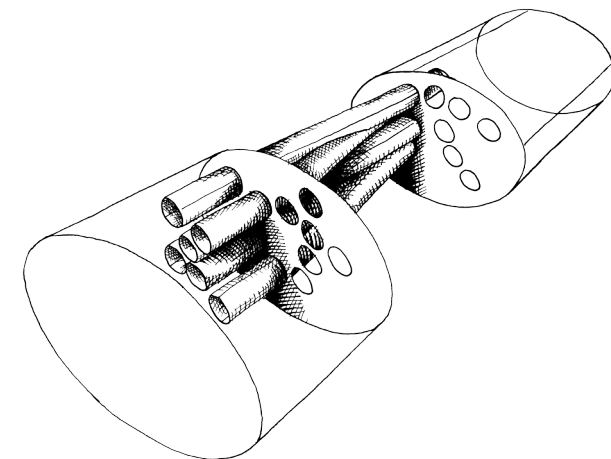


A REGENERATION STRATEGY FOR SPINAL CORD INJURY



Jonathan Nordblom

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To my best friend, Sarah

ABSTRACT

A severe traumatic spinal cord injury (SCI) frequently leads to a devastating and permanent disability. Due to glial scarring and an inhibitory local environment, regrowth of disrupted axons in the injured spinal cord beyond a lesion is obstructed, thus preventing reconnection with neurons at the other side. Many experimental strategies have been presented to limit the damage and improve outcome after SCI, but few options are available for the patient.

Neurons in the central nervous system may regenerate using a growth permissive medium, such as peripheral nerve grafts. This capacity has been used to bridge a spinal cord gap by facilitating regeneration of long tracts in the spinal cord through transplanted peripheral nerve grafts, aimed at redirecting the regenerating axons into growth permissive grey matter on the other side of the injury. This principle was demonstrated in 1996, when surgical transplantations combined with adjuvant acidic fibroblastic growth factor (FGF1) led to partial recovery of hind limb function.

The aim of this thesis was to develop a reproducible microsurgical method for precise placement of peripheral nerve grafts (PNGs), construct a biodegradable graft holder, assess the effect of controlled delivery of FGF1, evaluate potential regeneration of corticospinal tracts after spinal cord repair and investigate if it is possible to determine the cranial and caudal injury borders in patients with chronic and complete spinal cord injury.

Our experiments in the adult rat demonstrate that replacing a section of thoracic spinal cord with a graft holder filled with peripheral nerves induced a spinal cord regeneration of various axonal types, including corticospinal axons. Further, we provide evidence of axonal ingrowth into the caudal spinal cord by anterograde neural pathway tracing and electrophysiological studies. This regeneration induced a functional improvement and robust electrophysiological response in the hind limbs, paced-up by the addition of graded doses of FGF1. The thesis also demonstrates that the cranial and caudal injury borders of patients with thoracic chronic and complete SCI can be diagnosed with high accuracy, which may be important for future diagnosis in spinal cord injury.

In conclusion, we present a regeneration strategy for the transected spinal cord, primarily through the use of a biodegradable graft holder filled with individually directed peripheral nerve grafts in combination with FGF1.

LIST OF PUBLICATIONS

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

I: Jonathan Nordblom, Jonas K.E. Persson, Mikael Svensson and Per Mattsson

Peripheral Nerve Grafts in a Spinal Cord Prosthesis Result in Regeneration and Motor Evoked Potentials Following Spinal Cord Resection

Restorative Neurology and Neuroscience 2009, 27(4):285-295.

II: Jonathan Nordblom, Jonas K.E. Persson, Jonas Åberg, Hans Blom, Håkan Engqvist, Hjalmar Brismar, Johan Sjö Dahl, Anna Josephson, Arvid Frostell, Sebastian Thams, Lou Brundin, Mikael Svensson and Per Mattsson

FGF1 containing biodegradable device with peripheral nerve grafts induces corticospinal tract regeneration and motor evoked potentials after spinal cord resection

Restorative Neurology and Neuroscience 2012, 30(2):91-102.

III: Jonathan Nordblom, Per Mattsson, Sebastian Thams, Jonas K.E. Persson, Jonas Åberg, Håkan Engqvist, Lou Brundin and Mikael Svensson

Acidic Fibroblastic Growth Factor Promotes Spinal Cord Regeneration in a Transplantation Model using Peripheral Nerve Grafts

(Manuscript)

IV: Arvid Frostell, Per Mattsson, Jonas K.E. Persson, Björn Hedman, Jonathan Nordblom, Anders Lindenryd, Katarzyna Trok, Lou Brundin and Mikael Svensson

Neurophysiologic Evaluation of Segmental Motor Neuron Function of the Thoracic Spinal Cord in Chronic SCI

Spinal Cord 2012, 50(4):315-9.

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LIST OF ABBREVIATIONS

5-HT	5-Hydroxytryptamine (serotonin)
AIS	ASIA (American Spinal Injury Association) Impairment Scale
ALS	Amyotrophic lateral sclerosis
ASIA	American Spinal Injury Association
BBB	Basso, Beattie and Bresnahan scale for hind limb locomotor function
BDA	Biotinylated dextran amine
BDNF	Brain-derived neurotrophic factor
bid	Twice daily (<i>Lat.</i> bis in die)
BMSC	Bone marrow stromal cells also known as mesenchymal stem cells
C1-C7	Cervical vertebrae (or segments) 1-7
C3	C3-transferase, enzyme that inhibits Rho proteins
CaSO ₄	Calcium sulphate
CGRP	Calcitonin gene-related peptide
ChABC	Chondroitinase ABC enzyme
CNS	Central nervous system
CPGs	Central pattern generators
CSC	Calcium sulphate cement
CSF	Cerebrospinal fluid
CSPGs	Chondroitin sulphate proteoglycans
CST	Corticospinal tract
EMG	Electromyography
FDA	American Food and Drug Administration
FGF1	Fibroblast growth factor 1 or acidic fibroblast growth factor (aFGF)
GalC	Galactosylceramidase
GAP-43	Growth associated protein 43
GFAP	Glial fibrillary acidic protein
GTPases	A large family of enzymes that can bind and hydrolyze guanosine triphosphate (GTP)

Hz	Hertz
IL1 and 6	Interleukin 1 and 6
i.p.	Intraperitoneal (injection)
iPS	Induced pluripotent stemcell
IR	Immunoreactivity
i.v.	Intravenous (injection)
L1-5	Lumbar vertebrae (or segments) 1-5
mA	Milliampere
MAG	Myelin-associated glycoprotein
MEP	Motor evoked potentials
mg	Milligrams
MPR	Multiplanar reconstruction (of MR images to compensate for 3-D differences)
MUPs	Motor unit potentials
µg	Micrograms
µm	Micrometers
µv	Microvolt
NEX	Number of excitations (during MRI scanning)
NF	Neurofilament
NFL	National Football League
ng	Nanograms
NGF	Nerve growth factor
nm	Nanometers
Nogo	Neurite outgrowth inhibitor
NSC	Neural stem cells
NSCISC	National Spinal Cord Injury Statistical Center
O ₂	Oxygen
OECs	Olfactory ensheathing cells
OP	Operation
PBS	Phosphate buffered saline solution

PM	Pectoralis major muscle
PNG	Peripheral nerve grafts
PNM	Phrenic motor nucleus
PNS	Peripheral nervous system
Rho	Rho family of GTPase enzymes
S1-5	Sacral spinal cord segments 1-5
S100	Family of low molecular weight proteins, found in Schwann cells
SA	Serratus anterior muscle
SC	Schwann cells
s.c.	Subcutaneous (injection)
SCI	Spinal cord injury
SEP	Sensory evoked potentials
T	Tesla
T1-12	Thoracic vertebrae (or segments) 1-12
T2	T2-weighted MRI sequences; Basic MRI sequences where fat shows darker and water (and CSF) brighter
TE	Echo time (in MRI)
TH	Tyrosine hydroxylase
TNF alpha	Tumor necrosis factor alpha
TR	Repetition time (in MRI)
TSE	Turbo spin echo (in MRI)
VACHT	Vesicular acetylcholine transporter
vGLUT1	Vesicular glutamate transporter 1
w/v	Weight per volume percentage
Y27632	Rho-Associated Coil Kinase (ROCK) inhibitor

INTRODUCTION

The human spinal cord is cylindrical but slightly flattened dorsally and ventrally, approximately between 7 and 18 mm in diameter (enlargements in the cervical and lumbar parts) and on average 45 cm long in males and 42-43 cm in females, occupying the upper two thirds of the vertebral canal (Watson, 2009). The spinal cord is vital for motor, sensory and autonomic communication between the brain and the rest of the body. A severe traumatic spinal cord injury (SCI) typically leads to a devastating and permanent disability. Chronic spinal cord injury cannot heal spontaneously, nor be restored medically.

EPIDEMIOLOGY

Worldwide, traumatic spinal cord injury incidences of between 10.4 to 58 cases per million people are reported (van den Berg, Castellote, Mahillo-Fernandez, et al., 2010; Wyndaele and Wyndaele, 2006). With 40 cases per million people or about 12,000 new cases per year the number of people with SCI in the US was estimated to be approximately 265,000 in 2010 according to the National Spinal Cord Injury Statistical Center (NSCISC, 2011). The main causes are motor vehicle collisions, followed by falls, violence (primarily gunshot wounds), and sports. There is an overrepresentation of male gender (80.7% in the US), in which most injuries occur between the age of 16 and 30, with a global average age ranging from 31 to 50 years (NSCISC, 2011; van den Berg, Castellote, Mahillo-Fernandez, et al., 2010), reflecting the more risk taking behavior of the young male. There is also a second peak of incidence in elderly people, where the most common cause is falls (Pickett et al., 2006), reflecting a weaker spinal column combined with decreasing balance with increasing age. Incidence rates from the Stockholm region in Sweden seem to be in line with international statistics (20 cases per million per year), however with falls being the leading cause of injury, and SCI caused by violence uncommon (Divanoglou and Levi, 2009).

The most common site of SCI is the cervical region (up to 75%), almost exclusively so after falls in the elderly population (NSCISC, 2011; Pickett, et al., 2006), followed by thoracic and lumbar injuries (Pickett, et al., 2006; van den Berg, Castellote, Mahillo-Fernandez, et al., 2010). Reflecting the level of injury and severity of trauma, the most frequent

neurological categories are incomplete tetraplegia (39.5%), complete paraplegia (22.1%) incomplete paraplegia (21.7%) and complete tetraplegia (16.3%) (NSCISC, 2011).

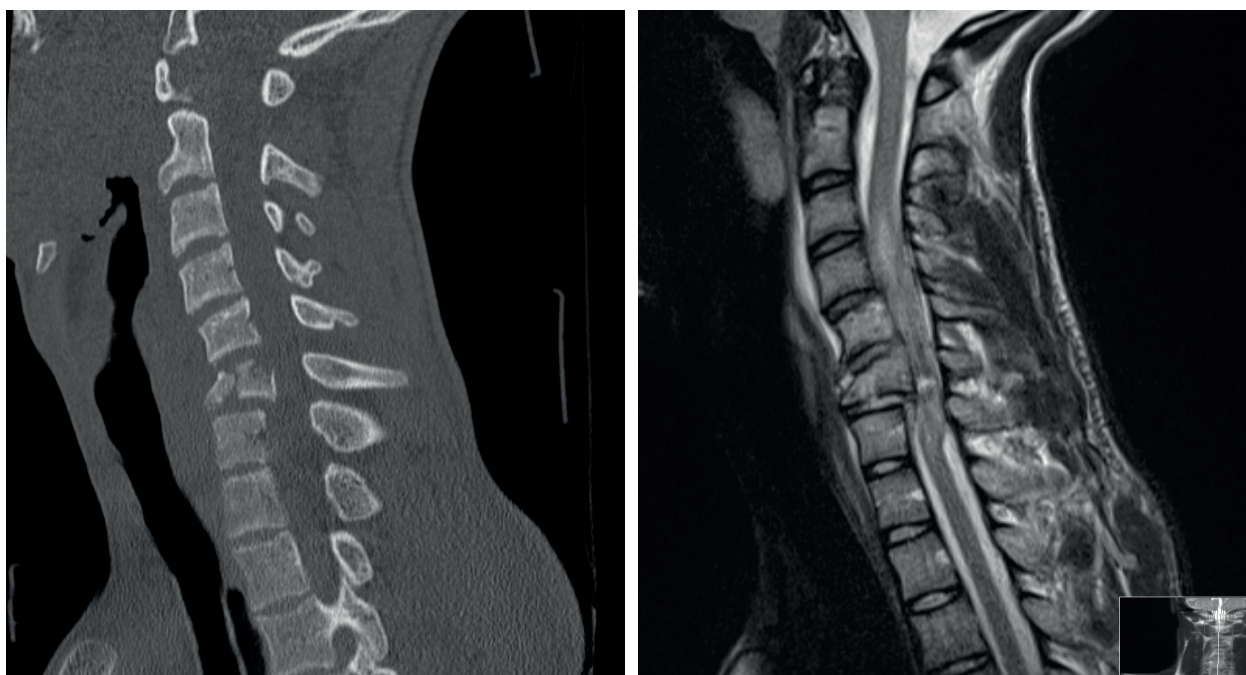


Figure 1. A seventeen year old male who fell 2.5 meters on his head causing axial trauma and overflexion of the cervical spine. Sagittal CT scan (1 hour after injury) and T2-weighted MRI scan (4.5 hours after injury) showing a comminuted flexion tear drop fracture of the sixth cervical vertebral body with a dorsal fragment compressing the spinal cord, causing local bleeding and spinal cord edema from C3 to C7. The patient was completely paralyzed below the sixth neurological cervical segment. The spinal cord was immediately decompressed and stabilized through removal of C4-C7 discs and fractured C5-C6 vertebral bodies with subsequent anterior fusion.

PATHOPHYSIOLOGY

Disruption of connectivity, level and complications

Since an injury to the spinal cord affects the ability of the brain to communicate with the body below the injury, the level of injury is of great functional importance for the patient, i.e., the higher the level, the worse the disability after a severe SCI. Trauma to the thoracic spinal cord affects the sensorimotor function of the lower extremities, but also often cause urinary bladder dysfunction, bowel dysfunction, sexual dysfunction and depending on injury level, cardiorespiratory complications (Gunduz and Binak, 2012). Both cervical and thoracic SCIs are frequently accompanied by infections from the urinary tract, respiratory tract and from pressure ulcers (Levi, Hultling, Nash, et al., 1995). These infec-

tions tend to recur and are potentially lethal complications if not treated properly. If the cervical spinal cord is traumatized, the arms and hands are affected in addition to the lower limbs (tetraplegia) and if the cervical spinal cord is severely affected above cervical segment C4, a ventilator is needed to maintain breathing since axonal connections between the brain stem and phrenic motor nuclei (PMN) which control the diaphragm are lost (Alilain et al., 2011). Advanced age, previous cardiopulmonary disease and pneumonia are also general predictors of the need for ventilator support after SCI (Casha and Christie, 2011).

One might think that ambulation is the subjective key problem after SCI, however paraplegics tend to rank sexual dysfunction and bladder/bowel dysfunction as an even greater problem, whereas quadriplegics rank hand and arm function higher (Anderson, 2004; Fisher et al., 2005). Both nociceptive and neuropathic pain is common after spinal cord injury, especially in elderly patients (Levi, Hultling, and Seiger, 1995; Teasell et al., 2010; Werhagen et al., 2004) and is an important factor adversely affecting quality of life. Chronic pain post SCI is frequently refractory to medical treatment and it has previously been reported that 37% of patients with low thoracic or lumbosacral SCI and 23% of patients with cervical or high thoracic SCI would be willing to sacrifice sexual, bowel and bladder function, as well as the hypothetical chance of recovered motor functions in exchange for pain relief (Nepomuceno et al., 1979).

Severity grade and classification

Depending on trauma severity and subsequent tissue damage, a spinal cord injury can manifest clinically as complete, in which motor or sensory (or autonomic) function does not exist below the injury level or incomplete, in which some to almost normal function below the injury level is seen. To describe the grade of completeness of traumatic SCI, the American Spinal Injury Association (ASIA) developed in 1982 (and revised in 2011) the now widely used ASIA Impairment scale ("AIS" or just "ASIA-scale") (ASIA, 2011; Kirshblum, Burns, et al., 2011; Kirshblum, Waring, et al., 2011; Maynard et al., 1997). The ASIA Impairment Scale is based on motor and sensory assessment of 20 key muscles in the upper and lower limbs (ten on each side) and 28 key dermatomes. The scale categorizes

SCI patients into five groups: ASIA-A (complete) when no motor or sensory function is preserved in the sacral segment S4-S5, ASIA-B (incomplete) when sensory but no motor function is preserved below the neurological level and includes the sacral segments S4-S5, ASIA-C (incomplete) when motor function is preserved below the neurological level and more than half of key muscles below the neurological level have a muscle grade less than 3 (unable to overcome gravity), ASIA-D (incomplete) when motor function is preserved below the neurological level and at least half of key muscles below the neurological level have a muscle grade of 3 or more (ability to move against gravity) and finally ASIA-E (incomplete) when motor and sensory function is normal (ASIA, 2011; Kirshblum, et al., 2011).

