarbital) and intracardially perfused with 50 ml Tyrode's solution containing 0.1 ml of Heparin, followed by 200 ml fixative (4% parafomaldehyde and 0.4% picric acid in PBS). Specimens were dissected, postfixed in similar fixative for one hour and then transferred to 10% sucrose solution. Frozen specimens were cut on a cryostat and sections were thaw-mounted on gelatin-coated slides. Sections were incubated with the primary antibody diluted in PBS with 0.3% Triton-X at 4°C over night. For confocal imaging of the specimen (Zeiss, LSM 510 Meta), primary antibodies were detected by incubation with secondary antibodies conjugated to Cy2, Cy3 or Cy5 (Jackson ImmunoResearch Laboratories, 1:50, 1:300 or 1:50 in PBS with 0.3% Triton-X, respectively) for 45 min at room temperature. For stereological evaluation primary antibodies were visualized using the avidin-biotin modification (Hsu et al., 1981) of the immunoperoxidase method of Sternberger (Sternberger, 1979). Specimens were incubated with biotinylated secondary antibodies (Jackson ImmunoResearch Laboratories, 1:200 in PBS with 0.3% Triton-X) for 1 h in room temperature, washed with PBS and then incubated for 1.5 h in avidin-biotin reagents (Vectastain Elite, Vector Laboratories, 5 µl of A + 5 µl of B per 1.0 ml, combined 90 min before tissue incubation). Sections were washed again with Tris-buffer and reacted with H_2O_2/3,3-diaminobenzidine (DAB) solution for 5 minutes, counterstained with cresyl violet and coverslipped.

Stereological number and length quantification (Paper I, III, IV and VI)

Stereology was used to obtain unbiased estimates of cell numbers, nerve fiber length and cell volume. The three-dimensional optical disector (Gundersen, 1986) and the two-dimensional surface of virtual spheres (Figure 2, Mouton et al., 2002) were used for cell counting and for fiber quantification, respectively. Both probes were used in a systematic sampling scheme, the fractionator (West and Gundersen, 1990). The optical disector evolved from the physical disector, in which pairs of adjacent slides are analyzed and objects are only counted if they are not present on the first section but appear on the second section (Sterio, 1984). This disector counting rule determines weather a unique point of each object such as the top, also called the "trailing edge" lies within the sampled volume. This rule assures that each object is only counted once. Later, the optical disector was developed, in which thick histological sections (30–40 µm) are instead "optically sectioned" with a narrow focal plane generated by a high magnification oil immersion objective (60x or 100x, 1.4NA). In practice, a rectangular counting frame (Stereologer™, SPA inc.) is superimposed on the field of view and then focused through the height of the disector. The numbers of cell nuclei that come into focus within the optical disector are counted. For quantification of nerve fibers, virtual spheres were superimposed on the field of view (Mouton et al., 2002). Virtual spheres were focused through the Z-axis and intersections between the surface of the sphere and nerve fibers were counted.

The fractionator method implies counting of objects within optical disectors that constitute a known fraction of the analyzed region. A systematic uniform sample of the entire set of sections (section sampling fraction [ssf], for example every 10^{th} section with a random starting section) which contains the whole area of interest is selected. Counting frames are systematically distributed with known x and y steps throughout the marked region from a random starting point. The area of the counting frame relative to the area associated with the x and y steps gives the second fraction (area sampling fraction (asf) = $_a$frame/ $_a$x, y steps). The height (h) of the optical disector relative to

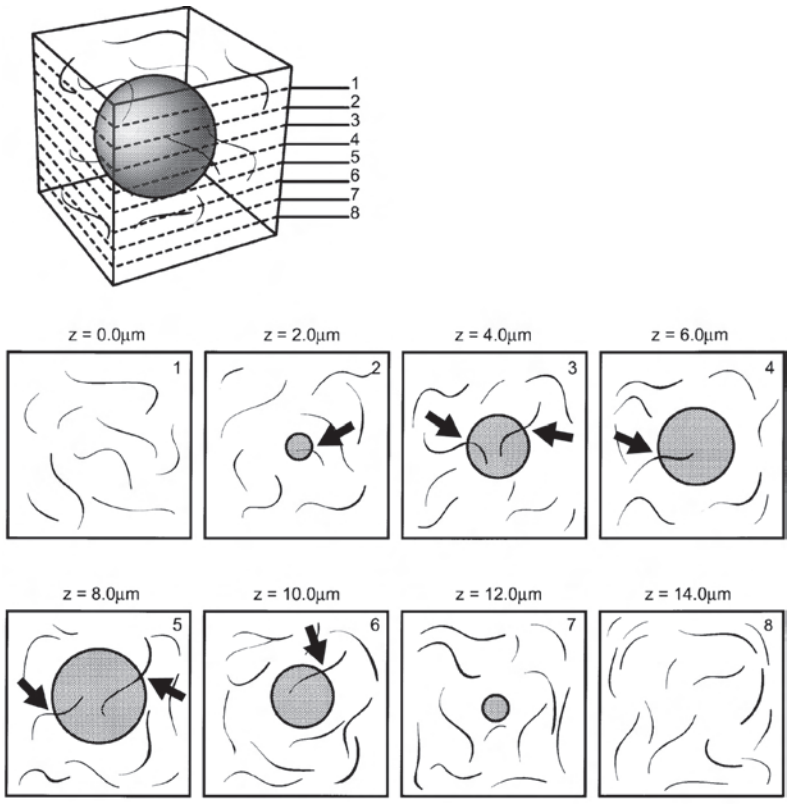

Figure 2. Schematic illustration of data collection using 3-D spheres. The focal plane is moved in 1 µm steps through the boxed area. Arrows indicate intersections between the surface of the sphere and nerve fibers. Illustration modified from (Mouton et al., 2002).

the thickness (t) of the section results in the third fraction (h / t). The total number of objects is calculated according to the formula

$$N_{total} = \sum Q - \cdot \frac{1}{ssf} \cdot \frac{1}{asf} \cdot \frac{t}{h} \qquad \text{where} \quad \sum Q -$$

is the number of neurons counted in the disectors.

