Universität Hamburg

# Murine neural stem cells engineered to express the neural adhesion molecule L1 under the control of the human GFAP promoter promote functional recovery after transplantation in a mouse spinal cord injury model in *Mus musculus* (Linneaus, 1758)

**DISSERTATION**

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# I Abstract

Spinal cord injury is a devastating neurological disorder. Thus, more research efforts on combinational approaches are needed to facilitate regenerative effects. The transplantation of neural stem cells (NSC) can promote survival or replacement of injured neurons, axonal growth, and reconnection with appropriate targets without tumor formation. However, grafted neural stem cells differentiate mainly into astrocytes with only a minor fraction differentiating into neurons or oligodendrocytes thus hampering their application.

Therefore, one aim of stem cell research is to improve neuronal differentiation of neural stem cells. Moreover, the predominant astrocytic differentiation could be taken advantage of in order to deliver therapeutic genes beneficial for regeneration. An ideal candidate for a therapeutic target gene is the gene encoding the neural adhesion molecule L1. L1 plays an important role in the development and regeneration of the central and peripheral nervous system and improves neuronal differentiation, cell survival and neurite outgrowth.

To realize these aims, transgenic mice which ectopically express L1 under the control of the human GFAP promoter were used. In these mice neural cell adhesion molecule L1 is ectopically expressed in radial glia and mature astrocytes possibly promoting survival or replacement of injured neurons, axonal growth, and reconnection of axons with appropriate targets. Consequently, neural stem cells overexpressing L1 under the influence of the human GFAP promoter (hGFAP-L1 NSCs) were derived from these mice, analyzed *in vitro*, and transplanted into a mouse model of spinal cord injury to evaluate their regenerative potential.

It could be shown that L1 was expressed in radial glial cells and astrocytes differentiated from these hGFAP-L1 NSCs. Furthermore, L1 expressing-radial glia cells (L1-imm cells) were immunoisolated from cultures of hGFAP-L1 NSCs applying L1 antibodies *in vitro*. Both hGFAP-L1 NSCs and L1-imm cells showed increased neuronal differentiation, enhanced migration, and decreased astrocytic differentiation when compared to wild type (WT) NSCs *in vitro*. Moreover, ectopic expression of L1 on the cell surface of astrocytes enhanced neuronal differentiation, decreased astrocytic differentiation, and enhanced neurite outgrowth of cocultured wild type neural stem cells *in vitro*.

Mice transplanted with hGFAP-L1 NSCs and L1-imm cells into the compression-lesioned spinal cord showed better locomotor functional recovery as compared to mice that had been injected with WT NSCs or sham-injected with PBS. A novel electrophysiological H/M reflex recording method could confirm these findings at low frequency stimulation.

Morphological analysis revealed increased neuronal differentiation and migration of transplanted cells as well as increased numbers of endogenous catecholaminergic (tyrosine hydroxylase $[TH]^{+}$) axons caudal to the lesion site in the hGFAP-L1 NSC and L1-imm cell group when compared to the WT NSC and PBS groups. Furthermore, soma size and synaptic coverage of host motoneurons increased caudal to the lesion site in the hGFAP-L1 NSC and L1-imm cell group in comparison to the other experimental groups. The host tissue showed a decreased microgial reaction after syngeneic transplantation of NSCs into the lesioned spinal cord but no difference in the glial reaction was observed.

These findings indicate that hGFAP-L1 NSCs and L1-imm cells are capable of improving functional recovery in a syngeneic transplantation paradigm by modulating regenerative processes in the injured spinal cord thus emphasizing the beneficial potential of the neural cell adhesion molecule L1.

# II Introduction

## 1 The human spinal cord

The spinal cord is part of the central nervous system (CNS). It is a long, fragile tube-like nervous tissue structure that reaches from the end of the brain stem almost to the bottom of the spine. It is encased by a series of back bones, the vertebrae, which allow for flexibility of the back and protect the delicate spinal tissue. In most adults, the spine is composed of 26 individual vertebrae. Like the brain, the spinal cord is covered by three layers of meninges, the pia mater, the arachnoid mater, and the dura mater. The spinal cord and meninges are surrounded by the spinal canal, which runs through the center of the spine (Figure 1A).

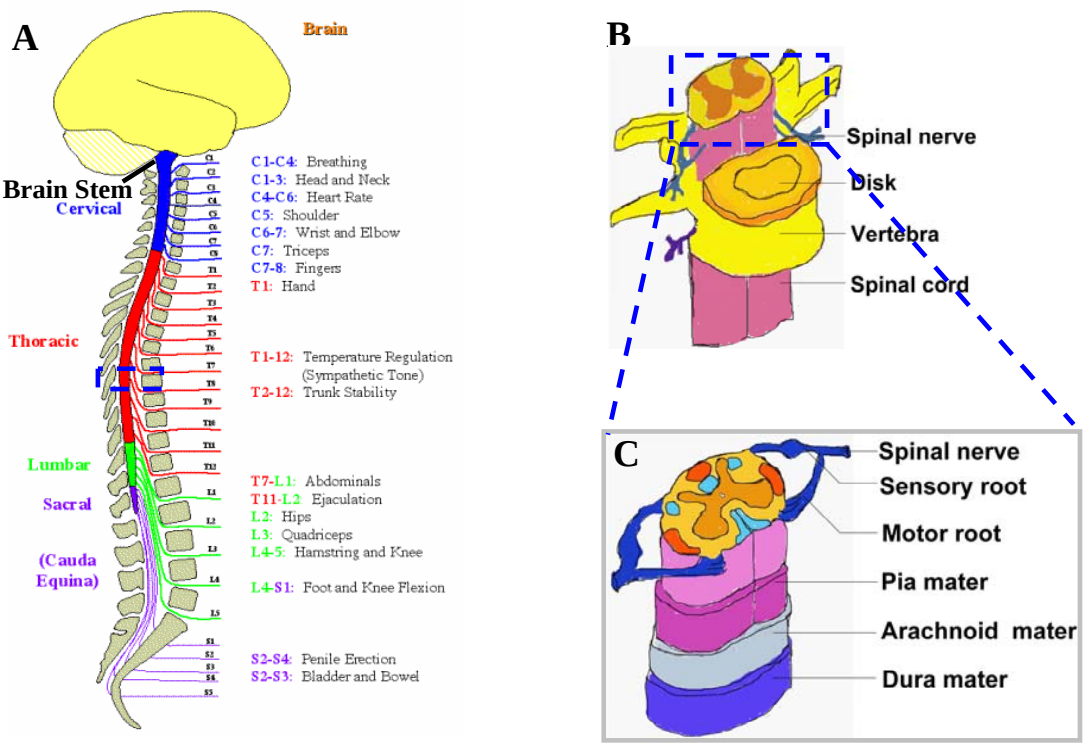


**Figure 1. Structure of spinal cord.** (A) The spine consists of a column of vertebrae. The spinal cord is encased in the spine and is arranged segmentally. (B) Between the vertebrae are disks (cartilage), which help cushion the spine and give it some flexibility. (C) 31 pairs of spinal nerves emerge from the spinal cord between the vertebrae in two short roots: one at the ventral (motor root) and one at the dorsal (sensory root) of the spinal cord. Spinal nerves are responsible for conveying information from and to specific parts of the body. Like the brain, the spinal cord is covered by three layers of meninges (pia, arachnoid, and dura mater). Figure 1A adapted from http://www.reeve.uci.edu/anatomy/scns.php with permission.

The spinal cord is segmentally arranged. The 31 segments in the spinal cord are grouped into 5 major divisions: cervical (8 segments), thoracic (12 segments), lumbar (5 segments), sacral (5 segments) and coccygeal (1 segment) (Figure 1A). At each spinal segment, sensory and motor roots emerge forming nerves that connect the spinal cord with specific parts of the body. The motor roots carry commands from the brain and spinal cord to effector organs of the body, particularly to skeletal muscles. The sensory roots provide information to the brain from receptor organs of the body (Figure 1C). Different segmental levels of the spinal cord control and receive information from different parts of the body. For example, cervical level 1-4 (C1-4) is involved in the control of breathing (Gray et al., 1995).

The organization of the spinal cord is elegant. Like the brain, it consists of gray and white matter. As seen in cross-section, the butterfly-shaped gray matter locates in the center of the spinal cord and contains the cell bodies of nerve cells (neurons). The ventral horns of the gray matter contain motor neurons, which convey information from the brain or spinal cord to muscles controlling movement. The dorsal horns consist of sensory nerve cells, which transmit sensory information through the spinal cord to the brain conveyed by sensory nerves from receptors in the body. The spinal cord conveys information between the brain and spinal cord through a number of long axonal pathways that mostly run in the white matter at the periphery of the spinal cord organized in: columns of nerve fibers that are extensions from nerve cells which transmit incoming sensory information from the rest of the body to the brain (ascending tracts); and columns that transmit outgoing impulses from the brain to the muscles (descending tracts). The main ascending pathways are indicated in red and the main descending pathways are indicated in blue (Figure 1C).

In summary, the spinal cord acts as the pathway that conducts incoming sensory information from the peripheral nervous system (both somatic and autonomic) and outgoing motor information between the brain and various effectors like skeletal muscles, cardiac muscle, smooth muscles, and glands. Furthermore, spinal cord is the center responsible for minor coordinating reflexes, such as the knee jerk reflex (Gray et al., 1995).

## 2 Spinal cord injury

The adult mammalian spinal cord is highly vulnerable to various insults. Spinal Cord Injury (SCI) is damage to the spinal cord that results in loss of functions such as mobility, feeling or control of bowel and bladder function below the level of the injury. SCI is a common cause of permanent disability and death in children and adult.

There are two common mechanisms of spinal cord injury. The first type of SCI is due to trauma, resulting in a bruise (called a contusion), a partial tear, or a complete tear (also called a transection) of the spinal cord. Frequent causes of traumatic SCI are automobile accidents, falls, gunshots, diving accidents, war injuries, etc. The second type of SCI is due to disease, such as polio, tumors, spina bifida, Friedreich's ataxia etc. Because the spinal cord contains bundles of nerve fibers carrying messages between the brain and the rest of the body, spinal cord injury of either traumatic or non-traumatic etiology often leads to the interruption of descending and ascending axonal tracts partially or completely isolating the segments of the spinal cord caudal to the level of injury from the brain which leads to devastating consequences: deficits in motor, sensory, or autonomic function below the level of the injury, including losses of voluntary movement and tactile sensibility, chronic pain, and spasticity. Therefore, the higher the injury occurs, the more severe the debilitation the patients will suffer from. For instance, injuries occurring at the lumbar level can result in paraplegia, the inability to move both lower limbs, as well as sexual and bladder dysfunction; cervical injuries can result in quadriplegia, the inability to move all four limbs (Bradbury et al., 2006; Thuret et al., 2006). Furthermore, the spinal cord does not show spontaneous activity like the brain. Thus, neuronal activity below the level of the injury is mainly controlled by limb movements and sensory stimuli from the moving limb. This leads to the relative lack of neuronal activity below the injury site in chronic SCI. The lack of neuronal activity is expected to interfere with most of the cellular mechanisms important for regeneration (Belegu et al., 2007).

In this study, we focus on traumatic SCI which can lead to a series of endogenous reactive changes in the normal structure of the spinal cord characterized by the death of cells, including neurons, oligodendrocytes, astrocytes and precursor cells, and the interruption of descending and ascending axonal tracts (Schwab et al., 1996; Horky et al., 2006).

Based on the timing of relevant reactive changes resulting from injury, the description of the mechanisms of SCI has conventionally been divided into the acute and the chronic phase. In order to maximize the benefits of any potential curative treatment, understanding the mechanisms contributing to severe spinal cord injury should be considered in developing regenerative treatments. Therefore, the temporal development of molecular and cellular reactions related to SCI must be optimally defined. Thus, three phases were defined: 1. Acute injury (primary and secondary phases) 2. Sub-chronic injury 3. Chronic injury. These are important in defining the time window during which therapeutic intervention might be beneficial (McDonald et al., 2002; Belegu et al., 2007).

## 2.1 Acute injury

### 2.1.1 Primary phase

In the acute phase of traumatic SCI, mechanical forces (contusion, compression, penetration or maceration) damage bone fragments, disc material, and ligaments. Direct compression of neural tissue by injured structures can damage both the central and peripheral nervous systems, as shown in Fig. 2.

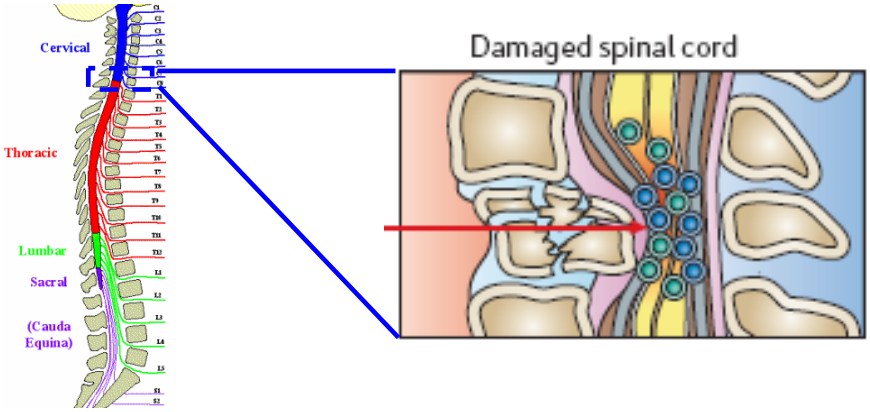


**Figure 2. Illustration of mechanical damages to the spinal cord.** Mechanical forces fracture or dislocate the vertebrae, disc material and ligaments of the spinal column that normally protect the spinal cord. Direct compression of neural tissue by fractured and displaced structures injures both the central and peripheral nervous systems. Figure adapted from Thuret S et al., 2006 with permission.

Blood vessels, which normally supply oxygen and nutrients necessary for the survival of neural cells and the function of the spinal cord, are damaged and may bleed. This bleeding (hemorrhage) can lead to hypoxic and ischemic events. Within minutes, the tissue swells rapidly to occupy the entire diameter of the spinal canal at the injury level.

Due to the bony nature of the wall of the vertebral canal, the swelling increases the pressure inside the spinal cord finally exceeding venous blood pressure which leads to deregulation of blood flow, release of toxic chemicals from disrupted neural membranes, and imbalance of extracelluar electrolytes. The immediate effect of trauma and these events is the spinal shock. The spinal shock, which generally resolves in 72 hours after SCI, is a state of transient physiological (rather than anatomical) depression of spinal cord function below the level of injury with associated loss of all sensorimotor functions (Atkinson et al., 1996; Belegu et al., 2007) which slows or completely blocks propagation of action potentials along axons. The duration of the shock correlates with the degree of encephalization of motor function, Encephalization is a measure of the extent to which the brain has increased in size to a degree greater than expected when taking body size into account (MacLean., 1990).

In the meantime, flaccid paralysis, including the bowel and bladder, is observed. These symptoms tend to last several hours to days until the reflex arcs below the level of the injury begin to function again (eg, bulbocavernosus reflex, muscle stretch reflex). But because of the direct interruption of the motor and sensory circuits in surrounding white matter at the lesion, different level of loss of motor and sensory function still remains (Tator et al., 1997; McDonald et al., 2002; Belegu et al., 2007).

### 2.1.2 Secondary phase

The initial insult to the spinal cord leads to a series of endogenous reactive pathophysiologic changes, mainly enhanced release of glutamate, severe oxidative stress, and inflammatory responses; these processes progressively destroying spinal cord tissue, are described as secondary injury (McDonald et al., 1999; Lee et al., 1999; McDonald et al., 2003).

Secondary injury is triggered by mechanical forces but the damages continually progress. A number of cell bodies and/or processes of neurons are directly damaged (McDonald et al., 1998; Belegu et al., 2007). These neurons might die and they are not replaced in most cases (although the adult spinal cord has endogenous stem cells). Furthermore, the injured cells, axons, and blood vessels release toxic chemicals attacking neighboring cells, and axons. The damaged cells release high level of glutamate, an excitatory neurotransmitter, which plays an important role in a disruptive excitotoxic process. In the normal intact spinal cord, only a small amount of glutamate

is secreted and binds to receptors on target neurons stimulating electrical activity in the target cells. In contrast, in the injured spinal cord, a large amount of glutamate is emitted from damaged spinal neurons, axons, and astrocytes and over-activates glutamate receptors on neighboring neurons. The over-activation of cells triggers a series of damaging events, including calcium influx and dyshomeostastis, which, results in increased protease activity (protease activity is fundamental to many key biological processes such as cell growth, cell death, blood clotting, matrix remodeling and immune defense), loss of mitochondrial function (the main function of the mitochondria is to create energy-rich metabolites (ATP) for cellular activity by the process of aerobic respiration), and increased oxidative stress (the production of peroxides and free radicals damages all components of the cell, including proteins, lipids, and DNA; severe oxidative stress can cause cell death and even moderate oxidation can trigger apoptosis, while more intense stress may cause necrosis). These changes can affect cell membrane components and lead to further neuronal excitotoxicity, which is mainly transduced by N-methyl-D-aspartate (NMDA) receptors. Another group of important cells, oligodendrocytes, are also vulnerable to excitotoxicity, mediated primarily by glutamate receptors called α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors and kainate receptors, especially under acidosis conditions (Matute et al., 1997; McDonald et al., 1998; Belegu et al., 2007).