Patient Name _____
 Examiner Name _____ Date/Time of Exam _____

ASIA INTERNATIONAL STANDARDS FOR NEUROLOGICAL CLASSIFICATION OF SPINAL CORD INJURY **ISCOS**

MOTOR
 KEY MUSCLES (scoring on reverse side)

	R	L
C5	<input type="checkbox"/>	<input type="checkbox"/>
C6	<input type="checkbox"/>	<input type="checkbox"/>
C7	<input type="checkbox"/>	<input type="checkbox"/>
C8	<input type="checkbox"/>	<input type="checkbox"/>
T1	<input type="checkbox"/>	<input type="checkbox"/>

UPPER LIMB TOTAL (MAXIMUM) ☐ + ☐ = ☐ (25) (25) (50)

Comments: _____

KEY MUSCLES
 L2 ☐ Hip flexors
 L3 ☐ Knee extensors
 L4 ☐ Ankle dorsiflexors
 L5 ☐ Long toe extensors
 S1 ☐ Ankle plantar flexors

(VAC) Voluntary anal contraction (Yes/No) ☐

LOWER LIMB TOTAL (MAXIMUM) ☐ + ☐ = ☐ (25) (25) (50)

SENSORY
 KEY SENSORY POINTS

0 = absent
 1 = altered
 2 = normal
 NT = not testable

Light Touch

	R	L
C2	<input type="checkbox"/>	<input type="checkbox"/>
C3	<input type="checkbox"/>	<input type="checkbox"/>
C4	<input type="checkbox"/>	<input type="checkbox"/>
C5	<input type="checkbox"/>	<input type="checkbox"/>
C6	<input type="checkbox"/>	<input type="checkbox"/>
C7	<input type="checkbox"/>	<input type="checkbox"/>
C8	<input type="checkbox"/>	<input type="checkbox"/>
T1	<input type="checkbox"/>	<input type="checkbox"/>
T2	<input type="checkbox"/>	<input type="checkbox"/>
T3	<input type="checkbox"/>	<input type="checkbox"/>
T4	<input type="checkbox"/>	<input type="checkbox"/>
T5	<input type="checkbox"/>	<input type="checkbox"/>
T6	<input type="checkbox"/>	<input type="checkbox"/>
T7	<input type="checkbox"/>	<input type="checkbox"/>
T8	<input type="checkbox"/>	<input type="checkbox"/>
T9	<input type="checkbox"/>	<input type="checkbox"/>
T10	<input type="checkbox"/>	<input type="checkbox"/>
T11	<input type="checkbox"/>	<input type="checkbox"/>
T12	<input type="checkbox"/>	<input type="checkbox"/>
L1	<input type="checkbox"/>	<input type="checkbox"/>
L2	<input type="checkbox"/>	<input type="checkbox"/>
L3	<input type="checkbox"/>	<input type="checkbox"/>
L4	<input type="checkbox"/>	<input type="checkbox"/>
L5	<input type="checkbox"/>	<input type="checkbox"/>
S1	<input type="checkbox"/>	<input type="checkbox"/>
S2	<input type="checkbox"/>	<input type="checkbox"/>
S3	<input type="checkbox"/>	<input type="checkbox"/>
S4-5	<input type="checkbox"/>	<input type="checkbox"/>

PIN PRICK

	R	L
C2	<input type="checkbox"/>	<input type="checkbox"/>
C3	<input type="checkbox"/>	<input type="checkbox"/>
C4	<input type="checkbox"/>	<input type="checkbox"/>
C5	<input type="checkbox"/>	<input type="checkbox"/>
C6	<input type="checkbox"/>	<input type="checkbox"/>
C7	<input type="checkbox"/>	<input type="checkbox"/>
C8	<input type="checkbox"/>	<input type="checkbox"/>
T1	<input type="checkbox"/>	<input type="checkbox"/>
T2	<input type="checkbox"/>	<input type="checkbox"/>
T3	<input type="checkbox"/>	<input type="checkbox"/>
T4	<input type="checkbox"/>	<input type="checkbox"/>
T5	<input type="checkbox"/>	<input type="checkbox"/>
T6	<input type="checkbox"/>	<input type="checkbox"/>
T7	<input type="checkbox"/>	<input type="checkbox"/>
T8	<input type="checkbox"/>	<input type="checkbox"/>
T9	<input type="checkbox"/>	<input type="checkbox"/>
T10	<input type="checkbox"/>	<input type="checkbox"/>
T11	<input type="checkbox"/>	<input type="checkbox"/>
T12	<input type="checkbox"/>	<input type="checkbox"/>
L1	<input type="checkbox"/>	<input type="checkbox"/>
L2	<input type="checkbox"/>	<input type="checkbox"/>
L3	<input type="checkbox"/>	<input type="checkbox"/>
L4	<input type="checkbox"/>	<input type="checkbox"/>
L5	<input type="checkbox"/>	<input type="checkbox"/>
S1	<input type="checkbox"/>	<input type="checkbox"/>
S2	<input type="checkbox"/>	<input type="checkbox"/>
S3	<input type="checkbox"/>	<input type="checkbox"/>
S4-5	<input type="checkbox"/>	<input type="checkbox"/>

TOTALS
 (MAXIMUM) (56) (56) (56) (56) =

(DAP) Deep anal pressure (yes/no) ☐
 PIN PRICK SCORE (max: 112)
 LIGHT TOUCH SCORE (max: 112)

NEUROLOGICAL LEVEL
 The most caudal segment with normal function

SENSORY R L
MOTOR ☐ ☐

SINGLE NEUROLOGICAL LEVEL ☐

COMPLETE OR INCOMPLETE?
 Incomplete = Any sensory or motor function in S4-S5
 ASIA IMPAIRMENT SCALE (AIS) ☐

ZONE OF PARTIAL PRESERVATION
 (in complete injuries only)
 Most caudal level with any innervation

SENSORY R L
MOTOR ☐ ☐

This form may be copied freely but should not be altered without permission from the American Spinal Injury Association. REV 04/11

Figure 2. International Standards for Neurological Classification of Spinal Cord Injury (AIS/ASIA-scale), revised 2011; Atlanta, GA. Reprinted 2011. With permission from the American Spinal Injury Association.

A limitation of the ASIA scale from a strict diagnostic point of view is that it does not take thoracic motor function (intercostal muscles, see figure 2) and trunk stability into account (i.e., the spinal cord efference from levels T2-L1). However, this diagnostic shortcoming matters less in today's clinical practice since it does not change therapeutic strategies. One clinical aspect of the level of thoracic injury is the occurrence of autonomic dysreflexia, more common in injuries above the T6 level i.e., above the level where splanchnic sympathetic outflow can be inhibited through efferent spinal cord signals, which is relevant due to cardiovascular complications (Gunduz and Binak, 2012).

Spinal shock, spontaneous recovery and chronic state

Knowledge of the energy level of the trauma, the ASIA score and the computerized tomography (CT) results upon arrival to the trauma unit with subsequent magnet resonance imaging (MRI) can often suggest the level and completeness of a spinal cord injury. However, the clinical phenomenon of spinal shock, a temporary state of flaccid paresis and bladder dysfunction with initially reduced or absent reflexes lasting days or longer (Ditunno et al., 2004), can mask residual neurological function. Therefore, ASIA scorings have to be performed repeatedly, particularly after 3 days when the prognostic value is greater (Burns and Ditunno, 2001; Burns et al., 2003; Furlan et al., 2008). Spontaneous neurological improvement is more likely among patients with incomplete injuries (AIS-B to AIS-D) and even more so among patients with some sparing of both motor and sensory functions (AIS-C and AIS-D), whereas spontaneous recovery in complete SCI (AIS-A) is very unlikely (Burns and Ditunno, 2001; Burns, et al., 2003; Fawcett et al., 2007).

Although a spinal cord injury is commonly labeled chronic at one year after injury, after which further improvement is seldom seen (Waters et al., 1993), a small percentage can show some recovery after 18 months or later (Fawcett, et al., 2007). The question as to when a spinal cord injury can be regarded as chronic, i.e., when there is very little chance of further improvement, is of importance not only for the patient's expectations but also for inclusion in future regenerative procedures, since an experimental procedure cannot be allowed to jeopardize a potential spontaneous recovery (Fawcett, et al., 2007; Furlan,

et al., 2008). The accurate assessment of level and completeness of spinal cord injury therefore seems to be of even greater importance for future clinical trials (Fawcett, et al., 2007).

BIOLOGY OF THE INJURED SPINAL CORD

Primary injury

The primary injury in SCI is initiated by mechanical trauma such as from dislocated bony fragments, traumatized discs, hemorrhages, columnar distraction or penetrating objects that concuss, contuse, distract or lacerate the spinal cord with transient or persistent compression (Tator, 1995). A combination of initial impact and persistent compression is the most common manifestation of traumatic SCI (Sekhon and Fehlings, 2001). Since it is a more vascularized and a softer tissue, grey matter is often more damaged than white matter (Tator, 1995; Young, 2002). The primary trauma results in death of neurons as well as damage and death of oligodendrocytes, astrocytes and endothelial cells. Microvascular tearing leads to hemorrhages, ischemia, edema and disturbance of nutrient supply, whereas tearing or complete disruption of axons causes membrane damage (at the nodes of Ranvier where myelinated axons are more vulnerable due to stretching), which also initiates degeneration of the distal part (Choo et al., 2008; Hagg and Oudega, 2006; Profyris et al., 2004).

Secondary injury

Secondary injury mechanisms begin right after the primary trauma and lead to continued deterioration during the acute, subacute and sometimes chronic phases of the spinal cord injury (Choo, et al., 2008; Hagg and Oudega, 2006; E. Park et al., 2004; Profyris, et al., 2004; Tator and Fehlings, 1991). Microvascular injury aggravated by persistent compression leads to ischemia around the injury center and an edema that spreads cranially and caudally at 24 - 48 hours after the injury (E. Park, et al., 2004; Tator and Fehlings, 1991). Hemorrhage and edema are also potential precursors for cystic transformation, a phenomenon that worsens the neurological deficits (Josephson et al., 2001; Tator and

Fehlings, 1991). Accumulation of excitatory amino acids such as glutamate contributes to additional cell death in both grey and white matter. Further, an increase of free radicals in the lesioned area disturbs the ATPase activity and leads to lipid peroxidation and cytoskeletal damage (Hagg and Oudega, 2006; E. Park, et al., 2004; Profyris, et al., 2004). Inflammation follows with infiltration of neutrophils, T-lymphocytes, activated macrophages and microglia which contributes to secondary destruction through the production of pro-inflammatory cytokines such as TNF alpha, interleukins, nitric oxide and glutamate (M. E. Schwab and Bartholdi, 1996).

After SCI, reactive hypertrophied astrocytes together with meningeal fibroblasts, oligodendrocyte precursors, myelin and oligodendrocyte debris form a gradually non-permissive glial scar, sealing off the lesion from the intact spinal cord (Fawcett and Asher, 1999; Fehlings and Hawryluk, 2010). Recent experiments in the rat suggest that a population of pericytes invade the injury zone and give rise to stromal cells that contribute to scar formation (Goritz et al., 2011). The reactive astrocytes, and to some extent also other glia cells, express molecules such as chondroitin sulphate proteoglycans (CSPGs) that inhibit neuronal outgrowth (Bradbury et al., 2002; Silver and Miller, 2004), and together with myelin-associated growth protein (MAG), oligodendrocyte-myelin glycoprotein (OMgp) make the glial scar not only a mechanical barrier, but also a chemical growth inhibitory barrier (Fawcett and Asher, 1999).

Another important growth repellant in the injured white matter and glial scar is the Nogo protein. After the non permissive properties of oligodendrocytes and CNS myelin were confirmed (M. E. Schwab and Caroni, 1988), the growth repellant oligodendrocyte protein called “Nogo” was cloned in year 2000 (Chen et al., 2000). CSPGs, MAG, OMgp, Nogo proteins and other inhibitory substances activate the small GTPase Rho, which regulates axonal growth downstream. An up-regulation of Rho leads to growth cone collapse and neurite growth inhibition (J. M. Schwab, Tuli, et al., 2006). Rho activation also contributes to apoptosis (McKerracher and Higuchi, 2006).

In spite of the posttraumatic glial scar, it is known that injured axons in the spinal cord have an inherent capacity of regrowth (David and Aguayo, 1981; Richardson et al., 1980)

and hence, large efforts have been made over the last decades to overcome the inhibiting barrier in hope of restoring electrical circuitry and neurological function. Although many strategies seem to be promising, no effective treatment has yet been established, and a combination of different strategies therefore seems to be a rational approach in the aspiration of achieving true regeneration (Bradbury and McMahon, 2006; Fehlings and Hawryluk, 2010; Kwon et al., 2010).

TREATMENT OF SPINAL CORD INJURY

Acute management and assessment

Current treatments after spinal cord injury aim at preventing worsening of an initial spinal cord trauma, counteracting secondary injury mechanisms, managing medical complications, providing rehabilitation and facilitating readjustment to a daily life. The stable bony structures and ligament apparatus of the vertebral column protect the spinal cord from manipulation and injury in everyday situations. Consequently, after a severe trauma with fractures and ligament ruptures there is a high risk that the vertebral column becomes unstable, meaning that the spinal cord independently of existent or nonexistent initial cord injury is left unprotected. As a result, minor movements or manipulations of the unstable columnar region in the neck or back can create a spinal cord injury or worsen an already existent one. Hence, when a spinal cord injury or a fracture of the vertebral column is suspected, the first important (pre-hospital) action is immobilization of the patient including a cervical collar, head immobilization, and a spinal board (Ahn et al., 2011). Transport of patients with acute traumatic SCI to a hospital center should occur without delay, and early transfer to a specialized center is preferable as it decreases overall mortality and complications (Ahn, et al., 2011; Parent et al., 2011).

Deficits of motor and sensory functions should be assessed according to ASIA standards, and after an initial scan with computerized tomography (CT), if suspicion of SCI or ligament rupture remains or is confirmed, MRI including sagittal T2 weighted sequences should be performed in the acute period for prognosis and guidance of further acute management (ASIA, 2011; Bozzo et al., 2011; Furlan, Noonan, Singh, et al., 2011; Goldberg and Kershah, 2010).

Acute treatment

Though clinical practice still varies, early decompression of the spinal cord including removal of dislocated bone fragments and hematomas is of great importance to save neurological function and should be carried out as soon as feasible, at the latest within 24 hours (Fehlings et al., 2010; Furlan, Noonan, Cadotte, et al., 2011). An unstable vertebral column needs to be stabilized surgically to avoid repeated trauma to the cord. Since ongoing ischemia may worsen secondary damage, hypotension should be aggressively avoided, and optimal respiration and oxygen saturation is mandatory (Casha and Christie, 2011). Mild systemic hypothermia (around 33 °C) slows basic enzymatic activity and reduces energy requirements of cells and may be neuroprotective also by reducing glutamate levels and apoptosis (Erecinska et al., 2003; Kwon, et al., 2010). After the NFL player Kevin Everett who suffered a cervical SCI in 2007 was treated with systemic hypothermia, the interest for application in SCI increased enormously. However, though pre-clinical and clinical results seems promising and the method is suggested to be safe, hypothermic treatment for SCI remains experimental and larger studies are needed to prove its true effect on neurological function (Dietrich et al., 2011; Kwon et al., 2011; Kwon, et al., 2010).

Medical treatment with a high dose of the anti-inflammatory glucocorticosteroid methylprednisolone, given within 8 hours and continuing 24 to 48 hours after SCI has been widely used over the last two decades since some positive effects on motor function were demonstrated in the multi center National Acute Spinal Cord Injury Studies (NASCIS) (Bracken, 2012; Bracken et al., 1990; Bracken et al., 1997). However, with steroid side effects and questioned strength of evidence, the use of methylprednisolone has become controversial. It is now less commonly used and is not recommended for routine use in SCI (Baptiste and Fehlings, 2007; Coleman et al., 2000; Hurlbert, 2000; Hurlbert and Hamilton, 2008).

Acute experimental treatments

In contrast to historical routine SCI care, which has largely served to stabilize the overall patient situation and prevent further mechanical impact with the exception of methylprednisolone, which is the only medical agent that has previously been used in standard

care for acute SCI (Hurlbert and Hamilton, 2008)), upcoming experimental treatments aim at limiting and modulating downstream negative secondary injuries on a molecular level through neuroprotection, promotion of axonal growth, blocking of inhibitory signaling, trophic factors and reduction of the glial scar (Fawcett, 2006; Kwon, et al., 2010; Nandoe Tewarie et al., 2010). In the last few years, a set of new experimental drugs have come up as potential SCI treatment candidates, some of which have, or are about to be tested clinically. Riluzole® is an orally administered sodium channel blocker that is approved by the American Food and Drug Administration (FDA) for use in amyotrophic lateral sclerosis (ALS). Riluzole® has been shown to promote cell survival and neurite outgrowth of sensory afferents *in vitro* and enhance motor recovery after experimental root avulsion injury with subsequent surgical reinsertion (Bergerot et al., 2004; Shortland et al., 2006). Influx of sodium with subsequent disturbed calcium hemostasis is suggested as a mechanism of damage to white matter in SCI (Rosenberg et al., 1999). Encouraged by preclinical data showing neuroprotection as evidenced by spared white matter and behavioral improvements, a multicenter study will evaluate administration of doses of Riluzole® approved by the FDA for ALS, within 12 hours after cervical and thoracic SCI (Cadotte and Fehlings, 2011; Kwon, et al., 2010).

Another neuroprotective drug is the tetracycline antibiotic Minocycline, that decreases glutamate-mediated excitotoxicity (Baptiste et al., 2004), acts as an immunomodulator by blocking microglial activation (Baptiste et al., 2005) and reduces oligodendrocyte death as well as axonal dieback (Stirling et al., 2004). Promising preclinical data has initiated a human clinical trial in Calgary, Canada where preliminary data suggests that i.v. administration within 12 hours after SCI is safe, and a larger Canadian multicenter study is therefore planned (Baptiste and Fehlings, 2007; Kwon, et al., 2010).

The endogenous NMDA blocker magnesium is neuroprotective, given that NMDA receptors in SCI are overstimulated by glutamate leading to massive calcium influx and cell death, and that magnesium is thought to inhibit glutamate release itself (Palmer, 2001; Vink and Cernak, 2000). Magnesium has in preclinical experiments improved locomotor function, however the experimental doses used have far exceeded approved human dosages. Clinical studies are therefore currently investigating administration of magnesium in

the lower doses that previously have been safe in trials of stroke and cardiac arrest (Kwon, et al., 2010).