Cell numbers can also be assessed combining the optical disector with volume estimation according to the Cavalieri principle. Multiplying the cell density with the volume of the investigated region results in the total number. Importantly the volume has to be measured in the sections following tissue processing, because tissue shrinkage or extension can change the reference volume. This phenomenon is also called the "reference trap" and would for example occur if sections are produced with a cryostat at a thickness of 40 µm and this value would be used to calculate the volume of the region, whereas cell density is assessed in optical disectors within processed tissue (in our experience 40 µm spinal cord sections shrink to an average thickness of 26 µm following tissue processing). Number estimates obtained by the fractionator method, which defines disector area and height as relative terms, are not affected by shrinkage (West et al., 1991). Moreover, whereas estimation of cell number by calculating cell density and region volume needs a well-defined anatomical region, the fractionator method avoids having to explicitly define boundaries of a region. Instead, the fractionator method requires that objects of interest are identifiable. This is a great advantage considering the anatomy of the spinal cord. However, there are possible sources of bias using the fractionator approach. For example there is the need to measure section thickness at every disector. Bias might be due to inaccurate microcater measurements of z drives of the microscope stage (Heidenhain, Traunreut, Germany, Z repeatability ± 0.1µm) and/or investigator bias when upper and lower surfaces of a section are defined. Another possible source of bias when using the optical

disector is that cell nuclei can be torn out of sections during sectioning and processing of tissue specimens. As a consequence cell densities will appear lower and variability will be higher in parts of the section close to the surface, a phenomenon that has been described as "lost caps" (Gundersen, 1986). To avoid underestimation of number counts we used a minimum of 2-µm thick "guard zones" that separated the optical disector from the section surface (Walters et al., 1999).

For the volume distribution, number weighted samples of cells are analyzed with the rotator. This technique is based on the principle that the length of an isotropic line from any fixed point in an object to the surface of the object is proportional to the mean object volume (Tandrup et al., 1997).

Lesion model (Paper II, III, IV, V and VI)

The most common injury models in rodent spinal cord injury research are contusion and transection models. We used a weight-drop contusion injury of the thoracic spinal cord as lesion model (Gruner, 1992; Widenfalk et al., 2001). Briefly, following incision through skin, subcutis and muscle, a laminectomy of T9 and the caudal half of T8 was performed (for the identification of thoracic vertebrae a vein crossing between processus spinosus of T4 and T5 is used). Next, the vertebral column was stabilized by clamps attached to the spinal processes cranial and caudal to the laminectomy and possible vertical movements were monitored by a sensor on the transverse process of T9. For the contusion injury a 10 g rod was raised to a height of 6.25, 12.5 or 25 mm above the dorsal surface of the spinal cord. It was then released and accelerated by the force of gravity until it hit the exposed spinal cord. The surface of the rod that hit the cord was 4.9 mm^2, round and flat. Spinal cord compression and velocity of the rod were recorded; the latter has been shown to be a reliable predictor of tissue damage and functional outcome (Gruner, 1992).

Recently, a new model for contusion injury, which controls the force applied to the spinal cord has been proposed to reduce variability (Scheff et al., 2003). An alternative to contusion injury is compression injury. In this model the spinal cord is squeezed with a modified aneurysm clip (Rivlin and Tator, 1978; Fehlings and Tator, 1995) or by placing a weight on the exposed spinal cord (Holtz et al., 1989).

In the acute phase, contusion and compression injuries lead to remarkably little gross damage, consisting of gray matter hemorrhage whereas the white matter appears to be morphologically intact within the first hours following injury (Balentine, 1978; Noble and Wrathall, 1989; Rosenberg and Wrathall, 1997). The lesion at the center initiates a cascade of centrifugal and rostro-caudal tissue destruction events. Thus, even interruption of fiber tracts following contusion injury is partially a secondary event. This is in contrast to the transection spinal cord injury model, where all nerve fiber tractotomies are a primary event. In addition, transection injuries destroys the integrity of the dura.

These differences have important implications when designing treatment strategies. While transection injuries are mainly suitable for studying the regeneration of interrupted fiber tracts, contusion injuries offer in principle three therapeutic interventions: protection, repair of spared fiber myelin sheaths and regeneration of interrupted fibers.

Functional magnetic resonance imaging (Paper II and VI)

Functional MRI visualizes active areas of the central nervous system based upon the premise that under normal circumstances neuronal activity and hemodynamics are linked in the brain (Horwitz et al., 2000; Heeger and Ress, 2002). In activated brain areas, blood flow increases in order

to provide increased oxygen and glucose to brain cells. The increase in blood flow is roughly proportional to the increased glucose demand (Fox and Raichle, 1986; Fox et al., 1988) while there appears to be overcompensation of the amount of oxygen needed. Hence the ratio of oxygenated to deoxygenated hemoglobin increases. Since paramagnetic deoxygenated hemoglobin attenuates the signal intensity (T2* signal) by introducing inhomogeneity in the magnetic field, its decrease causes an increased signal intensity. We used this BOLD (blood oxygen-level dependent) technique to observe cerebral activation in response to electrical stimulation of the forepaws or hindpaws. Briefly, rats were anesthetized with halothane, orally intubated and mechanically ventilated. Forelimbs and hindlimbs were stimulated by bipolar subcutaneous electrodes. Anesthesia was initiated with an intraperitoneal bolus (60 mg/kg) of α-chloralose and maintained by continuous infusion at a rate of 20 mg/kg/h. Pancuronium bromide (0.3-0.5 mg/kg/h i.p., Organon, Technika) was given for muscle relaxation. During fMRI experiments body temperature as measured by a rectal probe was maintained at 37°C ± 0.5°C by a laminar warm air stream. A spectrometer with a 4.7 T field strength magnet and a 40 cm diameter horizontal bore (Bruker Biospec Avance 47/40, Bruker, Karlsruhe, Germany) was used to acquire MRI images. The magnet was equipped with a 12 cm inner diameter self-shielded gradient system (max. gradient strength 200 mT/m; minimum inductive rise time 80 μs) and a circular resonator (inner diameter 35 mm; Bruker). A T2* weighted gradient echo spiral sequence (TR = 1010ms, TE = 40 ms, flip angle = 22.5 °, FoV = 4 cm x 4 cm, matrix = 64 x 64) was used for acquiring images for fMRI studies. Functional MRI experiments consist of repetitive rest periods and stimulation periods. During stimulation periods, electrical pulses with 1 mA are applied to extremities with a frequency of 3 Hz. Significant signal intensity changes between rest and stimulation periods were calculated using the software Stimulator (Strupp, 1996) or SPM99. Voxels with significant signal changes were color-coded.

