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On CNS Injury
and Olfactory Ensheathing Cell
Engraftment Strategies

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Stockholm, 2005

Cover: Striking differences of amounts of tyrosine hydroxylase-positive fibers (grey gradient) in the denervated striatum between different engraftment strategies. Co-grafts of olfactory ensheathing cells and fetal ventral mesencephalic tissue (right) resulted in more extensive dopaminergic reinnervation than co-grafts with astrocytes or ventral mesencephalic tissue lacking co-grafts (left), for details see paper V. Pictures by Saga Johansson. Pseudo pink colour was added in the right picture.

Back cover: Following spinal cord injury, the number of connexin 43 immunoreactive puncta (red) are markedly increased, especially in grey matter adjacent to the lesion epicenter. GFAP-positive astrocytic processes are shown in green and OX42-positive microglia in blue (details see paper I). The picture was selected as cover illustration for Journal of Comparative Neurology 489 (1).

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Dedicated to Joey, Lawrence and my parents

ABSTRACT

The intrinsic regenerative capacity of the adult mammalian central nervous system (CNS) is limited because of lack of nerve-growth stimulatory factors and presence of an insurmountable molecular environment for injured axons. This thesis aims to study neural regeneration following adult CNS injury, using rodent models of spinal cord injury, dorsal root avulsion, and Parkinson's disease. A focus is cell therapy with olfactory ensheathing cells (OEC), a unique type of glial cells assisting regeneration of olfactory axons from the periphery into the otherwise non-permissive CNS throughout adulthood.

To elucidate roles of intercellular communication via gap junctions after spinal cord injury, we investigated the expression patterns of principle gap junctional genes and proteins, connexin 43 (Cx43), Cx36, and Cx32. After a transection of the adult spinal cord, the levels of Cx43 mRNA and protein were upregulated within hours and lasted over 4 weeks post-injury primarily in astrocytes. In contrast, Cx36 and Cx32 mRNA and proteins were relatively sparse and mainly unchanged after spinal cord injury along the entire axis of the spinal cord. We suggest that long-term up-regulation of Cx43 may be one critical component in the rearrangement of the local astroglial network following spinal cord injury.

A growing number of repair strategies have shown neuroprotection/regeneration potential in CNS injury, including the use of various cell transplants, neurotrophic factors, blockade of inhibitory constituents, and stimulation of intrinsic growth capacity. Nevertheless, the degree of functional recovery is generally modest when individual approaches have been applied. Combinatorial treatments targeting multiple independent mechanisms may have additive effects. We evaluated the effects of OEC transplants alone and in combination with a cocktail treatment including acidic fibroblast growth factor (aFGF), chondroitinase ABC, and rolipram after spinal cord injury. Improvements of locomotor and sensory recovery were observed in behavioral tests and functional magnetic resonance imaging (MRI) in OEC-transplanted, cocktail-treated, and combination-treated rats. However, there was no robust evidence that the combination of OEC and the cocktail led to additive effects.

A lack of specific markers of OEC has hindered studies assessing survival and function of OEC following transplantation. We examined the usefulness of superparamagnetic iron oxide nanoparticles (SPION) as a cell label to allow in vivo tracking of grafted OEC by MRI. We found that labeled OEC were readily detectable in vivo for at least 2 months with exten¬sive migration in normal spinal cord as shown by MRI and histological markers. However, OEC showed limited migration in injured spinal cord and were not able to cross a glial scar region. In a similar experiment, we tracked grafted neural stem cells labeled by gold-coated SPION in the rat spinal cord. Gold-coated SPION exert powerful contrast-enhancing properties making it possible to detect cellular clusters of as few as about 20 cells.

In the model of dorsal root avulsion, we investigated whether peripheral nerve grafts combined with aFGF could improve reconnection of transected dorsal roots with the spinal cord as evaluated by somatosensory evoked potentials (SEPs). Four to twenty weeks after rhizotomy, most rats receiving such treatment had recovery of SEPs while none of the controls showed such recovery. The reappearing SEPs were eliminated by re-transection of the repaired roots, verifying their source as the regenerated roots.

In the unilateral 6OHDA denervation model of Parkinson's disease fetal ventral mesencephalic tissue (VM) grafts can partly restore dopamine innervation and counteract functional deficits, but the approach is hampered by limited graft survival and restricted dopaminergic reinnervation of striatum. We co-grafted VM with OEC to examine if OEC might promote survival and/or growth of grafted VM. The presence of OEC co-grafts improved dopamine cell survival, striatal reinnervation and functional recovery compared to VM graft alone, and caused a strikingly long-distance growth of graft-derived dopamine nerve fibers. Comparable results were observed in a co-culture system where OEC promoted dopamine cell survival and neurite elongation through mechanisms involving both releasable factors and direct cell contacts.

LIST OF PUBLICATIONS

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

I.   Lee I.H., Lindqvist E., Kiehn O., Widenfalk J., Olson L. Glial and neuronal connexin expression patterns in the rat spinal cord during development and following injury. Journal of Comparative Neurology, 2005, 489, 1-10.

II.   Lee L.M., Huang M.C., Chuang T.Y., Lee L.S., Cheng H. Lee I.H. Acidic FGF enhances functional regeneration of adult dorsal roots. Life Sciences, 2004, 74, 1937-1943.

III. Lee I.H., Bulte J.W.M., Schweinhardt P., Douglas T., Trifunovski A., Hofstetter C., Olson L., Spenger C. In vivo magnetic resonance tracking of olfactory ensheathing glia grafted into the rat spinal cord. Experimental Neurology, 2004, 187, 509-516.

IV.   Wang F.H., Lee I.H., Holmström N., Yoshitake T., Kim D.K., Muhammed M., Frisén J., Olson L., Spenger C., Kehr J. Magnetic resonance tracking of nanoparticle-labelled neural stem cells in the rat spinal cord. Manuscript.

V.   Johansson S., Lee I.H., Olson L., Spenger C. Olfactory ensheathing glial co-grafts improve functional recovery in 6-OHDA lesioned rats. Brain, 2005, 128 (12), in press.

VI.   Lee I.H., Endo T., Nissen J., Dominguez C., Lilja J., Xu X., Cao Y.H., Wiesenfeld-Hallin Z., Spenger C., Olson L. Olfactory ensheathing glia alone or combined with aFGF, chondroitinase and rolipram promote functional recovery after severe spinal cord contusion. Manuscript.

List of Abbreviations

6OHDA	6-hydroxydopamine
aFGF	acidic fibroblast growth factor
Au	gold
BOLD	blood oxygenation level-dependent
cAMP	cyclic adenosine monophosphate
CSPG	chondroitin sulphate proteoglycans
CNS	central nervous system
CX	connexin
DMEM	Dulbecco's Modified Eagle's medium
EPI	echo-planar imaging
GFP	green fluorescent protein
MION	monocrystalline iron oxide nanoparticles
MRI	magnetic resonance imaging
NSC	neural stem cells
OEC	olfactory ensheathing cells
PNS	peripheral nervous system
RARE	rapid acquisition with relaxation enhancement
SEPs	somatosensory evoked potentials
SPION	superparamagnetic iron-oxide nanoparticles
TH	tyrosine hydroxylase
VM	ventral mesencephalic tissue

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INTRODUCTION

Role of gap junctions in the central nervous system

Properties of gap junctions and connexins

Gap junctions are intercellular channels that directly connect cytoplasm of neighboring cells. They allow electrical as well as biochemical (ions and small molecules < 1 kDa) communication between adjacent cells (Simon and Goodenough, 1998). Gap junctions can mediate synchronous behavior of coupled cells and have a critical role in development, morphogenesis, and pattern formation (Kumar and Gilula, 1996).

Connexin expression in CNS

In the central nervous system (CNS), astrocytes are connected by a rich presence of gap junctions. The ubiquity of astrocytic gap junctions allows astrocytes to form a functional syncytium that permeates CNS parenchyma and links various regions in terms of flow of current, ions, and small metabolites (Mugnaini, 1986). The system acts as a K^+ sink following intense neuronal activity (Holthoff and Witte, 2000) and allows propagation of Ca^{2+} waves in response to activation (Cornell-Bell and Finkbeiner, 1991; Charles et al., 1992; Venance et al., 1997).

Neuronal gap junctions, or electrical synapses, allow functional coupling of a variety of neurons generating synchronous activity. The most studied electrical synapses occur among excitatory projection neurons of the inferior olivary nucleus, and among inhibitory interneurons of neocortex, hippocampus, and thalamus (Connors and Long, 2004). The properties of electrical synapses are strikingly different from those of chemical synapses (Bennett, 1977, 1997). For instance, the transmission of nerve impulses is bidirectional and fast without delay at the synaptic cleft, but less modifiable than chemical synapses. In the neonatal spinal cord, synchronization of spinal motor neuron oscillations gives rise to a stable locomotor rhythm (Tresch and Kiehn, 2000), and the number of these electrical synapses declines steeply during early development (Chang et al., 1999). The progressive loss of gap junctions among developing motor neurons may reduce their correlated activity, which in turn may trigger synaptic competition at neuromuscular synapses (Personius and Balice-Gordon, 2000). Recently, a novel ultrafast form of axoaxonal electrical coupling was demonstrated between rat hippocampal pyramidal cells, allowing the recruitment of neurons for fast oscillations by transmitting action potentials between axons. Thus, when such gap junctions are open, a presynaptic neuron will act upon many more postsynaptic cells than it would under baseline conditions (Schmitz et al., 2001).

Regulation of gap junctions in CNS diseases

Gap junctions have been implicated in several neurological disorders, including epilepsy (Perez-Velazquez et al., 1994; Valiante et al., 1995; Traub et al., 2001), brain tumors (Lin et al., 2002), brain ischemia (Siushansian et al., 2001; Rouach et al., 2002; Nakase et al., 2003), and neuropathic pain (Lin et al., 2002; Spataro et al., 2004). The junctional channels are unusual in that they typically remain open at rest, and only close under specific circumstances. However, the gating of these channels is complex. Gap junctions are targets for a number of cytokines, neurotransmitters, growth factors, and endogenous bioactive lipids (Rouach et al., 2002), and also sensitive to pH (Morley et al., 1996) and phosphorylation (Morley et al., 1996). Hence, they can be subject to different forms of modulation after CNS injury.

Little attention has been paid to the possible role of gap junctions in spinal cord injury. We therefore determined expression of Cx43, Cx36, and Cx32, which are the principle Cxs expressed by astrocytes (Yamamoto et al., 1990a, b; Dermietzel et al., 1991; Giaume et al., 1991), neurons (Belluardo et al., 2000; Rash et al., 2000), and oligodendrocytes (Dermietzel et al., 1989; Micevych and Abelson, 1991), respectively, in the developing, normal adult, and adult injured spinal cord. We asked whether severe spinal cord injury in adulthood would lead to changes in glial or neuronal connexin expression patterns and whether any such changes in response to injury might recapitulate expression patterns seen in the neonate.

Models of CNS injury

Spinal cord injury

Spinal cord injury leads to loss of mobility, sensation, and autonomic functions below the level of injury. As early as a century ago, Cajal described the ability of injured spinal axons to sprout and also found that regeneration failed. He wrote that, "Everything may die, nothing may be regenerated. It is for the science of the future to change, if possible, this harsh decree." (Cajal, 1928). Cajal's student, J. F. Tello, grafted pieces of peripheral nerve into the CNS and showed that CNS axons could regenerate in the nourishing environment of Schwann cells (Tello, 1911). In the 1980s, scientists observed that peripheral nerve grafts placed in adult rat spinal cord or brainstem induced ingrowth of axons from different types of neurons and elongation over distances of centimeters (Richardson et al., 1980; David and Aguayo, 1981), which disproved the concept that adult CNS neurons were unable to regenerate long axons. However, the regenerating axons failed to leave the peripheral nerve grafts and stopped elongating very soon upon encountering host CNS. The microenvironment of the adult mammalian CNS, in contrast to that of the peripheral nervous system (PNS), contains multiple potent inhibitory factors and structures which prevent axon growth (Schwab and Thoenen, 1985; Caroni and Schwab, 1988; Reier and Houle, 1988; Savio and Schwab, 1990; Snow et al., 1990; McKeon et al., 1991; Davies et al., 1997; Fawcett and Asher, 1999).