Subacute treatments

The monoclonal IgM antibody IN-1 was in the 1990's found to promote axonal growth with some long-distance axonal regeneration in the CNS and also improve functional recovery (Bregman et al., 1995; Schnell and Schwab, 1990). After its antigen target, the growth repellant protein Nogo had been isolated, anti-Nogo IgG antibodies for intrathecal administration were developed. In a clinical safety trial that started in 2006, ASIA-A patients with complete thoracic or cervical SCI have received anti-Nogo IgG antibodies between 4 and 14 days post-injury and the first phase of the trial has not showed any side effects so far (Kwon, et al., 2010; Zorner and Schwab, 2010).

Rho pathway inhibitors such as C3 transferase (C3) and Y27632 are promoting axonal growth by preventing downstream Rho inactivation of the growth cone and are also neuroprotective since Rho activation also leads to apoptosis (Dergham et al., 2002; Dubreuil et al., 2003). Functional improvements of behavioral recovery have been seen in preclinical experiments (Cadotte and Fehlings, 2011; Dergham, et al., 2002), and in a non-randomized multicenter study starting 2005 the Rho-antagonist Cethrin (BA-210) mixed with fibrin glue (Tiseel™) has been applied to the dura mater in AIS-A thoracic and cervical SCI patients within 7 days after injury. In this clinical trial, no major adverse events connected to Cethrin were seen and a quarter of 37 patients improved from ASIA-A to ASIA-B, C or D (Kwon, et al., 2010).

The bacterial enzyme Chondroitinase ABC (ChABC) has the ability to digest the growth inhibitory chondroitin sulphate proteoglycans (CSPGs) of the glial scar by partly removing their carbohydrate chains (Crespo et al., 2007; Silver and Miller, 2004). The experimental administration of ChABC promotes neural plasticity, sprouting of corticospinal and serotonergic fibers and functional improvement (Barritt et al., 2006; Bradbury, et al., 2002) and has also been found to be neuroprotective by sparing rubrospinal neurons after injury when administered one month after injury (Carter et al., 2011). By decreasing the CSPG

effect of the glial scar, ChABC has also been shown to be effective in combinatorial treatments, e.g., together with peripheral nerve grafts, Schwann cells or olfactory ensheathing cells (OECs, see below) (Alilain, et al., 2011; Bunge, 2008; Fouad et al., 2005; Houle et al., 2006). ChABC has yet not been tested clinically.

Neurotrophins are trophic proteins that are necessary for axonal growth during development. Further, they regulate neuronal survival, synaptic plasticity and neurotransmission (Jones et al., 2001). Receptors for Nerve growth factor (NGF) are found on CNS sensory axons, and infusion of NGF promotes growth of sensory grafts in the CNS (Oudega and Hagg, 1996). Receptors for Brain derived neurotrophic factor (BDNF) are widely expressed in the CNS, and deliverance of BDNF after SCI results in increased axonal growth and decreased neural atrophy (Kobayashi et al., 1997; Lu et al., 2005). Neurotrophin-3 promotes growth of corticospinal axons after SCI (Schnell et al., 1994) and treatment with NT-3 has also resulted in behavioral improvements in chronic SCI (Tuszynski et al., 2003).

Cell therapies in the subacute phase

Implantation of activated macrophages has experimentally been shown to promote partial recovery of motor function paralleled with positive electrophysiology (Rapalino et al., 1998). Autologous *ex-vivo* activated macrophages are believed to be both neuroprotective and neuroregenerative, probably due to the secretion of protective cytokines IL-1 beta and IL-6 together with brain derived neurotrophic factor (BDNF), while reducing the neurotoxic cytokine Tumor necrosis factor alpha (TNF alpha). A possible mechanism of enhanced regeneration could also be phagocytosis of myelin debris, which would leave space for and create a better regeneration environment for outgrowing axons (Bomstein et al., 2003). Clinically, a non-randomized Phase I study has been performed in Belgium and Israel, where ASIA-A patients with injuries within C5-T11 were given microinjections with activated macrophages into the spinal parenchyma at the border of the lesion within 14 days after injury. The results showed that the therapy was safe, however further trials are needed to investigate the usefulness of the procedure (Knoller et al., 2005; Schwartz and Yoles, 2006).

Neural stem cells (NSC) are progenitor cells that have the ability to divide repeatedly and differentiate into neurons, astrocytes or oligodendrocytes (Carpenter et al., 1999). Adult human NSCs have been isolated from several locations in the CNS including the lateral ventricle wall and hippocampus in the brain (Johansson, Svensson, et al., 1999; Kukekov et al., 1999). Transplanting embryonic human NSCs that are not restricted to a certain cell type involves the risk of teratoma development (Hentze et al., 2009; Sundberg et al., 2011) while activated endogenous stem cells tend to move towards an astrocytic lineage following spinal cord injury (Johansson, Momba, et al., 1999). Transplanted stem cells could theoretically give rise to new neurons though remyelination and secretion of growth factors seem to be a more reasonable treatment goal. Therefore, experiments have been performed to restrict multipotent NSCs in vitro to become committed to oligodendrocytic fate (oligodendrocyte progenitors) for transplantation into the spinal cord in the subacute stage of SCI (Keirstead et al., 2005b). In a primary FDA approved safety study, injections with human embryonal oligodendrocyte progenitor cells were to be administered to ASIA-A patients with injuries between thoracic segment T3 and T10 patients (GERON, 2009). However, the project was halted in 2011, allegedly for financial reasons, and no results have been reported except the absence of detected safety problems so far in the four treated patients (Pollack, 2011).

Pluripotent bone marrow derived stem cells (BMSC) are easy to access and grow well in tissue culture. BMSCs have been in vitro differentiated into neuronal-like cells and injected into contused spinal cords in preclinical experiments with resulting improvement in motor function, however with better results with injections one week after injury than immediately after injury (Hofstetter et al., 2002). Early clinical studies in Korea and Czech Republic have also shown that injecting autologous BMSCs seems to both be safe and to improve neurological functions to some extent (H. C. Park et al., 2005; Sykova et al., 2006; Yoon et al., 2007).

Schwann cells (SC) form the myelin sheaths around the peripheral nervous system (PNS). They are able to myelinate CNS axons, promote axonal regeneration (Duncan and Milward, 1995; Oudega and Xu, 2006) and are also thought to recruit endogenous host SC into the injured spinal cord (Biernaskie et al., 2007; Hill et al., 2006). However, SC alone can-

not promote outgrowing axons to overcome the inhibiting glial scar and reenter the host spinal cord, and hence combinatorial treatments seem to be needed to yield better results (Bunge, 2008; Oudega and Xu, 2006; Pearse et al., 2007). Clinical studies therefore would seem to need to investigate a combination of experimental strategies, which would be a challenge. Nevertheless, 33 ASIA-A and ASIA-B patients with chronic cervical and thoracic SCI have been enrolled in a clinical trial in Iran, where Schwann cells were harvested from the sural nerve and injected intramedullary. In this study, a 2-year follow up did not reveal any neurological deterioration or other major complications (Saber et al., 2011).

Treatment of chronic SCI

When the acute and subacute period of injury have passed and the patient moves toward a more stable and chronic situation, the focus in current clinical practice is set on maintaining remaining neurological functions, controlling pain, managing activities of daily living (ADL) and readjustment to an acceptable social and professional life. Secondary complications such as infections, pressure ulcers and spasticity have to be treated aggressively throughout the rest of a spinal cord patient's life to avoid long hospitalizations and death (van den Berg, Castellote, de Pedro-Cuesta, et al., 2010; Yeo et al., 1998). Especially complications from recurrent urinary tract infections with septicemia and renal failure, which in earlier decades used to be the most common cause of death after SCI (Breithaupt et al., 1961; Freed et al., 1966), and the frequent respiratory tract infections (Soden et al., 2000), but also cardiovascular complications and depression are potentially fatal complications that have to be treated conscientiously (Soden, et al., 2000; van den Berg, Castellote, de Pedro-Cuesta, et al., 2010).

Following a spinal cord contusion there will be nerve fibers which are axotomized, neuronal death as well as intact demyelinated axons (M. E. Schwab and Bartholdi, 1996). There is convincing data that only a very small but functionally important fraction of axons in descending motor tracts is needed (about 5%) to be intact to transmit cortical signals to the lower part of the spinal cord in order to execute good movement in the hind limbs (Bregman, et al., 1995). Moreover, in other models to repair the injured spinal cord, results suggest that only a small number of axons are needed across the SCI to induce some

cortically controlled movements (Bradbury, et al., 2002; Bregman, et al., 1995; GrandPre et al., 2002). Therefore, if only a fraction of the lost conductivity across the injury zone could be restored it would be rational to expect important neurological improvements.

Despite all the promising experimental research over the last decades, still no effective neuroregenerative treatment has been established. However, like in the acute and sub-acute stage, there are a number of experimental strategies that maintain the hope of future curative, or at least partly restorative treatments in chronic SCI. Olfactory ensheathing cells (OECs) are glial cells found in the nerve fiber layer in the olfactory bulb, in the nasal olfactory mucosa and surrounding the cranial olfactory nerve fibers, unique in the CNS to grow throughout life (G. A. Graziadei and P. P. Graziadei, 1979; P. P. Graziadei and G. A. Graziadei, 1979). Transplantations of OECs into the injured rat spinal cord have resulted in increased axonal growth and better functional recovery in rats 3 to 7 months after injury, with further improvement after treadmill step walking (Kubasak et al., 2008; Ramon-Cueto et al., 2000; Ramon-Cueto et al., 1998). The benefits of OECs have been thought to derive from growth permissive and growth stimulating properties, but unlike Schwann cell grafts (that exhibit similar growth promoting qualities), the OECs also have a unique ability to interact with astrocytes (Barnett and Riddell, 2007; Higginson and Barnett, 2011). A small clinical trial in Australia with injection of autologous OEC into the thoracic spinal cord (between segment T4 and T10) in chronic paraplegic patients has been shown to be safe without adverse events up to three years after treatment and led to neurological improvement in one case (Mackay-Sim et al., 2008; Mackay-Sim and St John, 2011). A large clinical study in China concluded that injections with human embryonic OEC in chronic SCI can improve neurological outcome regardless of patient age, and are also found to be safe in a minor trial (Huang et al., 2006; Huang et al., 2003). However, the validity and usefulness of these results has been questioned with respect to standard of design and safety (Dobkin et al., 2006). Clinical studies in Portugal, where olfactory mucosa has been transplanted into cervical and thoracic SCI (level C4-T12) concluded that it was relatively safe, and together with aggressive rehabilitation led to some neurological improvement (Lima et al., 2010; Lima et al., 2006).

Interestingly, it was recently reported that electric epidural stimulation of lumbosacral

segments in a patient with a complete and chronic motor SCI (ASIA-B) at the C7-T1 level combined with extensive training resulted in full weight-bearing standing and voluntary control over some leg movements during stimulation sessions (Harkema et al., 2011). Since the report, three additional patients with complete paraplegia have shown the same positive response to the training followed by epidural stimulation approach where positive effects were seen not only on voluntary locomotion, but also on bladder function, sexual function, temperature control and self-esteem (lecture by VR Edgerton at Karolinska Institute, April 2012). Hence, sensory input and spinal circuitry seems to be of great importance for regaining neurological functions after chronic SCI. It remains to be seen whether human central pattern generators could be modified through stimulation of outgrowth of proximal axons and whether modification of circuitry and central pattern generators (CPGs) through distal sensory input could actually facilitate the axonal bridging of a complete ASIA-A injury, however, intensive rehabilitation and epidural stimulation will probably play an important role in any future regenerative treatment.

If the descending and ascending tracts were to regenerate in a straight line and resemble the original neuroanatomy, the axons in the spinal cord tracts would have to grow far along the other side of the lesion, and there would also have to be specific exit signals for the axons in the white matter to leave the white matter and enter the neuron pools in the grey matter somewhere along the regeneration path. For a potential recovery, it seems reasonable to suggest a shorter route, where axons regenerating from white matter tracts across a spinal cord injury reach into the other side of the lesion and connect to grey matter neuron pools, establishing cortical control of accessible CPGs, which are believed to be responsible for coordinated locomotor function (Alstermark et al., 1987; Bradbury and McMahon, 2006; Raineteau and Schwab, 2001). Peripheral nerve grafts (PNGs) serve as a substrate that effectively stimulates regeneration in the peripheral and central nervous system (Alilain, et al., 2011; Cheng et al., 1996; Cote et al., 2011; Richardson, et al., 1980). Since the publication by Richardson et al. (Richardson, et al., 1980), several studies have confirmed the ability of peripheral nerves to promote axonal regeneration of CNS neurons (Cheng, et al., 1996; David and Aguayo, 1981, 1985; Houle, 1991; Y. S. Lee, Hsiao, et al., 2002; Siegal et al., 1990). In contrast to the inhibitory spinal cord white matter, the spinal

cord grey matter is an area believed to be more permeable to outgrowing axons (Siegal, et al., 1990). Therefore, one theory is that transplanted PNGs are more useful if directed from white matter to grey matter (white to grey matter strategy) when bridging an injury gap (Cheng, et al., 1996). In the work of Cheng and co-workers in 1996, the resected spinal cord was replaced by 18 peripheral nerve grafts directing descending motor tracts to the ventral horn on the caudal side of the lesion and ascending sensory tracts to the dorsal horn on the cranial side of the lesion with resulting partial restoration of hind limb function (Cheng, et al., 1996). The effects of Acidic fibroblast growth factor (FGF1) may, according to previous literature, be attributed to neuroprotection, improved regeneration or local modulation of the spinal cord injury milieu (Giacobini et al., 1991; Guest et al., 1997; Kuo et al., 2011; M. J. Lee et al., 2008; M. J. Lee et al., 2011; Y. S. Lee, Baratta, et al., 2002; Pataky et al., 2000; M. C. Tsai et al., 2008). If there is a potential clinical application of using PNGs, previous studies have suggested that acidic fibroblast growth factor (FGF1) is needed for functional recovery (Cheng, et al., 1996; Y. S. Lee, Hsiao, et al., 2002; Y. S. Lee et al., 2004; Y. S. Lee et al., 2010; E. C. Tsai et al., 2005).

From a clinical perspective, autologous PNGs are easy to harvest (e.g., from the sural nerve) and should not elicit autoimmune or toxic reactions. Hence, the question whether spinal cord repair through peripheral nerve grafts directed from white to grey matter actually can be done with high precision and in a reproducible way is very important. Also meaningful are the questions whether this procedure may actually also result in a regeneration of axons across a lesion gap in the spinal cord, providing a functional connection, and whether adjuvant FGF1 is needed to achieve this. An overview of spinal cord injury and treatment approaches is provided in figure 3.

PATIENT SELECTION AND EVALUATION IN CLINICAL SCI REPAIR TRIALS

The positive results after epidural cord stimulation (see above) raise the question how completeness in a spinal cord injury really should be defined. Very few patients with clinically motor and sensory complete SCI (ASIA-A) injuries show a clear discontinuity of the cord on MRI images, and for a safe diagnosis of neuroanatomical completeness clinical examination seems to need a complement with refined electrophysiological assessments

and MRI imaging where different tracts can be traced. Further, in future experimental clinical procedures, a main concern is to not worsen an already existing injury. Therefore, patients with complete lesions (ASIA-A) in the thoracic spinal cord should be the preferred patient group for clinical trials (Fawcett, et al., 2007; Lammertse et al., 2007; Steeves et al., 2007; Tuszynski et al., 2007). An additional loss of a thoracic segment (due to local adverse affects or surgery) in the thoracic spinal cord is less likely to affect important motor functions (i.e., affect ADL negatively) compared to a loss of additional cervical or lumbar neurological segments. Still, in order to effectively evaluate an experimental treatment of the injured thoracic spinal cord, it would be desirable to assess motor function and circuitry in, even with an improvement or deterioration of a few spinal cord segments.

Motor evoked potentials (MEPs) of the paraspinal muscles (Kuppuswamy et al., 2005) as well as intercostal muscles (Theodorou et al., 2003) are described as a method to evaluate motor function in thoracic SCI. Regarding the erector spinae muscles, Kuppuswamy et al. (Kuppuswamy, et al., 2005) noted that they extend more than one vertebral segment, making them less selective for evaluation of the thoracic cord. Electromyography (EMG) signals could be elicited several segments below the injury level, a phenomenon which is not seen in intercostal muscles (Theodorou, et al., 2003). Anatomical studies of human innervation of the intercostal muscles also support their isolated segmentation (Sakamoto et al., 1996). Every intercostal space has been shown to be innervated by its own intercostal nerve (i.e., the medial branch of the spinal nerve), and the muscle itself is isolated between the ribs (Sakamoto, et al., 1996). On the other hand, still no widely accepted method for assessing local function of the thoracic spinal cord exists other than pure sensory evaluation according to the ASIA scale (see figure 2). Moreover, it is suggested that a change in sensory level of three dermatomes over time is unusual and should be considered a rare event (of significant deterioration), and therefore could be used as a measure to track safety in thoracic ASIA-A SCI treatment trials (Harrop et al., 2009; Zariffa et al., 2011). Diagnosing the completeness and permanence, but also the sensory and motor levels of thoracic spinal cord injuries seems to be important for the evaluation of upcoming experimental procedures and even more so for deciding on patient inclusion, yet focus has in the past mainly been on sensory function.