Behavioral tests (Paper II, V and VI)

Functional recovery of spinal cord injured rats was assessed with different behavioral tests. Open field walking was scored according to the Basso, Beattie and Bresnahan rating scale (Basso et al., 1995). Individual rats were placed in an open field (75 x 125 cm) and observed for four minutes. Hindlimb function was scored from 0 to 21 (flaccid paralysis to normal gait). The integration of sensorimotor skills was tested in a gridway task (Behrmann et al., 1992). In this test animals are allowed to cross a 1.2 m long gridway three times. The number of hindlimb misplacements is counted and the three values averaged. Sensory function was tested by a hotplate test. Animals are placed on a hotplate preheated to 50°C for a maximum period of 60 s and the time that passes before the rat has licked both hindpaws is measured. Animals not displaying a reaction are removed after 60 seconds (Gale et al., 1985). Of note, the hotplate test does not purely assess sensory function because the read-out is a motor behavior.

Pain-like behavior in response to mechanical and thermal forepaw stimulation was also tested. For mechanical stimulation, the withdrawal threshold to graded mechanical touch/pressure by Von Frey hairs was estimated (Stoelting, Chicago, USA) (Hao et al., 1999). In ascending order, Von Frey hairs were applied on the palmar surface at a frequency of 0.5 s^{-1}. The mechanical threshold was defined as the lowest force which caused at least 3 withdrawals in 5 trials. Cold stimulation was accomplished by the application of ethyl chloride spray to the palmar surface. The response was ranked as 1 (no observable response), 2 (brief withdrawal and licking) or 3 (vocalization, prolonged withdrawal, licking, aversive reactions). Heat stimulation was carried out using the modified Hargreaves method (Hargreaves et al., 1988). In this method, heat is radiated to the palmar surface and the latency to withdrawal of the stimulated paw is measured.

In situ hybridization (Paper IV)

Animals were sacrificed by decapitation, tissue specimens removed and frozen on dry ice. Cryostat sections 14 µm thick were generated and thawed onto coated slides (ProbeOn, Fisher). Sections were hybridized with DNA oligoprobes complementary to rat mRNA for RhoA, RhoB, RhoC, Rac1, Cdc42 and Tc10. The sequences of theses genes were obtained from GenBank and 52 mer probes were designed to be complementary to the respective genes and not homologous to any other genes found in GenBank. As a negative control, we used a 50-mer random probe not complementary to any sequence deposited in Genbank (Nosrat and Olson, 1995). For in situ hybridization (Dagerlind et al., 1992; Widenfalk et al., 1997) probes were 3' end-labeled with [^{33}P] dATP (NEN Dupont) by terminal deoxynucleotidyl transferase (Amersham) and purified (QIAquick™Nucleotide Removal Kit Protocol, QIAGEN). Slides were incubated overnight (42° C) with 0,1 ml hybridization cocktail (50% formamide, 4xSSC, 1x Denhardt's solution, 1% Sarcosyl, 0,02M Na_3PO_4, pH 7.0, 10% dextransulphate, 0,06M DTT, 0,1 mg/ml sheared salmon sperm DNA and hot probe). Slides were then rinsed four times (45 min) in SSC at 60°C. During a fifth rinse the solution was allowed to adjust to room temperature. Further rinsing was carried out in distilled water and increasing concentrations of ethanol after which slides were air dried and exposed for two weeks to X-ray film (Kodak Biomax) for quantification. Slides were then dipped in photographic emulsion (Kodak NTB2, diluted 1:1 in H_2O), exposed for three weeks, developed, counterstained with cresyl violet and cover slipped. Quantification of probe labeling intensity as seen on the X-ray films was carried out by digital scanning of the films and measurements of the optical density of defined regions of interest, using appropriate software (public domain program ImageJ, v-1-31, National Institutes of Health; http://rsb.info.nih.gov/ij/). The program was calibrated for each film series using a ^{14}C-labeled polymer layer test slide (Amersham).

Cell culture (Paper V and VI)

For cell grafting studies we used two types of adult stem cells, mesenchymal stem cells, also called marrow stromal cells (MSCs, paper V) and neural stem cells (NSCs, paper VI). Mesenchymal stem cells, with the capability to form bone and cartilage, were first isolated from bone marrow by Friedenstein in the mid-1970s. At that time, whole bone marrow samples were placed in plastic culture dishes whereas in our experiments we flushed marrow out of femur and tibia using a syringe filled with medium. The medium consists of minimal essential medium with alpha modification (Gibco-BRL), 20% fetal calf serum (Atlanta Biologicals), 2 mM L-glutamine (Gibco-BRL), 100 units penicillin per ml, 100 µg streptomycin per ml, and 25 ng amphotericin B per ml (penicillin, streptomycin and amphotericin, Gibco-BRL). Bone marrow was filtered to remove bone chips and plated in 75-cm^2 flasks. Approximately 24 hours after plating, supernatant containing non-adherent cells was removed and fresh medium was added.