The commonly used rodent models of spinal cord injury include transection, contusion, and compression of the spinal cord. Transection involves opening the dura and partially or completely cut the spinal cord. Complete transection is perhaps the only technique to provide absolutely unambiguous anatomical and functional demonstration of nerve regeneration in repair protocols (Steward et al., 2003). However, most human spinal cord injuries are different from the transection model and more similar to the compression (Rivlin and Tator, 1978; Holtz et al., 1989; Martin et al., 1992; Fehlings and Tator, 1995) and contusion models (Somerson and Stokes, 1987; Beattie, 1992; Behrmann et al., 1992; Gruner, 1992; Young, 2002; Scheff et al., 2003) with preserved dura.

Dorsal root avulsion

Root avulsion is considered to be a type of PNS/CNS injury not amenable to surgery (Carlstedt, 1995; Hoffmann et al., 1996). It has long been held that the dorsal root entry zone of the spinal cord acts as an inhibitory barrier for axonal regeneration after section of the dorsal roots (Reier et al., 1983; Carlstedt, 1985; Liuzzi and Lasek, 1987). The avulsed rootlets may largely withdraw before a decision to operate has been made, making direct repair by repositioning difficult. Mounting evidence, however, indicate that dorsal root regeneration beyond the dorsal root entry zone can be enhanced by trophic factors (Houle and Johnson, 1989; Senut et al., 1995; Oudega

and Hagg, 1996; Zhang et al., 1998; Iwaya et al., 1999; Ramer et al., 2000), peripheral nerve grafts (Oudega et al., 1994), transplants of embryonic spinal cord (Tessler et al., 1988), dorsal root ganglia (Rosario et al., 1992), and OEC transplants (Ramon-Cueto and Nieto-Sampedro, 1994). Nevertheless, sensory axons reentering the spinal cord are typically few and long-distance projections are rare.

The 6OHDA lesion, a Parkinson's disease model

Parkinson's disease is characterized byprogressive degeneration of nigrostriatal dopamine neurons. The etiology is largely unknown, although there is increasing evidence for causative genetic factors in familial forms and genetic susceptibility factors in idiopathic forms. . To date, medications including levodopa and long-lasting dopamine agonists remain the most effective treatments. Grafting fetal ventral mesencephalic tissue (VM) to the adult dopamine-depleted striatum can partly restore dopamine innervation and counteract functional deficits in animal models of the disorder (Björklund and Stenevi, 1979; Perlow et al., 1979; Dunnett et al., 1997), and in some patients with severe Parkinson's disease. The most successful cases have been able to withdraw medical treatments (Lindvall et al., 1988; Björklund et al., 2003). However, dopamine cell transplantation protocols remain experimental (Lindvall and Hagell, 2000)because of limited availability of embryonic tissue, limited graft survival, restricted dopaminergic reinnervation of striatum, suboptimal functional effects and, sometimes, negative side-effects (Freed et al., 2001).

Repair strategies for spinal cord injury

During the last decades, remarkable progress in defining the molecular obstacles and inhibitors to axonal regeneration in CNS and developing means of circumventing them has been made. Current concepts for spinal cord repair can be generally classified into three categories: acute neuroprotection, the stimulation of neural regeneration and the induction of neural plasticity. The repair strategies include a variety of cell transplants (Li et al., 1997b; Xu et al., 1997; Ramon-Cueto et al., 1998; Hofstetter et al., 2002; Hofstetter et al., 2005), neurotrophic factors for axon growth (Schnell et al., 1994; Tuszynski et al., 1996; Kobayashi et al., 1997), blockade of inhibitory constituents such as the Nogo signaling system (Schnell and Schwab, 1990; Bregman et al., 1995; GrandPre et al., 2002), chondroitinase ABC (Yick et al., 2000; Bradbury et al., 2002), stimulation of intrinsic regenerative capacity by elevation of intraneuronal cAMP (Neumann et al., 2002; Qiu et al., 2002), inhibition of the Rho pathway (Dergham et al., 2002; Fournier et al., 2003), and combinatorial treatments targeting multiple independent mechanisms (Cheng et al., 1996; Coumans et al., 2001; Chau et al., 2004; Lu et al., 2004; Nikulina et al., 2004; Pearse et al., 2004; Fouad et al., 2005).

Olfactory ensheathing cells

In mammals the olfactory receptor neurons are unique to undergo continuous cell renewal throughout life and having the newly formed axons enter into CNS (Graziadei and Graziadei, 1979a; Graziadei and Graziadei, 1979b). New olfactory neurons, originated from neuroepithelial precursors located in the olfactory epithelium (Graziadei et al., 1979), extend their growing axons to the olfactory bulb and synapse with mitral cells within the bulb (Kosaka et al., 1998). The transition barrier zone between PNS and CNS is not found in olfactory system (Doucette, 1991). This property is thought in part to be due to OEC which ensheath olfactory axons both in the PNS and the CNS environment (Doucette, 1984; Doucette, 1993). Electron microscopic studies have shown that an OEC can ensheath hundreds to thousands of olfactory axons (Field et al., 2003). In

an experimental injury model of olfactory mucosa, "empty" channels within OEC cytoplasm following axonal degeneration were observed. OEC neither proliferated nor migrated but remained as conduits through which the subsequent regenerating axons grew (Williams et al., 2004).

Transplantation of OEC has been shown to support axonal extension through a lesion site and improve functional recovery following spinal cord injury (Franklin et al., 1996; Li et al., 1997b; Imaizumi et al., 1998; Ramon-Cueto et al., 1998) although exact mechanisms underlying the neuroprotective/regenerative properties of OEC are not fully understood. Several growth-promoting properties of OEC have been suggested, such as neurotrophic effects (Ramon-Cueto and Avila, 1998; Chuah and West, 2002; Moreno-Flores et al., 2002; Lipson et al., 2003; Chuah et al., 2004) and remyelination/ ensheathment of remaining demyelinated and/or regenerating axons (Franklin et al., 1996; Imaizumi et al., 1998; Barnett et al., 2000; Kato et al., 2000; Sasaki et al., 2004).

Acidic fibroblast growth factor

Acidic fibroblast growth factor (aFGF) consists of 140 amino acid residues, is present in relatively high amounts in the CNS, and possibly functions as a neurotrophic factor. It does not have a typical signal sequence, suggesting lack of a mechanism for extracellular secretion. In normal spinal cord, aFGF immunoreactivity is localized in the cytoplasm of ventral motor neurons and sensory fibers in the dorsal columns. After spinal cord injury, aFGF immunoreactivity increases in ventral motor neurons, intermediate lateral grey matter neurons and fasciculus cuneatus and gracilis caudal to the lesion, indicating that aFGF is anterogradely transported in ascending sensory fibers (Koshinaga et al., 1993). Application of exogenous aFGF promoted neuronal survival and axonal growth after spinal cord injury (Cheng et al., 1996; Guest et al., 1997; Teng et al., 1998; Lee et al., 2002; Tsai et al., 2005).

Chondroitinase ABC

Astrocytes and meningeal cells produce proteoglycans. Extracellular proteoglycans have been implicated as barriers to CNS axon extension in the developing rhombencephalic and mesencephalic midline and at the dorsal root entry zone of the spinal cord (Pindzola 1993). Chondroitin sulphate proteoglycans (CSPG) are the most abundant type of proteoglycans in the mammalian CNS and constitute potent inhibitors of axonal growth (Snow et al., 1990; McKeon et al., 1991; Davies et al., 1997). After spinal cord injury, CSPG are upregulated rapidly within hours and persistent for many months around the injury site (Lemons et al., 1999; McKeon et al., 1999; Tang et al., 2003). Chondroitinase ABC, extracted from the bacteria *Proteus vulgaris*, removes glycosaminoglycan sidechains from proteoglycans. It has been shown that chondroitinase ABC can enhance axonal regeneration in different models of CNS injuries (Yick et al., 2000; Moon et al., 2001; Bradbury et al., 2002; Pizzorusso et al., 2002; Chau et al., 2004).

Cyclic AMP and Rolipram

Preconditioning peripheral nerve injury prior to a dorsal column injury of the spinal cord can enhance regeneration of severed sensory axons into and beyond the lesion site (Neumann and Woolf, 1999). Injection of of the cyclic adenosine monophosphate (cAMP) analogue dibuturyl cAMP into dorsal root ganglion neurons days before spinal dorsal column injury mimicked a preconditioning peripheral nerve lesion and promoted regeneration of the central branches of primary sensory neurons into the spinal cord lesion (Neumann et al., 2002; Qiu et al., 2002). Cyclic

AMP itself can promote neurite growth (Cai et al., 1999; Cai et al., 2001; Kao et al., 2002; Gao et al., 2003), act as an axon guidance cue (Song et al., 1997; Song et al., 1998; Nishiyama et al., 2003), and effect growth-associated gene transcription (Shaywitz and Greenberg, 1999). Levels of cAMP inside cells are controlled by phosphodiesterases which hydrolyse cAMP to 5'-AMP, the only means of degrading this second messenger (Souness et al., 2000). Introduction of cAMP or an inhibitor of phosphodiesterases should lead to cAMP accumulation. Rolipram, an inhibitor of phosphodiesterase 4 (70-80% of phosphodiesterases in neural tissues, (Jin et al., 1999)), has been recently included as part of combinatorial treatments for spinal cord injury (Nikulina et al., 2004; Pearse et al., 2004).

Superparamagnetic nanoparticles for in vivo tracking of cell grafts

The use of iron nanoparticles for labeling cells and their in vivo MRI detection has been reported previously (Norman et al., 1992; Hawrylak et al., 1993). Early studies used particles in the micrometer range which could overload cells to be grafted and decrease their mobility (Norman et al., 1992). Recently, the size of labeling particles has been reduced to the nanometer range with only minimal effects on cell function and at the same time increased effects on MRI contrast by the incorporation of superparamagnetic compounds. Labeled cells have been detected by MR imaging of excised tissue *ex vivo* (Bulte et al., 1999; Franklin et al., 1999) and *in vivo* for a prolonged period of time (Bulte et al., 2001; Hoehn et al., 2002). Because of the small size, the particles are not hindered by lattice orientation and do not exhibit magnetic hysteresis, hence giving rise to reduced MRI signals in T2-weighted images. Non-invasive monitoring of grafted cells in vivo may help us to better understand not only the spatial but also the temporal distribution of cells used in experimental CNS repair strategies. In the thesis, we examined superparamagnetic iron oxide nanoparticles (SPION) with three different types of coatings: dextran (linear polysaccharide), dendrimer (branching, synthetic polymers), and gold. The nature of the coating as well as the size and the charge of the particles determine their stability, biodistribution and metabolism (Chouly et al., 1996).

AIMS

Focusing on spinal cord injury and possible protection and repair strategies, the aims of this thesis have been to:

Characterize the changes of gap junctional expression in spinal cords of neonatal, normal adult, and injured adult rats to see if events that follow injury may recapitulate development.