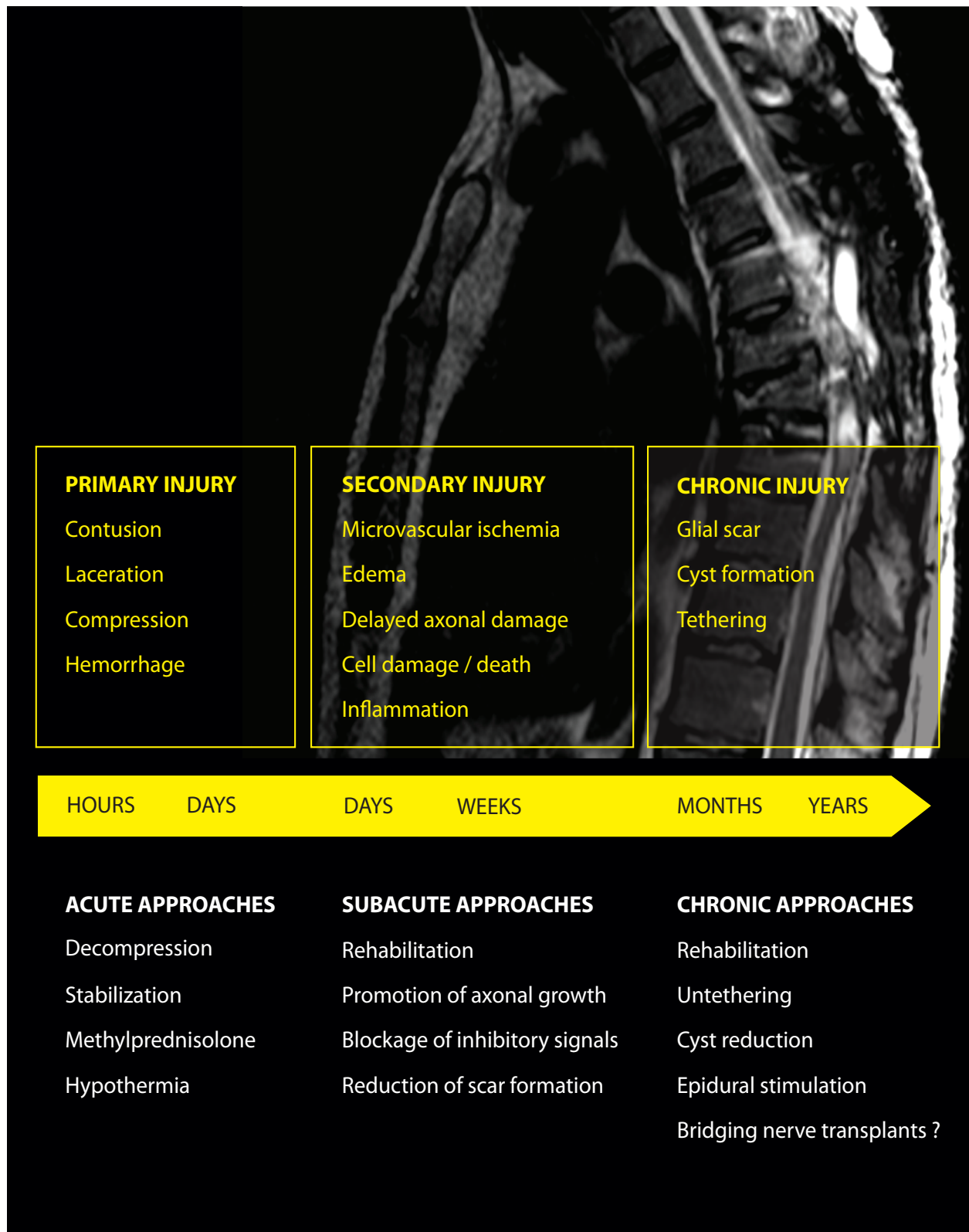


Figure 3. Injury mechanisms, time and approaches to save and restore the injured spinal cord.

AIMS OF THE STUDIES

The aim of this thesis was to:

1. Develop a microsurgical method for precise positioning of peripheral nerve grafts in a spinal cord resection gap.
2. Evaluate the effect of acidic fibroblast growth factor released from a biodegradable (calcium sulphate) device after spinal cord injury and subsequent repair with peripheral nerve grafts.
3. Investigate the possible involvement of corticospinal tract regeneration after spinal cord injury and repair with peripheral nerve grafts.
4. Investigate potential evidence for selective spinal cord tract guidance by meticulous positioning of peripheral nerve grafts.
5. Find a clinical method to neurophysiologically and radiologically demarcate the cranial and caudal borders of a chronic and complete spinal cord injury.

MATERIALS AND METHODS

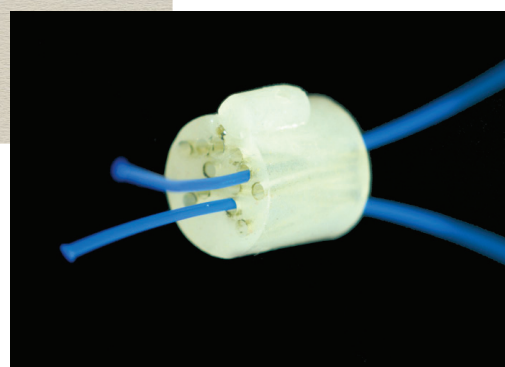
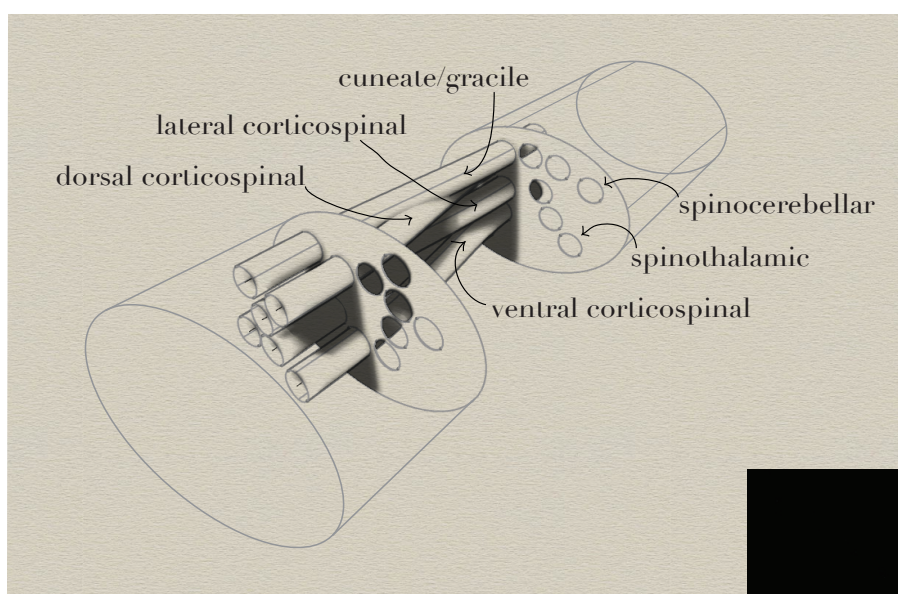
ANIMAL CARE AND ANESTHESIOLOGY (Papers I, II and III)

All experiments were approved by the Stockholm Animal Ethics Committee. Adult female 260-290 g Sprague Dawley rats (260-280 g in paper I and 270-290 g in papers II+IV, Scanbur®, Sollentuna, Sweden) were used in all experiments. All animals were kept in ventilated, humidity- and temperature controlled rooms with a 12-hour light per 24-hour cycle and received food pellets and water ad libitum, according to regulations at Karolinska Institutet. For spinal cord surgery, the animals were anesthetized with continuous isoflurane inhalation (2.2-2.7 %). For shorter cranial procedures (i.e., electrophysiology and intracranial tracing injections), re-lesion after electrophysiology, magnetic resonance imaging (MRI) and euthanasia, intraperitoneal ketamine and medetomidine (Ketalar®, 75 mg/kg + Dormitor®, 0.5 mg/kg) was used. The animals were kept warm (37 °C) with a thermostatic heating pad (Panlab, Cornelia, Spain) connected to a rectal probe (LSI Letica®HBI 102/2 instruments, Debiomed, Barcelona, Spain). Heart rate and oxygen saturation was measured in the paw, and oxygen flow was regulated to keep saturation above 95%. After spinal procedures, postoperative antibiotics were administered in the drinking water (Sulfadoxin 1,14 mg/ml and Trimetoprim 0.23 mg/ml; BorgalVet®, Intervet International B.V.). Postoperative analgesia was given subcutaneously for three days (Caprofen 5mg/kg BW once daily (bid first day); Rimadyl®Vet, Pfizer and Buprenorphine 0.05 mg/kg BW bid; Temgesic®, Schering-Plough). In spinal cord injured animals, urinary bladders were emptied manually twice daily.

DEVELOPMENT OF SPINAL CORD DEVICES FOR PERIPHERAL NERVE TRANSPLANTS (Papers I, II and III)

In order to direct peripheral nerve grafts from white to grey matter in a standardized and reproducible way, we developed a device containing 12 channels (which was the maximum number of channels to fit), where each channel represents one specific pathway of the spinal cord. Flexible wires (0.40 mm in diameter, the same dimension as a PNG) were positioned in a holder (Fig. 1a, paper I) in accordance with an anatomical map, where the

entrances represented the position of a white matter pathway and the exits represented the position of adjacent grey matter (Fig. 1a+b, paper I, Fig. 1a+b, paper II). A cylindrical device was then molded around the wires, which could be removed after the device had hardened. The devices measured 3.0 mm in length and 3.0 mm in diameter (Fig. 1c, paper I, Fig. 1c, paper II) containing 12 channels (0.38 mm in diameter) that could direct peripheral nerves from six white matter motor tracts (right and left dorsal corticospinal, lateral corticospinal and ventral corticospinal tracts) and six sensory tracts (right and left cuneate/gracile, spinocerebellar and spinothalamic tracts) to adjacent grey matter (see figure 4 below and Fig. 1a+b, paper I, Fig. 1a+b, paper II). Non-biodegradable dental cement (Bosworth Trim® Temporary Resin Acrylic, Bosworth Company, Skokie, IL, USA) was used as device material in paper I (see figure 5 below and Fig. 1c, paper I), and biodegradable calcium sulphate (CaSO_4) was used in papers II and III (Fig. 1 c, paper II). The rationale for using a biodegradable material was based on the idea that non-dissolving artificial material presented to CNS tissue over the long term may trigger a local immune response and act as a substrate for potential bacterial infections years after surgery. The nanoporous structure of the calcium sulphate also permitted loading and a controlled slow release of adjuvant FGF1 (Aberg et al., 2012).



Figures 4 (up) and 5 (right): the white-to-grey re-direction of three motor and three sensory pathways and a molded graft holder in dental cement (two channels indicated with blue sutures).

ACIDIC FIBROBLAST GROWTH FACTOR (Papers II and III)

Acidic fibroblast growth factor (FGF1) was used as adjuvant treatment in papers II and III. The biodegradable calcium sulphate devices used in these studies were incubated to absorb FGF1 (Protein Sciences, Meriden, CT, USA) in concentrations of either 0.05 mg/ml or 0.5 mg/ml (paper II) or 0 ng/ml, 5 ng/ml, 500 ng/ml or 50 µg/ml (paper III) for three days at +4 °C in a phosphate buffered saline solution (PBS, Sigma Aldrich, St Louis, MO, USA) with heparin (concentration 1:1 w/w, heparin sodium salt from porcine intestinal mucosa, Sigma Aldrich, St Louis, MO, USA). Adding heparin to FGF1 has previously been shown to stabilize and enhance the activity of this growth factor (Aberg, et al., 2012; Klint and Claesson-Welsh, 1999; Mohammadi et al., 2005). In paper II, the graft device absorbed approximately 10µl of FGF1/heparin fluid, corresponding to a total dose of 500 ng or 5.0 µg, depending on the FGF1 concentration. In paper III, the graft device absorption was measured for each concentration showing absorbed doses of 0 ng, 0.07 ng, 7 ng and 500 ng. Prepared graft devices were stored at -20 °C before surgery.

MICROSURGERY (Papers I, II and III)

Harvesting of peripheral nerves and spinal cord surgery

All surgery was performed at the experimental lab at the Department of Neurosurgery, Karolinska University Hospital in Stockholm. Anesthetized animals were shaved on the back and the skin was sterilized with chlorhexidine-alcohol solution. A Leica surgical microscope (Leica M651, Heerbrugg, Switzerland) was used for the operative procedure. For spinal cord repair, a dorsal midline skin incision was made from the mid scapular wing down to the L1 level. For transplantation with peripheral nerve grafts (PNGs), 12 autologous intercostal nerves were harvested through dissection on the right and left sides of the posterior thorax, and put in saline. Soft tissue and muscle tendons were detached from the T10-T12 spinal processes and laminae, and the vertebral column was immobilized perioperatively by the use of Cunningham™ Spinal Stereotaxic Adaptors (Harvard Apparatus, Holliston, MA, USA) attached on each side of the spinal column. A 3 mm wide T11 laminectomy (Fig. 2a, paper I) was performed with 1.0 and 0.5 mm high-speed

diamond drills (Anspach® e-Max 2, Palm Beach Gardens, FL, USA). Intermittent saline irrigation was used to prevent heat development, keep bleeding to a minimum and together with micro-suction maintain a clean operative field. Dural and arachnoid layers were removed with forceps and micro-scissors, and the dorsal vein gently coagulated with bipolar forceps to prevent bleeding during cord transection. For rats subjected to spinal cord injury, the spinal cord was completely transected at two sites 3 mm from each other at the level of the T11 vertebra, and complete resection of the 3 mm long segment was made using micro-scissors and micro-suction under high magnification (Fig. 2b, paper I). The injury gap was thoroughly re-inspected at high magnification to leave no doubt about its completeness. The twelve harvested PNGs were tied to 6.0 Prolene® sutures and pulled through the 12 channels of the graft device (made of dental cement in paper I and calcium sulphate in papers II and III). The grafts were then trimmed with micro-scissors at the entrance and exit of the channels under high magnification. The device (now containing 12 PNGs) was positioned in the injury gap with exact placement between the cranial and caudal spinal cord ends (Fig. 2c, paper I, Fig. 1d, paper II). A dorsal indicator in the lower midline of the device provided cranio-caudal and dorso-ventral orientation and was gently removed with a small bone rongeur after device placement. Muscle and skin were closed in layers with interrupted self-absorbable 4.0 Vicryl® and non-absorbable 3.0 Ethilone® sutures.

Cranial surgery

For registration of cortical motor evoked potentials and tracing injections into the motor cortex, anesthetized rats were placed in a stereotactic frame with ear pin holders. A midline skin incision was made to expose the skull, which was then drilled under intermittent saline irrigation to expose the epidural surface of the motor cortex on each side. After completed experimental procedures (i.e., electrophysiology or tracing injections) the skin was closed with interrupted 3.0 Ethilone® sutures.

EXPERIMENTAL GROUPS (Papers I, II and III)

In paper I (n=15), five rats were operated with laminectomy only (sham), five rats with spinal cord resection only (negative control) and five rats with spinal cord resection followed by repair using a dental cement device filled with 12 peripheral nerve grafts. Animals were kept alive for six months.

In paper II (n=48), four rats were sham operated, eight rats negative SCI-controls, eight operated with spinal cord resection and repair using a biodegradable CaSO_4 device containing 12 PNGs, eight operated with resection and repair with a device containing 500 ng FGF1 + 12 PNGs and eight operated with resection and repair with a device containing 5 μg FGF1 + 12 PNGs. These animals were kept alive for 20 weeks. In addition, 12 animals were used for tracing studies in which six rats were operated with spinal cord resection and repair with a device containing 500 ng FGF1 + 12 PNGs and six rats operated with spinal cord resection only (negative injury controls for tracing). The tracing animals were kept alive for ten weeks.

In paper III (n=30), rats were subjected to spinal cord resection only (n=6), operation with spinal cord resection and subsequent repair with vehicle soaked CaSO_4 device + 12 PNGs (n=6), device soaked in 0.07 ng FGF1 + 12 PNGs (n=6), 7 ng FGF1 + 12 PNGs (n=6) and 500 ng FGF1 + 12 PNGs (n=6). The animals in this study were kept alive for ten weeks. For an overview of experimental groups and analyses see Table 1.

Table 1. *Experimental groups*

Paper	III	III	III	II+III	II	I
Postop weeks	1	2	4	10	20	26
Treatment *= +PNGs				(n=12+30) 0 ng FGF1* 0.07ng FGF1* 7 ng FGF1* 500 ng FGF1* SCI only	(n=36) 500 ng FGF1* 5 µg FGF1* SCI only Sham	(n=15) PNGs SCI only Sham
Transcranial MEPs + SEPs						(n=15)
Transcortical MEPs	(n=24)	(n=23)	(n=29)		(n=34)	
Treatment *= +PNGs	0 ng FGF1* 0.07ng FGF1* 7 ng FGF1* 500 ng FGF1*	0 ng FGF1* 0.07ng FGF1* 7 ng FGF1* 500 ng FGF1*	0 ng FGF1* 0.07ng FGF1* 7 ng FGF1* 500 ng FGF1* SCI only		500 ng FGF1* 5 µg FGF1* SCI only Sham	
Re-lesion					(n=23)	(n=2)
Tracing				(n=12+29)		
BBB					(n=34)	(n=15)
Histology				(n=12+29)	(n=34)	(n=15)

ELECTROPHYSIOLOGY (Papers I, II and III)

Electrophysiology was carried out at 1, 2, 4 (paper III), 20 (paper II) and 26 (paper I) weeks after surgery, in anesthetized rats (see Table 1). A Medtronic Key Point was used for electrophysiology assessments (Software version 5.03, Minneapolis, MN, USA). In paper I, presence of somatosensory evoked potentials (SEP) and motor evoked potentials (MEP) were assessed through transcranial registration (SEP) and stimulation (MEP) respectively. Through a subcutaneous needle in the tail root, the major tail root nerve was electrically stimulated unilaterally at 3 Hz until the tail was clearly jerking (stimulus duration 0.2 ms, up to 30 mA, 150 stimulations). The averaged cortical SEPs were registered via a subcutaneous extracranial needle electrode, adjacent to the sensory cortex (posterior to the vertex in the midline) with a reference electrode placed subcutaneously at the nose. The low cut off filter was set to 10 Hz and high cut off filter was set to 5000 Hz. For MEPs, transcranial electrical stimulation was given through subcutaneous needles placed superficial to the motor cortex (anterior to the vertex in the midline) and MEPs were recorded through an intramuscular needle in the calf muscles with a reference electrode inserted 1 cm distal to the active electrode. Stimulus duration was 0.5 ms, and stimuli were gradually increased until reproducible responses were recorded. Stimulation thresholds (defined as the level of current needed for a clear MEP response in the target muscle with an amplitude above 50 μ V in at least five out of ten stimulations (Rossini et al., 1994)) and latency times (time from stimulation to response) were documented. No further recordings were registered above 40 mA, and stimulations were stopped at 70 mA. A subcutaneous needle electrode in the hind paw was used for grounding. A band pass filter set to 100-2000 Hz was used.