Isolation of MSCs as first described by Friedenstein, remains a commonly used method to isolate MSCs and separate them from hematopoietic stem cells. Bone marrow derived cell suspensions can be enriched for MSCs by cell sorting for different surface markers (Simmons and Torok-Storb, 1991; Haynesworth et al., 1992; Waller et al., 1995). None of these protocols, however, yields homogenous cell populations with well defined surface markers. Rather, MSCs are still commonly defined as a bone marrow derived cell population, isolated adherence to the plastic and able to differentiate into bone, cartilage and muscle. For transplantation studies the disadvantage of relatively poorly defined cellular properties of MSCs isolated by adherence to plastic is outweighed by the advantage of simple harvest and rapid expansion that diminishes the risk for contamination and infection.

Adult neural stem cells (NSCs) were harvested from spinal cords. Following removal of the meninges, spinal cord tissue was dissociated in 0.7 mg hyaluronic acid, 200 U DNAase and 1.33 mg trypsin per ml and incubated at 37°C for 30 min. The cell suspension was filtrated and centrifuged (200 G, 5 min). The resulting cell pellet was resuspended in 0.5 M sucrose in 0.5 × HBSS. Following a second centrifugation (750 G, 10 min), the pellet was resuspended in neurosphere culture medium based on DMEM F12 (Life Technologies) with glutamax and supplemented with 20 ng EGF, 20 ng bFGF, 20 µl B27, 100 U penicillin and 100 µg streptomycin per ml. Neural stem cells proliferate, attach to each other and eventually give rise to spherical clusters that float in suspension and form the so-called "neurospheres". At 7 days neurospheres were dissociated in 1.33 mg/ml trypsin and single cells were further cultured for 3 days into secondary neurospheres in 50% neurosphere conditioned medium and 50% fresh medium.

We used adult NSCs derived from the spinal cord rather than NSCs from the subventricular zone of the brain, because spinal cord-derived NSCs are involved in successful regeneration following spinal cord injury in fish and amphibians. In these animals the ependymal layer generates neuroblasts which proliferate and replace lost neurons (Yamada et al., 1997).

Viral transduction (Paper V and VI)

For marking of engrafted MSCs, a retroviral construct encoding green fluorescent protein (GFP, Clontech, Palo Alto, CA) was prepared using the LXSN vector (Clontech). For marking and induction of NSCs, a Moloney murine leukemia virus derived vector (Ory et al., 1996) encoding myc-tagged neurogenin2 followed by an internal ribosomal entry site and green fluorescent protein was constructed. The calcium phosphate precipitation method was used for stable transfection of Phoenix amphotropic packaging cells (Kinsella and Nolan, 1996) or the 293 GPG packaging cell line (Pear et al., 1993; Ory et al., 1996), respectively. The supernatants from the virus-producing cells were harvested and concentrated by centrifugation at 44,000 G for 90 min. MSCs and secondary neurospheres were transduced by adding viral supernatant and 8 µg polybrene per ml (Sigma).

Transplantation (Paper V and VI)

For cell transplantation into spinal cord lesions, we attached a glass pipette with a tip diameter of 100 µm to a 22-gauge needle (outer diameter 0.72 mm, inner diameter 0.15 mm) of a 10 µl Hamilton syringe using a PE-50 tube. We warmed an approximately 7-mm long piece of tube by hot air and then inserted first the glass tube and then the needle following careful distension of the tube endings with a small forceps. The syringe was configured to a stereotaxic frame. A microprocessor controlled pump infused the suspension at a rate of 0.5 µl/min (UltraMicroPump-II, Sarasota, Florida). The advantage of cell grafting with a glass capillary is enhanced graft survival and a decreased local glial response compared to transplantation with a metal canula (Nikkhah et al., 1994). However, the use of glass capillaries would be problematic in future human transplantation trials, since the tip of the glass capillaries might break during infusion and remain within the nervous tissue.

Results and Discussion

Anatomical link between the sensory and the sympathetic system (Paper I)

In humans, minor trauma to a limb may cause a chronic pain syndrome, which is disproportional to the severity and extent of the trauma. This disorder, also known as reflex sympathetic dystrophy, is characterized by chronic pain, tremor, edema and trophic changes of the skin. The symptoms commonly extend far beyond the original site of injury (Janig and Baron, 2003). Based on the observation that skin in involved areas is cold, this disorder was attributed to sympathetic hyperactivity, which was thought to excite nociceptive fibers (Leriche, 1923). Animal experiments identified DRGs, the peripheral nerve injury site and cutaneous nociceptors as possible sites of sympathetic-sensory coupling (Burchiel, 1984; Torebjork et al., 1995; Xie et al., 1995). Moreover, sympatholytic interventions typically relieve the symptoms in part of the patients (Blumberg et al., 1997). Recently, however, doubt was cast on the importance of peripheral sympathetic-sensory coupling for the development of sympathetic reflex dystrophy. Instead, dysregulation of supraspinal and spinal influences has been suggested to contribute to the development of sympathetic reflex dystrophy (Drummond, 2001; Janig and Baron, 2003).

In order to investigate spinal cord neurons that control the segmental sympathetic outflow, we injected pseudorabies into lumbar DRGs. The infection spread in a strictly retrograde transynaptic fashion via autonomic sympathetic ganglia to the intermediolateral cell columns ipsilateral to the injection. Infected presynaptic sympathetic neurons were found in lower thoracic and upper lumbar spinal cord segments. Later, infection spread to neurons in laminae I, III, IV and V of the ipsilateral dorsal horn and to neurons of the contralateral intermediolateral cell column. Infected lamina X neurons surrounding the central canal were seen between spinal cord segments L1 and S1. We then investigated the relation of infected neurons to primary sensory afferents by injecting Bartha-PRV and cholera toxin B simultaneously into lumbar DRGs. Interestingly, we found that infected neurons of laminae IV/LV, Clarke's column and lamina X were in close apposition to cholera toxin B labeled afferents. Parts of these contact sites were identified as synapses by the presence of the pre-synaptic zone protein Bassoon. Our findings in this study thus describe a novel segmental spinal cord circuit between primary sensory afferents and neurons which may regulate sympathetic outflow to the corresponding DRGs (Figure 3).