Following trauma, the inflammatory response occurs: inflammatory cells (including neutrophils, microglia, macrophages and T cells) react to signals from damaged tissue and invade the lesion through the disrupted blood-brain barrier (Belegu et al., 2007). In the healthy spinal cord, the blood-brain barrier (BBB) is both a physical barrier and a system of cellular transport mechanisms structure in the central nervous system. It maintains homeostasis by restricting the entrances of potentially harmful chemicals from the blood, and by allowing the entrance of essential nutrients between the bloodstream and the neural tissue itself; most types of inflammatory cells enter the CNS only rarely unless the BBB has been damaged by trauma or disease. As described, the spinal cord is surrounded by the meninges, when they are damaged and/or inflamed, the blood-brain barrier may be disrupted. This disruption may increase the penetration of various substances into the brain. These invading cells together with resident inflammatory cells have both harmful and beneficial properties concerning recovery of the injured spinal cord. For example, macrophages and monocytes, which appear to be the key immune cells in spinal cord injury, scavenge cellular debris. Moreover,

macrophages, monocytes, and microglial cells release a host of regulatory cytokines and chemokines that may help or prevent recovery after injury. Potentially beneficial substances released by these cells include the cytokines TGF-beta (transforming growth factor-beta) and GM-CSF (granulocyte-macrophage colony-stimulating factor) and several other growth factors. Apparently, detrimental products include cytokines such as TNF-alpha (tumor necrosis factor-alpha), IL-1-beta (interleukin-1-beta), and chemicals, for instance, free radicals, superoxides, and nitric oxide may contribute to oxidative damage (Matute et al., 1997; McDonald et al., 1998; Belegu et al., 2007).

Furthermore, astrocytes are activated, become hypertrophic and express several extracellular surface molecules and cytokines. Thus, reactive astrocytes, glial progenitors, microglia, macrophages, fibroblasts, and meningeal cells establish a dense cellular response initiating the process of scar formation (Belegu et al., 2007).

Collectively, the events that occur during secondary injury result in continued and selective cell death and demyelination in the previously intact tissue immediately adjacent to the injury epicenter, which leads to increased lesion size beyond the original site of injury (Jones et al., 2005).

### 2.2 Sub-chronic injury

The sub-chronic phase of spinal cord injury follows primary and secondary injury. The prominent feature of this stage is delayed cell death of degenerating oligodendrocytes in the white-matter protracting in a time-dependent longitudinal manner from the site of injury for weeks after the initial event (Crowe et al., 1997; Liu et al., 1997; Shuman et al., 1997; Abe et al., 1999; Li et al., 1999; Springer et al., 1999; Beattie et al., 2000; Casha et al., 2001).

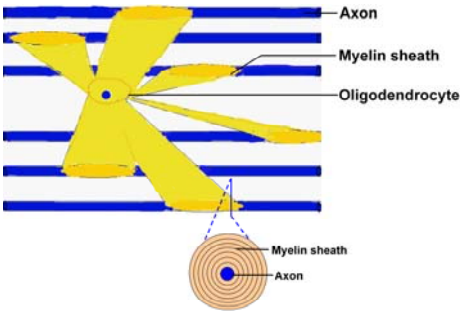


**Figure 3.** In the central nervous system, myelin is made by oligodendrocytes. Each oligodendrocyte wraps around an axon forming insulating myelin. Normal myelin is formed by approximately 30 wraps round an axon.

From a functional point of view, the delayed death of oligodendrocytes and the resulting demyelination of axons are critical events, because unmyelinated axons do not conduct electrical impulses physiologically. A wave of apoptosis of oligodendrocytes occurs in the white matter at about 7 days after injury. Interestingly, because oligodendrocytes myelinate multiple axon segments (Fig. 3) and loss of a single segment of myelin renders an axon dysfunctional, a large subset of axons crossing the lesion eventually become non-functional despite the axon remaining physically intact. Optimal strategies for rescuing nerve cells may be different from optimal strategies for saving oligodendrocytes (Casha et al., 2001).

And another prominent feature is the initiation formation and gradual maturation of a scar around the lesion that often appears impenetrable for regrowing axons (Bruce et al., 2000; Bunge et al., 1997; Fawcett and Asher, 1999; Fitch and Silver, 1999; Dawson et al., 2000; Jones et al., 2002a; Jones et al., 2003; Guest et al., 2005).

### 2.3 Chronic injury

After the acute and sub-chronic injury phase, spinal cord injuries proceed to the chronic SCI stage. In this stage, the scar, which consists of reactive astrocytes, meningeal fibroblast, microglia, macrophages, and accumulating cellular debris, is established and functions as a cellular barrier to axonal regeneration, as shown in Figure 4 (Fawcett et al., 1999; Jones et al., 2005; Belegu et al., 2007).

The scar tissue also contains secreted and transmembrane molecules inhibitory for axon growth, including chondroitin sulfate proteoglycans (CSPGs) (Lemons et al., 1999; McTigue et al., 2001; Plant et al., 2001; Jones et al., 2002a,b, 2003a,b; Qi et al., 2003; Tang et al., 2003), keratan sulfate proteoglycans (KSPGs) (Jones et al., 2002), myelin-associated inhibitory molecules including myelin-associated glycoprotein (MAG), myelin oligodendrocyte glycoprotein (MOG), oligodendrocyte myelin glycoprotein (OMgp), and Nogo-A (Mukhopadhyay et al., 1994; GrandPre et al., 2000; Sandvig et al., 2004; Buchli et al., 2005) as well as other inhibitory molecules including tenascins (Deckner et al., 2000), ephrins (Miranda et al., 1999; Rodger et al., 2001; Sandvig et al., 2004; Benson et al., 2005), and semaphorins (Pasterkamp et al., 2000; DeWinter et al., 2002; Moreau-Fauvarque et al., 2003; Sandvig et al., 2004). This extracellular and glial scar is thought to be a major impediment factor limiting axonal regeneration and leading

to misguided and aberrant axon arrangement after CNS injury (Fawcett et al., 1999; Grimpe et al., 2002).

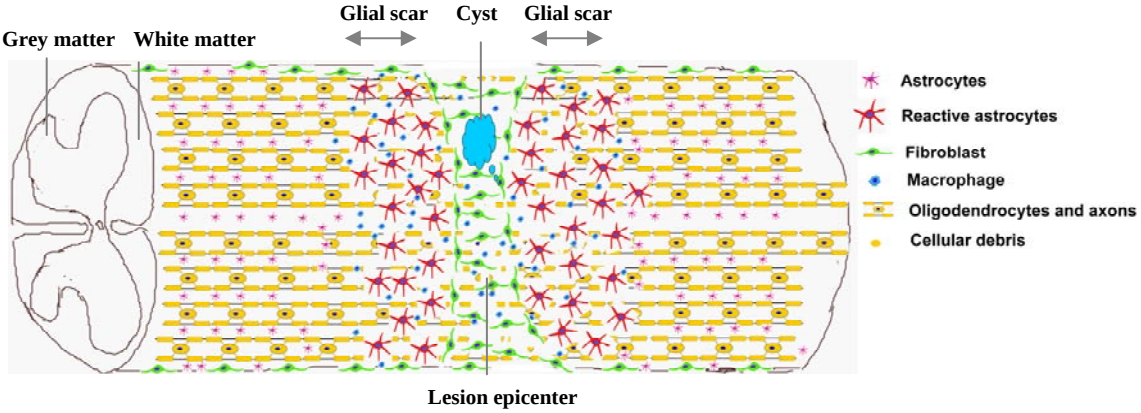


**Figure 4. Scheme of the chronic phase of spinal cord injury.** Injury to the adult CNS often results in the transection of nerve fibres and damage to the surrounding tissue. The distal ends of the severed axons are exposed to the damaged glial environment. During the early phase of injury, myelin-associated inhibitors from intact oligodendrocytes and myelin debris can restrict axonal regrowth. Recruitment of inflammatory cells and reactive astrocytes over time leads to the formation of a glial scar, often accompanied by a fluid-filled cyst. This scarring process is associated with the increased release of chondroitin sulphate proteoglycans, which can further limit regeneration. Together, these molecular inhibitors of the CNS glial environment present a hostile environment for axon repair.

The formation and maturation of the glial scar is often accompanied by a fluid-filled cyst (Silver et al., 2004; Yiu et al., 2006). In the context of chronic SCI, a cyst is an oval intramedullary lesion. Typically, spinal cord injury leads to the loss of central gray matter, cells die immediately and progressively. The loss of spinal cord tissue is not confined to the center of the lesion; it can spread up to one to one and one-half segmental levels of the spinal cord or sometimes even more leading to a central cavitation filled with cerebrospinal fluid (CSF). Twenty-five percent of patients with spinal cord injury will develop an enlarging spinal cord cyst (or syrinx) between 2 months and 30 years after their initial injury. These cysts can cause progressive weakness and numbness (Brodbelt et al., 2003).

During the entire acute and chronic phase of spinal cord injury, the distal axonal segment (the part disconnected from the neuronal cell body) retracts from postsynaptic neurons and undergoes degeneration (Becerra et al., 1995), while the proximal segment, which typically survives after injury, does not spontaneously regenerate. Therefore, inducing damaged axons to regrow, and to reconnect to appropriate targets is the one of the major goals in experimental research to restore function after spinal cord injury.

In addition to loss of motor and sensory function, other important dysfunction including pain and numbness, bladder, bowel, sexual dysfunction, and reduction of autonomic dysreflexia (blood pressure, heart rate, temperature regulation, and neuroendocrine dysfunction) (Belegu et al., 2007). Chronic impairments of these systems results in progressive aggravation of overall health. Understanding the detailed mechanisms contributing to this spinal cord injury should aid in developing regenerative treatments to offset such an enormous loss of function.

## 3 Therapy

It is well-recognized that SCI leads to the death of neurons, oligodendrocytes, astrocytes, and neural precursor cells, the interruption of descending and ascending axonal tracts leading to changes in the conduction of electrical impulses. The injury to the spinal cord can result in dramatic disability, including loss of voluntary movement and tactile sensibility and chronic pain and spasticity. Sometimes SCI is fatal (Belegu et al., 2007).

Despite these devastating damages, commonly observed pathological features show that there is only a limited physiologic capacity for endogenous spontaneous repair. First, there is little or no neurogenesis in the injured spinal cord to replace dead neurons, although the adult spinal cord contains neural stem/progenitor cells. Second, limited axonal sprouting occurs, but this process is predominantly hampered by molecular and cellular factors, and few injured nerve fibers regenerate to reconnect to their original targets over long distances in the adult mammalian central nervous system (Beattie et al., 1997; Yamamoto et al., 2001; Azari et al., 2005; Horky et al., 2006; Yang et al., 2006). So, although some spontaneous recovery was seen after spinal cord injury, it is often incomplete, therapeutic intervention is necessary. To enhance functional recovery, a combination of variable therapeutic interventions is very necessary and crucial.

Based on these ideas, attempts to promote recovery of the central nervous system CNS after injuries have focused on three main goals: i) the promotion of axonal growth (regeneration), ii) the protection of neural cells from cell death (neuroprotection), and iii) cell transplantation to generate new neurons and myelinating cells that will repopulate the injury site and integrate functionally into the host neural tissue to bridge any cysts or cavities and/or create a favorable environment for axon regeneration (Ziv et al., 2006).

To realize these goals, experimental therapies for regeneration in CNS injuries can be either achieved by replacing injured tissue by exogenous cell transplantation (*Cellular*

*therapeutic interventions*) or ameliorate neuronal cell death and enhance endogenous regeneration by the application of neurotrophic factors or growth factors and mitogens or inhibitors directed against inhibitory molecules or their receptors by direct application or vector-based gene therapy (*Molecular therapeutic interventions*) (Fig. 5).

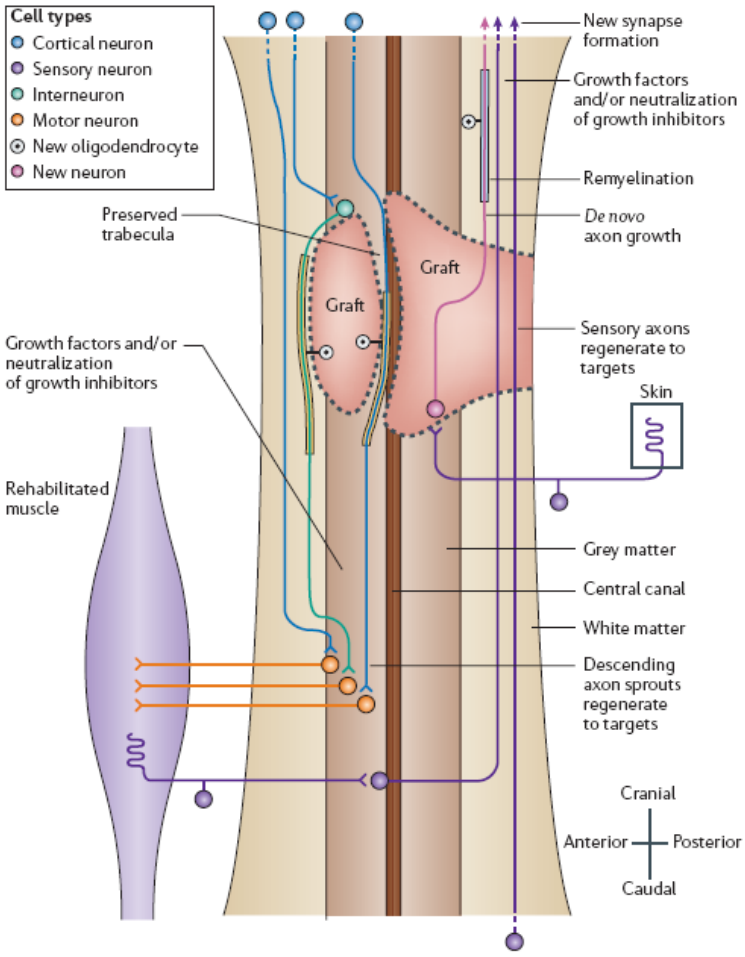


**Figure 5. Therapeutic intervention for SCI.** Scheme depicting a sagittal view through injured spinal cord after a potential combination of therapeutic interventions. Cellular grafts eliminate cyst and trabeculae are spared. Potential beneficial antibodies, peptides, or enzymes are applied to neutralize inhibitory molecules in the glial scar regions and in the intact spinal cord. Grafts replacing injured neurons and remyelinating cells by exogenous cell transplantation. Graft derived neurons can function as relay of neural circuits; Transplanted cells could also promote axonal regeneration and reconnect with appropriate targets. Furthermore, intervention may stabilize correct synapses and muscle atrophy to be reversed. Figure adapted from Thuret S et al., 2006 with permission.

### 3.1 Cellular therapeutic interventions

It is known that neural progenitor cells exist even in the spinal cord with the potential capacity to divide and differentiate into neurons, astrocytes, and oligodendrocyte, although they have only a very limited ability to generate new functional neural cells in

response to injury (Yamamoto et al., 2001; Azari et al., 2005; Horky et al., 2006; Yang et al., 2006).

Cell-based approaches involve transplantation of neural (e.g., neural stem cells or fate-restricted neuronal or glial progenitor cells, myelin-forming oligodendrocyte precursor cells, embryonic stem cells, embryonic CNS tissue and fetal spinal cord tissue, schwann cells, olfactory ensheathing glia) and non-neural tissue elements (marrow stromal cells, activated macrophages).

Cellular transplantation has several aims: to replace cell loss, to restore damaged neuronal pathways or reconstructing intraspinal synaptic circuitries by either regeneration or neuronal/glial replacement, and to provide a favorable environment to facilitate endogenous axon regeneration (Thuret et al., 2006).

### 3.1.1 Stem cells

A stem cell, regardless of the tissue of origin, has the capacity both to self-renew and to generate several types of differentiated progeny (multipotency) by asymmetric cell division (Gage, 2000). This means that at least one of the two progeny after a mitotic cell division remains a stem cell enabling a stem cell population to be retained being a prerequisite for the continuous production of new cells over long periods of time.

Mammalian embryogenesis elaborates distinct developmental stages in a strict temporal order. The vast majority of the somatic cells are derived from pluripotent precursors of the inner cell mass of the blastocyst which, when cultured *in vitro* are called embryonic stem cells (ESC). At around the time of gastrulation, distinct groups of precursors segregate from these ESCs into specific cell lineages found in the three germ layers ectoderm, mesoderm, and endoderm. Gradually these precursors mature into organ- and tissue-specific somatic stem cells which account for the progressive growth of the tissue mass during development (Faust et al., 1993). Somatic stem cells decrease in number with the tissue approaching maturity. In tissue where continuous cell replacement is necessary they reach a steady state level that is maintained throughout adult life. This has been clearly documented for the hemopoietic system, the epidermis, and the intestinal epithelium. The forebrain subventricular zone lining the lateral ventricles and the subgranular zone of the dentate gyrus of the hippocampus have been recognized as germinative zones proving the existence of neurogenesis and the presence of stem cells even in the adult mammalian CNS (Gage, 2000).