Investigate if peripheral nerve grafts combined with aFGF may induce functional recovery of transected dorsal roots in adult rats, evaluated by somatosensory evoked potentials.

Examine several types of superparamagnetic iron oxide nanoparticles as a cell label for tracking transplanted cells in vivo for a prolonged period of time in the normal and injured spinal cord.

Test whether OEC may influence survival and axon growth of fetal dopamine neurons when co-grafted into the denervated striatum of 6-OHDA lesioned rats.

Test the effects of delayed OEC transplantation on functional recovery after severe spinal cord contusion.

Test for additive effects of combining a cocktail including aFGF, chondroitinase ABC, and the phosphodiesterase inhibitor Rolipram with OEC grafts after spinal cord injury.

MATERIAL AND METHODS

Cell culture

Olfactory ensheathing cells

Primary OEC cultures were prepared from olfactory bulbs of adult 2 months old female outbred Sprague-Dawley or inbred Wistar-Furth rats (Scanbur, Sollentuna, Sweden). The nerve fiber and glomerular layers of olfactory bulbs were dissected, minced, and incubated with 0.1 % trypsin (Gibco) at 37˚C for 10 min. The dissociated cells were washed and resuspended in Dulbecco's Modified Eagle's medium (DMEM)/Ham's/F-12 (1:1 mixture, Sigma) supplemented with 10% fetal bovine serum (Gibco), 1% L-glutamate, and penicillin (100 units/ml, Gibco), streptomycin (100 μg/ml, Gibco) and amphotericin B (1.75 μg/ml, Gibco). After 54 hrs in uncoated Petri dishes, most fibroblasts and astrocytes become attached to the dish while OEC remain unattached for 96-120 h (Nash et al., 2001). OEC were thus collected from the supernatant, replated onto poly-D-lysine-coated (PDL, 0.1 mg/ml, Sigma) culture flasks, and allowed to grow to confluency for 14 days.

To determine purity of OEC in the primary cultures, additional cell cultures were plated onto PDL-coated, 2-well glass chamber slides (LabTek, Naperville IL) and processed for immunohistochemistry (see Histology). Triple staining was performed using primary antibodies raised against the low affinity neurotrophin receptor p75 (p75, mouse, 1:300, Abcam), glial fibrillary acidic protein (GFAP; chicken, 1:50, Abcam), and fibronectin (rabbit, 1:100, Chemicon). The purity of the cell cultures was determined by counting the immunoreactive cell types attached to the slides. Cells identified by p75-immunoreactivity were classified as OEC. These cells can also be diffusely GFAP or weakly fibronectin immunoreactive, as has been reported previously (Ramon-Cueto and Nieto-Sampedro, 1992; Nash et al., 2001); cells labeled strongly by fibronectin-antibodies alone were regarded as fibroblasts/meningeal cells; GFAP-positive but p75-negative cells as astrocytes. Five sample areas per well and six wells totally at 200X magnification were used to calculate the average percentage of each cell type. Using this protocol, out of the cell types that could be observed, we obtained 44% OEC, 51% fibroblasts, and 5% astrocytes in the OEC cultures. We did not further purify OEC because fibroblasts/meningeal cells mixed in the OEC transplants have been reported beneficial for repair of spinal cord injury (Li et al., 1998; Lakatos et al., 2003b).

Neural stem cells and viral transduction

Neural stem cells (NSC) were prepared from spinal cord of adult female Sprague-Dawley rats (Scanbur BK, Sollentuna, Sweden) as previously described (Johansson et al., 1999). After removal of the meninges, spinal cord tissue was dissociated at 37 °C for 30 min in a solution of 0.7 mg hyaluronic acid, 200 U Dnase and 1.33 mg trypsin per ml. After filtration (70 μm mesh) and centrifugation (200g for 5 min), cells were resuspended in 0.5 M sucrose in 0.5x Hank´s balanced salt solution. After a second centrifugation (750g for 10 min), the cell pellet was resuspended in neurosphere culture medium based on DMEM F12 (Invitrogen, Carlsbad, CA, USA) with Glutamax and supplemented with 20 ng epidermal growth factor, 20 ng basic fibroblast growth factor, 20 μl B27, 100 U penicillin and 100 μg streptomycin per ml. Neurospheres that

had formed after 7 days were dissociated in 1.33 mg/ml of trypsin and single cells were further cultured for 3 days into secondary neurospheres in 50% neurosphere-conditioned medium and 50% fresh medium. Vesicular stomatitis virus G protein-pseudotyped retroviral particles were prepared, which encoded enhanced green fluorescent protein (GFP) (Falk et al., 2002). Secondary neurospheres were infected 24 h after passage at a multiplicity of infection of 3.5. The efficiency of the transduction protocol is approximately 60%.

Cell labelling

In paper III, OEC were labeled with magnetodendrimers (MD-100, 25 μg Fe/ml, 20 nm in diameter) (Bulte et al., 2001; Strable et al., 2001) by co-incubation at 37˚C for 24 h before transplantation. In paper VI, OEC were labeled with 10 μM bromodeoxyuridine (BrdU, Sigma) by co-incubation for 48 h before grafting.

For neural stem cell labelling, cells were propagated in complete neurosphere culture medium for 6 h after viral transduction, and then incubated in neurosphere culture medium in the absence or presence of lipofection reagent TransFast (1 μg/μl, 1 h at 37 °C, Pregma) containing gold-coated monocrystalline iron oxide nanoparticles (Au-MION) at concentrations of 0.5; 2; 10 and 20 μg Fe/ ml at 37°C for 24 h or 48 h, washed with Hank's balanced salt solution, and harvested with 0.25% trypsin.

In vitro experiments

Co-culture of OEC and fetal dopamine neurons

OEC cultures were seeded onto PDL-coated 3.24 cm^2 cover slips placed in 6-well culture plates or onto PDL-coated membrane inserts suspended 0.8 mm above the well's bottom (4.2 cm^2 with a pore size of 1.0 micron, Falcon). After 7 days in culture the OEC had reached 70% confluency and a cell suspension of fetal VM was added to the cover slips. The mesencephalic cell suspension was prepared as described below for transplantation, 75 000 VM cells were added to each cover slip. The cultures were divided into three groups, (1) VM cells that were in direct contact with OEC, (2) VM cells on PDL-coated cover slips beneath a membrane with an OEC monolayer, (3) VM cells on PDL-coated cover slips with no OEC in the culture. The culture medium were switched from DMEM with 10 % FBS, to a culture medium consisting of 2/3 DMEM and 1/3 HBSS supplemented with 10 % FBS, 1.5 % glucose and 1 % Hepes when VM cells were added to the cultures. After 8 days in culture, cover slips were fixed in 2 % paraformaldehyde for 30 min, rinsed in PBS and processed for immunohistochemistry.

Cell proliferation assays of aFGF

Immortalized Bovine Capillary Endothelial-hTERT^{+} cells (Veitonmaki et al., 2003) were grown in gelatinised 10 cm culture dishes in DMEM (Sigma) supplemented with 10% fetal calf serum (Sigma). Cells were dispersed in 0.05% trypsin solution and resuspended in DMEM containing 1% fetal calf serum. A total of 10000 cells were added to each gelatinised well of a 24-well plate and incubated at 37°C for 1 h. Vehicle, positive control (bFGF 10 ng/ml), aFGF (50 ng/ml, fresh or incubated at body temperature for 2 weeks), and aFGF with chondroitinase ABC (Seikagaku and Sigma, both 110 units/mg protein; incubated for 2 weeks) were added in triplicates to each well. After 72 h incubation, cells were trypsinized, resuspended (Isoton II solution, Beckman), and counted.

Surgery

Spinal cord injury and cell transplantation

Adult 3 months old female outbred Sprague-Dawley and inbred Wistar-Furth rats (Scanbur, Sollentuna, Sweden) were kept on a 12:12-h day:night cycle with free access to food pellets and water. Experiments were approved by the Animal Research Ethics Committee of Stockholm.

The rats were subjected to general anesthesia with 2% isoflurane, and preoperative antibiotics (3 mg/kg sulfadoxin and 0.6 mg/kg trimethoprim, Borgal®; Hoechst AG) and an analgesic (buprenorphine 0.015 mg/kg, Temgesic®, Schering – Plough AB) administered subcutaneously. After vertebral laminectomy of T8-9, the spinal cord was contused by the impactor device (Gruner, 1992) using a 10 g weight drop from a height of 25 mm (paper VI), or cut by microscissors followed by lifting of the stumps to ensure complete transection (paper I, III).

The OEC suspension, 10^5 cells/µl in DMEM/Ham's/F-12, was infused at a speed of 0.5 µl/min (UltraMicroPump-II, Sarasota, FL) with a glass microcapillary (outer diameter 150 µm) mounted on a 10 µl Hamilton syringe with a polyethylene tubing. For spinal cord repair (paper VI), three midline intraspinal injections were made at the center, 2 mm rostral and 2 mm caudal to the contusion lesion using a stereotactic device. Each injection was delivered at 3 depths at 1.75, 1.25, 0.5 mm from the cord surface and 0.5 µl was given at each depth. Control groups were injected with medium vehicle in the same fashion. Postoperatively, all animals received analgesics for 2 days and prophylactic antibiotics for a week, thereafter if necessary. Bladders of the rats were manually emptied twice daily after injury.

Root transection and peripheral nerve grafting

Adult female Sprague-Dawley rats (230–260 g) were anesthetized with isoflurane (3 liters/ min), and subjected to left cervical hemilaminectomy to expose the left cervical 6th – 8th (C6–8) roots. After the dura was opened, the C6–8 dorsal and ventral roots were pulled tight and transected at their junction with the cervical cord. No proximal stumps remained in these roots. Each animal was randomly assigned to one of three groups, and each was subjected to a different treatment: i) The control group received transection of the left C6–8 roots only; ii) The nerve graft group had the same injury as the control group, but the roots were reanastomosed with intercostal nerve grafts. Fibrin glue containing vehicle was then applied. iii) The nerve graft with aFGF group underwent the same treatment as the nerve graft group except that additional aFGF (10 µg, a gift from Professor Y.H. Cao, KI, Stockholm, Sweden) was mixed into the fibrin glue. Autologous intercostal nerves were harvested and preserved in Hanks' balanced salt solution. One end of the intercostal nerve was anastomosed to the cut root with 10–O microsutures in an end-to-end fashion. The other end of the intercostal nerve was then approximated to the cord through a tiny pia incision. After C6–8 roots had been reconnected, fibrin glue was applied to the grafted area. The fibrin glue (Beriplast P, Germany) was prepared before use by mixing the fibrinogen (100 mg/ml) with apotinin solution (200 KIU/ ml) plus calcium chloride (8 mM) in the surgical area to form a glue cast. The final glue volume was about 10 µl.

6OHDA lesion and fetal VM transplantation

Adult female Sprague-Dawley rats (5-week old, body weight 150 g) were anesthetized with halothane and positioned in a stereotaxic frame. A unilateral striatal lesion was performed by injection of 6-OHDA (Sigma, 2 µg/µl in 0.9% NaCl containing 0.2mg/ml ascorbic acid) into the medial

forebrain bundle of the nigrostriatal pathway (coordinates 4.4 mm posterior and 1.2 mm lateral to bregma and 7.8 mm below the dural surface) using a micropump connected to a 10 μl Hamilton syringe at a rate of 1 μl/min with a total volume of 4 μl (totalling 8 μg 6-OHDA/animal). The syringe was withdrawn 2 min after the injection was completed. Animals received subcutaneous injections of buprenorphine (Temgesic, 0.3 mg/kg) every 12th h during the first two days after surgery to reduce pain.