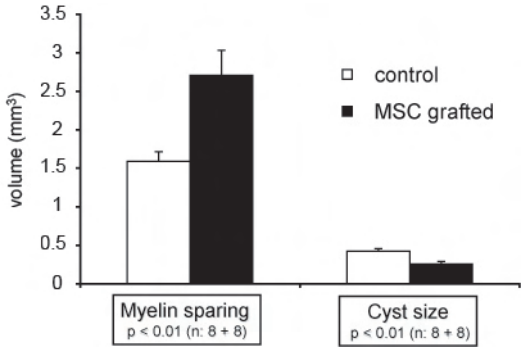

Figure 3. Schematic illustration of a segmental spinal cord circuit connecting primary afferent fibers (blue) via interneurons (green) to the presynaptic sympathetic neurons. Abbreviations: IML, intermediolateral cell column; py, pyramidal tract.

In the case of sympathetic reflex dystrophy the circuit we have identified would provide an anatomical link between the damaged sensory system and the sympathetic system that gives rise to symptoms: Peripheral nerve injury causes spontaneous firing of affected DRG neurons (Devor, 1983; Liu et al., 2000) which might in turn modulate the sympathetic outflow via the segmental spinal cord circuit described in the current study.

Spontaneous recovery of the sensory system following spinal cord injury (Paper II)

In patients, preserved appreciation for pin-prick (Crozier et al., 1991) predicts functional outcome following spinal cord injury. While sensory appreciation can be verbally communicated in human sensory tests, most animal tests evaluate motor behavior in response to sensory stimulation (Gale et al., 1985; Bradbury et al., 2002). For that reason behavioral sensory tests are not sufficient to make statements about pure sensory function.

We used functional MRI to monitor cortical activation in response to subcutaneous electrical hindpaw stimulation. The recovery of the cortical hindpaw representation following mild or moderate weight drop injury was compared to motor and sensory function estimated from behavioral tests. While moderate weight drop injury (12.5 mm) abolished cortical activation up to 16 weeks following injury, mild injury (6.25 mm) allowed for partial recovery of cortical BOLD-signaling in response to hindlimb stimulation 16 weeks following injury. These results were consistent with findings obtained by behavioral testing of sensory function. Thus, functional MRI and behavioral testing demonstrated that mild injury allowed for recovery of sensory function whereas moderate contusion injury was not followed by spontaneous recovery of sensory function. Surprisingly, the different levels of sensory recovery were not mirrored by the recovery of motor function in the rats. Thus, both mild and moderate injuries were followed by almost complete recovery of motor function. What could explain this pattern of recovery which differs from that seen in humans? In both humans and animals the lumbar central pattern generator is important for the generation of stepping movements (Grillner and Zangger, 1979). However, whereas in certain quadrupedals stepping of the hindpaws can be observed following removal of supraspinal input (Forssberg et al., 1980a, b; Barbeau and Rossignol, 1987), primates (Eidelberg, 1981; Eidelberg et al., 1981) and humans (Wernig and Muller, 1992; Dietz et al., 1994, 1995) cannot walk unassisted following complete spinal cord injury. In other words, meaningful hindpaw locomotion may need less supraspinal control in quadrupedals than in bipedals.

This finding has important implications for the validity of animal experiments that evaluate locomotor recovery following experimental treatment strategies. Activation and modification of lumbar locomotor control circuitry has been suggested to be the mechanism behind improved locomotor function in rodent spinal cord injury models. Recently, the beneficial effects of MSC transplantation on hindpaw locomotion have been partially related to this mechanism (Ankeny et al., 2004). However, increasing the activity of the lumbar central pattern generator would probably not result in significant improvement of human locomotor function. Human locomotor function requires supraspinal control via the corticospinal tract. In the spinal cord this motor tract is close to the lateral spinothalamic tracts. A lesion of the spinal cord involving this area will therefore damage both tracts and lead to decreased pin-prick sensation and paralysis (Crozier et al., 1991).

Assessment of sensory function with behavioral and functional MRI, which tests the conduction properties across the injury zone, might improve validity of rodent experimental spinal cord injury treatment strategies for clinical application.

Plasticity of motor system following spinal cord injury (paper III and IV) The inhibited ability to grow

Following contusion injury of the spinal cord, regeneration of interrupted nerve fibers is limited. This might be attributed to two factors: First, growth inhibiting properties of the scar environment that hinder fiber outgrowth and second, lack of intrinsic capacity to regenerate interrupted axons. Numerous studies have characterized growth-inhibiting molecules at a spinal cord injury

site such as Nogo (Caroni and Schwab, 1988; Bregman et al., 1995), myelin associated glycoprotein (McKerracher et al., 1994) and oligodendroycyte-myelin glycoprotein (Wang et al., 2002). In addition, increased amounts of extracellular matrix components such as chondroitin sulfate proteoglycans (Davies et al., 1999; Lemons et al., 1999; Jones et al., 2002) add to the physical barrier formed by the scar tissue and the development of cysts after injury can aggravate the situation (Josephson et al., 2001; Silver and Miller, 2004). The importance of the glial scar for inhibition of nerve fiber regeneration is further supported by studies of transection injuries with minimal trauma. Interruption of the corticospinal tract is then followed by successful long distance regeneration (Inoue et al., 1998; Iseda et al., 2004; Silver and Miller, 2004). However, the interruption of nerve fibers in traumatic spinal cord injury is not only a primary effect, like in a transection, rather, the white matter histologically appears largely intact immediately following a contusion (Balentine, 1978; Noble and Wrathall, 1989; Rosenberg and Wrathall, 1997). The lesion at the center triggers a cascade of tissue destruction events that severs fiber tracts. The question arises whether neurons whose axons have become interrupted by secondary damage also express growth associated molecules and make attempts to elongate their severed axons.