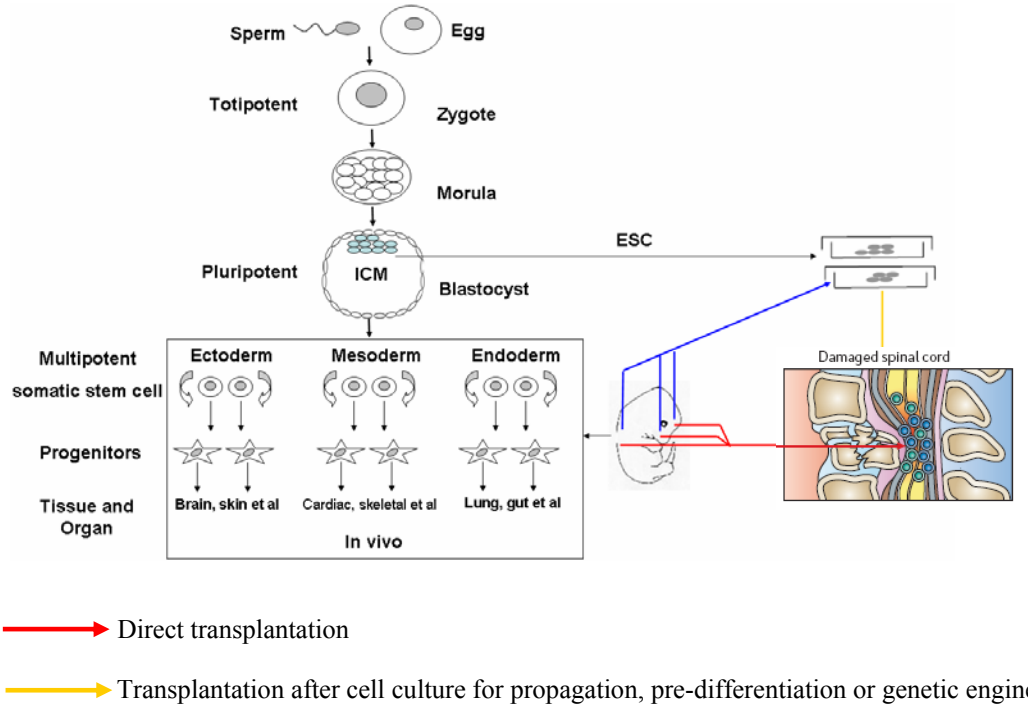


**Figure 6. Potential stem cell sources for cell replacement therapy for the injured spinal cord.** Zygotes and cells developing in their early cell divisions up to the morula stage are defined as totipotent because they can give rise to a complete organism and contribute to the placenta. Embryonic stem cells (ESC) are pluripotent cells that can give rise to every cell of the organism. Multipotent somatic stem cells, like CNS and hemopoietic stem cells, comprise the capacity of giving rise to many but not all cells originating from developmentally different germ layers. Stem/progenitor cells can be harvested: from the inner cell mass (ICM) layer, cultured as ESC; from the brain, spinal cord, olfactory system, bone marrow or umbilical cord of the fetus, cultured as embryonic somatic stem/progenitors, and from the adult organism, cultured as adult somatic stem/progenitors. Each of these cell populations can be propagated in cell culture and engineered to express beneficial molecules of interest, or pre-differentiated to a particular cell fate before transplantation in the injured spinal cord. Some of these cells could be transplanted directly into the injured spinal cord.

Recent animal studies have shown that stem cell transplantation strategies hold great promise to enhance functional recovery in SCI. There is no consensus yet on what kind of stem/progenitor cell is an ideal source for cellular grafts. Three major types of stem/progenitor cells have been applied in cell therapy in animal models of SCI: neural stem cells, embryonic stem cells, and bone marrow mesenchymal stem cells. These cells can potentially promote functional recovery by reconstituting damaged circuits, remyelinating axons, and increasing plasticity and/or axonal regeneration (Figure 6).

Also, endogenous stem/progenitor cells are present at the injury site and are actively dividing. Controlling their proliferation and fate might provide an alternative to transplantation.

#### 3.1.1.1 Neural stem cells, neuronal or glial progenitor cells

During development, neuroepithelial stem cells in the embryonic ventricular zone, an epithelial layer of neural stem and progenitor cells that lines the ventricles generate most of the neurons and glial cells in the brain. Once they exit the cell cycle, the immature, postmitotic neurons migrate out of the ventricular zone on the processes of radial glial cells, a specialized class of neural stem or progenitor cells that retain contact with both the ventricular and pial surfaces, to form the cortical plate, which eventually becomes the gray matter of the cerebral cortex (Kandel et al., 2000). The radial glia cells, initially only thought to be scaffolding cells for the migrating neurons on their way to the appropriate layer of the developing cortex, have recently been shown to comprise both stem cells and their more restricted mitotic derivatives (Gotz et al., 2002; Noctor et al., 2002; Malatesta et al., 2003; Rakic, 2003; Anthony et al., 2004). It is believed that radial glial cells are multipotent, both giving rise to neurons early in development and serving as a scaffold for migration, and later involuting to generate glial precursors and parenchymal astrocytes (Rakic et al., 2003).

As described, somatic stem cells decrease in number with approaching maturity. Subsequently, neurogenesis in the adult mammalian brain is restricted to the subventricular zone of the lateral ventricles and the subgranular zone of the dentate gyrus of the hippocampus (Gage, 2000). In both areas neural stem cells regenerate neurons in the adult mammalian brain.

Neural stem cells can be isolated from both embryonic and adult tissue and cultured *in vitro* by dissecting out a region of the fetal or adult brain that has been demonstrated to contain dividing cells *in vivo*, like the subventricular zone or the hippocampus in the adult or a larger variety of structures in the developing brain like the ganglionic eminence of the developing striatum or the spinal cord. Derived cells then are exposed to a mitogens such as fibroblast growth factor-2 (FGF-2) and/or epidermal growth factor (EGF) giving rise to either clonally growing spheroid bodies (neurospheres) in suspension culture or proliferating monolayer neural precursor cells on a matrix as a substrate for binding. After withdrawal of the mitogen, these precursors readily

differentiate to a various extent into all neural lineages (Gage, 2000). After transplantation into the CNS, neural precursor cells generated from different origins of the brain and propagated *in vitro* are able to rescue damaged host tissue and differentiate into appropriate site-specific cells for tissue replacement (Campbell et al., 1995; Lundberg et al., 1997; Björklund, 1999; Björklund et al., 2000).

Several features make neural stem/progenitor cells promising candidates for therapies to repair or treat neurological injuries and disorders: neural stem cells or fate-restricted neuronal or glial progenitor cells comprise a more homogeneous population without non-neural cells, they can be expanded *in vitro* for a limited amount of time, and they can be transplanted in a not terminally differentiated state enhancing their capacity for survival, migration, and differentiation *in vivo*. Moreover, they have the capacity to become neurons or glial cells after transplantation into the spinal cord (injured or intact) (Herrera et al., 2001; Cao et al., 2002; Cao et al., 2002; Han et al., 2002; Han, et al., 2004; Yan et al., 2004).

It is reported that *in vitro*-expanded neural stem cells derived from fetuses are able to generate neurons *in vivo* and improve motor function upon transplantation into an adult rat spinal-cord-contusion injury model (Ogawa et al., 2002; Teng et al., 2002). Human fetus-derived CNS stem cells grown as neurospheres survived, migrated, and differentiated into neurons and oligodendrocytes after long-term engraftment in spinal cord-injured immuno-suppressed mice associated with locomotor recovery. Importantly, the functional recovery was reversed when the transplanted cells were ablated indicating that the transplanted cells were responsible for recovery (Cummings et al., 2005). Moreover, human neural progenitors have also been transplanted into non-human primates after spinal cord contusion, which also result in modest functional locomotion improvement (Iwanami et al., 2005). Interestingly, implantation of the multicomponent polymer scaffold seeding with neural stem cells unit into an adult rat hemisection model of SCI increased the efficacy of cellular transplantation strategies and promoted long-term improvement in function (persistent for 1 year in some animals) relative to a lesion-control group (Teng et al., 2002). Moreover, it is reported that embryonic radial glia bridge spinal cord lesions and promote functional recovery following spinal cord injury (Cao et al., 2005; Hasegawa et al., 2005).

Transplantation of neuronal and glial restricted precursors into contusion SCI rat model improved bladder and motor function and decreased thermal hypersensitivity. The cells

survived and differentiated into neurons and glia cells, resulting in increased sparing/sprouting of descending pathways, which prevented sprouting of dorsal root axons, contributing to the recovery of function in lumbosacral circuitries (Mitsui et al ., 2005b).

These features suggest neural stem cell or fate-restricted neuronal or glial progenitor cell transplantation as a potential treatment for SCI. Another great advantage of neural stem cell transplantation when compared to ESC is that no tumour formation was reported. However, grafted neural stem cells differentiate mainly into astrocytes with only a minor fraction differentiating into neurons or oligodendrocytes. Thus, more research efforts on combinatorial approaches are needed to facilitate regenerative effects.

#### 3.1.1.2 Embryonic stem cells.

Embryonic stem cells (ESC) are pluripotent cells derived from the inner cell mass the of developing blastocyst (Martin et al., 1981; Evans et al., 1981) that can replicate indefinitely under appropriate culture conditions for potentially indefinite periods of time while maintaining genetic stability (Suda et al., 1987).

Many experiments have been done in effort to show that ESC can be used to repair or treat SCI. Firstly, it has been shown that ESC-derived neural progenitors transplanted into the rat spinal cord contusion SCI model, survived for several weeks and differentiated into astrocytes, oligodendrocytes, neurons, and led to axonal remyelination with partial functional recovery after transplantation (McDonald et al., 1999; Liu et al., 2000; Harper et al., 2004; Faulkner et al., 2005; Keirstead et al., 2005). Secondly, ESC can secrete neurotrophic factors which can prevent neural cell loss and induce proliferation of oligodendrocytes and myelination of regenerating axons after SCI (Enzmann et al., 2006). Moreover, ESCs were genetically manipulated to express functional markers or other genes of therapeutic value. Murine ESC engineered to express the neural cell adhesion molecule L1 transplanted into the compression-lesioned mouse spinal cord survived better and migrated rostrally and caudally from the lesion in contrast to control cells with corticospinal axons showing interdigitation with L1-overexpressong ESCs and extending into and, in some cases, beyond the lesion site (Chen et al., 2005). These therapeutic strategies may contribute to a successful treatment for spinal cord injury in the future (Hamada et al., 2006).

However, a potential danger of ESC-based therapy is the occurrence of teratocarcinomas after grafting (Björklund et al., 2002) even after transplantation of ES-derived neural precursor cells (Erdo et al., 2003). The formation of teratocarcinomas raises serious safety concerns about the potential use of human embryonic stem cells in the therapy of neurodegenerative diseases and spinal cord injury.

### 3.1.2 Embryonic CNS tissue

Primary fetal neuronal tissue has shown promise for the amelioration of certain neurologic conditions after transplantation into the human brain (Dunnett et al., 2000). It was observed that after transplantation of fetal spinal cord tissue into the lesion site, a number of host axons regrow into the transplant (Jakeman & Reier, 1991; Bregman et al., 1997). Furthermore, recovery of function after spinal cord injury was observed (Kunkel-Bagden et al., 1990; Reier et al., 1992; Bregman et al., 1993; Coumans et al., 2001; Bregman et al., 2002). Mechanisms underlying transplant-mediated recovery of function might be caused by the transplants acting as relays (transplanted neurons are innervated by proximal host neurons and project in turn to distal host neurons to transduce signals). In a clinical trial, tissue of fetal spinal cord was transplanted intraspinally to patients with syringomyelia. Neurophysiological feasibility and safety were assessed: cyst cavities were obliterated, but no functional improvement was observed (Falci et al., 1997).

However, the heterogeneity of the grafted tissue, the limited availability of sufficient amounts of human fetal tissue, and the poor survival of grafted cells limit the use of fetal tissue. For example, to collect a sufficient number of dopaminergic (DAergic) neurons for transplantation to treat one patient with Parkinson's disease, six to eight fetal donors per patient are needed, primarily because of the low post-transplantation survival rate of grafted fetal DAergic neurons. (Branton et al., 1999; Barinaga et al., 2000; Olanow et al., 2001; Williams et al., 2001; Li et al., 2002).

### 3.1.3 Other cell resources

There are some other cell resources, for example, Schwann cells, olfactory ensheathing cells, haematopoietic stem cells (HSCs), bone marrow stromal cells (BMSCs), also known as mesenchymal stem cells, and activated macrophages for cellular therapy of SCI.

After implantation of Schwann cells (SC), the myelin-forming glial cell of the peripheral nervous system, into the injured adult rat thoracic spinal cord, sensory and spinal axons with cell bodies near the grafts extend into these bridging grafts, become myelinated (Xu et al., 1995, 1997, 1999), are electrophysiologically active (Pinzon et al., 2001) promoting recovery of hindlimb function in some studies (Guest et al., 1996; Takami et al., 2002).

Olfactory ensheathing cells (OEC) are supporting cells that wrap around olfactory axons and facilitate their regeneration throughout the life of mammalian species. These cells are relatively easy to obtain from nasal biopsies and thus could provide a source of autologous cells for transplantation. In some cases, remyelination of axons and regeneration of damaged axons was reported along with a surprising degree of functional recovery (Li et al., 1997; Sasaki et al., 2006) after OEC implantation into several different acute and chronic models of rodent SCI. However, it is not yet clear whether they can be expanded in sufficient numbers for the application in human cell replacement strategies.

Implantation of HSCs (Koshizuka et al., 2004; Koda et al., 2005), BMSCs (Wu et al., 2003), and activated macrophages (Rapalino et al., 1998; Bomstein et al., 2003) has also been shown to result in partial recovery in paraplegic rats.

Although this progress has generated considerable enthusiasm about treating spinal cord trauma, there are many unresolved issues including the selection of the optimal source of cells, age of cells (embryonic versus adult), and transplantation method (for example, injection of suspensions or transfer within a cellular matrix), to be considered regarding the safety and efficacy of treatment (Boyd et al., 2005). Moreover, the functional outcomes reported must be interpreted with caution because many are primarily based on one evaluation protocol without including other behavioural/electrophysiological assessments (Reier et al., 2004).

### 3.2 Molecular therapies

#### 3.2.1 The neural cell adhesion molecule L1

The development of the spinal cord depends on a temporally and spatially precise regulation, including interactions of nerve cells with each other and their extracellular matrix environment. Cell adhesion molecules, which are displayed at the cell surface and mediate manifold functions, are very important for mediating these interactions.

They act as receptors for soluble factors and interact with extracellular matrix molecules and other cell adhesion molecules, involved in cell proliferation, migration, fate determination, guided neurite outgrowth, and synapse formation, stabilization, and modulation. These molecules are also important for cell survival, and, in particular, in regeneration of function after injury (Maness et al., 2007).

One of these cell adhesion molecules is L1, which is characterized by the extracellular L1-cassette, consisting of six Ig-like domains followed by five fibronectin type III repeats, a transmembrane region, and a short cytoplasmatic domain (Holm et al., 1996). In the CNS, L1 is restricted to neurons. It is detectable on unmyelinated axons of postmitotic neurons throughout adulthood. In the peripheral nervous system (PNS), L1 is also expressed by Schwann cells prior to myelination and under conditions of regeneration (Persohn and Schachner, 1987; Martini and Schachner, 1988; Bartsch et al., 1989). Moreover, the expression of L1 is increased in motor neurons by exercise and training, correlating with a BDNF response (Macias et al., 2002). Furthermore, L1 is not expressed in the nervous system only, but also in the hemopoietic cell lineage, in proliferating epithelial cells of the intestinal crypts, and in the cells of several peripheral tumors (Thor et al., 1987).

Mutations of the L1 gene can result in severe human neurological disorders and cause a number of related syndromes summarized as L1 syndrome, formerly known as CRASH-syndrome: hydrocephalus as a result of stenosis of the Aqueduct of Sylvius (HSAS), mental retardation, aphasia, shuffling gait, and adducted thumbs (MASA), and X-linked spastic paraplegia (XLSP). The CNS of patients with L1 mutations has anatomical malformations including enlarged ventricles or hydrocephalus, hypoplasia or aplasia of the corticospinal tract, corpus callosum, or septum pellucidum, and fusion of the thalami or colliculi (Dahme et al, 1997; Cohen et al, 1997).

From a functional point of view, the different mutations in the extracellular or intracellular domains of the L1 protein resulting in severe human neuropathological disorders indicate that L1 plays critical role for neural development (Kenwrick et al., 1999). The molecule is not only an adhesion molecule but also a receptor that turns on a number of intracellular messengers that stimulate its multifaceted functions. It is involved in neuronal migration, differentiation, neurite outgrowth, and myelination (Brummendorf et al., 1998; Dihné et al., 2003) as well as axon guidance, fasciculation, and regeneration (Castellani et al., 2000; Zhang et al., 2000). L1 exerts these functions

via homophilic and heterophilic cell interactions, its heterophilic binding partners are RGD-binding integrins and TAG-1/axonin-1, F3/F11/contactin, NCAM, CD9, CD24, and phosphacan (Silletti et al., 2000).