VM from embryonic day14.5 fetuses was dissected in DMEM. The tissue from 20 fetuses was pooled and trypsinated in 100 μl 0.5 % trypsin for 5 min at 37°C. After addition of 1.4 ml DMEM with 10 % fetal bovine serum the tissue was centrifuged at 1000 rpm for 5 min. The cell pellet was washed with DMEM, centrifuged at 2000 rpm for 2 min, resuspended in 500 μl DMEM and mechanically dissociated by trituration (10-20 strokes) using a Pasteur pipette, and finally a 23G needle. Thereafter the cells were counted in a Bürker chamber and the cell suspension was centrifuged for 7.5 min at 6000 rpm. The cell pellet was resuspended in DMEM. For co-transplantation, the cell suspension from the cultured OEC or astrocytes of the same origin was mixed with the embryonic VM suspension.

Animals were randomly assigned to receive 100 000 embryonic cells together with 200 000 OEC (OEC/VM co-grafts) or with 200 000 astrocytes (astrocyte/VM co-grafts), or 200 000 OEC alone (OEC grafts) or 200 000 astrocytes alone or 100 000 embryonic cells alone (VM grafts). The grafts were placed in the lower lateral part of the upper medial quadrant of striatum (coordinates 0.5 mm anterior and 2.0 lateral to bregma, and 5.0 mm below the dural surface) by a stereotaxic frame. Injections were performed at a rate of 1 μl/min. The syringe was slowly withdrawn 5 min after the injection was completed to minimize back-flow. Non-grafted controls received an injection of DMEM at the same coordinates used for grafting. The animals received subcutaneous injections of analgesics buprenorphine every 12th h during the first two days after surgery, and they were sacrificed 8.5 weeks after transplantation. From the day prior to transplantation, the rats received a daily injection of cyclosporin A (10 mg/kg, Sandimmun, diluted in Ringer´s solution with an addition of 2 mg/kg doxycyclin) to avoid graft rejection.

Implantation of intrathecal catheter

After T8/9 laminectomy and the impact injury at T9 (paper VI), a small dural incision was made with a 27-gauge needle at the rostral end of the T8 laminectomy. A 32-gauge polyurethane catheter (ReCathCo LLC, Allison Park, PA) was advanced intrathecally and caudally until its tip was located directly above the contusion site. The 32G catheter was connected to a 200-μl minipump (model 2002, 200-μl, Alzet, Cupertino, CA) with cyanoacrylate glue. The tubing was fixed with sutures around the glue. The minipump delivered either aFGF in a dose of 0.7 μg/ kg/ day (R&D) and chondroitinase ABC 0.14 units/ kg/ day (Seikagaku and Sigma, 110 units/mg protein) in artificial cerebrospinal fluid (CSF, Harvard Apparatus, Holliston, MA) or artificial CSF alone at a speed of 0.5 μl/hr for 2 weeks immediately after injury.

Behavioral tests

Spinal cord injured rats

All animals were tested weekly for open field walking using the Basso, Beattie, and Bresnahan (BBB) locomotor rating scale (Basso et al., 1995) and a subscore designed to detect addi-

tional details of locomotor function (paw placement, toe clearance, instability, and tail position) (Lankhorst et al., 1999).

At 9 weeks postinjury (paper VI), we evaluated the responses to mechanical, heat and cold stimulation of forepaws and hindpaws. Rats were placed in testing chambers with a metal mesh floor for 15 min to habituate. For mechanical stimulation, a set of calibrated nylon monofilaments (von Frey hairs, Stoelting, IL) was applied to the glabrous skin of the paws with increasing force until the animal withdrew the limb. Each monofilament was applied 5 times. The withdrawal threshold was recorded as the least force at which animals withdrew the paw at least 3 out of 5 consecutive stimuli. For heat stimulation, a radiant heat source (IITC, Woodland Hills, CA) was aimed at the glabrous surface of the paws through the metal mesh floor and the latency to withdraw the stimulated paw was measured. For cold stimulation, ethyl chloride (Medikema AB, Perstorp, Sweden) was sprayed to the glabrous surface of the paws. The hindpaw responses were classified as: 0 – no response; 1 – startle response without paw withdrawal; 2 – withdrawal of the paw; 3 – withdrawal of the paw combined with flinching and licking the paw and/or vocalization. The forepaw responses were classified as: 0 – no response; 1 – paw withdrawal; 2 – shaking or licking; 3 – repeated shaking, licking or vocalization. Note that scoring of response to cold stimulation is slightly different in hindpaws and forepaws.

6OHDA lesioned rats

Amphetamine- and apomorphine-induced rotational behaviour was used to confirm completeness of the lesion and to determine effects of engraftment protocols. Rats were placed in plastic rotometer bowls and connected to a computerized system registering the number of turns. After 10 min, when spontaneous rotational behaviour had ceased, amphetamine (2 mg/kg i.p. diluted in 0.9 % NaCl) or apomorphine (0.05 mg/kg s.c. in the flank region, diluted in 0.9% NaCl) was injected. Rotational behaviour was followed for another 70 (apomorphine) or 90 (amphetamine) min. Rotational tests were carried out 2 and 4 weeks post-lesion. Care was taken to select only animals with at least 7.0 turns/min after amphetamine injection and that responded to apomorphine with totally > 450 turns and the characteristic two-peak rotation curve (Herrera-Marschitz and Ungerstedt, 1984). Behavioural recovery was assessed by amphetamine-induced rotations 2, 3, and 7 weeks after grafting, and by apomorphine-induced rotations 8 weeks after grafting.

Somatosensory evoked potentials

Cortical SEPs were recorded before the dorsal root operation and every four weeks afterwards for a total of 20 weeks (paper II). Under isoflurane anesthesia, the cranium was exposed and 4 small-diameter holes were drilled over the somatosensory cortex bilaterally, representing the forelimbs. The stereotaxic coordinates were: 1 mm anterior to bregma, 3 mm lateral to the sagittal suture, and 2 mm posterior to bregma, 3 mm lateral to the sagittal suture bilaterally (Paxinos and Watson, 1997). Silver electrodes were glued to the burr-holes to serve as epidural recording electrodes and cortical SEPs were recorded under light isoflurane anesthesia (0.75-1 liter/min). A reference needle electrode was inserted into the nasion. The forepaw was stimulated using a pair of needle electrodes placed in thenar muscles at a frequency of 2.11 Hz and for a duration of 0.2 msec. Current (2.0-5.0 mA) was passed between the two needle electrodes at a level that evoked maximum cortical responses. The SEPs were amplified (amplification range: 10mA) and filtered (band pass, 20-2,000 Hz), and one hundred consecutive responses were averaged (Medelec system, Synergy). To verify the origin of reappearing SEPs, if any, rats showing recovery

of SEPs underwent re-transection of the repaired dorsal roots and SEPs recorded one week after re-transection of the repaired roots.

Magnetic resonance imaging

MRI scans were obtained (paper III) as described earlier (Fraidakis et al., 1998). Briefly, a spectrometer with a 4.7 Tesla field strength magnet and a 40-cm horizontal bore diameter (Bruker Biospec Avance 47/40, Bruker, Karlsruhe, Germany) was employed. A commercially available double tuned 1H/31P 40-mm surface coil with a planar circular detection area was used. To model MR imaging of labeled OEC suspensions, a series of different concentrations of cells (10^2, 10^3, 2.5×10^3, 5×10^3, 10^4, 2×10^4 and 5×10^4 cells) was injected into 0.5% agarose gel. The agarose plate was imaged using gradient echo and spin echo pulse sequences. These images were obtained with a field of view of 10 x 10 cm and a 1 mm slice thickness.

At 1, 8, and 12 weeks postgrafting, grafted rats were fixed onto the MRI rig with a tooth holder in supine position, aligned by securing the tail to the rig with adhesive tape and sustained laterally using Styrofoam. Anesthesia was induced using isoflurane (1-1.2 %) administered through a mask adapted to the mouthpiece of the rig. Body temperature was kept within the range of 36.5-37.5 ˚C using a temperature-controlled air stream around the body of the rat. The main sequence applied for spinal cord imaging was a Bruker implementation of rapid acquisition with relaxation enhancement (RARE) imaging (Hennig et al., 1986). For T2-weighted images the following parameters were used: RARE factor 8, TR 2500 ms, average TE from the RARE echo train 61 ms, Field of view 6 x 6 cm, matrix 512 x 512 pixels, slice thickness 1 mm.

Functional magnectic resonance imaging

We measured brain activity in spinal cord injured rats stimulated at the hindlimbs, using blood oxygenation level-dependent (BOLD) fMRI (paper VI), which is based on the correlation of neuronal activity and hemodynamic changes in the CNS (Horwitz et al., 2000; Heeger and Ress, 2002). At 16 weeks postinjury, grafted rats were anesthetized with isoflurane, intubated orally and ventilated by a mechanical ventilator (Ugo Basile 7025 Rodent Ventilator). Following an injection of a bolus of 0.05 mg α-chloralose with 2 mg pavulon, isoflurane anaesthesia was shifted to intravenous anesthesia of alpha-chloralose (20 mg/kg/hour) and pavulon (4 mg/kg/hour) through a tail vein using a microinjection pump. Bipolar stimulation electrodes (28 gauge, Grass Telefactor) were inserted subcutaneously 3 – 4 mm apart in both Achilles tendons. The rat was placed in supine position on a custom-built rig and positioned in the MR scanner. Rectal temperature was monitored and maintained at 37 ± 0.5 °C by a manually controlled warm-air system. The imaging experiments were performed using a 4.7 T spectrometer with a horizontal bore (Bruker Biospec Avance 47/40, Bruker, Karlsruhe Germany) and a RF coil with an inner diameter of 35 mm for signal transmission and detection. Data were analyzed using appropriate software (Paravision v. 3.0.2). Seven adjacent transverse 1 mm slices with a field of view of 40 mm were placed and centered at 6 mm posterior to the rhinal fissure. The same geometry was used for the collection of both fMRI data and anatomical high-resolution images, except the latter were expanded into 27 1-mm slices covering the whole brain to transform the images into a common stereotatic template (spatial normalization) (Schweinhardt et al., 2003). Functional MRI data were acquired using a single shot echo-planar imaging (EPI) sequence (TR/TE = 1500/20 ms, matrix size = 64x64 yielding an in-plane resolution of [0.625, 0.625] mm/pixel). For high-resolution anatomical data, a T2 weighted spin-echo RARE sequence was employed (number of aver-

age = 4, RARE factor = 4, TR/TEeff = 3000/25 ms, matrix size = 256x256 yielding a in-plane resolution of [0.156, 0.156] mm/pixel). Pulsed currents of 1 and 2 mA at a frequency of 3 Hz were given to both hindpaws.

We collected 467 repeated slice packages using the paradigm: 40.5 s rest and 19.5 s stimulation alternated 10 times during one session. Every rat was exposed to 4 fMRI sessions with 1 and 2 mA twice each. Statistical parametric maps were calculated using the general linear model method (Friston et al., 1995) as implemented in the SPM2 software (the Welcome department of Cognitive Neurology, London). All fMRI data were spatially smoothed by convolving the data with a Gaussian kernel to smooth random Gaussian fields, and filtered with a kernel that matched the size of the activation (Rosenfeld and Kak, 1982). The full width half maximum was 10 mm, which corresponds to approximately 2 (in-plane) voxels. A t-value for each voxel was calculated for the desired level of significance ($P < 0.001$, uncorrected) as considered to be activated. The significant voxels were then superimposed onto the corresponding high-resolution anatomical slices. The total number of the activated voxels in cortex, the beta estimates of center of the cluster with the highest significance (the maximal voxel), and the spatial coordination of the maximal voxel after normalization were evaluated for interindividual comparison. The beta estimates describe contrast difference for the voxels, which can be considered as a measure of the BOLD response intensity.