First we analyzed the expression of small GTP-binding proteins belonging to the Rho family (Wherlock and Mellor, 2002). The constellation of different Rho-kinases controls growth cone advance and retraction through growth cone collapse. We found that retrogradely labeled axotomized pyramidal cells upregulated the expression of Tc10. This particular Rho-kinase has been shown to promote axonal outgrowth and regeneration (Tanabe et al., 2000). The extent of regeneration of severed anterogradely labeled corticospinal tract axons was then quantified. By using a stereological technique that ensures sampling of fibers independent of their orientation we found that corticospinal tract fibers display excessive sprouting following contusion injury. Regenerative outgrowth of proximal fiber stumps was obvious 12 weeks after injury 1.6–2.4 mm rostral to the injury center. However, this sprouting was not associated with successful regeneration of interrupted fibers beyond the injury site, rather fibers elongated laterally in the spinal cord and formed a "central neuroma" rostral to the injury site. In conclusion, interrupted corticospinal tract fibers display a marked, quantifiable intrinsic capacity to elongate following a crush injury of the spinal cord. However, regenerating fibers are not able to navigate through the injury zone and thus newly formed sprouts were found in the rostral zone of the injury.

The well-known intrinsic capacity of lesioned CST fibers to regenerate suggests it might not be helpful to design treatment strategies which nonspecifically boost outgrowth of severed nerve fibers. On the contrary, promotion of uncontrolled fiber outgrowth might have dramatic side effects (see also paper VI). However, it might be valuable to stimulate the outgrowth of specific neuron populations using specific combinations of growth factors in a locally restricted manner which also

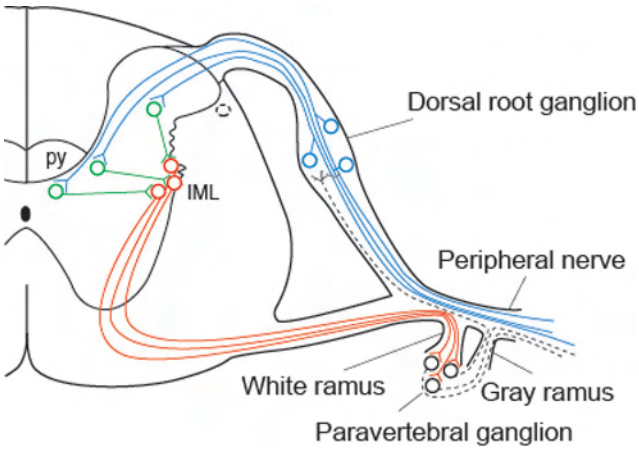

Figure 4. Grafting of MSCs to into a spinal cord contusion injury leads to increased sparing of myelin at the injury site. Also, a significant decrease of the cyst size is observed with MSC treatment.

guides growth, e.g. along a chemical gradient of trophic support. The results of these studies also emphasizes the need to focus on the removal of growth inhibiting properties of scar tissue (Silver and Miller, 2004) and to provide guidance for outgrowing nerve fibers through the lesion zone (Pettegrew and Windle, 1976; Cheng et al., 1996; Bunge, 2001).

Influence of stem cell grafts on local pathology (Paper V and VI)

A number of studies has demonstrated beneficial effects of stem cell transplantation on functional outcome following spinal cord injury. We grafted two different types of stem cells directly into the lesion zone and analyzed possible reparative and regenerative effects.

Marrow stromal cells (MSCs) were harvested from femur and tibia of adult rats, expanded *in vitro* and delivered to the injured spinal cord. MSCs survived well provided that they were grafted 1 week following injury. Transplantation of MSCs reduced the formation of cysts and also increased white matter sparing (Figure 4, Abrams et al., 2003). Histology revealed that engrafted MSCs formed bundles that bridged the epicenter of the injury. These cell aggregates provided directional guidance to regrowing NF-positive fibers (Figure 5). Engrafted MSCs might have facilitated the regeneration of these fibers directly via their growth promoting surface which expresses N-cadherin (Puch et al., 2001). N-cadherin is known to enhance neurite extension *in vitro* and *in vivo* (Schense et al., 2000). Alternatively, engrafted MSCs promoted outgrowth of NF-positive fibers indirectly: Cell aggregates formed by engrafted cells contained host derived nestin-positive and GFAP-negative immature astrocytes. During development immature astrocytes guide outgrowing pioneer corticospinal axons (Joosten and Gribnau, 1989; Gorgels, 1991) and they also appear to be involved in successful regeneration of corticospinal tract fibers following transection injury with minimal trauma (Iseda et al., 2004).

Neural stem cells (NSCs) were harvested from the spinal cord of adult rats. Following expansion in vitro, they were grafted into a weight drop lesion site. Transplantation of neural stem cells did neither alter the size of the cyst at the lesion site nor change the amount of spared white matter. The majority of engrafted cells gave rise to astrocytes and they were mainly located in areas containing debris and macrophages (Figure 6). However, grafting of NSCs had a dramatic effect on fiber outgrowth. Using unbiased stereology, we estimated that grafting of NSCs doubled the amount of CGRP-immunoreactive sensory axons in lamina III and BDA-labeled corticospinal tract fibers in spinal cord segments immediately above injury. In contrast to MSCs, which appeared to provide a growth supportive scaffold for fiber elongation, NSCs stimulated outgrowth of fibers that where not in direct contact with the grafted cells. This effect was therefore probably mediated via release of growth factors, as astrocytes constitute a known source of multiple neurotrophic factors, especially in areas of CNS trauma (Brown et al., 2004).

In conclusion, mesenchymal and neural stem cells behave in very distinct ways following transplantation into a spinal cord injury zone. Whereas MSCs provide growth-supportive scaffolds for regrowing nerve fibers, NSCs seem to produce trophic support in the form of released growth factors which promote uncontrolled sprouting of different fiber systems.

Sprouting of nerve fibers, gain or pain?