Interestingly, several features make L1 a promising candidate for therapies to repair or treat SCI. During the development of the spinal cord, L1, associated with beta1 integrin, mediates axonal outgrowth in the embryonic spinal cord (Blackmore, 2006). L1 also modulates nerve-growth-factor-induced CGRP-IR fiber sprouting (Chaudhry, 2006) and serves as guidance for axonal pathfinding and fasciculation of thalamocortical projections (Wiencken-Barger et al., 2004). Mice lacking L1 have deficits in locomotion (Jakeman et al., 2006). *In vitro* substrate-bound L1 also promotes neuronal attachment and neurite outgrowth in the presence of astrocytes and fibroblasts (Webb et al., 2001). Ectopic expression of L1 in astrocytes in transgenic mice not only leads to better learning and memory and higher flexibility in relearning (Mohajeri et al., 1996), but also results in faster progression of corticospinal axon growth in development (Ourednik et al., 2001). Furthermore, it enhances cell survival (Chen et al., 1999) and synaptic plasticity (Luthi et al., 1996; Saghatelyan et al., 2004). A reduced number of neurons in the hippocampus of adult L1-deficient mice raised the possibility that L1 may be involved in neurogenesis (Demyanenko et al., 1999).

Particularly pertinent for the therapy of spinal cord injury are the following experimental findings: in the lesioned spinal cord, L1 expression is increased surrounding the lesion site in neonatal rats with complete spinal cord transection (Kubasak et al., 2005). Furthermore, it can overcome inhibitory cues *in vitro* as represented by the repellent activity of semaphorin3A (Castellani et al., 2000) and the inhibitory chondroitin sulfate proteoglycan NG2 (Jones et al, 2003a). Delivery of soluble cell adhesion molecule L1-Fc promoted locomotor recovery in rats after spinal cord injury (Roonprapunt et al., 2003). Recently, adeno-associated virus-mediated L1 expression promoted functional recovery after spinal cord injury (Chen et al., 2007). The effect of L1 in spinal cord injury has also been investigated by transplantation of cell adhesion molecule L1-transfected embryonic stem cells. The transplantation could enhance survival of transplanted cells and support limited regrowth of corticospinal tract axons in mice after spinal cord injury (Chen et al., 2005). Based on these findings, the neural cell adhesion molecule L1 is a promising candidate for therapeutic intervention of SCI.

### 3.2.2 Neurotrophic factors

Therapies for regeneration in spinal cord injuries can either ameliorate neuronal cell death or enhance endogenous regeneration by the application of neurotrophic factors. Neurotrophic factors are proteins that bind to receptors on the cell surface resulting in the activation of cellular proliferation and/or differentiation thus regulating a variety of cellular processes. They have been implicated in the pathophysiology of injury to the central nervous system with the ability to promote survival of neural cells, neurite outgrowth, synaptic plasticity, and neurotransmission. It is reported that axonal regeneration can readily occur despite the inhibitory environment generated by acute SCI when a sufficient number of growth-promoting factors are present at the injury site, including cell adhesion molecules and growth factors (Jones et al., 2003a).

Various neurotrophic factors have been studied in animal models. Different methods have been tested: direct infusion and cellular delivery of individual or a combination of neurotrophic factors. The application of these neurotrophic factors induced growth of sensory and motor axons, enhanced remyelination, and promoted functional recovery. These treatments included the application of brain-derived neurotrophic factor (BDNF) (McTigue et al., 1998; Liu et al., 1999; Jin et al., 2000; Namiki et al., 2000; Lu et al., 2001; Shumsky et al., 2003; Tobias et al., 2003; Zhou & Shine, 2003; Sharma et al., 2006), basic fibroblast growth factor (bFGF) (Lee et al., 1999), glial cell-derived neurotrophic factor (GDNF) (Zhou & Shine., 2003; Blesch & Tuszynski, 2003; Sharma et al., 2006), nerve growth factor (NGF) (Grill et al., 1997a; Tuszynski et al., 1996; Namiki et al., 2000; Tuszynski et al., 2002), and neurotrophin 3 (NT3) (Grill et al., 1997b; McTigue et al., 1998; Shumsky et al., 2003; Tobias et al., 2003; Tuszynski et al., 2003; Mitsui et al., 2005), and NT4 and NT5 (Blesch et al., 2004).

Unfortunately, clinical trials using systemic delivery of growth factors for various disorders have failed either as a result of lack of efficacy or unacceptable side effects, probably due to the solubility, diffusibility, and multifaceted function of growth factors. Growth factors must be delivered in sufficient quantities to have an effect and their distribution must be restricted to the site at which they are needed. Moreover, growth factors need to bind their corresponding receptors to exert functions. Therefore, it's necessary to investigate the expression of appropriate receptors to optimize the time window for the delivery of growth factors (Thuret et al., 2006).

### 3.2.3 Neuroprotective intervention

As cells undergo cell death after SCI, limiting the evolution of cell death through neuroprotective measures could also promote regeneration**.**

Glucocorticoid steroids like methylprednisolone sodium succinate, are applying intravenously for clinical use in acute SCI (Hall et al., 2004). Although clinical trials with methylprednisolone have demonstrated modest and potentially therapeutic benefits, its safety and efficiency have been challenged recently and need to be reevaluated (Hall et al., 2004; Coleman et al. 2000; Hurlbert 2000; Fehlings et al., 2005; Short et al., 2000; Bracken et al., 2001). Other approaches have also been shown to limit the evolution of cell death through neuroprotective measures and to improve hindlimb function in mouse and rat models of SCI, for instance, neutralization of CD95L (Demjen et al., 2004) and administration of minocycline (Lee et al. 2003; Teng et al., 2004; Stirling et al., 2004; Blight et al., 2006).

### 3.2.4 Extracellular matrix modifiers

After spinal cord injury inhibitory molecules are present that make the local environment non-permissive with abundant extrinsic growth-inhibitory cues. Central neurons could regenerate axons and reconnect with appropriate targets if a permissive environment was provided to balance and exceed inhibitory signals. Various strategies have been developed to overcome signals inhibitory to axon growth.

It has been shown that acute degradation of the growth-inhibitory chondroitin sulphate by treatment with chondroitinase ABC improves axonal growth after SCI (Moon et al., 2001; Bradbury et al., 2002). Surgical removal of the inhibitory scar tissue may foster a relatively permissive environment for axon regeneration. However, this approach will inevitably introduce further tissue damage and produce a second, unfavorable cellular response (Ye and Houle, 1997; Lu et al., 2002; Storer et al., 2003a; Storer et al., 2003b; Tobias et al., 2003; Tuszynski et al., 2003). Future works for efficacy and safety of chondroitinase ABC in non-human primate models of SCI still need to be performed.

### 3.2.5 Modulation of interactions with myelin inhibitors

Intact and injured CNS myelin contains several growth inhibitory molecules (including Nogo-A, myelin-associated glycoprotein, oligodendrocyte myelin glycoprotein, chondroitin sulphate and ephrin B3). Various therapies have been developed to target

and overcome these inhibitors of axon growth. Experimental therapies for regeneration in SCI can be ameliorated by the application of inhibitors directed against inhibitory molecules or their receptors. For instance, application of therapeutics against Nogo, which is highly expressed in CNS oligodendrocytes, can enhance axonal growth and functional recovery in rodent models of SCI (Schnell & Schwab, 1993; Bregman et al., 1995; von Meyenburg et al., 1998; Thallmair et al., 1998; Z'Graggen et al., 1998; Fouad et al., 2004). The functional outcomes of anti-Nogo therapeutics were also confirmed by genetic deletion of Nogo-A Nogo receptor which resulted in CNS axonal growth and functional recovery after SCI (Song et al., 2004; Zheng et al., 2005).

In conclusion, the pathophysiology of SCI indicates that repair of lost function requires multifaceted or combinatorial strategies to facilitate the regenerative effects for optimal recovery of function. Ideal grafts would not only affect the repair of damaged host spinal cord tissue by becoming integral components of the host cyto-architecture and circuitry but would also provide exogenous therapeutic gene products beneficial for either regeneration by endogenous stem cells or the integration of the grafted exogenous cells.

## 4 The aim of this study

As described, the transplantation of neural stem cells can promote survival or replacement of injured neurons, axonal growth, and reconnection with appropriate targets without tumor formation. Neural stem cell transplantation is a promising approach for cell replacement therapy after spinal cord injury. However, grafted neural stem cells differentiate mainly into astrocytes with only a minor fraction differentiating into neurons or oligodendrocytes thus hamper their application.

The first aim of this study is to improve neuronal differentiation of neural stem cells *in vitro* and *in vivo.* The second aim of this study is to make full use of the predominant astrocytic differentiation of neural stem cells, to deliver an exogenous therapeutic target gene beneficial for regeneration.

An ideal candidate for an exogenous therapeutic target gene is the neural adhesion molecule L1. It plays an important role in the development and regeneration of the central and peripheral nervous systems and improves cell survival, neurite outgrowth and neuronal differentiation.

To realize these aims, transgenic mice which ectopically express L1 under the control of the human GFAP promoter were generated. The expression of the neural cell adhesion molecule L1 is driven by the human GFAP promoter (Kordower et al. 1997; Malatesta et al., 2003), which leads to ectopic expression of neural cell adhesion molecule L1 in radial glia and mature astrocytes possibly promoting survival or replacement of injured neurons, axonal growth, and reconnection of axons with appropriate targets. Thus, neural stem cells overexpressing L1 under the influence of the human GFAP promoter were analyzed *in vitro* and transplanted into a mouse model for spinal cord injury to evaluate their regenerative potential.

# III Materials and methods

## 1 Materials

### 1.1 Chemicals

If not indicated otherwise, all chemicals were obtained from the following companies in p.a. quality: Merck (Darmstadt, Germany), Serva (Heidelberg, Germany) and Sigma (Deisenhofen, Germany). Molecular weight standards were obtained from Invitrogen (Karlsruhe, Germany). Oligonucleotides were ordered from metabion (Munich, Germany). Cell culture material was ordered from Nunc (Roskilde, Denmark) and Invitrogen.

### 1.2 Solutions and buffers

(In alphabetical order)

| | | | |
|---|---|---|---|
| Antibody buffer 1 | 0.3 | % (w/v) | bovine serum albumine (BSA) |
| *(Immunocytochemistry for live cell staining)* | | | in PBS pH 7.4 |
| | 0.02 | % (w/v) | Triton X-100 |
| Antibody buffer 2 | 0.5 | %w/v | lambda-carrageenan in PBS, pH 7.4 |
| | 0.02 | % w/v | sodium azide |
| *(Immunocytochemistry for fixed cell staining)* | | | |
| Blocking buffer 1 | 3 | % (w/v) | BSA in PBS pH 7.4 |
| | 0. 2 | % (w/v) | Triton X-100 |
| *(Immunocytochemistry for live cell staining)* | | | |
| Blocking buffer 2 | 0.2 | % (v/v) | Triton X-100 |
| | 0.02 | % (w/v) | sodium azide |
| | 5 | % (v/v) | normal goat or donkey serum |
| *(Immunocytochemistry for fixed cell staining)* | | | |
| Blocking buffer | 5 | % (w/v) | instant milk powder in PBS |
| *(Western Blot)* | | | |
| Blotting buffer | 25 | mM | Tris |
| *(Western Blot)* | 192 | mM | Glycine |
| BrdU | 10 | µmol | BrdU in HBSS |

| | | | |
|---|---|---|---|
| DAPI | 50 | µg/ml | in PBS pH 7.4 |
| DNA-sample buffer (5x) | 20 | % (w/v) | glycerol in TAE buffer |
| *(DNA-gels)* | 0,025 | % (w/v) | orange G |
| dNTP-stock solutions | 20 | mM each | dATP, dCTP, dGTP, dTTP |
| *(PCR)* | | | |
| Electroporation buffer | 20 | mM | HEPES |
| | 137 | mM | sodium chloride |
| | 5 | mM | potassium chloride |
| | 0.7 | mM | $Na_2HPO_4$ |
| | 6 | mM | dextrose |
| Ethidiumbromide- | 10 | µg/ml | ethidiumbromide in 1xTAE |
| staining solution *(DNA-gels)* | | | |
| $HBSS^-$ | 5.33 | mM | KCl |
| | 0.44 | mM | $KH_2PO_4$ |
| | 4.00 | mM | $NaHCO_3$ |
| | 138.0 | mM | NaCl |
| | 0.3 | mM | $Na_2HPO_4$ |
| | 5.6 | mM | D-glucose |
| Paraformaldehyde buffer | 4 | % | paraformaldehyde |
| | | | in 0.1 M cacodylate buffer, pH 7.3 |
| | 0.1 | % | $CaCl_2$ |
| Phosphate buffered saline | 137 | mM | NaCl |
| (PBS) | 2.7 | mM | KCl |
| | 8.0 | mM | $Na_2HPO_4$ |
| | 1.5 | mM | $KH_2PO_4$ |
| Poly-L-lysine | 100 | µg/ml | in PBS pH 7.4 |
| (PLL) | 200 | µg/ml | in PBS pH 7.4 |
| RIPA-buffer | 50 | mM | Tris-HCl, pH 7.4 |
| *(cell lysis)* | 1 | % (w/v) | Triton X-100 |
| | 150 | mM | NaCl |
| | 1 | mM | EGTA |
| | 1 | mM | $Na_3VO_4$ |

| | | | |
|---|---|---|---|
| Running Gel 10% (8%) | 3.92 | ml (4.89 ml) | deionized water |
| *(protein gels)* | 5.26 | ml (5.26 ml) | 1 M Tris pH 8.8 |
| | 0.14 | ml (0.14 ml) | 10% SDS |
| | 4.70 | ml (3.73 ml) | 30% Acrylamide – Bis 29:1 |
| | 70.0 | µl (70 µl) | 10% APS |
| | 7.00 | µl (7 µl) | TEMED |
| Sample buffer (5x) | 0.312 | M | Tris-HCl pH 6.8 |
| *(protein-gels)* | 10 | % (w/v) | SDS |
| | 5 | % (w/v) | β-Mercaptoethanol |
| | 50 | % (v/v) | Glycerol |
| | 0.13 | % (w/v) | Bromphenol blue |
| SDS running buffer (10x) | 0.25 | M | Tris-HCl, pH 8.3 |
| *(protein-gels)* | 1.92 | M | glycine |
| | 1 | M | SDS |
| Stacking Gel 5% | 3.77 | ml | deionized water |
| *(protein gels)* | 0.32 | ml | |
| | 1 | M | Tris pH 6.8 |
| | 0.05 | ml | 10% SDS |
| | 0.83 | ml | 30% Acrylamide – Bis 29:1 |
| | 25.0 | µl | 10% APS |
| | 7.00 | µl | TEMED |
| TAE (50x) | 2 | M | Tris-Acetat, pH8.0 |
| *(DNA-gels)* | 100 | mM | EDTA |
| Tail lysis buffer | 50 | mM | Tris-HCl, pH 8.0 |
| | 50 | mM | KCl |
| | 2.5 | mM | EDTA |
| | 0.45 | % (v/v) | NP40 |
| | 0.45 | % (v/v) | Tween 20 |
| | 0.1 | mg/ml | Proteinase K |

### 1.3 Cell culture media

Media were purchased from Invitrogen and supplemented as described

| | | | |
|---|---|---|---|
| NSC-medium | DMEM/F12 supplemented with | | |
| | 1 | × | B27-supplement |
| | 0,6 | % | D-glucose |
| | 2 | mM | L-glutamine |
| | 3 | mM | sodium bicarbonate |
| | 5 | mM | HEPES |
| | 50 | U/ml | penicillin |
| | 50 | µg/ml | streptomycin |

| | | | |
|---|---|---|---|
| NSC-medium | DMEM/F12 supplemented with | | |
| with growth factors | 1 | × | B27-supplement |
| | 0,6 | % | D-glucose |
| | 2 | mM | L-glutamine |
| | 3 | mM | sodium bicarbonate |
| | 5 | mM | HEPES |
| | 50 | U/ml | penicillin |
| | 50 | µg/ml | streptomycin |
| | 20 | ng/ml | basic fibroblast growth factor (bFGF) |
| | 20 | ng/ml | epidermal growth factor (EGF) |
| | | | (PreproTech, Rocky Hill, NY, USA) |

### 1.4 Antibodies

#### 1.4.1 Primary Antibodies

| | |
|---|---|
| anti-β-tubulin III | Rabbit monoclonal antibody Tuj1 (Sigma) |
| | IH: 1:1000 |
| anti-BrdU | mouse monoclonal antibody (Developmental Studies Hybridoma Bank) |
| | IH: 1:100 |
| anti-CaMKII | mouse monoclonal antibody (Sigma) |
| | IH: 1:1000 |
| anti-ChAT | goat polyclonal antibody (Millipore) |

| | |
|---|---|
| | IH: 1:100 |
| anti-CNPase | mouse monoclonal antibody (Sigma) |
| | IH: 1:1000 |
| anti-DCX | goat polyclonal antibody (Santa Cruz Biotechnology) |
| | IH: 1:200 |
| anti-GFAP | rabbit polyclonal antibody (Dako) |
| | IH: 1:1000 |
| anti-GAD-65/67 | rabbit polyclonal antibody (Sigma) |
| | IH: 1:500 |
| anti-GAPDH | mouse monoclonal antibody (Millipore) |
| anti-Iba-1 | rabbit polyclonal antibody (Wako Chemicals) |
| | IH: 1:1500 |
| anti-L1 | rat monoclonal antibody |
| | IH: 1:100 |
| anti-L1 | rabbit polyclonal antibody (Rathjen and Schachner, 1984) |
| | IH: 1:100 |
| anti-nestin | mouse monoclonal antibody (Developmental Studies Hybridoma Bank) |
| | IH: 1:50 |
| anti-RC-2 | mouse monoclonal antibody (Developmental Studies Hybridoma Bank) |
| | IH: 1:10 |
| anti-5-HT | guinea pig polyclonal antibody (Millipore) |
| | IH: 1:1000 |
| anti-TH | rabbit monoclonal antibody (Millipore) |
| | IH: 1:800 |
| anti-VGAT | mouse monoclonal antibody (Synaptic Systems) |
| | IH: 1:1000 |
| anti-VGLUT1 | mouse monoclonal antibody (Synaptic Systems) |
| | IH: 1:500 |

| | |
|---|---|
| anti-VGLUT2 | mouse monoclonal antibody (Synaptic Systems) IH: 1:500 |

### 1.4.2 Secondary antibodies

For indirect immunofluorescence: Cy2™, Cy3™, and Cy5™ conjugated antibodies (diluted 1:200 - 1:300) against goat, rabbit, mouse, donkey, and guinea pig antibodies (Jackson ImmunoResearch via Dianova, Hamburg, Germany) were used.