In paper II and III, direct cortical stimulation replaced the transcranial stimulation for MEP assessment to reduce stimulation intensity needed and possible artifacts. Presence of MEPs in forelimbs as well as hind limbs was registered in all investigated animals. After dural exposure, the motor cortex was identified using anatomical landmarks, and stimulated via a bipolar stimulator probe (Neurosign® Magstim, UK). Recording needles were inserted bilaterally into the hamstring muscles and forearm muscles, with the reference needles inserted into the respective muscle tendon about 1-2 cm distal to the active

electrodes. The response in all four extremities were simultaneously recorded after stimulation of the right as well as left motor cortex. Stimulation was given with four pulses, stimulation duration 0.2 ms, interstimulus interval 2 ms, and stimuli were gradually elevated until reproducible responses were recorded. If no MEPs could be triggered, stimulation was interrupted at 30 mA. A subcutaneous needle electrode in the tail root was used for grounding and a band pass filter set to 10-2000 Hz was used.

Retransection for assurance of true MEPs

After positive MEP recordings in the hind limbs, animals were re-lesioned with a complete spinal cord transection at the level of T8, with subsequent new MEP recordings to confirm that the registered positive potentials had been caused by electrical current actually initiated and generated above the T8-level, and to exclude that the current had traveled outside the spinal cord to the hind limbs. In paper I, one animal in each group with positive MEPs was re-lesioned, and in paper II all positive animals were re-lesioned. In addition, MEP-recordings from the upper extremities were simultaneously registered in the latter study, to likewise ensure that stimulation was given correctly after the re-lesion. In paper III, no re-lesions were performed after positive MEPs, since the animals were subjected to anterograde tracing studies after completed MEP sessions.

MAGNETIC RESONANCE IMAGING OF THE RAT SPINAL CORD (Paper II)

Five animals were scanned with magnetic resonance imaging (MRI) at one day or 10 weeks postoperatively. After anesthesia, MRI scans were performed using a Bruker® Bio-Spec Avance 47/40 spectrometer with a 4.7 T horizontal magnet with a 40 cm bore, used with a flat surface coil of 20 mm diameter. Images were acquired employing rare sequences (Hennig et al., 1986) with a field of view of 30 x 30 mm with an effective echo time (TE) of 48.62 ms and a repetition time (TR) of 2.5 s. A rare-factor of 8 was used with a rare maximum of 4. The slice thickness was 1 mm and 192 in phase code and 256 in read out. Number of excitations (NEX): 4.

TISSUE PROCESSING (Papers I, II and III)

Sections for immunohistochemistry (Papers I, II and III)

After full anesthesia, all animals were perfused with body warm isotone saline (37 °C) through intracardiac infusion, followed by the infusion of cold formaldehyde (4% w/v). The spinal cords including the lesion areas were removed by meticulous dissection. For tracing animals, the brains were also carefully removed (papers II and III). The spinal cord devices made of dental cement (paper I) were cut transversely in the middle and removed from the cord and its adherent transplanted nerves. The 12 transplants were then cut with micro-scissors and put in 1.5% glutaraldehyde / 4% formaldehyde for later semithin sectioning (0.5-1 μm , see below), leaving 12 nerve stumps on each (i.e., cranial and caudal) spinal cord surface. These cranial and caudal cord segments (including their adherent transplant nerve stumps) were fixated in formaldehyde for two hours, rinsed in phosphate buffer with 17% (w/v) sucrose overnight for cryoprotection, and longitudinally sectioned in a cryostat into 16 μm thick sections.

In paper II (where biodegradable CaSO_4 devices was used), a 12 mm segment of the spinal cord (with the injury zone placed in the middle) was cut transversely in 1 mm slices in a tissue matrix (Braintree Scientific, MA, USA) in half of the rats. Of these transverse slices, every second slice was put in glutaraldehyde / paraformaldehyde for later semithin sectioning. Every other transverse slice was postfixed in paraformaldehyde (4% w/v) for two hours, rinsed in phosphate buffer with 17% (w/v) sucrose overnight for subsequent cryostat sections (14 μm), and mounted on slides. In half of the animals, 60 μm longitudinal free floating cryostat sections were made of the harvested spinal cord segments after postfixation and cryoprotection.

In paper III, harvested spinal cords from injury control animals and animals repaired with a device containing 500 ng FGF1, were sectioned into 30 μm longitudinal cryostat sections. In the other experimental groups (0 ng, 0.07 ng and 7 ng FGF1) the repair area and adjacent spinal cord (cranial and caudal) was divided into 5 mm segments by transverse cutting (in the tissue matrix) for subsequent transverse cryostat sections (14 μm), and mounted on slides. The sections from the removed brain were 14 μm thick.

Semithin sections (Papers I and II)

After intracardiac perfusion with formaldehyde, peripheral nerve grafts collected from the channels of the dental cement device (paper I) as well as 1 mm transverse slices of the spinal cord including the degraded CaSO_4 device (paper II) were postfixed in formaldehyde (4%) with glutaraldehyde (1.5%) in phosphate buffer (0.15M, pH 7.4) overnight. They were then rinsed in phosphate buffer (20 min x 3) and immersed in osmium tetroxide (1%) for two hours, then rinsed in phosphate buffer, dehydrated in a graded series of ethanol to acetone and embedded in agar resin 100. Semithin sections (0.5-1 μm) were cut on an ultratome, stained with toluidine blue dye (Chroma-Gesellschaft GmbH & Co. d-48161 Munster, Germany) and mounted with Pertex® (Histolab products, Gothenburg, Sweden) and cover glass. The morphological characteristics of the tissue were analyzed with light microscopy (x100 magnification, oil immersion, Leica Microsystems DM 4000B, Wetzlar GmbH, Heidelberg, Germany).

IMMUNOHISTOCHEMISTRY (Papers I, II and III)

All cryostat sections (14 μm , 16 μm , 30 μm and 60 μm , papers I, II and III) were incubated in PBS with 1% bovine serum albumin (BSA, Sigma, St. Louis, USA), 0.3 % Triton-X-100 (Sigma, St. Louis, USA) and 0.1% sodium azide in phosphate buffer (0.15M, pH 7.4) for 1 hour at room temperature. The sections were incubated with primary antibodies at 4 °C overnight except the free flowing thick longitudinal sections (60 μm) that were incubated with anti-NF (1:200, see table) at 4 °C in PBS for three days to ensure good penetration. After incubation with primary antibody, the sections were rinsed and routine protocols for the avidin-biotin complex (ABC) technique and indirect immunofluorescence (IF) were used to visualize the immunoreactivity (Svensson and Aldskogius, 1993). For the ABC technique, sections were incubated with biotinylated secondary antibodies (Vectastain® ABC KIT, Vector, Burlingame, CA, USA) for one hour. The sections were then rinsed in Tris-HCl-buffer (IBI, Shelton, UK) and immunoreactivity was detected with diaminobenzidine (DAB KIT, Vector, Burlingame, CA, USA). Sections were rinsed in Tris-HCl buffer followed by dehydration in successively higher concentrations of ethanol to xylene and mounted in Pertex®

(Histolab AB, Gothenburg, Sweden) on slides (SuperFrost®, Menzel-Gläser, Braunschweig, Germany). Sections designated for immunofluorescence were incubated with secondary antibodies for one hour in room temperature, rinsed and mounted in glycerol or Mowiol® (Calbiochem, CA, US) for analysis with fluorescence microscopy (Leica Microsystems DM 4000B, Wetzlar GmbH, Heidelberg, Germany) or confocal microscopy (Leica Microsystem TCS SP2, Heidelberg, Germany and Carl Zeiss LSM 5 Exciter, Jena, Germany). An exception concerning secondary incubation times was made for the 60 µm longitudinal sections in study II which, to assure sufficient penetration were incubated with secondary antibodies (Alexa 488, 1:500, see table 2, paper III) overnight at room temperature. For primary and secondary antibodies/fluorocromes used, see tables 2 and 3.

Table 2. *Primary antibodies*

Antibody	Type	Species	Concentration	Source
Anti-Gap-43	Monoclonal	Mouse	1:100	Chemicon /Millipore
Anti-vGLUT1	Polyclonal	Guinea pig	1:1000	Chemicon /Millipore
Anti-5-HT	Polyclonal	Rabbit	1:5000	Sigma
Anti-VACht	Polyclonal	Rabbit	1:1000	Sigma
Anti-S100	Polyclonal	Rabbit	1:500	Dako
Anti-GalC	Monoclonal	Mouse	1:500	Chemicon /Millipore
Anti-TH	Monoclonal	Mouse	1:100	Chemicon /Millipore
Anti-CGRP	Polyclonal	Rabbit	1:500	Chemicon /Millipore
Anti-panNF	Monoclonal	Mouse	1:200	Zymed, Invitrogen
Anti-Synaptophysin	Polyclonal	Rabbit	1:100	Chemicon /Millipore
Anti-GFAP	polyclonal	Rabbit	1:1000	Dako

Table 3. *Secondary antibodies*

Flouorochrome	Directed against	Species	Concentration	Source
Alexa 488	Mouse IgG	Donkey	1:500	Invitrogen, Molecular Probes
Cy-3	Rabbit IgG	Donkey	1:1000	Jackson Laboratories
Cy-3	Guinea Pig IgG	Donkey	1:500	Jackson Laboratories

ANTEROGRADE TRACING (Papers II and III)

Seven weeks after primary surgery, animals subjected to anterograde tracing with biotinylated dextran amine (BDA) were anesthetized and the cerebral cortex exposed. A glass micropipette connected to a stereotactic frame and controlled with air pressure was used. In accordance with previous electrophysiological identification of the motor cortex (MEP trigger points), four shallow injections of 0.5 μ L 10% BDA (MW 10000; Molecular Probes, Leiden, The Netherlands) were made (1.2 mm deep) into the right and left motor cortex, labeling motor cortex neurons projecting toward the hind limbs. Three weeks later (10 weeks post grafting) the animals were terminated for histology. For visualization of the BDA tracer, transverse sections (14 μ m), longitudinal sections (30 μ m) and free-floating 60 μ m sections were incubated with avidine conjugated to Alexa 488 (1:100, Invitrogen, Molecular Probes, Eugene, Oregon, USA) for one hour or four hours (60 μ m free-floating sections) at room temperature. Omission of primary or secondary antibodies was used as negative controls. After incubation with secondary antibodies all sections were rinsed and mounted on glass with Mowiol®. Analysis of immunohistochemistry was made in the confocal microscope.

FUNCTIONAL EVALUATION OF HIND LIMB LOCOMOTION (Papers I and II)

Locomotor recovery was scored weekly by two observers using the Basso, Beattie and Bresnahan score (BBB) (Basso et al., 1995). The score ranges from 0 (flaccid paralysis of the hind limbs) to 21 (normal gait).

PATIENT SELECTION AND CLINICAL EXAMINATION (Paper IV)

The clinical study (paper IV) was approved by the local human ethics committee at the Karolinska University Hospital, and all applicable institutional and governmental regulations concerning the ethical use of human volunteers were followed. Written and informed consent was obtained from all subjects. Five male ASIA-A patients between 18-65 years with thoracic SCI from blunt trauma were enrolled one to four years after injury. Medical records were checked from injury to present to ensure that no transitions concerning ASIA classification had taken place. A neurological examination of each patient was performed according to the ASIA protocol (ASIA, 2011) by the same senior neurologist prior to each electrophysiological exam to determine the clinical injury level and the presence of spasticity.

MRI OF SCI PATIENTS (Paper IV)

All MRI examinations were performed according to the same protocol. A 1.5T MRI scan was performed using a whole-body MRI scanner (Philips Intera Master, Best, The Netherlands) with a 15-channel spine coil (Medical Advances, Wisconsin, USA). A three-plane localizer was obtained covering the spinal canal. The diagnostic imaging protocol was acquired in a sagittal plane using a customized 3D TSE T2 weighted isotropic voxel pulse sequence. The voxel size was 1.0 x 1.0 x 1.0 mm, TE 120 ms, TR 2 s, flip angle (FA) 90 degrees, echo train length 97 ms, profile order linear Y, field of view 300 mm, bandwidth 653 Hz, flow compensation sensitized, fold-over direction AP and number of signals averaged 1. Multiplanar reconstruction (MPR) was performed on a Philips workstation (Philips Easy Vision R3.5, Best, The Netherlands). Through MPR reconstruction individual 3D - differences between patients (i.e. scoliosis, see Fig. 4 a-e, paper IV) were compensated for when performing sagittal measurements). For exact assessment of the spinal cord dimensions on MR images, scanning of a saline immersed paraffin piston model with a known outer diameter was included in every examination. The length of the radiological discontinuity of the spinal cord was determined by cranio-caudal examination of the spinal cord in the transverse plane (axial T2-weighted images). The cranial marker was set where signs

of neural tissue and exiting spinal nerves disappeared and the caudal marker where these signs reappeared. The sagittal MRI images (MPR reconstructed) were scaled to the same relative size using Adobe Photoshop CS4. Rotation, brightness and contrast were also adjusted to facilitate comparison (Fig. 4 a-e, Paper IV).

CLINICAL ELECTROPHYSIOLOGY (Paper IV)

A senior neurophysiologist, blinded to the clinical examination and MRI results, performed all electrophysiological examinations. Two persons counted intercostal spaces, and validation of intercostal levels was obtained through a small indicator (a vitamin E capsule) between two ribs during MRI examination. Electromyography (EMG) recordings were obtained bilaterally from a bipolar needle inserted into the intercostal muscles at rest, during voluntary activation through head lift (assessment of the cranial border of the spinal cord lesion) and spastic activation of lower limbs (assessment of the caudal border of the spinal cord lesion). Care was taken to exclude the pectoralis major (PM) and serratus anterior muscles (SA). After insertion of registration needles (Fig. 1, Paper IV), specific activation of PM and SA was performed to evaluate their contribution in every specific needle position. Loss of activation was consistent with denervation due to anterior horn damage and loss of alpha motor neurons, root avulsion or spinal nerve injury. Electrophysiological results were translated into number of denervated segments. This was defined as the number of whole spinal segments showing complete denervation, i.e., bilateral absence of voluntary or spastically generated motor unit potentials (MUPs) combined with presence of fibrillations and/or positive sharp waves during EMG examination. When denervation was unilateral in one segment, or if one segment presented a mixed result (fibrillations and either spastic MUPs or voluntary MUPs), it was considered as 0.5 denervated segments. Sensation at needle insertion was also recorded and used to define sensory level.

STATISTICS

Statistical significance was calculated using Student's T-test, Fishers exact test, ANOVA, and the Pearson product-moment correlation coefficient using Statistica 10, Graph Pad Prism 5.0 and Microsoft Excel for Mac 2008 software.

RESULTS

The animals tolerated the surgical procedures and postoperative periods well in spite of various initial animal care problems. During the initial development of the methods, severe weight loss, autophagy and urinary tract infections were seen in several animals. Contractures due to paralysis of the hind limbs were seen throughout the studies, an unsatisfactory finding, specifically since it possibly hindered locomotor recovery. With shorter operation times (2 vs. 5 hours) and smaller surgical exposures, weight loss and autophagy became less of a problem. Urinary tract infections could effectively be reduced with prophylactic trimethoprim/sulfadoxine antibiotics or treated with quinolone antibiotics. To counteract contracture development, a recent addition in the animal cages of a wire mesh net to walk on has profoundly decreased the severity of contractions, with resultant clearer movement patterns and better locomotor scores (unpublished data).

MACROSCOPIC POST MORTEM FINDINGS AND MRI (Papers I, II and III)

Our graft holding devices were tolerated well and showed no signs of migration. The dental cement device was well in place six months after surgery and covered with scar tissue dorsally. After removing the device material, the transplanted nerve grafts were exposed,



Figure 6. Transplanted nerve grafts after removal of the graft holder.

appearing to be attached to the spinal cord surfaces (figure 6/Fig. 2D, paper I). The nerve transplants were macroscopically at their original positions (in accordance with the pre-operative mapping of ascending and descending tracts and intended guiding into grey matter positions), however assessment of the projection of each graft onto the exact subpopulation of neurons was not possible in the dissection microscope. The implanted biodegradable

calcium sulphate (CaSO_4) graft device (papers II and III) seemed to be well in place the first post-operative day on MRI, showing a high water content signal (Fig. 2b, paper II). MRI at

10 weeks postoperatively showed grafts running in the gap in the divided cord (Fig. 2c, paper II). The CaSO_4 devices were found to be completely degraded/dissolved in all animals at dissection 10 and 20 weeks post surgery (Fig. 2a and c, paper II). There were no signs of infection, spine deformities or other unexpected macroscopic findings (Fig. 2a, paper II).

ELECTROPHYSIOLOGY IN ANIMALS (Papers I, II and III)

Transcranial motor- and sensory evoked potentials at six months after repair (Paper I)

For an overview of electrophysiology results see Table 4 below. Clear and reproducible motor evoked potentials (MEP) were registered six months after surgical repair with implantation of the dental cement device carrying 12 peripheral nerve grafts (PNG) in four out of five repaired animals and in all sham animals, with varying amplitudes and latency times (Fig. 4, paper I). Latency times were 22.4 ± 5.1 ms (mean \pm SD) in repaired animals (Fig. 4b, paper I) compared with sham-operated animals, where latency times were significantly shorter (15.9 ± 3.1 ms, $p < 0.049$, student's t-test). In all control animals (resection without repair) and re-lesioned rats (transection at T8 level), MEPs were absent (Fig. 4a, paper I). In all the sham-operated animals, there were reproducible cortical sensory evoked potentials SEP when stimulating at the left and right tail base respectively with symmetric intra-individual latencies between 17 and 21 ms, however no SEPs could be recorded in repaired animals or injury controls (SCI only).