In situ hybridization

Animals were sacrificed by decapitation at 4 hours and 1, 3, 7, 14 or 28 days after spinal cord injury (paper I). Additional normal neonatal P2 and normal adult rats were included. Adult spinal cords were divided into 9 segments (7-mm long) from cervical to sacral levels, such that the level of transection or laminectomy was positioned between segment 5 and 6. They were frozen on dry ice, and 14 μm coronal cryostat sections collected. In situ hybridization was carried out with radioactive oligonucleotide probes (Dagerlind et al., 1992). Two different 48-mer probes for rat Cx43 (nucleotides 421-468 and 704-751, GenBank accession number NM_012567.1), rat Cx36 (nucleotides 1515-1562, GenBank accession number Y16898), mouse Cx36 (nucleotides 1513-1560, GenBank accession number AF226992), and rat Cx32 (nucleotides 118-165 and 573-620, GenBank accession number X04070) were synthesized (DNA Technologies; Aarhus, Denmark) and used. The probes were found to be complementary only to the intended genes when compared to known mRNA sequences deposited in GenBank. A control 50-mer random probe not complementary to any known sequence was added (Nosrat and Olson, 1995). After 3' end-labeling with [^{33}P]dATP (Dupont NEN) by terminal deoxynucleotidyl transferase (Amersham), probes were purified (QIAquick Nucleotide Removal Kit Protocol, Qiagen). Slides were incubated overnight (42°C) with 0.1 ml of hybridization mixture containing 50% formamide, 4x SSC (0.15 M NaCl, 0.015 sodium citrate, pH 7.0), 1x Denhardt's solution, 1% Sarcosyl, 0.02M Na_3PO_4, pH 7.0, 10% dextransulfate, 0.06 M DTT, 0.1 mg/ml sheared salmon sperm DNA, and hot probes. Slides were then rinsed four times (45 min) in 1x SSC at 60°C. During a fifth rinse in 1x SSC, the bath was allowed to adjust to room temperature. Further rinsing was performed in distilled water and increasing concentrations of ethanol. Slides were then air dried and exposed for 1 week (Cx43) or 4 weeks (Cx36 and Cx32) on x-ray films (Hyperfilm-β-max, Amersham) for quantification (see below). Afterwards, slides were dipped in emulsion (Kodak NTB2, diluted 1:1 with water), exposed and developed after another 4 (Cx43) or 8 weeks (Cx36 and Cx32), counterstained with cresyl violet, and mounted (Entellan, Merck). All hybridization was performed under high-strin-

gency conditions. The control probe was hybridized and processed together with the other probes and gave rise to no specific pattern of hybridization signals.

Optical densities of X-ray films were quantified by image analysis (ImageJ version 1.32, National Institutes of Health, Bethesda, MD). The program was calibrated for each film series using a ^{14}C-labeled polymer layer test slide (Amersham). The quantification was performed blindly for all probes and the results were confirmed by microscopic observations on emulsion-dipped slides. Four sections per spinal cord segment were averaged, resulting in one value per spinal segment. Each value was presented as a percentage of the same segment of the normal spinal cord. Occasionally artifacts precluded quantification of certain sections and only sections devoid of artifacts were measured. The whole cross section of the spinal cord, excluding meninges, was measured for all probes.

Stereology

In paper V, numbers of tyrosine hydroxylase (TH)-positive neurons in grafts, lengths of TH-immunoreactive fibers in striatum and numbers of TH+ neurons within the lesioned substantia nigra were determined using computerized image analysis (Nikon microscope and Stereologer™, SPA inc.). The software utilizes a three-dimentional "optical dissector" for systemic sampling, the "fractionator" sampling method (Gundersen, 1986). For cell counting, for example, every 10th section of the substantia nigra were systematically sampled (section sampling fraction [ssf] = 1/10) after randomly selecting the first section. A rectangular counting frame, "the dissector", with known area was superimposed on the field of view by the computer software, and then the numbers of cell nuclei that come into focus within the height of the optical dissector was counted. Counting frames were systematically distributed with known x and y steps throughout the marked region from a random starting point. The area of the counting frame relative to the area associated with the x and y steps gives the second fraction (area sampling fraction [asf]). The height of the optical dissector relative to the thickness of the section results in the third fraction (thickness [t]/ height [h]). The total number of neurons is given by $N_{total} = \sum Q^- \cdot [1/ssf] \cdot [1/asf] \cdot [t/h]$ where Q^- is the number of neurons counted in the dissectors. Number estimates obtained by the fractionator method are free of assumptions about volume of the investigated region and are unaffected by tissue shrinkage.

The total length of TH-immunoreactive fibers was measured by superimposing a virtual 3D-probe in the shape of a sphere with a diameter of 10 µm in the rectangular dissector (Mouton et al., 2002). Fibers that traverse the virtual sphere surface at any angle were counted. Again, the counting frames, with the virtual spheres, were distributed with known x and y steps throughout the marked region. The total length of fibers/volume is given by $L_{total} = 2 \cdot \sum Q^- \cdot [v/a] \cdot [1/ssf] \cdot [1/asf] \cdot [t/h]$ where [v/a] is the volume of the dissector relative to the surface area of the probe and Q^- is the number of fibers transversing the probe surface.

The area of interest was manually outlined using a low magnification objective (4 x or 10 x lens. Cell or fiber counts were performed with an oil immersion high magnification objective (60 x or 100 x lens, numerical aperture = 1.4). Around eight animals from each group were randomly chosen for stereology.

Histology

At the end of the experiments, animals were anesthetized by intraperitoneal injection of pento-barbital (50 mg/kg), and intracardially perfused with 50 ml physiological saline containing 10 units/ml heparin followed by 200 ml ice-cold fixative (4 % paraformaldehyde and 0.4 % picric acid in 0.1M phosphate buffer, PBS, pH 7.4). The specimens were dissected, post-fixed for 1 h at room temperature, and kept in 10% sucrose overnight at 4°C. Frozen specimens were sectioned at 14 or 40 μm thickness on a cryostat. The 40 μm-thick sections were pre-treated with 2 M HCl for 30 min at 37°C followed by PBS rinse to enhance penetration of antibodies. The endogenous peroxidase activity was quenched by 0.3 % H_2O_2 and 30% methanol in PBS for 10 min followed by PBS rinse. The sections were preincubated in blocking solution containing 10% Donkey serum (Sigma) and 0.3% Triton X-100 in PBS for 1 h at room temperature, and incubated with primary antibodies diluted in 0.3% Triton X-100 (0.6 % for 40 μm-thick sections) in 0.1 M PBS overnight (48 h incubation for 40 μm-thick sections) at 4 °C. The sections were then rinsed and incubated with appropriate secondary antibodies conjugated with Cy2, Cy3, Cy5 (1:50, 1:300, 1:50; Jackson ImmunoResearch) or biotin (Hsu et al., 1981), or peroxidase (Sternberger, 1979) for 1 h at room temperature. Biotinylated secondary antibodies were further incubated with avi-din-biotin-horseradish peroxidase reagents (Vectastain Elite, Vector Laboratories, 5 μl A + 5 μl B per 1 ml, combined 90 min before incubation) for 1.5 h. Peroxidase complexes were visualized by reaction with H_2O_2/3,3-diaminobenzidine (DAB) solution for 5 min. The slides were rinsed again, counterstained if needed, mounted (anti-fading agent 2.5% triethylenediamine (Sigma) for fluorescent slides), coverslipped, and analyzed using fluorescence microscope (Zeiss Axio-phot) or confocal laser microscope (Zeiss LSM510 Meta, Zeiss, Germany). Images were digitally processed (Adobe Photoshop CS, version 8.0) to remove microscopically verified artifacts and to enhance contrast if necessary.

RESULTS

Upregulation of astroglial gap junctions after spinal cord injury (Paper I)

Cx43, Cx36, Cx32 in neonatal, adult normal and transected spinal cord

Cx43 mRNA signals and punctate Cx43-like immunoreactivity were robust in cells in grey matter, in and around the ependyma, and in leptomeningeal membranes in neonatal spinal cord. In the adult spinal cord, Cx43 mRNA signals and immunoreactivity were more abundant in grey than in white matter, mainly parallel to GFAP-positive astrocytes and consistent with earlier studies (Ochalski et al., 1997). After spinal cord injury, Cx43 mRNA signals and immunoreactivity were rapidly upregulated from 4 hours post-injury to at least 4 weeks post-injury ($P < 0.05$). Cx43-like immunoreactivity was mostly co-localized with GFAP rather than with OX-42, indicating such upregulation was mainly in astrocytes rather than microglia. Sham surgery (laminectomy groups) led to a transient and mild elevation of Cx43. The increased signals were spatially restricted within around 20 mm to the transection site with a peak in the rostral stump adjacent to the lesion, where the signals reached more than 3 times normal levels at 4 weeks post-injury.

Cx36 mRNA signals were strong in developing neurons in grey matter of the neonatal spinal cord. In contrast, the signals greatly declined in the adult spinal cord and appeared barely above background over grey matter. The sparse mRNA signals and Cx36-like immunoreactivity were confined to the cytoplasm of neurons in the adult spinal cord, consistent with earlier studies (Rash et al., 2000). After spinal cord injury, there were no obvious changes of Cx36 mRNA over the 4 weeks post-injury. Along the rostro-caudal axis, Cx36 mRNA signals appeared slightly regulated in thoracic segments adjacent to the lesion, suggesting that there were mild changes of mRNA levels around the lesion epicenter rather than in remote neuronal pools.

Cx32 mRNA signals were mainly found in cells located in ventral and lateral funiculi of the neonatal spinal cord. These positions correspond to the localization of developing oligodendroglial cells in the developing spinal cord. In adulthood, in contrast to Cx43 and Cx36, Cx32 mRNA signals were more abundant in white than grey matter. Immunohistochemistry of Cx32 co-localized with the oligodendrocytic marker PLP, consistent with earlier studies (Scherer et al., 1995; Li et al., 1997a). After spinal cord injury, we did not observe significant changes of Cx32 mRNA signals throughout the 4 weeks post-injury period compared to normal or sham rats.

Acidic FGF enhances functional recovery after dorsal root avulsion (Paper II)

Stimulation of the forepaws of normal rats produce characteristic cortical evoked potentials with positive (P1)- negative (N1)- positive (P2) waveforms. The initial P1 was previously attributed to ascending activity in the thalamocortical white matter, whereas the P2 was ascribed to activity in the cortical cells (Perl and Whitlock, 1955). The P2 waveforms displayed a peak latency of 10.87 ± 1.47 msec (mean values $\pm$ standard error of the means) and peak amplitude of 3.92 ± 2.78 μV (n=25). In terms of a percentage of the respective values on the contralateral (control) side, the latency in normal rats was $104.01 \pm 15.26\%$, while that of amplitude was $120.95 \pm 58.80\%$ (n=25).

After rhizotomy, the cortical SEPs completely disappeared in all control rats during the 20-week post-injury period. In contrast, 3 of 5 nerve graft rats and 6 of 7 nerve graft with aFGF rats regained cortical SEPs. The period required for SEPs recovery varied from 4 to 20 weeks post-operation. Of particular note is that the percentage of SEPs recovery in the nerve graft with aFGF group was significantly higher than that in the rhizotomy control group ($P< 0.01$, Fisher exact test). The other paired comparisons did not show significant differences.