## 2 Methods

### 2.1 Animals

Female C57BL/6J mice were used at the ages of 3–4 months. The animals were kept under a standard 12-h light-dark cycle at constant temperature (22 °C) with food and water *ad libitum*. Animal treatments, data acquisition, and analyses were performed in a blinded manner. Mice ectopically expressing L1 under the control of human GFAP promoter and C57BL/6J mice ubiquitously expressing enhanced green fluorescent protein (GFP) under the control of the chicken actin promotor were used. Genotyping of the mice was performed using PCR assays. The animals were kept under standard laboratory conditions. All experiments were conducted in accordance with the German and European Community laws on protection of experimental animals, and the procedures used were approved by the responsible committee of The State of Hamburg. Numbers of animals studied in different experimental groups and at different time periods after surgery are given in the text and figures.

### 2.2 Genotyping

Transgenic offspring was genotyped by polymerase chain reaction (PCR) using primers 5'-pGFAP-L1 gta cca cct gcc tca tgc ag, 3'-pGFAP-L1 tcg tcc agc gga act cca ct. L1-C 5’ GGT AGG CAG GAG ATA AGG TCA 3’ L1-D 5’ CAG TCA TTG ATC CTG GAG TGC 3’. DNA was isolated from tail cuts by incubating the tissues overnight in tail lysis buffer at 55 °C. A standard genomic PCR was run as described below. These conditions amplified in hGFAP-L1 mice a fragment of 500 base pairs which was visualized using 1% agarose gel electrophoresis and ethidium bromide.

### 2.2.1 Standard genomic PCR

Amplification of DNA fragments was performed in a 50 µl reaction mix with thin-walled PCR tubes in MWG-PCR cyclers. *Taq-Polymerase* and the appropriate reaction buffer were obtained from Stratagene. The following reaction mixture was used:

| | |
|---|---|
| Template | 1 µl |
| Primer 1 (10pM) | 2 µl |
| Primer 2 (10pM) | 2 µl |
| Nucleotides (dNTPs) | 5 µl (20mM) |
| PCR-buffer (10 x) | 5 µl |
| *Taq*-Polymerase | 1 U |
| dd$H_2O$ | ad 50 µl |

The PCR was performed with the following step gradient:

| | | |
|---|---|---|
| 1) Initial denaturing 95°C | 5 | min |
| 2) Denaturing 95°C | 45 | sec |
| 3) Annealing $T_m$ 68°C | 1.5 | min |
| 4) Synthesis 72°C | 1 | min |
| 5) Termination 72°C | 5 | min |
| 6) Cooling 4°C | | |

The amplification procedure (steps 2-4) was repeated 30 times. The melting temperature of the primers depends on the GC content and was calculated by the following formula:

$$T_m = 4 \times (G+C) + 2 \times (A+T)$$

If the two primers had different melting temperatures, the lower of both was used. Afterwards, the quality of the PCR product was monitored by gel electrophoresis and the PCR product was purified with the rapid PCR purification kit.

### 2.2.2 DNA Gel-electrophoresis

DNA fragments were separated by horizontal electrophoresis chambers (BioRad) using agarose gels. Agarose gels were prepared by heating 1-2 % (w/v) agarose (Invitrogen) in 1× TAE buffer, depending on the size of DNA fragments. The gel was covered with 1× TAE buffer and the DNA samples were pipetted in the sample pockets. DNA sample buffer was added to the probes and the gel was run at constant voltage (10V/cm gel

length) until the orange G dye had reached the end of the gel. Afterwards, the gel was stained in an ethidiumbromide staining solution for 20 min. Finally gels were documented using the E.A.S.Y. UV-light documentation system (Herolab, Wiesloh, Germany).

### 2.3 Culture of neural stem/progenitor cells

Stem cells were isolated from embryonic day 14 (E14) embryos. 14-day-old embryos from breeding of ectopically expressing L1 mice under the control of human GFAP promoter with C57BL/6J mice ubiquitously expressing enhanced green fluorescent protein (GFP) under the control of the chicken actin promotor were used (Okabe et al., 1997). Genotyping of the embryos was performed using polymerase chain reaction (PCR) assays. The genotype of GFP-transgenic embryos was determined by analyzing tail biopsies for the presence of GFP fluorescence. Lateral and medial ganglionic eminences were removed and placed into a defined medium composed of a 1:1 mixture of Dulbecco's Modified Eagle's Medium and F-12 supplemented with glucose (0.6%), sodium bicarbonate (3 mM), B27 (2%; Gibco, New York, USA), glutamine (2 mM), HEPES buffer (5 mM), epidermal growth factor (EGF; 20 ng/ml; TEBU, Offenbach, Germany), and fibroblast growth factor-2 (FGF-2; 20 ng/ml; TEBU). The tissue was mechanically dissociated using a fire-polished Pasteur pipette in NSC-medium and cells were plated in uncoated tissue culture flasks at a density of 200,000 cells/ml. After 6 days *in vitro* cells were passaged for the first time with a seeding density of 50,000 cell/ml. From the first passage onward, neurospheres were passaged every $5^{th}$ day. Vital cells were determined by 0.5% Trypan blue dye (Invitrogen) exclusion.

### 2.4 Proliferation and differentiation assays

Proliferation and differentiation experiments were performed with neurospheres between passages 3 and 6.

For measurement of overall proliferation of neural precursor cells, dissociated precursor cells were plated on PLL pre-coated coverslip, 5-bromo-2-deoxyuridine (BrdU) (10 µmol; Sigma) was administered in EGF–FGF-containing culture medium 8 hr before cells were fixed with 4% paraformaldehyde. The percentage of $BrdU^+$ cells was determined.

For measurement of differentiation, neurospheres were mechanically dissociated and plated at a density of 50,000 cells/ml onto 15 mm glass coverslips coated with 0.01% PLL. Precursor cells were first maintained in an undifferentiated state for 1-2 days after plating in EGF–FGF containing serum-free culture medium. Growth factors were then removed, and precursor cells were allowed to differentiate for an additional 7 d. Seven days after withdraw of growth factors, coverslips were washed in PBS, and cells were fixed for 30 min in 4% paraformaldehyde in PBS.

### 2.5 Immunocytochemistry and Immunohistochemistry

#### 2.5.1 Immunocytochemistry of living cells

Coverslips with the attached cells were washed with PBS and placed on Parafilm in a humid chamber. 100 µl of PBS containing the primary antibody in the appropriate dilution were added on the coverslips and incubated at 4°C for 20 min. Afterwards, coverslips were put into 24-well dishes and washed twice with PBS. Then coverslips were put on Parafilm in the humid chamber again, covered with 100 µl $HBSS^-$ containing the fluorescent dye-coupled secondary antibody in a 1:200 dilution, and incubated at 37°C for 20 min in the dark. Finally, coverslips were washed twice with PBS, fixed and mounted on objectives with Fluoromount G medium (Polysciences, Warrington, PA, USA).

#### 2.5.2 Immunocytochemistry of fixed cells

For immunocytochemistry of fixed cells, cultured cells were washed in PBS, pH 7.3 for three times and fixed for 15 minutes in 4% paraformaldehyde before incubation in PBS containing 0.2% v/v Triton X-100, 0.02% w/v sodium azide and 5% v/v normal goat serum (if the secondary antibody was raised in goat, if not appropriate serum was used, e.g. donkey) for 30 minutes. Primary antibodies, applied at 4°C overnight, were polyclonal rabbit antibodies to β–III tubulin (Tuj1) (1:1,000; Sigma), RC2 (1:10; Developmental Studies Hybridoma Bank, Iowa City, IA), nestin (1:50; Developmental Studies Hybridoma Bank, Iowa City, IA), 2',3'-cyclic nucleotide 3' phosphodiesterase (CNPase; 1:1,000), and polyclonal rabbit antibodies to GFAP (1:1000; Dako, Carpinteria, CA), L1 (1:1,000), and a monoclonal rat antibody to L1 (1:100; Chemicon). After washing in PBS, appropriate secondary antibodies were applied for 1 hour at room temperature. Finally, coverslips were washed twice with PBS. To determine total cell

numbers *in vitro*, cells were counterstained with DAPI (Sigma) for 2 min and the ratio of cell type-specific marker-positive cells of all $DAPI^+$ cells was calculated. For BrdU staining, DNA was denatured with with 2 M HCl for 30 min at 37°C. Monoclonal mouse antibody to BrdU (1:100; Developmental Studies Hybridoma Bank, Iowa City, IA) was administered overnight at 4°C. For Immunocytochemistry of living cells, fixation was omitted, and cells were washed and incubated with primary antibody on ice for 20 min, followed by washing and incubation with secondary antibody for 15 min on ice. Subsequently, cells were fixed in 4% paraformaldehyde in PBS and mounted on objectives with Fluoromount G medium (Polysciences, Warrington, PA, USA). For negative controls, primary antibody was omitted. Specimens were examined with a fluorescence (Axioplan 2; Carl Zeiss Microimaging, Thornwood, NY, USA) or confocal laser-scanning microscope (Leica SP2, Leica Microsystem Heidelberg GmbH; Fluroview 1000, Olympus).

### 2.5.3 Immunohistochemistry of tissue sections

For tissue fixation and sectioning, mice were anesthetized and transcardially perfused with fixative consisting of 4% formaldehyde and 0.1% $CaCl_2$ in 0.1 M cacodylate buffer, pH 7.3, for 15 min at room temperature (RT). Spinal cords were removed following fixation, placed in 15% buffered sucrose (pH 7.3), and refrigerated at 4°C for approximately 48h. Serial longitudinal sections were cut in a cryostat (CM3050; Leica, Nussloch, Germany). 25 µm thick Sections were collected on SuperFrost Plus glass slides (Roth, Karlsruhe, Germany). Sampling of sections was always performed in a standard sequence so that sections 250 µm apart were present on each slide.

Sections were processed for the immunocytochemical procedures as described previously (Irintchev et al., 2005; Jakovcevski I et al., 2007). Photographic documentation was made on confocal laser-scanning microscope (Leica SP2, Leica Microsystem Heidelberg GmbH; Fluroview 1000, Olympus) or an Axiophot 2 microscope equipped with digital camera AxioCam HRC and AxioVision software (Zeiss). The following antibodies were used: goat anti- doublecortin (DCX) (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-choline acetyltransferase antibody (ChAT) (1:100; Millipore, Hofheim, Germany), rabbit anti-tyrosine hydroxylase (TH) (1:800; Millipore), rabbit anti-ionized binding calcium adapter molecule 1 (Iba-1) (1:1500; Wako Chemicals, Richmond, VA), rabbit anti-glial

fibrillary acidic protein (GFAP) (1:1000; DakoCytomation), rabbit anti-neuronal class III ß-tubulin (Tuj1) (1:2000; Covance, Berkeley, CA), rabbit anti-glutamate decarboxylase (GAD) (GAD-65/67, 1:500; Sigma), mouse antivesicular GABA transporter (VGAT) (1:1000; Synaptic Systems, Gottingen, Germany), mouse anti-vesicular glutamate transporter 1 (VGLUT1) (1:500; Synaptic Systems), mouse anti-vesicular glutamate transporter 2 (VGLUT2) (1:500; Synaptic Systems), mouse anti-CNPase (1:1000; Sigma), mouse anti-CamKII (1:1,000; Sigma) and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1000; Millipore). The following cyanine 2 (Cy2)-, Cy3-, and Cy5-conjugated secondary antibodies were used for immunofluorescence: goat anti-rabbit, goat anti-mouse, donkey anti-goat, donkey anti-rabbit, donkey anti-mouse, and donkey anti-guinea pig (Jackson ImmunoResearch via Dianova, Hamburg, Germany). The images were processed using Adobe Photoshop 7.0 software (Adobe Systems, San Jose, CA).

### 2.6 Neurosphere migration assay

A 15-mm coverslips was coated with 200 µg/ml poly-L-lysine (PLL). The coverslips were placed on a 24-well dish. Single non-adherent spheres of similar diameters (100–300 µm) were picked up individually using a 10 µl pipette tip and seeded in 5 µl droplets on PLL-coated glass coverslips for 20 min. After 20 min of adhesion, the medium was adjusted to 500 µl per well with either the NSC culture medium or the NSC culture medium with EGF or FGF-2. Migrating cells were followed for a 5 d period. At several time points (4 hr, 24 hr, 5 d), the longest distance between the edge of a sphere and the leading edge of the outgrowth was measured in each quarter using an inverted microscope. At least 40 spheres were analysed for each time point, and the experiments were performed in triplicate.

### 2.7 Immunoisolation of L1-positive cells

For L1-immunoisolation, plastic petri dishes were coated with a goat anti- rabbit IgG antibody (80 µl antibody, 1.3 mg/ml [Jackson Immunoresearch Laboratories, West Grove, PA]) in 10 ml Tris/HCl (100mM, pH 9.5) for 24 h at 4°C; After rinsing with PBS, the dishes were incubated with bovine serum albumin solution (0.2% in phosphate-buffered saline) for 24 h at 4°C; After blocking, a rabbit anti-L1 antibody was added and incubated overnight at 4°C (diluted in 0.2% BSA in PBS, final antibody

concentration 5µg/ml). After incubation overnight at 4°C and rinsing with PBS, the antibody-coated dishes were used for immunoisolation.

For dissociation, neurospheres were incubated for 10 min at 37°C in 500µl Accutase (Invitrogen). Then, neurospheres were rinsed with NSC culture medium and were mechanically dissociated. After centrifugation, cells were resuspended in bovine serum albumin (BSA) (0.02% in PBS) and added to the L1-coated petri dish. Immunoisolation was performed at room temperature for 1.5 h. Then, nonadherent cells were washed off with PBS. Adherent cells were harvested using Accutase (Invitrogen) for 1 minute at room temperature and were suspended in NSC culture, spun down, and resuspended in NSC culture medium with growth factors. To characterize the properties of the selected cells, cells were cultured in neural stem cell culture medium on poly-L-lysine-coated coverslips for 6 hours, 2 and 6 days. We performed immunocytochemical staining using antibodies against L1, radial cell type-specific marker proteins including RC2, nestin, neuronal marker protein β–III tubulin, and astrocytic marker protein GFAP.

### 2.8 Spinal cord injury and cell transplantation

#### 2.8.1 Spinal cord injury surgical procedures

For surgery, the mice were anesthetized by intraperitoneal injections of ketamine and xylazine [100 mg of Ketanest (Parke-Davis/Pfizer, Karlsruhe, Germany) and 5 mg of Rompun (Bayer, Leverkusen, Germany), per kilogram of body weight]. Laminectomy was performed at the T7–T9 level with mouse laminectomy forceps (Fine Science Tools, Heidelberg, Germany). A mouse spinal cord compression device was used to elicit compression injury (Curtis et al., 1993; Steward et al., 2003). Compression force (degree of closure of the forceps) and duration were controlled by an electromagnetic device. The spinal cord was maximally compressed (100%, according to the operational definition of Curtis et al., 1993) for 1 s by a time-controlled current flow through the electromagnetic device. The skin was then surgically closed using 6-0 nylon stitches (Ethicon, Norderstedt, Germany). After the operation, mice were kept in a heated room (35°C) for several hours to prevent hypothermia and thereafter singly housed in a temperature-controlled (22°C) room with water and standard food provided *ad libitum*. During the postoperative time period, the bladders of the animals were manually voided twice daily (Apostolova et al., 2006; Jakovcevski et al., 2007).