Transcortical motor evoked potentials at 20 weeks after repair (Paper II)

Twenty weeks after SCI repair a significant number of animals with CaSO_4 device + PNG showed positive MEPs in the hind limbs and all animals with an FGF1 loaded device + PNG showed positive bilateral MEPs (Fig. 4, paper II). Thresholds were higher and latency times longer compared to sham animals. MEPs were registered in the forelimbs (papers II and III) in all animals with a preserved lateralization, i.e., unilateral motor cortex stimulation resulted in a contralateral response in the forelimb. MEPs from the hind limbs generally also presented with a clear lateral preference, in some animals only in one leg (unilateral

response) and in other animals in both legs (bilateral response). In animals repaired with CaSO_4 device + PNGs only (no adjuvant FGF1), five out of eight animals presented MEPs in hamstrings bilaterally (Fig. 5, paper II), i.e., a significant improvement in comparison with control animals (SCI without repair, $p=0.031$, Fisher's exact test, $df = 1$). The mean stimulatory threshold was 12.2 ± 1.1 mA and mean latency time was 15.6 ± 2.6 ms. Two animals presented with a unilateral response and one animal failed to trigger potentials at all in the lower extremities. Twenty weeks after repair, all animals subjected to PNGs + FGF1 treatment ($n=8+8$) presented MEPs in hamstrings bilaterally independent of dose (500 ng or 5 μg) of FGF1, which was a significantly better result compared to negative controls (SCI only, $n=6$, $p=0.001$, Fisher's exact test, $df = 1$), but also better than the group repaired with PNGs without adjuvant FGF1 ($p=0.028$, Fisher's exact test, $df = 1$).

Stimulatory thresholds with PNG + 500 ng FGF1 and PNG + 5 μg FGF1 were 14.3 ± 0.75 mA and 12.3 ± 0.6 mA respectively and the mean latency time 12.3 ± 1.7 ms and 18.5 ± 2.5 ms. Reproducible cortical MEPs were recorded bilaterally in all four sham cases ($n=4$). Stimulation thresholds were 5.0 ± 0.2 mA (mean \pm SEM) and latency time 11.6 ± 1.1 ms (mean \pm SEM) (Fig. 4, paper II). Bilateral MEP responses could not be induced in any of the 6 investigated animals in the control group (SCI without grafting, Fig. 5, paper II). However, 2 of these animals showed a MEP response in one leg (stimulatory threshold 13.0 ± 3.0 mA, latency time 11.1 ± 0.75 ms). Animals that presented MEPs at assessment 20 weeks after surgery were re-lesioned three segments above the repair zone (T8), which in every case resulted in a complete loss of MEP responses in the hind limbs (Fig. 4, paper II), but preserved MEPs in both arms.

Transcortical motor evoked potentials at one, two and four weeks after repair (Paper III)

Cortical MEP assessment of repaired animals (PNGs + 0 ng, 0.07 ng, 7 ng or 500 ng FGF1) after one, two and four weeks after surgery (paper III) showed no MEPs in any group at one week after surgery (Fig. 1, paper III), some MEPs responses in all repair groups after two and four weeks and bilateral responses in all animals receiving PNGs and either 7 ng FGF1 or 500 ng FGF1, but none of the investigated control animals showed any sign of MEP recovery in the hind limbs after stimulation up to 30 mA at four weeks post

surgery (Fig. 1, paper III). Electrophysiology results presented as the ratios of number of legs with positive MEPs in one group of animals / number of examined legs in the group two weeks after surgery was 42% in animals repaired with (PNGs + 0 ng FGF1), 42% (PNGs + 0.07 ng FGF1), 75% (PNGs + 7 ng FGF1) and 73% (PNGs + 500 ng FGF1, Fig. 1, paper III). The corresponding numbers at four weeks were, 67% (PNGs + 0 ng FGF1), 58% (PNGs + 0.07 ng FGF1), 100% (PNGs + 7 ng FGF1) and 100% (PNGs + 500 ng FGF1) of examined legs showed positive MEPs (Fig. 1, paper III). The treatment groups with 7 ng or 500 ng FGF1 showed significantly better MEP results when compared with the 0.07 ng group ($p=0.0063$, Fisher's exact test) as well as the 0 ng group ($p=0.0107$, Fisher's exact test).

In more detail, two weeks after surgical repair two animals in the group receiving PNG grafts with 0 ng FGF1 showed bilateral MEPs and one additional animal showed unilateral MEPs. Stimulation thresholds were 12.0 ± 1.5 mA (mean \pm SEM) and latency times of 10.3 ± 1.2 ms (mean \pm SEM). In the group with 0.07 ng FGF1, two animals showed bilateral MEPs and one additional animal showed unilateral positive MEPs. Stimulatory thresholds were 11.1 ± 1.8 mA (mean \pm SEM) and latency times 11.0 ± 0.6 ms (mean \pm SEM). In the group with 7 ng FGF1, three animals showed bilateral MEPs and an additional three animals showed unilateral positive MEPs. Stimulatory thresholds were 9.2 ± 1.0 mA (mean \pm SEM) and latency times of 13.5 ± 3.5 ms (mean \pm SEM). In the group with 500 ng FGF1, two animals showed bilateral MEPs and an additional three animals showed unilateral positive MEPs. Stimulatory thresholds were 12.8 ± 2.0 mA (mean \pm SEM) and latency times 10.7 ± 0.5 ms (mean \pm SEM).

At four weeks after repair, in the group receiving PNGs + 0 ng FGF1, the same two animals (positive at week two) showed bilateral MEPs and an additional three animals in the same group showed unilateral MEPs with stimulation thresholds of 14.4 ± 1.3 mA (mean \pm SEM) and latency times of 11.2 ± 0.2 ms (mean \pm SEM). In the group with 0.07 ng FGF1, the same two animals showed bilateral MEPs after four weeks and an additional three animals showed unilateral MEPs. Stimulatory thresholds were 11.3 ± 0.8 mA (mean \pm SEM) and latency times 11.8 ± 0.9 ms (mean \pm SEM). In the group with 7 ng FGF1, all animals showed bilateral MEPs after four weeks, with a stimulation threshold of 12.0 ± 1.6 mA (mean \pm SEM) and latency time 12.1 ± 1.0 ms (mean \pm SEM). Finally, also in the group with 500 ng

FGF1, all animals showed bilateral MEPs after four weeks, with stimulation thresholds of 14.2 ± 2.1 mA (mean \pm SEM) and latency times of 11.9 ± 1.5 ms (mean \pm SEM).

Table 4. Overview of electrophysiology results

Paper	Treatment	Sham	SCI only	PNGs	0.07 ng FGF1*	7 ng FGF1*	500 ng FGF1*	5 μ g FGF1*
I	MEP latency	15,9 \pm 3,1 ms	-	22,4 \pm 5,1 ms				
	Main results	Clear and reproducible MEPs six months after surgical repair with implantation of dental cement device + 12 PNGs in four out of five repaired animals and in all sham animals.						
II	Stimulatory thresholds	5.0 \pm 0.2 mA	(13.0 \pm 3.0 mA)	12.2 \pm 1.1 mA			14.3 \pm 0.75 mA	12.3 \pm 0.6 mA
	MEP Latency	11.6 \pm 1.1 ms	(11.1 \pm 0.75 ms)	15.6 \pm 2.6 ms			12.3 \pm 1.7 ms	18.5 \pm 2.5 ms
	Main results	20 weeks after surgical repair with implantation CaSO4 device + 12 PNGs all animals with adjuvant FGF1 treatment (n=8+8) presented bilateral MEPs, significantly better than negative controls (p=0.001), but also better than treatment with PNGs but without FGF1.						
III	Stimulatory Thresholds		-	14.4 \pm 1.3 mA	11.3 \pm 0.8mA	12.0 \pm 1.6mA	14.2 \pm 2.1mA	
	MEP latency		-	11.2 \pm 0.2 ms	11.8 \pm 0.9 ms	12.1 \pm 1.0 ms	11.9 \pm 1.5 ms	
	Main results	Four weeks after surgical repair repair with implantation CaSO4 device + 12 PNGs, treatment groups with 7 ng or 500 ng FGF1 showed significantly better MEP results than 0.07 ng group (p=0.0063) as well as the group repaired with PNGs without FGF1 (p=0.0107). Some reappearance of MEPs were seen already after two weeks in all repair groups.						

*= +PNGs. Treatment groups with better results marked with a double lined frame.

MORPHOLOGY OF TRANSPLANTED PERIPHERAL NERVE GRAFTS

(Papers I and II)

High-resolution microscopy of semithin transverse sections showed that transplanted nerve grafts six months and 20 weeks after implantation in all animals were filled with numerous de novo axons, which had regenerated through the nerve grafts (Fig. 3, paper I, Fig. 6d-f, paper II). The axons were of varying size, varying degree of myelination and arranged in fascicles. The nerve grafts also contained blood vessels. No obvious differences were found among the graft positions with respect to axon or myelin presentation, since the whole transverse area was covered with myelinated axons.

IMMUNOHISTOCHEMISTRY (Papers I, II and III)

Neurofilament (NF) positive axons were found in the white as well as grey matter of all animals. In repaired animals in paper I (SCI + PNGs in dental cement devices), transplanted nerves adherent to the spinal cord surfaces contained numerous NF-positive axons. Analysis of the transition zone between the nerve grafts and the spinal cord showed that the nerve grafts with their axon bundles proceeded a short distance into the spinal cord (~0.5 mm), in line with the macroscopic adherence. At the ends of the nerve grafts, some NF-positive axons appeared to leave the nerve graft to proceed further into the spinal cord (Fig. 2E, F, paper I). There was no consistency as to where these fibers were found with respect to cranial or caudal ends of the lesion or to positioning of the nerve graft. In line with these findings, 20 weeks after repair with a biodegradable CaSO_4 device (paper II), all nerve grafts within the injury zone contained numerous NF-positive axons, most likely representing regenerating axons, either ascending or descending (Fig. 6b, paper II). Caudal to the graft area numerous NF labeled axons appeared in the grey matter (Fig. 6c, paper II). These axons had no clear organization and were projected in all directions in a turning and winding manner, resembling sprouting axons. Even though neurofilament staining cannot separate between sprouting ascending sensory axons from the transected caudal surface and descending fibers entering the caudal spinal cord, we suggest that some of these fibers represent regenerating axons in line with the tracing results showing corticospinal regeneration (see below). All repaired animals showed numerous NF-positive axons, however neither their presence nor pattern could be correlated to the dose of adjuvant FGF1.

Glial fibrillary acidic protein (GFAP) immunoreactivity (IR) demonstrated scattered astrocytes in all groups in the normal spinal cord or more than 1 mm away from any repair area in the various treatment groups (Fig. 7, paper II). Within the degraded repair area in treatment groups only a few astrocytes were found. At the cranial and caudal resection borders in spinal cord resected animals, there was an increased GFAP-IR regardless of repair strategy, with no obvious difference among the groups.

In order to evaluate the lesion area in detail, we performed a series of immunohistochemical stains on transverse and longitudinal sections of the spinal cord 10 weeks after repair

(paper III) using conventional markers for regeneration, i.e., markers for axons, growth cones and neurotransmitters. Animals from experimental groups with 0 ng, 0.07 ng, 7 ng, 500 ng FGF1 and negative controls were studied. At this time point, we could not detect a significant histological difference between the different groups receiving FGF1, however, it cannot be ruled out that there were differences at earlier stages.

Projection of regenerating axons through the lesion area and into distal spinal cord segments

The overall presence of regenerating axons was studied using an antibody directed against the regeneration-associated marker growth associated protein 43 (GAP-43), commonly associated with growth cones and regenerating axons (Skene and Virag, 1989). In cross sections cranial to the lesion (lower thoracic levels) the staining for GAP-43 was intense, especially in the dorsal horn, the dorso-ventral gray matter junction and around the central canal (not shown). In the lesion area, GAP-43 staining was observed in all nerve grafts, but the density and intensity of labeled fibers showed variation between individual grafts (Fig. 3A, paper III). Furthermore, all nerve grafts stained positive for NF, but the staining was more evenly distributed amongst the grafts compared to GAP-43 staining (Fig. 2A, left picture, paper III). In longitudinal sections, GAP-43 showed strong staining in nerve grafts just cranial to the lesion and throughout the entire distal portion of the spinal cord (not shown). In neighboring sections, NF was present throughout the cranial portion of the spinal cord with a peak in intensity just cranial to and within the lesion area (not shown). At high magnification at the lesion area, we observed numerous axons and growth cone-like structures displaying a “turning and winding” morphology (in line with results from paper II, Fig. 6), usually associated with outgrowing axons (not shown).

Schwann cells maintained within the nerve grafts

One reason for using peripheral nerve grafts in the biodegradable device was to provide a source of Schwann cells, which have previously been associated with enhanced regeneration of CNS axons (Oudega and Xu, 2006). Schwann cells introduced into the lesioned spinal cord are expected to associate with axons and promote axonal growth. Accordingly,

all grafts intensely stained for the Schwann cell marker S100 (Fig. 2C, left picture, paper III), thus indicating either that a large portion of the Schwann cells in the nerve grafts remained in situ, or indicating possible recruitment of endogenous Schwann cells into the spinal cord (Biernaskie, et al., 2007; Hill, et al., 2006). Oligodendrocytes on the other hand, have been shown to directly inhibit growth of regeneration CNS axons, hence constituting an obstacle for long distance regeneration (M. E. Schwab and Caroni, 1988). In the lesioned area and nerve grafts, little or no immunoreactivity for the oligodendrocyte marker galactosylceramidase (GalC) was observed (Fig. 2C, right picture, paper III).

Differential regeneration of spinal tracts

In the design of the biodegradable device we took the anatomical positioning of individual descending and ascending spinal cord tracts into consideration. Our aim was to provide optimal conditions for regeneration of the different tracts into anatomically relevant positions in the grey matter. We therefore characterized regeneration of individual tracts using immunohistochemistry for various neurotransmitters.

Tyrosine hydroxylase (TH) is an enzyme expressed by dopaminergic and adrenergic/noradrenergic neurons. In the spinal cord TH mainly labels descending noradrenergic fibers, such as locus ceruleus, and sympathetic preganglionic fibers. In distal thoracic cross sections, cranial to the repair area, scattered TH positive elements (including nerve terminals) were observed in the dorsal and ventral horns with the highest intensity in the lateral horn and around the central canal (not shown). In transverse sections of the lesion area, distinct immunoreactivity was detected in a subpopulation of axons, with certain preference for ventrally located grafts (Fig. 2B, paper III).

Calcitonin gene related peptide (CGRP) is a neuropeptide expressed by, among others, motor neurons and a subpopulation of dorsal root ganglion neurons. Accordingly, immunoreactivity for CGRP was seen in motor neuron somata and the dorsal horn of proximal spinal cord segments (not shown). In contrast to the TH staining, sections from the lesion area showed some preference for CGRP positive axons in dorsally located nerve grafts (Fig. 2B, right pictures, paper III). There were CGRP positive axons also in some ventral grafts,

but to a lesser extent. In longitudinal sections, CGRP positive axons heavily infiltrated the injury zone and distal spinal cord segments (not shown).

The vesicular glutamate transporter 1 (vGLUT1) marks axon terminals belonging to the lateral CST, spinal interneurons and primary afferents. In spinal cord cross sections cranial to the lesion, we observed a pattern similar to that of the uninjured spinal cord with dense innervation of the dorsal and ventral horn (not shown). However, the lesion area, the nerve grafts and the distal spinal cord segment contained little or no vGLUT1 immunoreactivity (Fig. 2B, paper III and data not shown).

5-hydroxytryptamine (5-HT, serotonin) was used to label descending bulbospinal tracts. In cranial sections the 5-HT positive nerve terminals were concentrated in the ventral horn, in particular around motor neurons, and in the region around the central canal (not shown). Staining was absent or sparsely detected in the nerve grafts and lesion area (Fig. 3C, paper III). In longitudinal sections, the 5-HT immunoreactivity was intense cranial to the lesion area, but with rather sparse infiltration into the lesion area and distal segments (not shown).

Vesicular acetylcholine transporter (VACht) was used to label motor neurons, cholinergic interneurons and parasympathetic neurons. Immunoreactivity was detected in motor neuron somata and in presynaptic terminals in the dorsal and ventral horns of spinal cord segments cranial to the lesion (not shown). Staining was absent or sparse in nerve grafts. Sparse or no VACht positive elements were detected in and distal to the injury zone (Fig. 3D, paper III).

ANTEROGRADE TRACING AND SYNAPTOPHYSIN (Papers II and III)

The results obtained with electrophysiology indicated long distance regeneration of descending spinal motor tracts. In order to characterize neuroanatomical correlates of such regeneration (possibility of corticospinally derived regeneration through the spinal cord device), presence of the anterograde tracer BDA in the spinal cord three weeks after injection into the motor cortex (ten weeks after repair) was investigated. Animals without grafting were used as controls. In both groups, numerous pyramidal cells and distinct

tracts of labeled fibers were detected in the cortex and deeper regions down toward the brain stem (Fig. 6g, paper II, Fig. 4A, paper III). Furthermore, we found columns with BDA positive fibers in the spinal cord, contralateral to the injection side, above the lesion area, representing uninjured parts of the corticospinal tract (Fig. 6h, paper II). Numerous BDA-containing fibers below the site of injury were found in all animals treated with grafting and FGF1 in the lower thoracic cord. In the spinal cord, BDA positive axon-like structures were observed at the anticipated location for the lateral CST dorsolaterally to the central canal (Fig. 4B and C, paper III). These fibers were detected more than 3 mm caudal to the injury indicating axonal elongation beyond the site of injury in the subjacent one or two cord segments (Fig. 6i, paper II).

BDA fibers were found in both central and peripheral parts of the caudal cord, which is in line with the findings of Tsai and co-workers, who repaired the spinal cord with obliquely projected nerve graft or spinal cord fusion and found BDA traced fibers in both grey and white matter (E. C. Tsai, et al., 2005). In the caudal spinal cord segment, BDA positive fibers could be detected in central regions in close association and with synaptophysin (Fig. 4 D-F, paper III) and GAP-43 positive elements (not shown). No BDA was detected in the spinal cord below the lesion in control animals (Fig. 6j, paper II).