We investigated the effects of excessive sprouting on functional outcome in animals that were grafted with NSCs. We found that extensive sprouting of the proximal stumps of the corticospinal tract did not correlate to improvement of hindpaw motor function. This is in line with the observation that the excessive sprouting did not lead to successful regeneration of nerve fibers beyond the

injury center. However, the sprouting corticospinal tract fibers advanced slightly closer to the injury center in spinal cords that had received cell grafts than in animals that had not receive cell grafts.

Transplantation of NSCs also gave rise to sprouting of thin CGRP-immunoreactive fibers into Rexed's lamina III. Twice as many CGRP-positive nerve fibers were detected in lamina III in animals that had received NSCs compared to animals that did not receive cell grafts. Unlike the sprouting of CST fibers, the degree of sprouting of CGRP-immunroeactive fibers was positively correlated with a severe side effect of NSC-grafting: Animals that received NSC grafts developed allodynia in response to mechanical, heat and cold stimulation of their forepaws. The severity of mechanical allodynia was positively correlated to the amount of CGRP-positive fiber sprouting in individual animals.

In conclusion, whereas sprouting of corticospinal tract fibers was not associated with beneficial treatment effects on motor recovery, sprouting of CGRP-positive fibers was associated with development of allodynia of the forepaws. We found that the number of graft-derived astrocytes correlated with the severity of allodynia. Therefore we suppressed astrolial differentiation of engrafted NSCs by transducing them with Neurogenin2, a helix-loop-helix transcription factor that inhibits astroglial differentiation. Transplantation of neurgenin2-transduced NSCs did not cause allodynia of the forepaws but instead allowed for partial sensory recovery of the hindlimbs as demonstrated by behavioral tests and functional MRI. Moreover, neurgenin2-transduced NSCs gave rise to significantly more oligodendrocytes compared to naïve NSCs. The number of graft-derived oligodendrocytes was positively correlated to the amount of spared white matter at the injury center. Importantly, we identified graft-derived oligodendrocytes as a source of myelin forming cells.

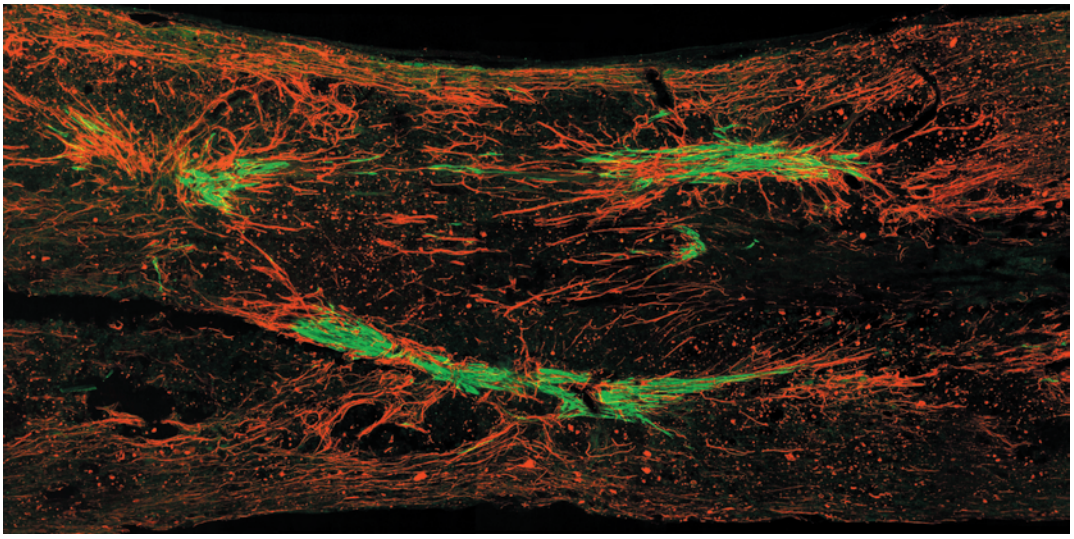

Figure 5. Longitudinal section of the spinal cord centered above the epicenter of a contusion injury. MSCs (green) for bundles that bridge the lesion zone. Aggregates formed by engrafted cells provide directional guidance for regrowing NF-positive (red) nerve fibers.