### 2.8.2 Cell transplantation procedures

Four groups of mice were examined: (*i*) C57BL/6J mice that had received neural stem/precursor cells ectopically expressing L1 under the control of human GFAP promoter (hGFAP-L1 group) 4 days after SCI; (*ii*) mice transplanted with L1 expressing radial glia-like cells 4 days after SCI (L1-imm group); (*iii*) mice that were transplanted with wild type neural stem/precursor cells 4 days after SCI (WT group); (*iv*) mice that were sham-injected with PBS 4 days after SCI (PBS group). On the day of transplantation, neural progenitors/stem cells were dissociated by accutase and resuspended in PBS at a density of 100,000 viable cells per µl and 1 µl was injected 0.5 mm both rostral and caudal to the lesion site, 1 mm deep into spinal cord. Viability (>80%) was determined by Trypan blue exclusion. All animal experiments were approved by the University and State of Hamburg Animal Care Committees.

## 2.9 Motoneuron soma size and quantification of perisomatic terminals

Estimations of soma areas and perisomatic terminals were performed as described previously (Apostolova et al., 2006; Jakovcevski I et al., 2007). Longitudinal spinal cord sections stained with antibodies against ChAT were examined under a fluorescence microscope to select sections that contained motoneuron cell bodies at a distance of at least 500 µm distal from the lesion scar. Stacks of 1µm-thick images were obtained on a LSM 510 confocal microscope (Zeiss) using a 63x, 1.5 oil-immersion objective and digital resolution of 512 x 512 pixel. Four adjacent stacks (frame size, 153 x 153 µm) were obtained consecutively in a rostrocaudal direction so that motoneurons located both close and remote to the lesion scar were sampled. One image per cell at the level of the largest cell body cross-sectional area was used to measure soma area, perimeter, and number of perisomatic terminals. Areas and perimeters were measured using the Image Tool 2.0 software program (University of Texas, San Antonio, TX). Linear density was calculated as number of perisomatic terminals per unit length.

## 2.10 Analysis of motor function

### 2.10.1 Locomotor rating

The recovery of ground locomotion was evaluated using the Basso, Beattie, Bresnahan (BBB) rat rating scale (Basso et al., 1995) as modified for the mouse by Joshi and Fehlings (2002) and more recent modifications of the rating scale for mice (Engesser-

Cesar et al., 2005; Li et al., 2006) were used. Scoring was performed by one and the same investigator. Motor performance of each animal was evaluated during free movement in an open-field arena placed 90 cm above ground to aid close observations of the mice (Joshi and Fehlings, 2002). Rating of each animal was later performed by analysis of video recordings of beam walking (see below) observed at slow playback speed. Assessment was performed at 1, 3, and 6 weeks after injury. Scores for the left and right extremities were averaged (Apostolova et al., 2006; Jakovcevski et al., 2007).

### 2.10.2 Single-frame motion analysis

Mice were trained to perform a classical beam-walking test. In this test, the animal walks unforced from one end of a horizontal beam (900 mm length, 40 mm width) toward its home cage located at the other end of the beam. A left- and right-side view of each animal during two consecutive walking trials were captured before the operation with a Panasonic (Hamburg, Germany) NV-DS12 camera at 25 frames per second and recorded on videotape (video recorder SVL-SE 830; Sony, Cologne, Germany). The recordings were repeated 1, 3, and 6 weeks after spinal cord lesion. The video sequences were digitized and examined with VirtualDub software. Selected frames in which the animals were seen in defined phases of locomotion (see below) were used for measurements performed with the University of Texas Health Science Center at San Antonia Image Tool 2.0 software. Two parameters, which we designated foot-stepping angle and rump-height index, were measured (Apostolova et al., 2006; Jakovcevski et al., 2007).

### 2.10.3 Foot-stepping angle

The foot stepping angle is defined by a line parallel to the dorsal surface of the hindpaw and the horizontal line. The angle is measured with respect to the posterior aspect at the beginning of the stance phase. In intact mice, this phase is well defined and the angle is smaller than 20°. The average of three to five measurements per animal, extremity, and trial was found to be representative for the individual animals. After spinal cord injury and severe loss of locomotor abilities, the mice drag behind their hindlimbs with dorsal paw surfaces facing the beam surface. The angle is increased to >150°. In severely disabled mice, video frames for analysis were selected from three to five different “step cycles” delineated by the forelimbs. The video frames in which the angle appeared to have its lowest values for individual “cycles,” typically after a visible attempt to flex the

extremity, were selected for measurements. In less severely disabled mice that performed stepping of variable quality, the angle was measured on dorsal or ventral placement of the paw on the ground after a swing phase or after a forward sliding of the paw over the beam surface. Step cycles were defined, depending on the capabilities of individual animals, according to the criteria outlined above for intact or severely disabled mice. The values for the left and right leg of individual mice were averaged. Foot-stepping angle was defined as and proved to be a numerical parameter allowing objective assessment of the plantar stepping ability, one of the major behavioral aspects assessed by BBB score (Apostolova et al., 2006; Jakovcevski et al., 2007).

### 2.10.4 Rump-height index

The second parameter, the rump-height index, was estimated from the recordings used for measurements of the foot-stepping angle. The parameter is defined as height of the rump, i.e., the vertical distance from the dorsal aspect of the animal's tail base to the beam, normalized to the thickness of the beam measured along the same vertical line. For each animal and trial, at least three frames in which the rump height was maximal during different step cycles, defined according to the stepping ability of the animal as described above, were used for measurements. The rump-height index is a numerical estimate of the ability to support body weight. This ability requires coordination in different joints of both hind extremities and is influenced by various factors such as stepping art (plantar vs dorsal), muscle strength, and spasticity (Apostolova et al., 2006; Jakovcevski et al., 2007).

### 2.10.5 Limb extension–flexion ratio

The limb extension–flexion ratio was evaluated from video recordings of voluntary movements of the mice performed during the "pencil" test. An intact mouse, when held by its tail and allowed to grasp a pencil with its forepaws, tries to catch the object with its hind paws and performs cycling flexion–extension movements with the hind limbs. For the spinal cord injury paradigm, left and right-side view videos were recorded for each animal. The extension and flexion length of the extremity (distance from the most distal midpoint of the paw to a fixed, well discernible point on the animal's body, e.g., the tail base) were measured for at least three extension–flexion cycles per animal and time point. Mean values for the two extremities from one animal were averaged. The

extension–flexion ratio is a numerical estimate of the animal's ability to initiate and perform voluntary, non weight-bearing movements. Such movements require connectivity of the spinal cord to supraspinal motor control centers but, in the form evaluated here, no coordination or precision (Apostolova et al., 2006; Jakovcevski et al., 2007).

### 2.10.6 Ladder-climbing test

The mice were placed at the bottom rungs of the ladder, and climbing was video recorded from a position "below" the ladder, i.e., viewing the ventral aspect of the animals. The video recordings were observed at slow-speed playback, and the number of correct steps (correct placing of the hind paw and sustained position until the next forward move) over 36 rungs were counted. Intact animals typically stepped on every second rung, i.e., using < 20 correct steps (averaged for the two extremities) to cross the 36-rung distance. Injured mice made between 0 and 10 correct steps depending on the injury severity and degree of recovery. These initial observations indicated that the ladder climbing test could provide an opportunity for quantitative evaluation of complex motor behavior for the whole span of functional capabilities, from the non-injured state to complete paralysis.

Grid walking and horizontal ladder crossing have been used as tests for evaluation of "skilled walking," which is dependent on descending motor control and ascending flow of proprioceptive and tactile information (Soblosky et al., 1997; Metz et al., 2000). In these tests, the animals walk across a grid or a horizontal ladder with rungs of equal or uneven spacing. The number of mistakes, misses, or slips per crossing or per unit time are used for evaluation. However, in cases of severe paraplegia, these tests are useless because the animals drag behind their legs and slips are rarely seen. As a ladder, a 4-mm-thick frame made of a Resopal plate (96 cm long, 12 cm wide, with central incision of 88x6 cm; Resopal, Gross-Umstadt, Germany) was used to which 43 round wooden rungs (100 x 2 mm) were glued at equal intervals (2 cm). The ladder was fixed in an inclined position (55°) using a Plexiglas platform. The inclined position of the ladder provides body weight support for disabled mice and thus aids climbing. Also, the inclination of the ladder makes sliding of the paralyzed legs along the rungs impossible, and the extremities constantly protrude through the inter-rung spaces except in cases of correct and sustained placing of the paw (Apostolova et al., 2006; Jakovcevski et al., 2007).

### 2.10.7 Recovery indices

The recovery index (RI) is an individual animal estimate for any given parameter described above and is calculated (percentage) as follows: RI= $[(X_{7+n}-X_7)/(X_0-X_{7)}]x100$, where $X_0$, $X_{7,}$ and $X_{7+n}$ are values before operation, 7 d after injury, and a time point n days after the spinal cord injury, respectively. In simpler terms, this measure estimates gain of function ($X_{7+n}-X_7$) as a fraction of the functional loss ($X_0-X_7$) induced by the operation. It may attain 0 or negative values if no improvement or additional impairment occurs during the observation time period. The index cannot be calculated only if the operation causes no change in the value ($X_0-X_7=0$). The recovery index is a meaningful and comprehensive parameter allowing better comparisons within one investigation and between results of different laboratories. Calculation of recovery indices is absolutely necessary if a parameter is prone to variability as a result of individual animal variability in body constitution and behavioral traits. An example is the rump-height index defined above. The index values are influenced not only by functional impairment but also by the animal's body size and the beam thickness. Overall recovery indices were calculated, on an individual animal basis, as means of recovery indices for individual parameters. The overall index is an estimate of the general condition of the treated animals based on individual objective measures. It is taken as a "clinical score" for individual mice similar to the BBB score, which is based on assessment of different aspects of locomotion. Recovery indices are to be interpreted in conjunction with analyses of all individual parameters measured to unequivocally identify the bases for the overall functional outcome (Apostolova et al., 2006; Jakovcevski et al., 2007).

### 2.10.8 Rate depression of the H-reflex Electromyography recordings

The EMG recordings were performed as described by Hyun Joon Lee in our lab (Lee HJ et al., 2008). Briefly, mice were anesthetized by intraperitoneal injections of ketamine and xylazine ketamin-xylazin mixture. Forelimbs and hindlimbs fixed mice were placed on a warm water pad (37°C) driven by a temperature regulating pump system (TP472 T/pump, Gaymar Industries, Orchard Park, NY, USA) to maintain body temperature. The sciatic nerve was stimulated using bipolar electrical pulses of 0.2 ms duration to elicit reflex responses with electronic pulses at 6 weeks after spinal cord injury (Model 2100 Isolated Pulse Stimulator, A-M systems, Calsborg, WA, USA). The signals of M- and H-responses measured in plantar muscle during repetitive stimulation of the sciatic

nerve from the recording electrode were transmitted to a differential amplifier (P55 General Purpose AC Preamplifier, Grass Technologies, West Warwick, RI, USA), magnified (x 1000) and filtered (300 Hz high pass and 1 kHz low pass). The latencies of the responses were measured as time elapsed between trigger and peak of each waveform. Stimulus intensity was gradually increased until both M- and H-waves with latencies of approximately 2 and 5 ms, respectively. After the threshold measurement, stimulus intensity was further increased until maximal and stable H-responses were elicited. Thereafter, stimulation continued at the defined suprathreshold level at frequencies of 0.1, 0.3, 0.5, 1, 2, 10, 20Hz. The amplified analog signal was delivered to an A/D converter (ADC42, Pico Technology, Cambridgeshire, U.K.) and the data were analyzed using the Picoscope data acquisition software (PicoScope 5, Pico Technology, Cambridgeshire, U.K.). The amplitudes of M- and H-waves were measured as peak-to-peak values and averaged to calculate H/M ratios.

### 2.11 Western blot analysis

Protein samples were heated at 95°C for 5 min in SDS sample buffer and loaded to the gel. Gel was run the at constant voltage of 60V for 20 min and then 100V until the bromphenol blue line migrated to the bottom of the gel. Proteins were transferred at constant voltage 80V for 150 min at 4°C from SDS-polyacrylamide gel onto a nitrocellulose membrane (Protran, Nitrocellulose BA 85, Schleicher & Schüll, Dassel, Germany) using a Mini Transblot apparatus (BioRad) as described in the manufacturer's protocol. Immunoreactivity was visualized by enhanced chemiluminescence detection system (ECL).

### 2.12 Statistical analysis

All numerical data are presented as group mean values with SEM. Parametric or nonparametric tests were used for comparisons, as indicated in the text and figure legends. Analyses were performed using the SPSS 11.5 software package (SPSS, Chicago, IL). The threshold value for acceptance of differences was 5%.

# IV Results

## 1 The cell adhesion molecule L1 is ectopically expressed on neural stem cells and neural stem cell-derived radial glial cells and astrocytes under the influence of the human GFAP promoter *in vitro*.

As the aim of this study was to analyze the influence of neural stem cells ectopically expressing the neural cell adhesion molecule L1 under the influence of the human GFAP-promoter on regeneration after spinal cord injury in mice, neural stem cells were isolated from transgenic and wild type mice that had been generated as described (Diedrich et al., 2008).Transgenic and wild type adult mice and fetuses were anatomically indistinguishable. Transgenic offspring was genotyped by polymerase chain reaction (PCR). The ganglionic eminences were isolated from individual transgenic and wild type fetuses as described under Materials and Methods. The tissues were dispersed into a single cell suspension and the cells were seeded in EGF and FGF2-containing medium. Within 5–7 days, the cells proliferated, forming clusters of cells (neurospheres). After about 5 days *in vitro*, the neurospheres were mechanically dissociated and the single cells grew into neurospheres again. For immunohistochemical analysis, neurospheres were dissociated and grown as monolayer on PLL-coated coverslips.

The expression of L1 in neural stem cells expressing L1 under the influence of the human GFAP-promoter (hGFAP-L1 NSCs) and wild type controls (WT NSCs) was analyzed by western blot and immunohistochemical analysis. Because L1 expression is driven by the human GFAP promoter, L1 is predicted to be expressed by radial glial cells, a specialized neural stem cells giving rise to most of the cortical neurons that additionally acts as a scaffold for migrating neuroblasts and astrocytes (Fig. 7G). Firstly, L1 expression of undifferentiated, proliferating neural stem cells was determined. In hGFAP-L1 NSC cultures, immunocytochemical double staining antibodies against L1 and cell type-specific markers showed that L1 was co-expressed with the radial glia marker RC2 (Fig. 7B) and nestin (Fig. 7C) in a subset of cells (around 5%), which suggested that L1 was ectopically expressed on radial glia cells and that the cultures were heterogeneous. Moreover, few cells co-expressed L1 and the astrocytic marker GFAP (Fig. 7D). In undifferentiated WT NSCs, L1 expression was not detectable. One week after induction of differentiation by withdrawal of FGF-2 and EGF, differentiated astrocytes from hGFAP-L1 NSC cultures showed immunohistochemical staining with

antibodies against L1 (Fig. 7E), whereas, there was no detectable L1 expression in astrocytes differentiated from WT NSCs. Furthermore, L1 was detectable in radial glia and astrocytes derived from hGFAP–L1 NSCs after passaging more than 16 times, suggesting that the cells retain L1 expression and phenotypic stability. Even after cryopreservation, the hGFAP–L1 stem cell-derived radial glia and astrocytes also expressed L1 (data not shown) indicating the stability of the transgene. Moreover, L1 expression in radial glia and asrocytes derived from hGFAP–L1 NSC was also confirmed by Western blot analysis before and after differentiation *in vitro* showing that L1 expression was enhanced in hGFAP-L1 versus WT NSCs before, 4, and 7 days after differentiation (Fig. 7F).

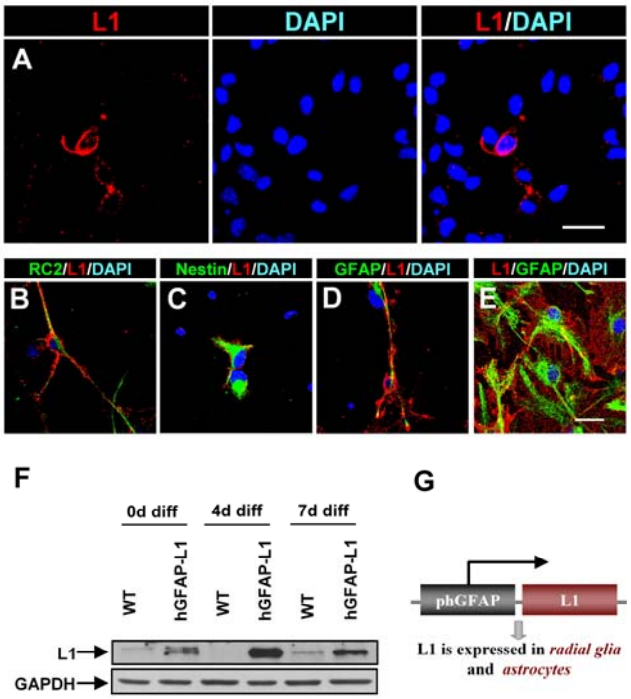


**Figure 7. Cell adhesion molecule L1 expressed under the influence of the human GFAP promoter is ectopically express on neural stem cell-derived radial glia cells and astrocytes *in vitro*.** (A) Immunohistochemical analysis of the expression of L1 (red) on proliferating neural stem cells (NSC) derived from embryonic hGFAP-L1 transgenic mice *in vitro*. (B-D) Immunohistochemical doublestaining of proliferating hGFAP-L1 NSCs with antibodies against L1 (red) and the radial glia marker RC2 (B, green), neural stem cell marker nestin (C, green), and astrocytic marker GFAP (D, green), suggesting that L1 is ectopically expressed on radial glia cells and astrocytes. (E) Immunohistochemical doublestaining of hGFAP-L1 NSCs with antibodies against L1 (red) and GFAP (green) Seven days after induction of differentiation by withdrawal of FGF-2 and EGF. Nuclei were counter-stained with DAPI. Scale bar, A-E, 20 µm. (F) Western blot analysis of L1 expression in WT and hGFAP-L1 NSC before and after differentiation *in vitro*. Note that L1 expression was enhanced in hGFAP-L1 versus WT NSC before, 4 and 7 days after differentiation. Western blot analysis of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is shown as a loading control. (G) Scheme illustrating transgenic expression of L1 under the influence of the human GFAP promoter.