The reappearing SEPs were eliminated upon re-transection of the repaired roots, thus verifying that they originated from the regrown axons rather than from reinnervation via adjacent intact roots. The regenerated SEPs, however, were often evoked with greater electrical stimulation, but amplitudes varied widely. The waveforms and interside comparison of latencies (105.6 ± 9.55 %) did not show significant differences from those of normal rats.

In vivo tracking of cells grafted into the rat spinal cord (Paper III, IV)

OEC labeling with dendrimer-coated iron oxide nanoparticles

After 24 hours incubation with magnetodendrimers MD-100 nanoparticles, more than 99% of the cells in the OEC primary culture showed iron-containing vesicles in the cytoplasm demonstrated by Prussian blue staining with DAB enhancement. The cell morphology and survival did not suggest any signs of degeneration compared with unlabeled cells. The labeled cells generated low T_2-weighted signals, and the intensity was related to the number of labeled cells. A cell cluster with a minimum of 5×10^3 labeled cells led to distinct MRI contrast allowing detection.

The labeled OEC were easily detected by MRI in normal spinal cords up to 2 months postgrafting, as a distinctive dark area in T_2-weighted images. The MR contrast extended predominantly along the dorsal column. Maximal contrast extension was 8 mm at 1 week postgrafting and 9 mm at 4 weeks postgrafting, which then remained up to 2-3 months. In contrast, labeled dead OEC were localized within 2 mm of the injection site up to 4 weeks postgrafting. The injection of unlabeled live OEC generated either no MRI contrast change or a minor contrast change less marked than that caused by MD-100. We assumed that this minimal contrast change at the injection site of unlabeled cells could be due to mild hemorrhage. The MRI contrast signals were spatially correlated with that obtained using histological staining for iron (DAB-enhanced Prussian blue stain, Pearson correlation coefficient 0.694; $P < 0.05$) and grafted OEC (p75-immunoreactive, Pearson correlation coefficient 0.802; $P < 0.01$). The two histological markers, p75 and DAB-enhanced Prussian blue, were correlated to each other as well (Pearson correlation coefficient 0.826; $P < 0.01$).

When MD-100 labeled OEC were injected into either the rostral or caudal stump of the transected spinal cord, the injection site could always be distinguished as a dark area. However, less significant, though visible changes of hypointense MRI contrast patterns, were found in both stumps of the transected cord at 1 week postgrafting, regardless of which stump had been deposited with labeled OEC. Parallel to the extended MR contrast within the injected stump, the contrast in the non-injected stump also elongated (maximum of 22 mm at eight weeks postgrafting). Postmortem DAB-enhanced Prussian blue stain showed that a large number of cells with DAB reaction product were present along the white matter tracts in both stumps as MR signals. Furthermore, such stained cells were also present in the control transected cord given only medium injection. These cells were also ED1-immunoreactive, suggesting that they were activated microglia/ macrophages. It thus appeared that after spinal cord transection, reactive microglia/macrophages

accumulated endogenous hemorrhage (hemosiderin) products as well as possibly phagocytized exogenous MD-100 labeled OEC. The two compartments of ferric ions (MD-100 and hemosiderin products) could not be clearly distinguished by Prussian blue stain, or by MRI. We propose that these microglia/ macrophages were detected by MRI after they had accumulated hemorrhage (hemosiderin) products or phagocytized MD-100 released by dead OEC (for the injected stump). Nevertheless, Prussian blue stain was correlated to the MRI contrast in the stump of transplantation (Pearson correlation coefficient 0.628, $P < 0.05$), but not in the non-injected stump. The p75 immunoreactivity showed that fewer OEC survived in the transected spinal cord than in the normal spinal cord, and OEC showed limited migration in the stump of transplantation (maximal 4.1 mm at 8 weeks postgrafting), without significant change of distance between 1 and 8 weeks postgrafting. Importantly, grafted cells failed to cross the transection gap and there were no p75-immunoreactive cells migrating into the opposite stump up to 8 weeks postgrafting. This was also observed in the control transected cord with delivery of unlabeled OEC, indicating that the migratory ability of OEC was not limited by iron loading.

NSC labelled with gold- and dextran-coated iron oxide nanoparticles

Both gold (Au)- and dextran-coated monocrystalline iron oxide nanoparticles (MION), tested in agar colloidal suspension, caused a dose-dependent attenuation of MRI signal intensities. Au-MION showed a clear suppression of T_2-weighted signals by 16.2% of control (water/agar) levels already at a concentration of 0.001 μg Fe/μl, whereas dextran-MION induced a similar effect (13.3% attenuation) first at concentration of 0.01 μg Fe/μl. Accordingly, at 0.03 μg Fe/μl levels, Au-MION caused an almost complete attenuation in MRI signal intensity, whereas dextran-MION still permitted detection of about 34.6% of T_2-weighted signals originating from water/agar.

A silver enhancement histochemical protocol was used to examine the presence of Au-MION in GFP-expressing neural stem cells (NSC). The maximal efficacy (80%) of NSC labelling with Au-MION was obtained at the concentration of 10 μg Fe/ml for 24 h incubation period. Prolonged incubation to 48 h or higher (20 μg Fe/ml) nanoparticle concentration had no enhancing effect on loading efficiency. Silver staining was restricted only to cytosol and cell membrane, indicating that Au-MION did not penetrate to the nucleus. The nanoparticle-labelled and control cells showed similar morphologies and survival rates. MRI scans of control and Au-MION labelled cells revealed that as few as 20 labelled cells resulted in 7.1% attenuation of T_2-weighted signals, while 200 labelled cells caused 21.6% attenuation compared to 200 non-labelled cells.

To compare the behaviour of Au- and Dextran-MION *in vivo*, colloidal suspensions of the two types of nanoparticles were infused into the rat spinal cord. The T_2-weighted signal intensities, reflecting diffusion and clearance of iron oxide nanoparticles from the spinal cord, were monitored for up to 1 month. For Dextran-MION, the diffusion volume quickly increased along the longitudinal axis of the spinal cord with time. Consequently, the corresponding MRI signal was markedly attenuated already after 2 days, and completely diminished at 1 month post-injection. However, during the same period, the diffusion volume of Au-MION did not change and the particles showed intensive suppression of T_2-weighted signals even 1 month after implantation. The GFP-NSC labelled with Au-MION were infused into the rat spinal cord and detected by MRI at 1 h, 48 h and 1 month using the same protocol as that for scanning of directly infused nanoparticles. The labelled cells were confined to the grafted site as a distinct dark area, without

notable migration from the original site of infusion. In control, saline infused rats, no changes in MRI signals were observed during the study period.

The MRI findings were closely correlated to those obtained by histological staining of Au-MION (silver enhancement) and GFP immunoreactivity for GFP-NSC.

However, several silver-stained cells were also immunopositive for ED1, indicating that Au-MION from dead stem cells were transferred to microglia and macrophages.

OEC co-grafts improve functional recovery in 6-OHDA lesioned rats (Paper V)

Amphetamine- and apomorphine -induced rotational behaviour

Significant reduction of the number of amphetamine- and apomorphine- induced rotations was seen in all groups that received fetal VM grafts (100 000 cells) alone or in combination with OEC or astrocytes. Importantly, OEC/VM co-grafted rats had a greater reduction in amphetamine-induced rotations than animals given single VM grafts ($P < 0.01$). Furthermore, rats with OEC/VM co-grafts demonstrated a significantly better decrease in both amphetamine and apomorphine-induced rotations compared to animals with astrocyte/VM co-grafts at all times. Animals that received a single astrocyte graft showed no significant reduction of rotational behaviour, and this was also the case for non-grafted controls. However, animals that received a single OEC graft showed a modest, but significant reduction of amphetamine-induced rotational behaviour 7 weeks after transplantation ($P < 0.05$), but no reduction in the number of apomorphine-induced rotations 8 weeks after transplantation, compared to non-grafted controls.

Number of TH-positive neurons in substantia nigra

The number of dopamine nerve cells in substantia nigra, as defined by TH immunoreactivity, was determined by stereology. The values obtained on the lesioned side were expressed as percentage of neurons found on the intact contralateral side. Consistent with the rotational responses to apomorphine, few TH+ neurons were observed within substantia nigra on the lesioned side, ranging between 1 and 6 % of the number of TH-immunoreactive neurons found on the intact side for individual animals. There was no significant difference in TH+ cell numbers between the different treatment groups or in non-grafted controls, demonstrating that the lesions in all animals were complete or close to complete.

Intra-striatal graft analysis

Histological analysis of the brains 8 weeks post-grafting revealed viable grafts in striatum. Immunohistochemistry demonstrated surviving TH+ neurons, GFAP+ astrocytes and p75+ OEC as expected in the respective grafts. A dense TH-positive fiber network was present within the VM grafts and also extended to variable distances into host striatum. Only in animals with OEC co-grafts could a TH+ fiber network be observed within the lateralmost peripheral part of striatum. A few TH+ fibers could be observed in the peripheral part of striatum in animals with a single VM grafts and co-grafts with astrocytes. No TH+ cells were observed in animals grafted with astrocytes alone, OEC alone or in non-grafted, sham-injected controls, neither were any TH+ fibers observed within the respective graft areas. However, in all groups a very small number of TH-immunoreactive fibers that appeared to be residual fibers spared from the lesion were occasionally seen randomly scattered in striatum, reflecting the very low number (1-6%) of remaining dopamine neurons found in substantia nigra.

Co-grafted OEC, identified by p75 immunoreactivity, were found mainly confined at the graft site, although we cannot completely rule out the possibility that some OEC could have lost p75 immunoreactivity after grafting and migrated outside the graft site. No p75+ cells were found in host striatum in animals grafted with VM alone or in non-grafted controls.

To further characterize TH+ neurons in the grafts, we used Girk2 (a marker for dopamine neurons of substantia nigra) and CB (a marker for dopamine neurons of the ventral tegmental area) immunoreactivity. Analysis of the number of Girk2+ and CB+ cells in the grafts showed that co-grafting VM with OEC significantly increased the percentage of TH+ neurons that express Girk2. The percentage of TH+/CB+ neurons did not significantly differ among different groups, suggesting that dopamine neurons of substantia nigra rather than those of the ventral tegmental area were stimulated by the presence of OEC co-grafts.

Effects of OEC on dopamine neurons in vitro

Analysis of TH-positive cell numbers showed that both direct contact with OEC and OEC conditioned medium significantly increased the number of TH+ neurons in culture by 255% ($P < 0.001$) and 182% ($P < 0.05$), respectively, compared to control VM cultures. The number of primary neurites, total number of branching points and length of longest neurite for TH-positive neurons revealed that direct contact with OEC increased neurite length and decreased primary branching of neurites. There was a significant increase in length of neurite extension ($P < 0.005$) and decreased branching points ($P < 0.001$) from neurons cultured in direct contact with OEC monolayers compared to control cultures. TH-positive nerve cells grown in OEC conditioned medium appeared to be intermediate between the control and direct cell contact conditions.

OEC alone or combined with aFGF, chondroitinase and Rolipram promote functional recovery after severe spinal cord injury (Paper VI)

Activity of aFGF

In order to test bioactivity of aFGF for prolonged periods at body temperature we used a bovine capillary endothelial cell line assay. Acidic FGF at a concentration of 50 ng/ml increased cell counts approximately 2.5-fold. For comparison, basic FGF used at a concentration of 10 ng/ml increased the number of endothelial cells in the same assay approximately 2-fold. We next determined that storage of aFGF at 37° for two weeks prior to the assay did not significantly decrease bioactivity. Finally, we tested if mixing aFGF with chondroitinase ABC, would affect aFGF bioactivity, and found that full aFGF activity was maintained also in the presence of the enzyme. The activity of chondroitinase ABC kept at 37˚C for weeks has been tested by others previously showing that more than 75 % enzymatic activity remains after 2 weeks of incubation (Chau et al., 2004).