FUNCTIONAL SCORING (Papers I and II)

All sham-operated animals presented with functional scoring of 21 points (normal gait), at all observations. In animals subjected to resection of the spinal cord in paper I, with or without repair, the BBB score was 0 during the first eight weeks and further vacillated between 0 and 1 during the remaining four months (data not shown). In paper II, all animals subjected to spinal cord resection scored 0 on the BBB scale in the first postoperative weeks (Fig. 3, paper II). From postoperative week 6-20, the repaired animals (CaSO₄-device + PNGs) presented with scores between 0 and 4, with significantly higher scores in repair groups in comparison to controls ($p=0.0001$, One Way ANOVA, Bonferroni's multiple comparison test). Controls without repair scored 0 the first three weeks and throughout the remaining test period 0-1. Study III focused on assessment of electrophysiology and histology. Since multiple other surgical procedures were performed and no major BBB

findings were expected during the short survival times, animals were not subjected to functional scoring.

CLINICAL NEUROLOGIC STATUS, ELECTROPHYSIOLOGY AND MRI (Paper IV)

Five male thoracic ASIA-A patients aged 32-50 years (median 35 years) were included, 1-4 years post injury (median 3 years). Three patients were injured in motorcycle accidents, one patient in a car accident and one in an accident with a hang-glider. All patients had presented as AIS-A in their first trauma assessment, and had remained ASIA-A throughout the clinical course. None of the patients had a clinical history of frequent autonomic dysreflexia. Clinical examination confirmed ASIA-A grades, and neurological levels were T3-T6 (median T5). Lower body spasticity was present in all patients.

Clinical electrophysiology

Three distinct patterns were recognized regarding thoracic spinal cord motor function (using EMG registrations from bipolar needles in the intercostal muscles): Normal, voluntarily activated motor unit potentials appeared above the clinical level of SCI (Fig. 2a, paper IV). In contrast, a varying number of intercostal spaces presented with spontaneous EMG activity with fibrillation potentials and positive sharp waves at (or close to) the level of sensory loss (Fig. 2b, paper IV). Below the level of injury there were normal MUPs (Fig. 2c, paper IV), generated in concert with spastic activation of lower limbs. EMG registrations and number of denervated segments are presented in figure 3, paper IV. Sensation of the needle insertion was used to determine the sensory level. The sensory level was consistent with motor injury level in two patients, one segment above voluntary motor level in two patients and one segment below motor level in one patient (Fig. 3, paper IV).

MRI of chronic thoracic SCI in patients

MRI was performed in all patients to give an anatomical overview of the individual injury and facilitate interpretation of results from neurophysiology. In spite of presence of titanium instrumentation in all patients due to previous internal spine stabilization, high quality

images could be obtained. The anatomical extent of the SCI varied. The discontinuation of normal spinal cord as seen on MRI was 13 - 60 mm, with a median found at 30 mm (Fig. 4, paper IV).

Comparing electrophysiology and MRI

The number of denervated segments was plotted against the length of the spinal cord discontinuity as judged by MRI (Fig. 5, paper IV). The Pearson product-moment correlation coefficient for number of denervated segments and length of lesion was $r = 0.97$ ($p < 0.01$), indicating a strong correlation between length of lesion and number of denervated segments.

DISCUSSION

Although several promising regeneration strategies have been presented in recent years (Bunge, 2008; Cadotte and Fehlings, 2010; Cote, et al., 2011; Kwon, et al., 2010; J. M. Schwab, Brechtel, et al., 2006) spinal cord injury (SCI) remains a major challenge. Most strategies (single or combinatorial) aim at modulating the spinal cord injury through, for example, the rescue of remaining functional cord (neurons, glia, axons), improving the regenerative capability of axons or creating a more permissive environment. Few options are being evaluated for the chronic and completely damaged spinal cord, except for the infrequently used procedure of surgical cordectomy in selected ASIA-A cases with syringomyelia and spasticity (Ewelt et al., 2010; Laxton and Perrin, 2006).

A NEW MODEL FOR SURGICAL REPAIR OF THE SPINAL CORD

Inherent capability of axons to grow into peripheral nerve grafts

Although the exact mechanisms are unknown, the capacity of axotomized neurons of the mammalian central nervous system (CNS) to regenerate into a peripheral nerve environment is no longer a controversy. Since the publication by Richardson et al. (Richardson, et al., 1980), in which CNS axons from the transected thoracic spinal cord were shown to grow into sciatic peripheral nerve grafts, several studies have confirmed the ability of peripheral nerves to promote axonal regeneration of CNS neurons (Cheng, et al., 1996; David and Aguayo, 1981, 1985; Houle, 1991). Although axonal growth from the CNS into a peripheral nerve graft proceeds rapidly, the axonal elongation will be hampered again at the PNG-CNS interface, if the graft is reinserted into the white matter of the spinal cord.

The rationale for our multichannel device was therefore based on Cheng and Olson's work in 1996, where a 5 mm completely resected spinal cord segment was replaced by 18 peripheral nerve grafts, directing descending motor tracts to the ventral horn on the caudal side of the lesion and ascending sensory tracts to the dorsal horn on the cranial side of the lesion (Cheng, et al., 1996). This repair model was intended to bypass the problem of growth inhibitory white matter, and the guidance of outgrowing axons to grey substance resulted in partial functional recovery of the hind limbs if the transplantations were com-

bined with adjuvant acidic fibroblast growth factor (FGF1). Later reports by other groups have supported the strategy (Y. S. Lee, Hsiao, et al., 2002; E. C. Tsai, et al., 2005). Nevertheless, the intricate microsurgical procedure - freehand positioning of multiple PNGs, one by one directed from white tracts to grey substance with their transverse ends onto the transversely cut few millimeter thick spinal cord - has been found difficult to repeat.

Microsurgical strategy and potential impact

To overcome the microsurgical variations expected by freehand positioning, we developed a nerve graft holder (device), which was evaluated in this thesis. When developing the device, we measured the size of the peripheral nerve grafts and planned their positioning according to a rat spinal cord map (Fig. 1B, paper I). We realized that a maximum of six nerve grafts could fit in on each side of the midline within the spinal cord device, and hence altogether 12 nerve grafts were transplanted. Six nerve graft routes were designed to guide the descending motor (corticospinal) tracts and six routes were designed for main ascending sensory tracts (Fig. 1B, paper I). Curved projections were obtained through a molding procedure in which wires with diameters equivalent to nerve grafts were used. After hardening of the mold, the wires were removed for later (perioperative) replacement by nerve grafts.

Practical considerations limited the selection of pathways. Nerve grafts were redirected to the closest grey matter area to enable redirection of as many transplants/pathways as possible, since the ideal connection targets within the grey matter are unknown, and possibly further away. Moreover, the exact functional importance of the various descending motor tracts is also not very well known. In the rat, there are some indications that the functional score of the hind limbs on the BBB scale (Basso, et al., 1995) is dependent on the intact rubrospinal tract (Loy et al., 2002; Schucht et al., 2002), whereas other studies indicate that the restitution of BBB scores after spinal cord regeneration may be related to the corticospinal tract (CST) (Y. S. Lee, et al., 2004; E. C. Tsai, et al., 2005). When testing a rat for grid-walk, results seem to be dependent primarily on corticospinal tracts, however in these experiments the dorsal funiculus was injured and hence sensory impairment might be the main reason for loss of grid walk abilities (Schucht, et al., 2002). Therefore,

the individual functional contribution of the various descending tracts in the rat seems to be complex. Maps of descending motor tracts in human reveal different locations compared to the rat (Watson, 2009). Because of the complexity in functional spinal cord regeneration, the ranking of desirable tracts to regenerate in the human is difficult to ascertain. However, it seems reasonable to believe that rubrospinal and corticospinal tract regeneration is important. Regardless of which tracts are chosen in the human, each device would have to be tailor-made since the coordinates of the tracts vary along the spinal cord. The post-mortem studies revealed no migration of the devices and the peripheral nerve grafts were at their original positions and incorporated in the original spinal cord (Fig. 2D, paper I), signifying a reproducible and accurate model for spinal cord repair with peripheral nerve grafts. Nevertheless, even with a pre-made device for PNG positioning, the procedure is challenging and care must be taken to enable high surgical precision.

ELECTROPHYSIOLOGY

In the current thesis, much of the regeneration evidence relies on electrophysiological evaluation. The animals were examined with motor evoked potential (MEPs) at various time points after different repair modules. The motor evoked potentials were generated from a depolarization of cortical neurons and following electric propagation along the axons. The response was picked up in the lower limb muscles by an inserted needle. The depolarization of muscle cells around the needle tip generated the electric potentials with a certain latency time and amplitude. The latency time reflected the number of synapses needed to cross for the propagation as well as the maturity (e.g., degree of axonal myelination) along the electric pathway. Latency times were significantly longer in repaired animals compared to sham animals after six months, which may reflect a more immature conduction or added synaptic contacts in the caudal spinal cord. The amplitude was dependent on the number of depolarized neurons (stimulation dependent) as well as the needle tip location in the muscle (registration dependent). Large variations in amplitudes are reported from MEPs examinations in the same subject (Wassermann et al., 2008), even with fixed stimulation and registration coordinates. In our studies, the coordinates of stimulation and registration among the animals were similar, but not identical. Therefore,

MEPs was reported present or absent (yes or no answer), without amplitude comparisons.

Transcranial and transcortical electrical stimulation

Two different stimulation procedures were used in the study. First we used transcranial stimulation, a method that is commonly used in the patient (Wassermann, et al., 2008), and secondly we used direct cortical stimulation where we stimulated directly on the cortex after a small craniectomy. The advantage of cortical stimulation was the lower current needed (decreasing overall artifacts), and moreover, the successfully stimulated cortex spot also served as an indicator of where the anterograde biotinylated dextrane amine tracer (BDA, used to track the corticospinal tracts) should subsequently be injected. However, both procedures generated unambiguous spinal cord specific propagation of potentials, since re-lesioning of the spinal cord made the MEPs disappear.

Contribution from corticospinal fibers

Positive MEPs had been generated from the same location in the motor cortex as the subsequent BDA injections were made, and the stimulation site also correlated well with standard neuroanatomical descriptions of the rat's brain cortical organization (Paxinos and Watson, 2007). Even though it therefore is likely that the CST fibers conduct some of the signals that give rise to the positive MEPs in this study, other reasonable MEP generating pathways are possible. Such pathways could be polysynaptic circuits through brainstem derived signals or propriospinal axons in the spinal cord traversing the injury zone in the PNG, as well as other long tract terminations in neuron pools in the caudal spinal cord before connecting to the secondary motor neurons targeting hind limb muscles. If outgrowing CST fibers would terminate directly onto alpha motor neurons of the hamstring muscles (in the rat innervated mainly by the L5 segment (Manzano and McComas, 1988), the regeneration distance from the cranial side of the injury into the caudal cord would have to be about 20 mm (post mortem examination of distance from cranial side of injury to L5 segment, data not shown). Interestingly, recurrent MEPs appeared already at two weeks after reconstruction with higher doses of FGF1 treatment (Paper III), which seems to be too early for an effect related to true regeneration of long tracts all the way to the

hamstring motor neuron pools/L5 segment. However, considering a regeneration pace of 1 mm/day, crossing the 3 mm gap in the spinal cord and entering the spinal cord should be possible in 14 days, even with an initial axonal die-back in the cranial cord. The explanation for this early electrophysiological effect may relate to a shorter regenerative process (as suggested above) with contact and synapse formation with more proximal neuronal circuits such as central pattern generators that possess preserved conductivity to more caudal segments of the spinal cord (see below).

Some animals were examined with somatosensory evoked potentials (SEPs) after the repair procedure. No cortical registrations could be observed in these animals. It may either be due to the absence of effective treatment in these animals, difficulties for the ascending sensory regeneration to make contact with higher centers or merely the fact that SEPs are more sensitive to surrounding disturbances and because of their smaller amplitudes more difficult to detect than MEPs. The most probable mechanism for potential cortical sensation after the repair procedure used in this thesis would be ascending regeneration in the peripheral nerve grafts and ingrowth into the grey matter in the cranial spinal cord, where synaptic contacts are made with already existing pathways to the cortex. An ascending regeneration of sensation in the grey matter up to brainstem locations would seem to be less likely. In the future, the use of functional MRI may add information regarding possible regeneration of ascending sensory tracts and cranial connections after spinal cord repair.

TRACING AND HISTOLOGY

Tracing of the dorsal corticospinal tract with biotinylated dextrane amine was robust and reproducible in the current study. After spinal cord repair the tracer was clearly present several millimeters into the caudal cord in a turning and winding pattern of axon-like structures that were also associated with synaptophysin. It can be concluded therefore that corticospinal axons are able to regenerate through peripheral nerve grafts to enter the distal spinal cord. These CST fibers are likely to contribute to the observed electrophysiological response, but alternative explanations may be possible (see electrophysiology section above). It is furthermore likely that the traced corticospinal axons are also involved in

locomotor recovery, as previous reports suggest a high correlation between corticospinal regeneration and locomotor recovery (Y. S. Lee, et al., 2004; E. C. Tsai, et al., 2005).

Regeneration of axons and selective guidance

The peripheral nerve grafts were filled with *de novo* regenerated and well-myelinated axons at several weeks after repair. These axons were also positive for neurofilament stains and could be followed through the grafts and into the spinal cord. The origins of these fibers could differ, producing either ascending or descending regeneration, but their presence confirms that peripheral nerve grafts serve as an excellent milieu for spinal cord tract regeneration. Other strategies to enable spinal cord tracts to regenerate across a spinal cord gap or cavity have focused on creating a growth permissive environment (Bunge, 2008; Keirstead et al., 2005a; Li et al., 2009; Novikov et al., 2002; Oudega et al., 2005), thereby achieving regeneration into the spinal cord. However, without the benefit of specific guidance these strategies rely on random growth across the lesion.

This raised the question whether random growth is as useful as specific guidance through peripheral nerve grafts. Further, it was unclear whether selective peripheral nerve graft transplantations actually constitute a guidance of selective tracts or rather of a number of peripheral nerves with their transverse endings positioned against spinal cord surface merely serving as a general growth substrate for all tracts, and traces of various tracts therefore would be found in several nerve grafts. The immunohistochemistry from the transverse sections of the repair area including the peripheral nerve grafts showed that nerve grafts were filled with axons of different origins. For example, some nerve grafts stained for thyroxin hydroxylase and others stained for calcitonin gene related peptide, but the substances could not be detected in the same grafts. It therefore seems reasonable to believe that the surgical placement of a peripheral nerve graft ending determines which tract will regenerate through it. Thus, it may be possible to guide specific functions by careful positioning of a nerve graft, in line with a recent study describing selective re-innervation of the diaphragm after peripheral nerve grafting (Alilain, et al., 2011).

Central pattern generators

The repair strategy used in the current studies will likely allow a fraction of axons to regenerate through the SCI area and into the other side of the spinal cord. If the descending and ascending tracts could overcome the inhibitory white matter and regenerate straight (resembling the original neuroanatomy), the spinal cord tracts would have to grow far on the other side of the lesion. Moreover, specific signals for the axons to leave the white matter somewhere along the regeneration and enter to the neuron pools in the grey matter would be needed. In the current studies, we hypothesized that the regeneration of white matter tracts across the spinal cord must reach into the other side of the lesion and connect to neuron pools in the grey matter for the re-establishment of cortical control of already existing central pattern generators (CPGs), which are believed to be responsible for coordinated locomotor function (Alstermark, et al., 1987; Bradbury and McMahon, 2006; Raineteau and Schwab, 2001).

However, the improvement in functional recovery following various repair strategies such as inhibition of axon repelling factors, utilization of regeneration promoting factors or transplantation of cells may in incomplete contusion models be explained to some extent by local modulation within CPGs or sprouting of intact axons - even if there is evidence of cortically induced movements (Alstermark, et al., 1987; Bareyre et al., 2004; McKenna and Whishaw, 1999; Raineteau and Schwab, 2001; Weidner et al., 2001; Z'Graggen et al., 2000; Z'Graggen et al., 1998). Rarely, there is evidence of cortex-controlled movements. There is also good evidence that movements below a complete SCI may be achieved by physiotherapy alone, explained by modulation of local reflex patterns and pain transmission (Behrman and Harkema, 2000; Fouad and Pearson, 2004). Furthermore, clinical experiments have shown that physiotherapy combined with epidural stimulation below the injury can elicit voluntary movements in chronic paraplegia (Edgerton and Harkema, 2011; Harkema, et al., 2011), attesting to the importance of local modulation below the injury.

Our studies use peripheral nerve grafts to bridge a spinal cord injury, a strategy that previously has resulted in an improvement in functional recovery if combined with local application of acidic fibroblast growth factor, but not without (Cheng, et al., 1996; Y. S. Lee, Hsiao, et al., 2002). Furthermore, our findings show that bridging a SCI with nerve grafts

will result in cortex to hind limb electrophysiologic contact, which indicates that FGF1 either modulates central pattern generators in the caudal spinal cord or influences the regeneration from descending tracts, perhaps at the transitional zone of nerve grafts entering the spinal cord. Our findings support the view that axonal regeneration in the CNS can allow a modest recovery of function after complete spinal cord injury. Whether these regenerating long tracts improve functional recovery directly or through the stimulation of local circuits needs to be studied further.

Locomotor recovery

The most common way to measure recovery of locomotor function after spinal cord injury in the rat is the use of the BBB (Basso, Beattie and Bresnahan) scale (Basso, et al., 1995). Schucht and co-workers present a number of rats in which selective injuries of the spinal cord were made and correlated to the BBB-score. The data shows a strong correlation between an anatomically intact rubrospinal tract and maintained BBB, whereas destruction of the dorsal corticospinal tracts did not reduce the BBB-score (Schucht, et al., 2002). Lee et al. applied a Fluoro-Gold™ capsule to the re-transected caudal spinal cord months after repair with peripheral nerve grafts. The number of fluoro-gold positive neurons in the motor cortex had a strong correlation to the BBB recovery of the animals (Y. S. Lee, et al., 2004). Thus, it seems as if the rubrospinal tract may be important for BBB scores in the normal rat, whereas the dorsal corticospinal tract may be important in the recovery of BBB after spinal cord repair. In the current study, we demonstrate clear dorsal corticospinal tract regeneration by tracing studies. It seems likely that these fibers contribute to the recovery of BBB observed.