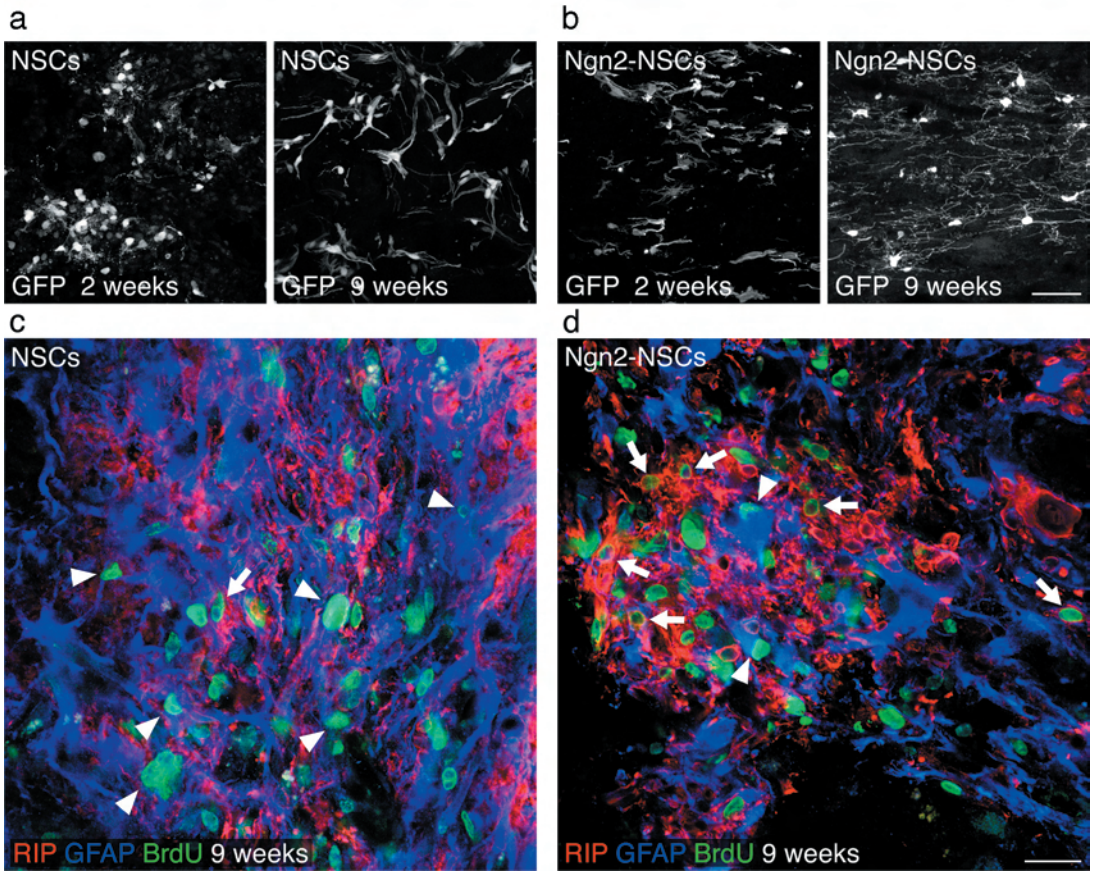

Figure 6. Histological characterization of engrafted NSCs. (a) NSCs exhibit a rather immature cell morphology two weeks after transplantation and acquire stellate cell morphology at 9 weeks. (b) Two weeks post injection Ngn2-NSCs extend thickened and less structured processes which develop into either long, fine arborizing processes or short, thick ones at 9 weeks. (c) The majority of NSCs give rise to GFAP-immunoreactive astrocytes (arrowheads) but some RIP-immunoreactive oligodendrocytes are also seen (arrow). (d) Compared to NSCs, Ngn2-NSCs generate more oligodendrocytes (arrows) and fewer astrocytes (arrowheads).

Future perspectives

The ultimate goal of experimental spinal cord injury research is to develop safe treatment strategies for human spinal cord victims. Whereas current clinical treatment combines immediate delivery of high doses of methylprednisolone (Crozier et al., 1991) and surgical stabilization of the vertebral column, future strategies will probably need to combine several of the recent experimentally founded techniques:

Immediate neuroprotection

Traumatic spinal cord injury leads to direct destruction of neural cells by necrosis but also induces apoptosis, a programmed pathway to neuronal death (Beattie et al., 2000). This secondary damage is triggered by factors such as hypoxia, free radicals and excitatory amino acids (Beattie et al., 2002). These factors are targets for neuroprotective treatments strategies, which aim to stop the cascade of secondary tissue damage. Blocking the common intracellular pathway for apoptosis is another promising approach reduce the amount of secondary damage (Li et al., 2000; Arataki et al., 2005).

Taken together, neuroprotective treatments appear promising leads, awaiting careful clinical trials. Successful treatment should preserve original cytoarchitecture and hence reduce the need for subsequent reparative and regenerative treatments. In addition, protective treatment would facilitate early ambulation and rehabilitation of the spinal cord injured individual. However, beneficial effects of currently available clinical neuroprotective treatments are very limited at best. Also, the effect of neuroprotective treatment strategies is likely to be limited in severe spinal cord trauma and in chronically injured spinal cord victims.

Incomplete injuries: reparation

Incomplete spinal cord injuries are typically composed of a central cavity surrounded by a rim of spared nerve fibers. Part of the spared axons lack adequate myelination and therefore show impaired conduction properties (Waxman, 1989). Cell transplantation therapies using Schwann cells, olfactory ensheathing cells, mesenchymal stem cells or neural stem cells have been suggested for the repair of demyelinated CNS axons.

Which cell type would be most appropriate to use in human trials? Currently a phase I clinical trial is performed at the Grifith University in Brisbane, where 12 – 20 million autologous olfactory ensheathing cells are transplanted into the site of injury. Like Schwann cells and marrow stromal cells, olfactory ensheathing cells also have the advantage of possible autologous transplantation. This circumvents risks associated with allografts such as graft rejection and transmission of infectious diseases. For both Schwann cells (Takami et al., 2002) and marrow stromal cells (Chopp et al., 2000; Wu et al., 2003; Ankeny et al., 2004; Ohta et al., 2004; Zurita and Vaquero, 2004; Kamada et al., 2005; Neuhuber et al., 2005) there is accumulating evidence of beneficial effects following grafting to an injury site, while adverse side effects have not yet been described. Autologous transplantation of neural stem cells might be difficult, and more importantly grafted neural stem cells may lead to uncontrolled sprouting of different fiber systems, including pain fibers. Finally, should we try to promote nerve fiber outgrowth in incomplete traumatic spinal cord injuries? So far there is insufficient experimental evidence that stimulation of nerve fiber outgrowth and subsequent successful regeneration of interrupted nerve fibers might have been the underlying mechanism of recovery seen in treatment strategies for traumatic spinal cord injury. If nerve fiber stimulation is chosen then also removal of growth inhibiting properties of the glial scar (Silver and Miller, 2004) and appropriate guidance of outgrowing fibers needs to be provided (Bunge, 2001). However this approach seems to be better suited for complete lesions.

Complete injuries: regeneration

In complete injuries scar tissue that impedes regeneration of nerve fibers can be removed using a sharp device. Guiding structures such as peripheral nerve bridges or biodegradable polymers can then be used to bridge the gap. This approach needs surgical removal of traumatized tissue. MRI could help to detecting possibly spared tissue at the injury site. MRI, is an excellent tool to visualize spinal cord pathology (Imhof and Fuchsjager, 2002). There is an accumulating body of evidence that providing guidance and a growth-supporting environment is a prerequisite for successful regeneration.

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