Distribution after intrathecal delivery

A 34-mer oligonucleotide, which could readily be detected in tissues by radioactive in situ hybridization, was used to test how far macromolecules would spread from the site of intrathecal infusion using a the two-week osmotic pump delivery system. We found wide spreading of the oligonucleotide in the subdural space along the entire length of the spinal cord when the tip of the delivering cannula was placed over the site of injury at T9. At this level there was also evidence of penetration of the tracer into spinal cord tissue, with gray matter being more strongly labeled than

white matter. Less, if any penetration of the tracer into spinal cord parenchyma was seen at sites rostral or caudal to the site of injury. Strikingly, the tracer oligonucleotide had also reached the liquor-spaces of the brain and was found both in the pial surface and in the walls of the ventricles. There was little if any penetration of this particular tracer into intact brain parenchyma.

Sensorimotor behavioral evaluations

OEC alone. Delivery of OEC alone to the injured area of the spinal cord 2 weeks after injury led to significant modest improvement of the open field BBB score compared to vehicle-injected controls. The OEC-treated animals performed better than controls from the first week after grafting, i.e. three weeks after injury, suggesting neuroprotective effects of the implanted cells. Injured animals that can not perform consistent forelimb-hindlimb coordination never proceed above BBB 13 and their possible continuing recovery of more subtle motor function can only be revealed by the BBB subscore rating scale (Lankhorst et al., 1999) which was therefore also used. The difference between the two groups became more impressive on the subscore scale. On this scale, the OEC-grafted group continuously improved until 16 weeks after injury, while the control group did not improve until 13 weeks postinjury, at which time minimal scores were obtained.

The withdrawal threshold to mechanical stimulation was tested using von-Frey hair filaments at 9 weeks after injury, hence 7 weeks after cell grafting. At this time there was hyperreflexia of the hindlimbs in injured controls. OEC grafting restored the withdrawal threshold to normal levels, suggesting that increased descending inhibitory supraspinal control reached the lumbar spinal cord. There were no significant effects of the OEC grafts on responses to either cold or heat. Indeed, all injured animals (treated or not) showed similar degrees of hyperreflexia to temperature stimulation compared to normal animals at 9 weeks postinjury. In addition, the forelimb withdrawal responses to mechanical and temperature stimulations of all injured rats did not differ significantly from normal rats, indicating that no observable neuropathic pain had been induced by the treatments.

Drug cocktail and combinatorial treatments. We next tested if delivery of three compounds previously shown beneficial in experimental spinal cord injury treatment protocols, aFGF, chondroitinase ABC, and Rolipram, might exert additive effects when delivered together, and in combination with OEC grafts. Since the drug cocktail was given from the day of injury, an additional control group was needed. At the end of a 2 week-delivery, we routinely injected 30 µl artificial CSF intrathecally to verify the patency of the tubing system. Although the 32-gauge polyurethane intrathecal catheterization has the advantage of no notable influence on functions in rats (Sakura et al., 1996), we noted it had a high occlusion rate (around 50%) while implanted following severe spinal cord injury for 2 weeks (although the exact time of occlusion could not be determined). Thus the numbers of rats in these groups were relatively small after exclusion of those with occluded catheters.

The two treatment groups in this experiment both reached modestly better BBB scores than the specific control group. The improvement by the cocktail was observed already from the end of the 2 weeks of delivery, suggesting neuroprotective effects. However, there seemed to be no significant difference between the drug cocktail group and the cocktail + OEC group. While there may thus be no additive effect of the two treatments it must be taken into account that the cocktail + OEC group had to undergo additional surgery, i.e. removal of scar tissues at 2 weeks postinjury and engraftment of OEC, compared to the drug cocktail and the control groups. When the

performance of these three groups were analyzed using the subscore, both treated groups clearly stood out as improved compared to the control group which received no scores at all throughout the 14 weeks of observation time.

The hindlimb withdrawal threshold to von Frey hairs was significantly higher in the cocktail + OEC group than in the injured control group with delayed vehicle injection. Similarly, the drug cocktail group displayed a higher withdrawal threshold than its acute vehicle control group. The hindlimb responses to cold appeared increased in all three groups of this experiment compared to normal animals, although differences between individual groups were not significant at 9 week postinjury. Similarly, the withdrawal latencies to heat appeared shorter than normal in injured groups without obvious differences between these groups.

Functional MRI

To avoid ambiguities in determining sensory function by behavioral tests due to possible simultaneous motor impairments and hyperreflexia of the animals, we also used fMRI to monitor BOLD responses in cortex cerebri following electrical stimulation of the hindpaws. These observations are not dependent on a motor read-out.

Robust BOLD responses were elicited in corresponding sensorimotor cortex on both sides in normal rats in response to bilateral hindpaw stimulation (Spenger et al., 2000). Cortical BOLD signals were observed in 6 out of 9 OEC-grafted rats (67%) and in 3 out of 8 controls (38%). The percentage of animals having BOLD activity was significantly decreased in the control, but not in the OEC-grafted group compared to the normal. Interestingly, the main hind limb representation was devoid of activity after severe spinal cord injury, instead, BOLD signals were recorded at the medially located cingulated gyrus with anterior-posterior extension (Hofstetter et al., 2005). The aberrant activation area is akin to that activated by tail stimulation (Spenger et al., 2000), suggesting plastic changes of neural connectivity after injury. Rarely, less strong signals were found in the more lateral regions to the original representation. The total number of the activated voxels in cortex was decreased in both OEC-grafted and control groups compared to the normal, but was not significantly different by OEC engraftment. Similarly, the beta estimates of the maximal voxel with the highest significance (T value, data not shown) and the spatial coordinates of the maximal voxel in x-axis dimension (the distance to sagittal suture) did not show significant differences between the OEC-grafted and the control group. The correlation coefficients between BOLD signals and various behavioral results were not significant, as we previously observed "numb rats walk" (Hofstetter et al., 2005), suggesting differential spared/regenerative capacity of sensory and motor systems.

CONCLUDING REMARKS

Role of increased astrocytic gap junctions after spinal cord injury (Paper I)

Rohlmann et al. (Rohlmann et al., 1994) and Laskawi et al. (Laskawi et al., 1997) showed rapidly elevated Cx43 proteins in the facial nucleus and motor cortex following peripheral facial nerve lesions. Theriault et al. (Theriault et al., 1997) reported altered Cx43 immunoreactivity in the form of epitope masking after acute SCI, suggesting that spinal cord astrocytes undergo a phase of Cx modification. To date, however, most studies have focused on ischemic injury models and contradictory results of Cx regulations after ischemia have been described. In an *in vitro* brain

ischemic model, astrocytic gap junctions remained open during and after injury (Cotrina et al., 1998). Nevertheless, the role of gap junctions in ischemia remains poorly understood (Rouach et al., 2002).

The increase of Cx43 mRNA and protein levels after spinal cord injury suggests an increased number of astrocytic gap junctions. The functional consequences of enhanced gap junctional communication have long been a matter of debate. An increased intercellular communication may buffer potentially harmful levels of ions such as K+ (Holthoff and Witte, 2000) and metabolites or transmitters, and maintain a homeostatic CNS environment. On the other hand, an increase in intercellular communication may propagate death signals and kill healthy neighboring cells that would have otherwise survived injury (Lin et al., 1998). Functional analyses of gap junctions have been done mainly in vitro. The relative lack of selective gap junctional blockers prohibits the determination of their modes of action. To better understand the role of specific Cxs, transgenic mice have also been employed. Cx43 null mutant mice die shortly after birth (Reaume et al., 1995). By using organotypic brain slices from such Cx43 null mutant newborns, as well as by using antisense oligodeoxynucleotides, Frantseva et al. observed a decrease of cell death after impact injury compared to control mice, suggesting that gap junctions may enhance neuronal vulnerability to traumatic injury (Frantseva et al., 2002). In a brain ischemic injury model, Cx43 heterozygote knockout mice were however shown to have a larger infarct volume 4 days post-injury compared to wild type mice (Siushansian et al., 2001). Moreover, mice lacking Cx43 specifically in astrocytes, generated using the cre-recombinase system (Theis et al., 2003), show increased apoptosis and infarct volume 4 days after brain ischemia, suggesting a neuroprotective role of astrocytic gap junctions in acute stroke (Nakase et al., 2003).

Rewiring a Periphery-CNS junction (Paper II)

Our results demonstrate that anatomical reconstruction by peripheral nerve grafts without aFGF leads to recovery of SEPs in 3 out of 5 rats; however, the statistical difference is not significant. Peripheral nerve grafts combined with aFGF markedly improved the outcome, so that 6 out of 7 rats regained SEPs after treatment. Transganglionic tracing by cholera toxin B-conjugated horseradish peroxidase showed that regenerated sensory axons from the repaired dorsal root grew into the spinal cord (Huang et al., 2002).

The mechanism by which aFGF promotes such sensory regeneration is unclear. Acidic FGF is present in the majority of dorsal root ganglion neurons of all size classes during late embryonic and postnatal stages, suggesting that aFGF is associated with the maturation process of the sensory system (Elde et al., 1991; Oellig et al., 1995). Our previous studies demonstrated that aFGF delivered by fibrin glue in combination with peripheral nerve grafts enhanced functional restoration in adult rats subjected to spinal cord transection (Cheng et al., 1996) and to ventral root transection (Huang et al., 2003). This reparative strategy has been shown to promote regeneration of corticospinal and several bulbospinal tracts after spinal cord transection (Cheng et al., 1996), and to increase survival of motor neurons after ventral root transection (Huang et al., 2003).

SPION advantages and disadvantages (Paper III)

Dendrimer-coated SPION are non-specifically endocytosed by the cell membrane and accumulate in cytoplasmic endosomes (Bulte et al., 2001). The labeling procedure is simple and robust. The high contrast effects on MRI are easily detected within an experimental time frame of about 1-2 hours per animal, which is ideal for short and repetitive in vivo MRI.

However, we found a disadvantage of SPION as applied to the severely injured spinal cord. In the transected cord with either medium injection or an OEC graft, hemorrhage products gave rise to extensive iron deposits that we could not distinguish from iron-containing nanoparticles by Prussian blue staining. The hemorrhage resulted in T_2-weighted hypointensity the degree of which depended on the degree of injury. This interferes in the detection of labeled cells and complicates the interpretation of MRI. Since the hypointense contrast was detected in both the injected and non-injected stumps for the live labeled cell injections, and cells were not found to cross the transection zone, the hypointense contrast in the non-injected stump is likely derived from hemorrhage. In the injected stump, however, the MR contrast correlated relatively well to DAB-enhanced Prussian blue staining, which is presumably a combination of nanoparticles and hemorrhage. A quantitative mismatch between the distributions of MR contrast and histological markers can also be due to redistribution of nanoparticles, e.g. to macrophages (Cadusseau and Peschanski, 1989; Hawrylak et al., 1993). Severe injuries may cause massive inflammatory infiltration and exacerbate metabolism of the biodegradable iron particles. Therefore, it is important to determine whether cell labels remain colocalized with cell transplants especially under pathological conditions.

The nanoparticle coating matters (Paper IV)

Au-MION were shown to exert powerful contrast-enhancing properties in MRI. The effect was stronger than that of Dextran-MION of the same size and concentration. As little as 20 cells could be detected under optimal conditions using T_2 weighted spin-echo imaging. Hence the golden shell of the Au-MION might have further attributed to the T_2 relaxation by a possible disturbance of the local magnetic field caused by electromagnetic interaction with the applied radio signal (Eddy current) during excitation.