All grafted animals presented (paper II) with BBB scores between 0 and 4 from the 6th postoperative week. This was significantly better than injured animals without repair suggesting that a limited functional regeneration had occurred. The reported BBB scores in treated animals are somewhat lower than BBB scores reported by Tsai and coworkers (BBB of 4-5) (E. C. Tsai, et al., 2005) or Lee et al (BBB around 7) (Y. S. Lee, Hsiao, et al., 2002; Y. S. Lee, et al., 2004) after spinal cord injury repair with PNGs and FGF1. However, it is difficult to grade repair strategies comparing treatments groups in separate papers, due to

possible differences in, for example, the origin of rats, and the care or rehabilitation milieu (Garrison et al., 2011; Y. S. Lee, et al., 2010). Functional recovery after CNS injury is also reported to differ depending on rat strain (Reid et al., 2010). The current groups of rats suffered from severe contractures and fixed joints, and a possibly emerging BBB increase could have been disguised. They were not treated with physiotherapy.

There is also a controversy whether improvements in BBB scores reflect functional locomotion due to long tract regeneration or to reflex-like movements (Privat et al., 2000). Improved locomotor ability can occur in an animal with a completely transected spinal cord without supraspinal input, provided that the animal undergoes treadmill training (Lovely et al., 1986, 1990; Thota et al., 2001). However, without such training and without intervention designed to stimulate regeneration, functional improvement is minimal. Thus, the improvement in locomotion of treated rats (without treadmill training) compared with controls in this study indicates that the positive effect is associated with long tract regeneration. Moreover, the complete loss of recovered function and MEPs caudal to the original transection induced by bi-polar stimulation of the motor cortex after re-transection at level T8 provides further evidence that the mechanism of locomotor recovery after repair results from regeneration of long tracts.

It seems reasonable to suggest that either regeneration of the spinal cord alone or rehabilitation alone are not sufficient for a functional recovery. Therefore, the treatment for a chronic and complete spinal cord injury should focus on both a regenerative intervention as well as intensive rehabilitation to train and stimulate newly formed neuronal circuits. The current study demonstrates a specific regeneration therapy for spinal cord tracts, demonstrated by tracing studies and electrophysiology. The functional scores (BBB) in this study were somewhat lower than in other studies, perhaps due to a less than satisfactory rehabilitation setting. It is probable that interventions such as postoperative placement of animals in cages with a metal wire mesh on the bottom, reported to act as physiotherapy and to prevent flexion contractures in peripheral hind limb lesions (Ramsey et al., 2010; Strasberg et al., 1996), could prevent contracture development and improve locomotor recovery.

ACIDIC FIBROBLAST GROWTH FACTOR

FGF1 is a growth factor that has been shown to improve regeneration in the CNS (Guest, et al., 1997; Pataky, et al., 2000) as well as improve neuronal survival (Y. S. Lee, Baratta, et al., 2002). According to previous literature, the effect of FGF1 may be attributed to neuro-protection, improved regeneration or local modulation of the spinal cord injury milieu (Giacobini, et al., 1991; Guest, et al., 1997; Kuo, et al., 2011; M. J. Lee, et al., 2008; M. J. Lee, et al., 2011; M. C. Tsai, et al., 2008). FGF1 occurs naturally in the spinal cord (Koshinaga et al., 1993) and is reported to be important for the development of the central nervous system (Dono, 2003).

Cheng, and later Lee, reported that a precondition for recovery was local administration of adjuvant FGF1 (Cheng, et al., 1996; Y. S. Lee, Hsiao, et al., 2002), which was also used by Tsai and Tator (E. C. Tsai, et al., 2005). FGF1 indeed improved electrophysiology results in our studies (Papers II and III), however made no behavioral difference (Paper II), and it is interesting that PNG transplantations without FGF1 also led to substantial regeneration (Papers I, II, III). Explanations for the FGF1 independent response in our series could reflect different guidance, more precise guidance and more stable attachment of grafts with our surgical method, or merely different strains of rats. Further, the animals suffered from fixed joints and severe contractures which may have disguised otherwise emerging movements and separation of treatment groups. The higher FGF1 doses (Papers II and III) were chosen to assure an FGF1 release similar to or above the FGF1 dose employed by Cheng and co-workers.

COMBINATION OF TREATMENTS

There is much data to confirm that PNGs are a successful substrate for regeneration stimulation of the peripheral or central nervous system (Alilain, et al., 2011; Cheng, et al., 1996; Cote, et al., 2011; Richardson, et al., 1980). Therefore we evaluated spinal cord bridging repair strategies based on PNGs in a biodegradable graft holder in combination with different doses of FGF1 (i.e., there were no groups with empty channels), and the beneficial effect of FGF1 addition to the graft holder could only be attributed to PNG/FGF1 in combi-

nation and hence we could not separate between the individual contributions from PNGs and FGF1. In the future, it would be interesting to investigate the impact of the individual FGF1 and PNGs components (e.g. also with collagen tubes or cultured Schwann cells) in spinal cord repair with the multi channel device.

Further combinations

The pathophysiology of SCI is complex and it is likely that an effective future clinical treatment will require combinations of several strategies such as neuroprotection, growth factors and microsurgical methods to achieve a platform for axonal regrowth. Furthermore, there is promise that bioengineering technology utilizing cell therapy strategies, including Schwann cells, olfactory ensheathing glial cells, stem/progenitor cells, induced pluripotent stem cells (iPS) or transplantation of peripheral nerves at the site of injury, can promote regeneration of the injured spinal cord (Cote, et al., 2011; Salewski et al., 2010; Tetzlaff et al., 2011). Our data demonstrate that the presence of positive MEPs alone is insufficient to ensure a locomotor recovery and further support the literature suggesting that future repair strategies will consist of both a surgical and pharmacological approach, as well as physiotherapy. An overall combinatorial approach to treat SCI may eventually lead to effective treatment protocols in humans (Bradbury and McMahon, 2006; Bunge, 2008; Kwon, et al., 2010).

THE MATERIAL PROPERTIES OF THE PNG HOLDER (Papers I, II and III)

Although dental cement is strong and easy to use, it lacks important properties such as porosity and biodegradability to avoid potential long-term reactions against materials implanted into the CNS. We therefore looked for a material that permitted a complex injection molding process (Papers II and III), sufficient mechanical strength for handling during surgery, biocompatibility, biodegradability and an ability to permit loading and controlled release of an active substance, in this case FGF1. Injectable ceramics, traditionally used in orthopedic applications, are biocompatible, resorbable, and are also naturally porous in their structure (Dorozhkin, 2010; Thomas and Puleo, 2009). Calcium sulfate cement (CSC), also known as plaster of Paris, has a long history of use in medical devices. Its biocompat-

ibility in vivo of CSC has been shown in various clinical applications (Bajada et al., 2007; Clayer, 2008; Thomas and Puleo, 2009; Yu et al., 2009). The porosity and resorption rate (35-40 days in vitro), makes CaSO_4 cement an excellent drug carrier (Aberg, et al., 2012). Several studies report the use of CSC for local delivery of antibiotics (Mousset et al., 1995; Nelson et al., 2002; Richelsoph et al., 2007) and other drugs such as growth factors (Mamidwar et al., 2008; Thomas and Puleo, 2009) as it provokes very little immunological response. The mechanical properties, dissolution rate, FGF1 absorption and slow release capacity of the calcium sulfate device in vitro (Aberg, et al., 2012) made it a good candidate for in vivo experiments.

Post-mortem findings demonstrated that the device was well degraded and integrated with the spinal cord at ten and twenty weeks after repair and no apparent reaction against the material was observed. Nevertheless, the micromilieu in a spinal cord contusion area partly consists of hemorrhage and various axonal repellents, and future experiments might accordingly benefit from a cocktail of agents to neutralize the inhibiting factors. Therefore, a future similar set up with biodegradable CaSO_4 carrying other adjuvant pharmacological agents alone, or in combination with FGF1 (Aberg, et al., 2012), could be even more effective. Moreover, various degradable or non-degradable biomaterials have been tested as guidance channels or delivery systems for cellular and non-cellular neuroprotective or neuroregenerative agents in experimental SCI (Li, et al., 2009; Moore et al., 2006; Novikov, et al., 2002; Pritchard et al., 2010; Wang et al., 2011) and future devices made according to the same principles, but with other materials might be even better carriers of adjuvant treatments.

THE DEFINITION OF UPPER AND LOWER BORDERS IN COMPLETE AND CHRONIC SCI

In any experimental clinical repair procedure for SCI, avoidance of significant neurologic deterioration must be the highest priority. Accordingly, patients with complete lesions (ASIA-A) in the thoracic spinal cord should be the preferred patient group for clinical trials since additional loss of a thoracic segment or partial segment is less likely to cause major (motor) harm or affect ADLs (Fawcett, et al., 2007; Lammertse, et al., 2007; Steeves, et

al., 2007; Tuszynski, et al., 2007). A safe method of diagnosing thoracic spinal cord injury completeness as well as level is needed both for patient selection (definitive preoperative diagnosis) and for postoperative evaluation. Potential postoperative improvement in motor function and circuitry, even with an improvement of just a few spinal cord segments (leading to no obvious clinical motor improvement) would certainly be of great interest. The results from our clinical study suggest that a thorough electrophysiological examination may provide a detailed map of the cranial and caudal margins of a thoracic SCI. After activation of the motor neurons in the ventral horn, EMG registrations from corresponding myotomes reflect the viability of the spinal cord. Cranial to the injury, motor neurons were activated voluntarily, and caudal to the injury the motor neurons were activated by induced spasticity in the lower extremities, a method that to our knowledge has not previously been described. Further, we found an MRI sequence for spinal cord imaging which delineates the injury zone with a high correlation ($r=0.97$) to the functional evaluation.

The patients presented with a permanent denervation pattern in the injury zone with positive sharp waves and fibrillation potentials, without signs of re-innervation. This pattern likely arose from spontaneous firing from up-regulated acetyl choline receptors a few weeks after depressed neural input, which remains until re-innervation occurs (Brown et al., 2002). The denervation pattern was interpreted as an indicator of permanently devitalized motor neurons in the corresponding ventral horn (confirming chronically injured spinal cord tissue), although a component of simultaneous peripheral nerve injury cannot be ruled out. It should be mentioned that thoracic motor neuron pools in incomplete SCI could be evaluated in the same way as in ASIA-A SCI. The method will not however discriminate between different ASIA-classes since it does not target long fiber tracts crossing the injury zone. The electrophysiology study in paper IV was performed to demarcate upper and lower motor levels in thoracic SCI. For a complete evaluation of SCI from a neurophysiological point of view, other assessments evaluating sensory or autonomic functions need to be performed (Ellaway et al., 2004).

No adverse events were seen in any of the 5 patients. Positioning needles into the intercostal muscles understandably involve the risk of causing an iatrogenic pneumothorax and has been reported in a few cases of cervical root stimulation (Hawley, 2000). It is impor-

tant to avoid too deep an insertion and therefore intercostal needle-EMG should be performed by an experienced neurophysiologist. Ultrasound guidance could also be used to avoid erroneous needle insertion.

SURGICAL TRANSECTION AND CLINICAL REPAIR OF SCI

Following a spinal cord contusion there may be nerve fibers which are axotomized, neuronal death as well as intact demyelinated axons (M. E. Schwab and Bartholdi, 1996).

There is convincing data that in descending motor tracts, only a very small (but functionally important) fraction of intact axons are needed (about 5%) to transmit cortical signals to the lower part of the spinal cord in order to execute good motions in the hind limbs (Bregman, et al., 1995). Since all nerve grafts in the current study contained well myelinated axons, it is unclear which of the tracts provided the positive MEPs. In the future, eleven of the twelve tracts may be plugged to assess the impact of each individual tract. Moreover, in other models to repair the injured spinal cord, axons have been traced across the spinal cord injury site and the results suggest that only a small number of axons are needed across the SCI to induce some cortically controlled movements (Bradbury, et al., 2002; Bregman, et al., 1995; GrandPre, et al., 2002). Thus, in patients and animals subjected to a chronic SCI resulting in permanent paraplegia, we speculate that no or almost no intact axons remain to connect the cortex with the caudal part of the spinal cord.

At the same time, we cannot rule out that the contused area following a SCI may be too hostile and repellant for nerve regeneration and perhaps will benefit from excision, in the patients with no or almost no remaining axons, i.e., with chronic paraplegia or tetraplegia (see above). The regeneration in the spinal cord may in these cases benefit from being directed toward desired neuron pools by replacing injured spinal cord matter and debris with peripheral nerve grafts. Consequently, if the method to repair the completely damaged spinal cord by resection of the damaged part and insertion of a peripheral nerve graft holder reaches the patient, there will be a high demand on the diagnosis of complete injury as well as the upper and lower limit of the damaged zone. A theory of how spinal cord could be facilitated in patients is provided in figure 7.

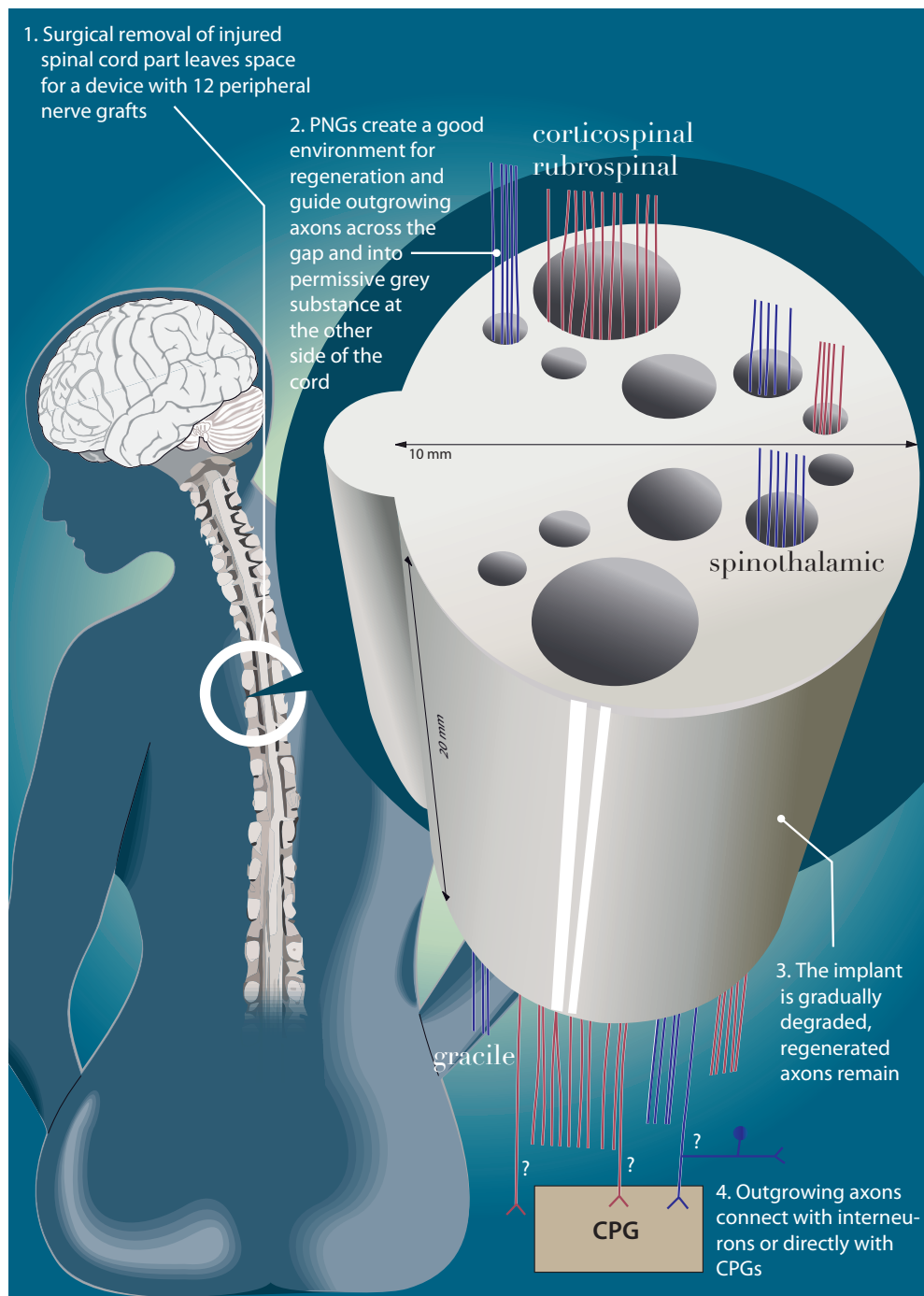


Figure 7. Theory of how spinal cord regeneration could be facilitated in patients; however, many questions remain unresolved, for example how outgrowing axons interact with central pattern generators, and how this interaction could be augmented. With permission from Jonas Askergren, Ny Teknik.

CONCLUSIONS

1. Using the new spinal cord device, peripheral nerve grafts may be precisely positioned at desired locations at a transected spinal cord surface.
2. Acidic fibroblast growth factor released from a biodegradable device after spinal cord injury and repair with peripheral nerve grafts shortens the time to reappearance of motor evoked potentials.
3. Axons of the corticospinal tract regenerate through peripheral nerve grafts and into the caudal spinal cord in the current repair model.
4. Different axons enter the peripheral nerve grafts depending on positioning, which may increase the incentive for precise placement of nerve grafts in an attempt to selectively guide longitudinal tracts.
5. Assessment with intercostal electromyogram and MRI can demarcate the cranial and caudal lesion borders in patients with chronic and complete thoracic spinal cord injury.

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