## 2 Immunoisolation and immunocytochemical characterization of L1 expressing cells derived from hGFAP-L1 NSC with L1 antibodies

In undifferentiated hGFAP-L1 NSC cultures, L1 expressing $RC2^+$ and $nestin^+$ radial glia cells account for about 4.9% of the cultured neural stem cells. It is believed that radial glial cells are multipotent, both giving rise to neurons early in development and serving as a scaffold for migration, later involuting to generate glial precursors and parenchymal astrocytes (Rakic et al., 2003). As L1 is involved in neuronal migration, neurite outgrowth, and regeneration, it seemed useful to enhance the fraction of L1 expressing radial glial cells in NSC cultures for transplantation in SCI injury. For molecules expressed at the cell surface like L1, immunoisolation is an efficient method for the purification of large numbers of positive cells (Jüngling et al., 2003). Because the aim of this study was to enhance functional regeneration, in the following experiments *in vitro* and *in vivo* hGFAP-L1-NSCs and WT NSCs derived from mice ubiquitously expressing enhanced green fluorescent protein (GFP) allowing for reliable detection of transplanted cells in the host.

### 2.1 Efficiency of immunoisolation of hGFAP-L1 NSCs and wild type neurosphere-derived L1-positive cells

The cell suspension of dissociated hGFAP-L1 NSCs or WT NSCs was subjected to immunoisolation using L1 polyclonal antibodies. During immunoisolation, L1-expressing cells adhered to the antibody-coated surface of the culture dish. Non-adherent cells were washed off with PBS. Adherent cells were harvested and resuspended in neural stem cell culture medium for further characterization and propagation (Fig. 8A).

The total number of cells before and the number of cells after immunoisolation with the L1 antibody was counted to determine the efficiency of the immunoisolation. The mean fraction of L1-selected cells derived from hGFAP-L1 neurospheres was 4.9±1.7%, the mean fraction of L1-selected cells derived from wild type neurospheres was 1.9±0.9% (Fig. 8B). The $L1^+$ cells selected from wild type neurospheres did not proliferate, indicating that they comprise a subpopulation of cells differentiating toward a neuronal lineage and thus expressing L1.

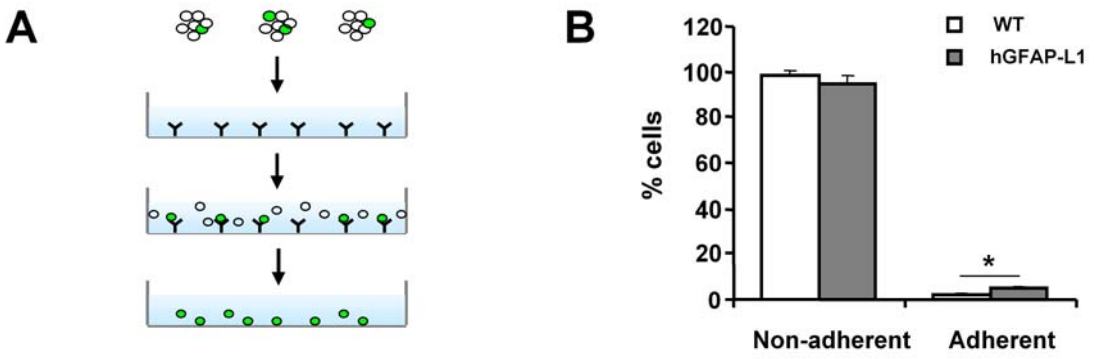


**Figure 8. Purification of L1 expressing cells derived from hGFAP-L1 NSCs or WT NSCs by L1-immunoisolation** (A) Scheme demonstrating the experimental procedure for immunoisolation applied in this study: 1. In vitro proliferation of NSCs as neurospheres in the presence of EGF and FGF2. 2. Dissociation of neurospheres and plating on culture dishes coated with polyclonal L1 antibody. 3. Non-adherent cells are discarded and adherent L1-expressing cells are resuspended and cultured for further experiments. (B) Mean percentage of adherent immunoisolated and non-adherent cells is shown (mean ± s.e.m.). Note the enhanced number of adherent cells in hGFAP-L1 NSCs (* $p < 0.05$).

## 2.2 Neurosphere-derived cells immunoisolated with L1 antibodies express L1 cell adhesion molecule on the cell surface

To confirm L1 expression in immunoisolated NSCs, immunocytochemical live staining was performed using a monoclonal antibody against L1 at 6 hours, 2 and 6 days after immunoisolation and plating of adherent cells on the PLL-coated coverslips. Immunocatochemical analysis revealed that almost all isolated cells showed L1 expression on the cell surface (Fig. 9). The majority of cells showed a typical bipolar morphology with elaborated neuritic processes after 6 days in culture (Fig. 9).

Thus, three types of neural stem/precursor cells were analyzed in the following experiments: (i) neural stem/precursor cells ectopically expressing L1 under the control of human GFAP promoter (***hGFAP-L1 NSCs***); (ii) neural stem/precursor cells ectopically expressing L1 under the control of the human GFAP promoter immunoisolated with L1 antibodies (***L1-imm cells***); (iii) wild type neural stem/precursor cells (***WT NSCs***);

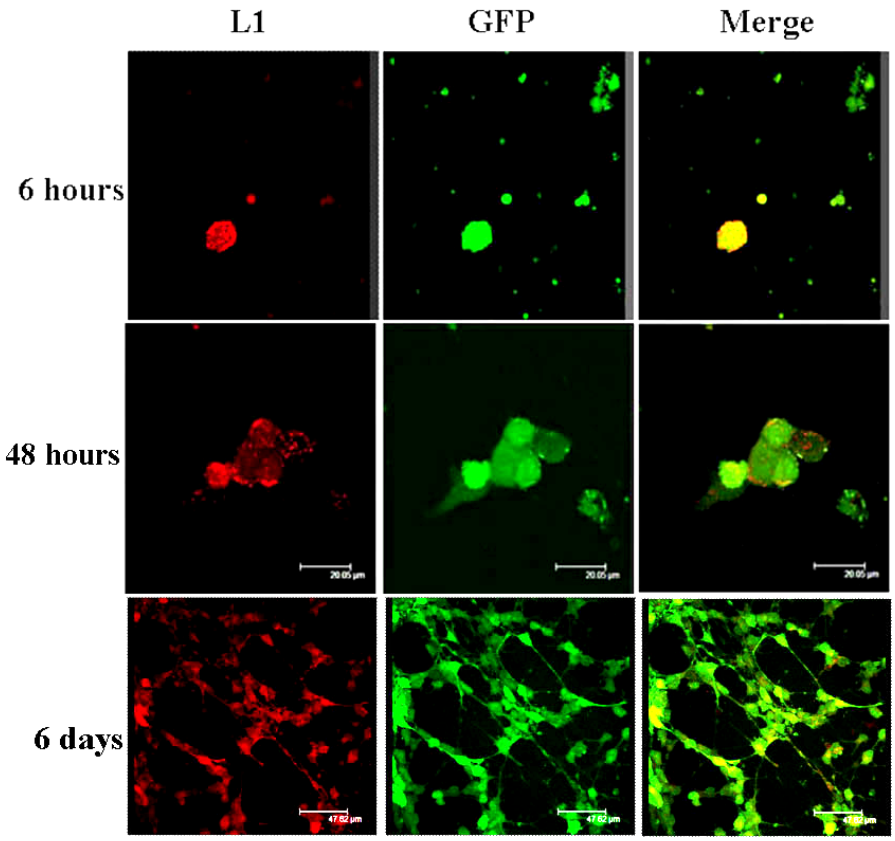


**Figure 9. Immunocytochemical characterization of cells immunoisolated from hGFAP-L1 NSCs with an L1 antibody. hGFAP-L1 NSCs expressing** enhanced green fluorescent protein (GFP) were immunoisolated as described above and cultured as monolayer on poly-L-lysine coated coverslips. Live cell immunostaining of cells with anti-L1 antibody after six hours, two days, and six days *in vitro* culture. Scale bars: 20µm (six hours, two days), 45µm (six days).

## 2.3 Immunocytochemical characterization of L1-imm cells revealed expression of radial glia marker proteins nestin and RC2

To characterize the properties of the L1-imm cells immunocytochemical staining in proliferating adherent cell cultures under the influence of EGF and FGF2 was performed using antibodies against radial cell type-specific marker proteins including RC2, nestin (Fig.10A), neuronal marker protein β–III tubulin, and the astrocytic marker protein GFAP. The majority of these cells were positive for nestin (91±3.1%) and RC2 (82±5.7%) (Fig.10B). β–III tubulin-positive (Tuj1) neurons and GFAP-positive astrocytes were rarely detected (data not shown).

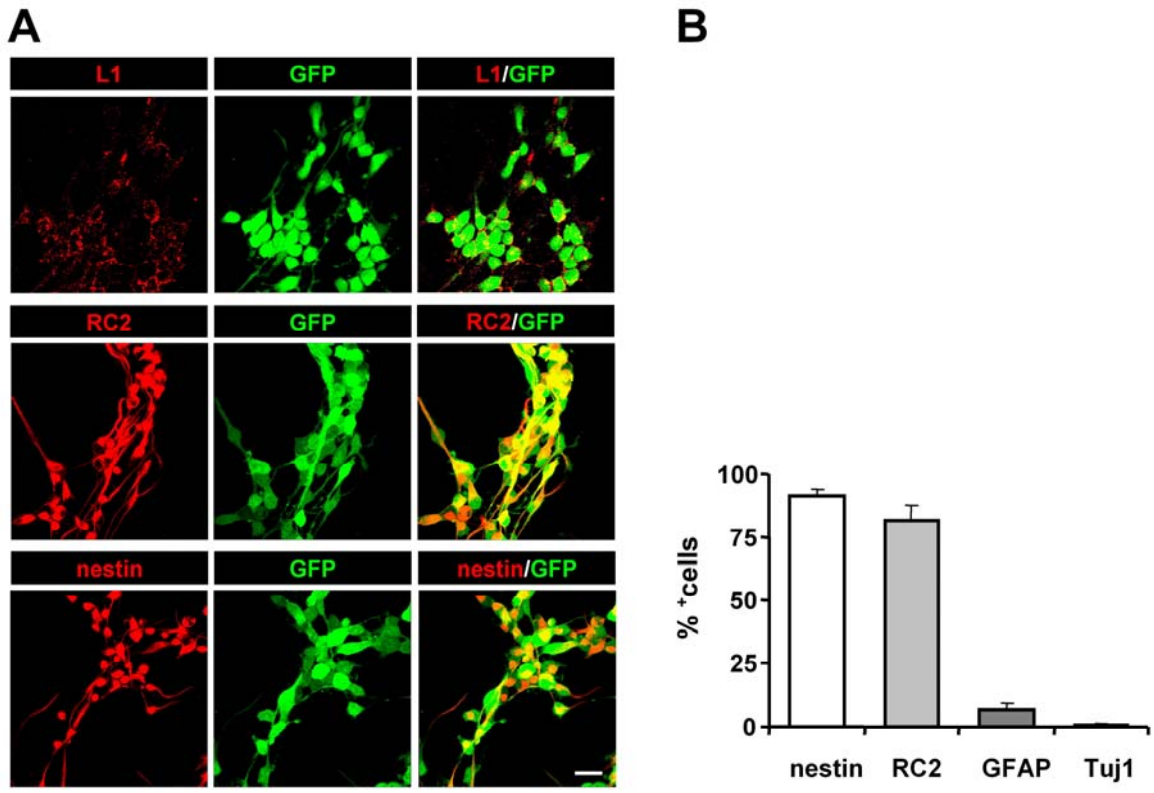


**Figure 10. Immunocytochemical characterization of L1 expressing cells derived from hGFAP-L1 NSCs by immunoisolation.** (A) Immunostaining of enhanced green fluorescent protein (GFP) expressing hGFAP-L1 NSCs cells purified by L1-immunoisolation (L1-imm cells) and cultured for 6 days on poly-L-lysine-coated culture dishes. Immunoisolated $GFP^+$ cells express L1 (upper), RC2 (middle) and nestin (lower). Scale bar, 20 μm. (B) Mean fraction of $marker^+$ cells isolated by L1-immunoisolation. Note that 91±3 % and 82±6% of cells were nestin and RC2-positive cells, respectively, whereas GFAP and Tuj1-positive account for a low percentage only (mean ± s.e.m. are displayed).

## 3 Analysis of the influence of ectopic expression of L1 under the control of human GFAP promoter in neural precursor cells on proliferation, differentiation, and migration

To investigate the effects of L1, proliferation, differentiation and migration was analyzed in hGFAP-L1 NSCs, L1-imm cells and WT NSCs, *in vitro*.

### 3.1 Neural precursor cells derived from hGFAP-L1 transgenic mice show decreased proliferation when compared to neural precursor cells derived from WT mice

To determine proliferation of neural precursor cells, dissociated cell cultures from hGFAP-L1 NSCs, L1-imm cells and WT NSCs were cultured on PLL-coated coverslips under the influence of FGF-2 and EGF and labeled with BrdU for eight hours. The percentages of $BrdU^+$ precursor cells of all cells were subsequently determined. Offering PLL as a substrate was preferred since it was shown that PLL has no significant effect on the proliferation of neural stem/precursor cells (Dihné et al., 2003). hGFAP-L1 NSCs and L1-imm cells showed a significant reduction of $BrdU^+$ precursor cells to a level of 77 ± 10% and 67±9%, respectively, when compared with wild type cells, which were set to 100% (Fig. 11).

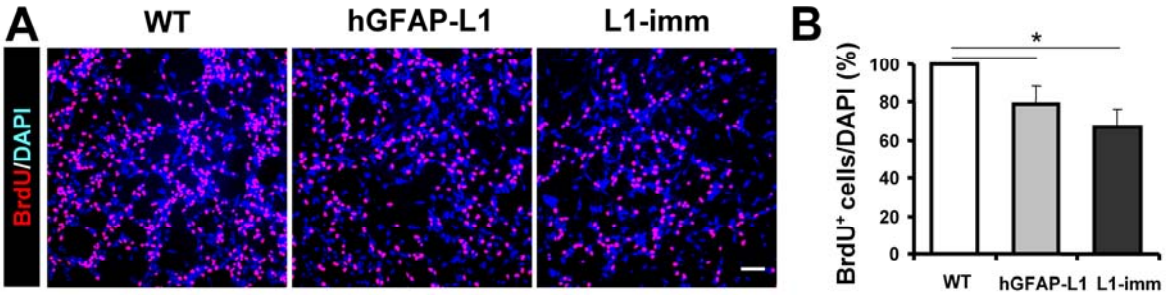


**Figure 11. Ectopic expression of L1 in neural precursor cells inhibits precursor cell proliferation.** To measure the influence of L1 expression on proliferation, dissociated precursor cells were plated on PLL-coated coverslips. A BrdU pulse was administered for 8h, and the percentage of $BrdU^+$ cells was determined. (A) Photomicrographs show a reduced fraction of $BrdU^+$ precursor cells in hGFAP-L1 NSCs and L1-imm cells when compared with WT NSCs. (B) Percentages of $BrdU^+$ cells of all cells in GFAP-L1 NSCs and L1-imm cells are shown in relation to the fraction of $BrdU^+$ cells in WT NSCs, which was set to 100%. Mean values ± s.e.m. are shown (* $p < 0.05$, Student's t-test was performed for statistical analysis). Scale bar, 20 µm.

### 3.2 hGFAP–L1 NSCs and L1-imm cells show enhanced neuronal differentiation compared to WT NSCs

To investigate whether ectopic expression of L1 under the control of human GFAP promoter in neural precursors may affect differentiation of precursor cells, hGFAP–L1 NSCs, L1-imm cells, and WT NSCs were assessed for their ability to differentiate into all of the major neural phenotypes (neurons, astrocytes, and oligodendrocytes). Dissociated cell cultures from hGFAP–L1 NSCs, L1-imm cells, and WT NSCs growing

on PLL-coated coverslips differentiated after withdrawal of the growth factors FGF-2 and EGF. Seven days after induction of differentiation, neurons were identified immunohistochemical analysis with antibodies against the marker protein β–III tubulin (Tuj1), mature astrocytes by antibodies against glial fibrillary acidic protein (GFAP), and oligodendrocytes by antibodies against 2', 3'-cyclic nucleotide 3' phosphodiesterase (CNPase).