Au-MION offer additional advantages when used to label cells. Au-MION, identified by silver enhancement staining for gold, can avoid indistinct iron staining (Prussian blue) of haemorrhage products, as we have shown previously (Lee et al., 2004). When macrophages phagocytize red blood cell debris, they will give rise to positive Prussian blue staining for iron. A selective staining for gold can be used for Au-MION to avoid false-positive signals obtained by Prussian blue staining.

Our data suggest that the grafted neural stem cells do not migrate efficiently in the intact rat spinal cord. However, very few cells appeared to have survived until the 1 month time point, as revealed by GFP immunoreactivity, making it difficult to draw conclusions regarding the cell migration. One possible explanation is that the survived cells could have down-regulated their GFP transgene. However, the occurrence of a number of ED1-positive reactive microglial cells at the site of implantation suggests that at 1 month after implantation, a substantial portion of Au-MION material had been transferred to microglia. The gold surface of the nanoparticles provides an inert shell protecting nanoparticles from rapid dissolution in cytoplasmic endosomes and loss of MRI signalling attributes. The persisting intensity of the MRI signals at one month postsur-

gery indicates that the Au-MION nanoparticles were able to resist also the digestive machinery of the macrophages. In addition, golden surface on the iron oxide core of the particles offers a convenient route for further functionalization of nanoparticles via thiol-coupling and conjugation of biomolecules.

Influence of OEC on dopamin neuron growth in denervated striatum (Paper V)

We demonstrate a unique ability of OEC to stimulate long-distance neurite growth from grafted dopamine neurons in the denervated host striatum. In the peripheral striatal compartments, we found that the TH+ nerve fiber length was remarkably increased by 10 times in OEC/VM co-grafted rats compared to VM grafted rats (100 000 VM cells). Equally remarkably, these fibers were present at distances from the grafted neurons that were almost not at all reached by TH neurons in single VM grafts. Comparable results were observed in the co-culture system; OEC promote dopamine cell survival and neurite elongation. The extended innervation of the denervated host striatum is a long sought-for effect, which has not been reported before for rodent allografts. Human embryonic dopamine neurons grafted to rodent hosts can show more extended innervation patterns which might be related to the extended period of development in humans (Strömberg et al., 1986; Brundin et al., 1988b; Strömberg et al., 1989; Wictorin et al., 1992).

The number of grafted neurons is known to be closely correlated to the reduction of amphetamine- and apomorphine-induced behaviour (Brundin et al., 1985; Brundin et al., 1988a; Rioux et al., 1991; Nakao et al., 1994). When we increased the number of grafted VM cells from 100 000 to 250 000, we observed an enhanced reduction of drug-induced rotations in animals that received VM grafts alone. Except for observations 2 weeks after grafting, the enhanced effect of adding OEC on rotational behaviour that we observed when 100 000 embryonic cells were used, was not evident when we increased the number of grafted dopamine neurons. The reduction in drug-induced rotational behaviour (Brundin et al., 1988b) has been shown to follow a saturation curve, where after a certain degree of dopaminergic reinnervation (Björklund et al., 1980) or neuronal survival (Brundin et al., 1988a), further increases have no additional effect. Presumably, the high number of neurons grafted in the second experiment was sufficient to almost completely reverse the lesion-induced rotational behaviour already in the group that received single VM grafts, thus masking any possible additional effects of OEC co-grafts. Although the survival-promoting effect of co-grafting with OEC is not evident when the number of grafted VM cells increase, the ability to support widespread neurite growth was not diminished. Thus the OEC-induced effects on dopamine cell survival and neurite extension are separate events.

The mechanisms underlying the growth-promoting ability of OEC have yet to be clarified. While co-grafted OEC stimulate TH+ neurites to grow extensively beyond the grafted site, the OEC themselves were primarily confined to the site of engraftment. Others and we have shown that OEC grafts, identified by p75 immunoreactivity or by lentiviral-transferred green fluorescent protein, exhibit limited migration from a severe lesion and do not extend across the astrocytic barrier (Ruitenberg et al., 2002; Lee et al., 2004). Li and Raisman proposed that the effect of OEC grafted into the focally lesioned spinal cord was to form a "patch" of peripheral myelination across which the cut central axons regenerated and continued to grow along their original central pathway with the resumption of oligodendrocytic myelination (Li et al., 1998). We suggest that OEC grafts in the lesioned striatum are primarily confined close to the injection site and akin to the previous observations, although the possibility that some OEC could have lost p75 immunoreactivity and migrated outside the graft can not be completely excluded. Our observations fur-

ther suggest that OEC act on fetal dopamine neurons by providing a cell-cell contact-mediated, and/or paracrine stimulation of dopamine neurons boosting neurites to overcome the inhibitory gliotic region, and/or stimulate neurites to grow longer distances, or to grow for a longer-than-normal time to reach the striatal periphery. In our co-culture experiment, dopamine neurons cultured in direct contact with an OEC monolayer were less branched and had a more fusiform morphology, suggesting that OEC may retard dopamine neuroblast maturation, thus prolonging the period of active nerve fiber growth. Growth-promoting properties of OEC may result from the combination of several factors: OEC induce little astrocytic response and chondroitin sulphate proteoglycan expression following transplantation into adult CNS, providing a more permissive substrate for nerve outgrowth (Lakatos et al., 2000; Lakatos et al., 2003a). OEC also express membrane surface molecules, neurite-promoting molecules, soluble neuregulins and neurotrophic factors that may contribute to the observed effects in the co-grafting situation (Ramon-Cueto and Avila, 1998; Chuah and West, 2002; Moreno-Flores et al., 2002; Lipson et al., 2003; Chuah et al., 2004).

Combinatorial spinal cord repair strategies (Paper VI)

Methodological considerations. The standardized 25 mm weight drop on the spinal cord causes a reproducible, permanent motor deficiency in which the different types of control animals rarely exceed a BBB score of about 11. Since effects of different treatments in this model are often modest, ranging from 1-2 points on the BBB scale, we added a BBB subscore scale to better differentiate less obvious motor differences between groups. Preparation and delivery of OEC followed published protocols. In designing the cocktail delivery protocol, we needed to use dual osmotic pumps, since rolipram had to be dissolved using DMSO, and could be delivered subcutaneously, while aFGF and chondroitinase ABC was instead mixed and delivered directly to the site of injury. We therefore tested bioactivity of aFGF during prolonged exposure to body temperature and mixed with the enzyme to ensure that full activity was maintained. As a further test of the second osmotic delivery system we mapped the distribution of an oligonucleotide delivered in the same way and found widespread presence in the liquor spaces and adjacent walls of the brain and spinal cord. However, parenchymal penetration was only evident at the site of spinal cord injury.

Behavioral improvements. As expected, there was a robust, statistically significant, albeit modest improvement of outcome on the BBB score when OEC were grafted to the site of injury two weeks after the injury. This improvement was seen already one week after cell treatment, suggesting a protective effect. The OEC-treated animals maintained higher BBB scores than controls throughout the 16 weeks observation period. Thus we followed effects of OEC grafts for 14 weeks, which may be too short to determine final outcome of the procedure, particularly if eventually axon regeneration and/or synaptic plasticity at the cord or brain level is also involved. Using the BBB subscore (Lankhorst et al., 1999) that takes into account additional details of locomotor function of the hind limbs, such as paw placement, toe clearance, instability and tail position, the difference between OEC treated animals and controls became striking. The shape of the subscore curve over time also indicates that further improvement might occur after the 16 week end point of our observations. Studies with von Frey hairs at 9 weeks showed that OEC also normalized withdrawal thresholds, while cold or heat responses were not influenced by the grafting.

Delivery of the triple-drug cocktail appeared to have effects similar to the OEC treatment, although somewhat less robust. Effects were again seen from early time points after injury, sug-

gesting a neuroprotective role of the drug combination. Again, effects could also be detected by the BBB subscore. Perhaps unexpectedly, the combination of OEC and the drug cocktail did not lead to additive effects on behavior. While this may suggest that the effects of both treatments alone to protect from additional secondary damage does not help, it should be kept in mind that the combined procedure is more demanding for the animal. Thus a possible additive effect might have been masked by the more complicated surgery that these animals had to undergo. Evaluation of axon tracing experiments and spinal cord immunohistochemistry is needed to clarify these issues.

Functional MRI evaluations. We have previous experience of fMRI as a tool to determine the degree of sensory function across a site of injury in the spinal cord, the advantage being that the data are not dependent on a motoric behavior read-out. The percentage of presence of BOLD signals following severe spinal cord injury tends to increase in OEC-grafted group (67%) compared to the control (37%) although not significant. Previous studies have also indicated that there may be dissociations between sensory and motor deficits and recovery in rats. In fact, rats may display an almost normal gait as determined by BBB scoring, yet show very small BOLD signals in response to electric stimulation of the hind limbs (Hofstetter et al., 2005).

ACKNOWLEDGEMENTS

me stereology and bringing up fun chats; **Johan Lilja** for his brilliance in physics and software-writing for the fMRI experiment; Jeff Blackinton, **Petra Schweinhardt**, **Silvia Bürvenich**, and **Martin Werme**.

 Collaborators, colleagues and friends at Karolinska Institute: **Ole Kiehn** for his insightful comments for the experiment of gap junctions; **Bertil Fredholm** at the Department of Physiology and Pharmacology, for offering his expert advice on cAMP and adenosine functions in the CNS; **Fu-Hua Wang** and **Jan Kehr** for thoughtful and prompt revision of the gold-nanoparticles manuscript at the last minute; **David Xu** for teaching me the operation of confocal microscopy; **Niklas Holmström** and **Jonas Frisén** at the Department of Cell and Molecular Biology; **Niklas Lilja, Tua Finnman, Margareta Almström, Helena Ericsson, Anna Eriksson,** Annika Mattson at the animal department for their great assistance in animal care; **Eric Westman** and **Thomas Klason** at the MR center for the facility setup and computer assistance; **Göran Sandberg** and **Peter Wallén**, the student teaching organizers; **Tommy Nord** and **Lars Flemström; Cecilia Dominguez , Xiao-Jun Xu, Jing-Xia Hao, Zsuzsanna Wiesenfeld-Hallin** at Department of Neurotec and **Johan Nissen** and **Yi-Hai Cao** at the Department of Microbiology and Tumor Biology Center, for collaboration on the combinatorial treatments for spinal cord injury. Collaborators outside Karolinska Institute**: Ming-Chao Huang** at Taipei Veterans General Hospital for his delicate microsurgery for spinal root repair, and **Jeff Bulte** at John Hopkins Medical Institution for his well-advised comments for the superparamagnetic nanoparticles experiment.

I also want to thank a couple of friends outside of work: **Zong-Pei Chen** for her amusing companionship, from nightlife in Stockholm to reading *Cell*; **Kristina Trolle** for teaching me Swedish (I was lazy though) and **Mat Trolle** for giving me hearty suggestions about parenting; **Jeanette Wallstrom** for her gracious hospitality and artistic handy skills and **Erik Wallström** for showing me around at Department of Neurology, Karolinska Hospital; **Yuan-Hwa Chou, See-Tong Pang, Wen-Hui Weng** for helping me to adjust to life in Stockholm when I first arrived; **Ming-Lang Chen**, a director and a writer, for his transcendent works and the long-term friendship; **Yuxuan Jin** for her beautiful photography and hospitality at New York city; my former colleagues at Department of Neurology, Taipei Veterans General Hospital: **Yi-Chung Li** and **Wei-Ta Chen** for their encouragement and warmth; the Chief **Zin-An Wu, Bing-Wen Soong, Shang-Yeong Kwan, and Kon-Ping Lin** for their thought-provoking discussions.

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