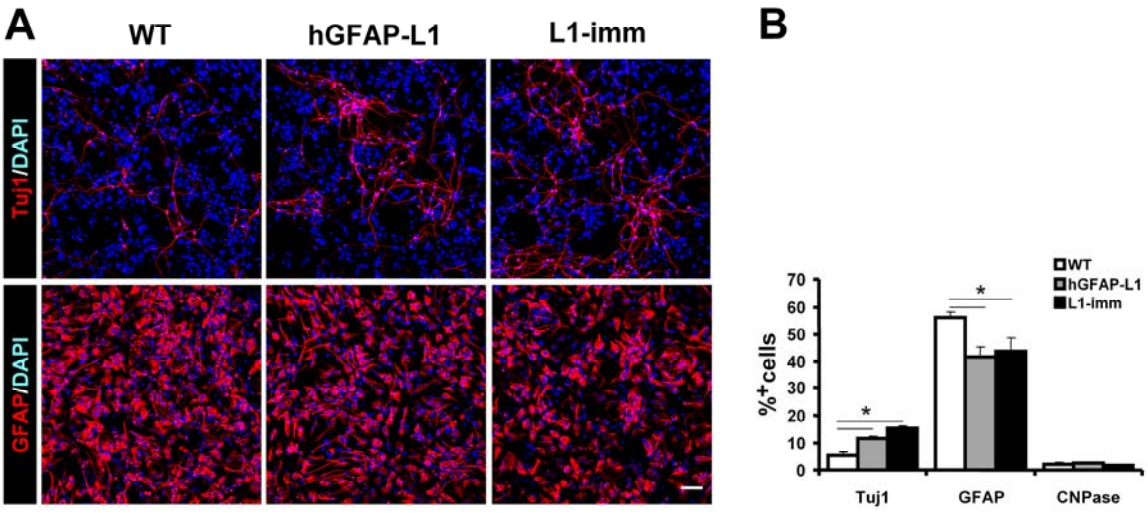


**Figure 12. Ectopic expression of L1 in hGFAP-L1 NSCs and L1-imm cells enhances neuronal differentiation and decreases astrocytic differentiation.** Dissociated neural precursor cells were plated on PLL-coated substrates. Seven days after induction of differentiation by growth factor withdrawal. (A) Neuronal differentiation was assessed by immunohistochemical analysis with antibodies against β-III-tubulin (Tuj1, red) and astrocytic differentiation was measured by immunohistochemical analysis with antibodies against GFAP (red). Nuclei were counterstained with DAPI (blue). Scale bar, 20 μm. (B). For statistical analysis, the percentage of marker$^+$ cells of all cells was determined. Mean ± s.e.m. are displayed. * $p < 0.05$. Student's t-test was performed for statistical analysis. Note the increased percentage of β-III-tubulin+ neurons and decreased percentages of GFAP$^+$ astrocytes in hGFAP-L1 NSCs and L1-Imm cells when compared to WT NSCs. The percentage of CNPase$^+$ oligodendrocytes did not differ between the groups.

After withdrawal of EGF and FGF-2, about 11.6±1.2% and 15.3±1.0% of cells acquired neuronal phenotypes as shown by Tuj1 immunoreactivity in the hGFAP-L1 and L1-imm groups, respectively as compared to 5.4±1.5% of cells in WT NSCs (Fig. 12A, B). In contrast, the percentages of GFAP$^+$ astrocytes were decreased in hGFAP-L1 NSCs and L1-imm cells (41.5±3.8% and 43.6±4.9%, respectively) as compared to 56.2±1.9% of GFAP$^+$ astrocytes in WT NSCs (Fig. 12A, B). Very few cells expressed the

oligodendrocyte marker protein CNPase: 2.6±0.5% and 1.6±0.6% of cells differentiated into CNPase positive oligodendrocytic profiles in hGFAP-L1 and L1-imm cells as compared to 2.1±1.1% of cells in WT NSCs. As cells of all neural lineages could be derived from stem cells derived from hGFAP-L1 transgenic animals, the isolated cells are multipotent. Thus, L1 increases neuronal and decreases astrocytic differentiation of neural precursor cells *in vitro.*

### 3.3 hGFAP-L1 NSCs and L1-imm cells show enhanced migration *in vitro* compared to WT NSCs

To investigate whether ectopic expression of L1 under the control of the human GFAP promoter in neural precursor cells had an effect on migration of cells in adherent cell culture, neurospheres generated from hGFAP-L1 NSCs, L1-imm cells and WT NSCs were plated on PLL-coated substrate to compare their migratory behavior. The migration distance was evaluated 24 hours after plating (Fig. 13A). Cells migrating from hGFAP-L1 and L1-imm neurospheres were found to migrate further than cells from WT neurospheres, when cultured in a medium without EGF and bFGF. The average migration distance of hGFAP-L1, L1-imm and WT neurospheres were 240±35 µm, 250±47 µm, and 110±22 µm, respectively (Fig. 13B). Thus, ectopic expression of L1 in neural stem/precursor cells enhances the migration of neurosphere-derived cells *in vitro*.

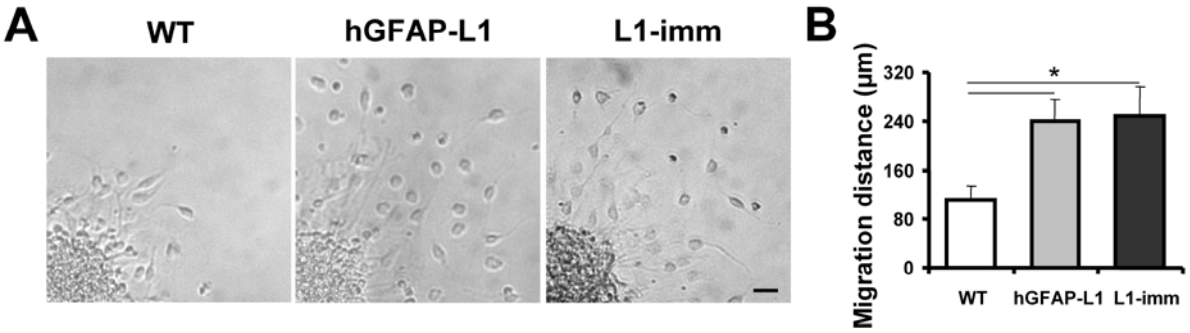


**Figure 13. Ectopic expression of L1 in neural precursor cells enhances the migration of neurosphere-derived cells.** We measured whether ectopic expression of L1 in neural precursor cells enhances the migration of neurosphere-derived cells cultured on PLL precoated coverslips, which permits radial migration of neurosphere-derived cells *in vitro*. Cells from hGFAP-L1 NSC, L1-imm and WT neurospheres were allowed to attach and cultured in a medium without both EGF and bFGF for 24 h. (A) There was a significant increase in the migration distance from hGFAP-L1 and L1-imm neurospheres compared with the WT neurospheres. (B) Mean migration distance ± s.e.m. is displayed. ∗$p<0.05$. Scale bar, 30 µm.

## 4. L1 expressed by transgenic astrocytes enhances neuronal differentiation, decreases astrocytic differentiation, and enhances neurite outgrowth of wild type neural precursor cells *in vitro*.

Because L1 expression is driven by the human GFAP promoter in neural stem cells derived from transgenic mice, L1 is expressed by differentiated astrocytes derived from these cells (Fig. 7D, E). To investigate whether L1 expressed by astrocytes could affect differentiation and neurite outgrowth of neural stem cells derived from wild type mice, WT and hGFAP-L1 NSCs expressing green fluorescent protein ($GFP^+$ cells) were cultured as a monolayer. Ten days after induction of differentiation by growth factor withdrawal, the majority of stem cells had differentiated into astrocytes in both groups. Astrocytes differentiated from hGFAP-L1 NSCs ($L1^+$ astrocytes) expressed L1 (as shown in Fig.7D, E) while astrocytes derived from WT NSCs (WT astrocytes) did not. WT NSCs not expressing GFP ($GFP^-$ cells) were seeded onto the differentiated astrocytes and were allowed to differentiate for another seven days (Fig. 14A). Neurons were immunocytochemically identified by their expression of β–III tubulin (Tuj1), mature astrocytes by their expression of GFAP, and oligodendrocytes by their expression of CNPase (Fig. 14B). 11.2±1.1% of $GFP^-$ cells acquired a neuronal phenotype when cultured on $GFP^+$ $L1^+$ expressing astrocytes as compared to 4.9±1.4% of cells in $GFP^+$ WT astrocytes. Furthermore, 40.1±3.2% $GFP^-$ cells differentiated into astrocytes when cultured on $GFP^+$ $L1^+$ astrocytes as compared to 53.7±2.1% of $GFP^-$ cells when cultured on $GFP^+$ WT astrocytes (Fig. 14B). Only few cells differentiated into oligodendrocytes in both groups (2.3±1.1% of $GFP^-$ cells cultured on $GFP^+$ $L1^+$ astrocytes as compared to 2.8±0.2% of $GFP^-$ cells cultured on $GFP^+$ WT astrocytes). In addition, neurons derived from $GFP^-$ cells cultured on $GFP^+$ $L1^+$ astrocytes showed an enhanced average neurite length (100.34±10.7 µm) when compared to $GFP^-$ cells cultured on $GFP^+$ WT astrocytes (66.5±4.6µm) (Fig. 14B).

Thus, ectopic expression of the neural cell adhesion molecule L1 on the cell surface of astrocytes enhances neuronal differentiation, decreases astrocytic differentiation, and enhances neurite outgrowth of cocultured wild type neural stem cells *in vitro*.

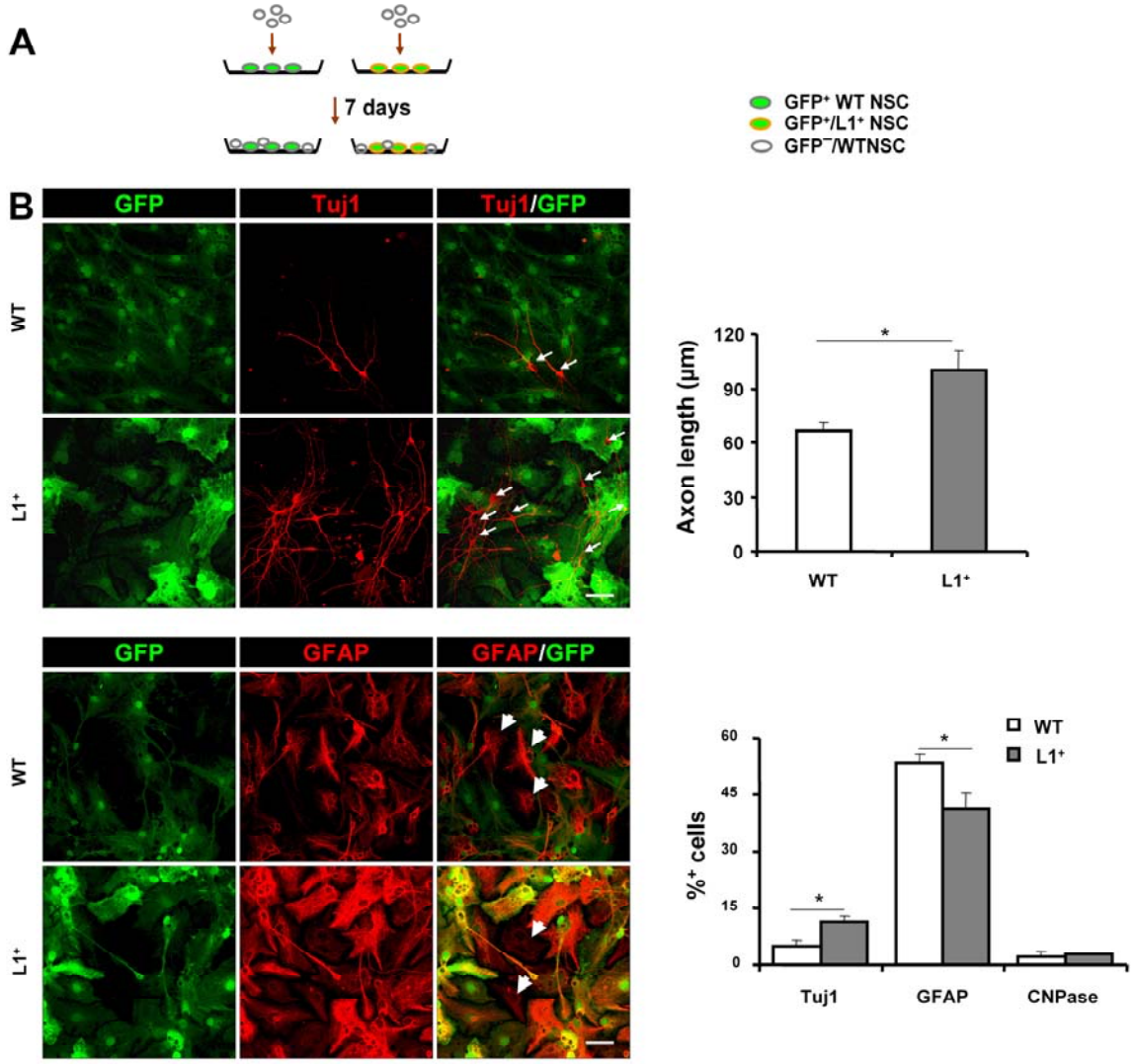


**Figure 14. L1 expressed by transgenic astrocytes derived from hGFAP-L1 NSCs enhance neuronal differentiation, decrease astrocytic differentiation, and enhance neurite outgrowth of neurons derived from cocultured WT NSCs *in vitro*.** (A) Schematic drawing illustrating the experimental procedure. WT and hGFAP-L1 NSCs expressing enhanced green fluorescent protein ($GFP^+$ cells) were cultured as a monolayer. Ten days after differentiation by growth factor withdrawal, differentiated cells had mainly differentiated into astrocytes in both groups. Astrocytes differentiated from hGFAP-L1 NSCs expressed L1 ($L1^+/GFP^+$) while astrocytes derived from WT NSCs did not ($WT/GFP^+$). Non-GFP expressing WT NSCs ($WT/GFP^-$) were seeded onto differentiated astrocytes of both groups and differentiated for another seven days. (B) Immunocytochemical analysis revealed $Tuj1^+/GFP^-$ neurons (arrows) and $GFAP^+/GFP^-$ astrocytes (arrowheads) on $WT/GFP^+$ or $L1^+/GFP^+$ astrocytes and the axon length of $Tuj1^+$-$GFP^-$neurons was determined. The percentages of $Tuj1^+/GFP^-$, $GFAP^+/GFP^-$, and $CNPase^+/GFP^-$ cells of all $GFP^-$ cells are displayed (mean ± s.e.m., *$p < 0.05$). Scale bar, 20 µm.

## 5 Spinal cord injury, transplantation, assessment of functional recovery and morphologic analysis

To investigate whether hGFAP-L1 NSCs and L1-imm cells had a beneficial effect on functional recovery after transplantation in a mouse spinal cord lesion paradigm when compared to WT NSCs or PBS, an electromagnetic mouse spinal cord compression device was used to elicit a compression injury in female WT C57BL/6J mice as previously described (Curtis et al., 1993). Briefly, the spinal cord was maximally compressed for 1 s by a time-controlled current flow through the electromagnetic device.

NSCs were dissociated and resuspended in PBS at a density of 100,000 viable cells per µl. Then, 1 µl of 100,000 viable cells or PBS as a control were injected 0.5 mm both rostral and caudal to the lesion site, 1 mm deep into spinal cord four days after spinal cord injury (Fig. 15A).

Four groups of mice were examined: (*i*) C57BL/6J mice transplanted with hGFAP-L1 NSCs, referred to as hGFAP-L1 group; (*ii*) mice transplanted with L1-imm cells, referred to as L1-imm group; (*iii*) mice transplanted with WT NSCs, referred to as WT group; (*iv*) mice that were sham-injected with PBS, referred to as PBS group. All transplanted NSCs were derived from mice ubiquitously expressing GFP and could thus be reliably identified by their green fluorescence *in vivo*.

Assessment of functional recovery from spinal cord contusion was evaluated using the Basso, Beattie, Bresnahan (BBB) rating scale (Basso et al., 1995), modified for mice (Joshi and Fehlings, 2002), and a novel single-frame motion analysis (Apostolova et al., 2006). This method includes evaluation of four parameters in three different tests: beam walking (foot-stepping angle and rump-height index), voluntary movements without body weight support (extension–flexion ratio), and inclined ladder climbing (number of correct steps). Assessment was performed before and at one, three, and six weeks after the injury. Values for the left and right extremities were averaged. Recovery indices were used as a measure of functional recovery on an individual animal level. Moreover, mice transplanted with hGFAP-L1 NSCs, L1-imm cells, WT NSCs, or PBS were sacrificed four and eight weeks after spinal cord injury for morphological analysis.

## 5.1 Survival and distribution of hGFAP-L1 NSCs, L1-imm cells, and WT NSCs in the compression-lesioned mouse spinal cord after transplantation

Eight weeks after transplantation, cells derived from transplanted NSCs were detected mainly around the scar tissue that had developed at the lesion-site while and small number of graft-derived cells had migrated into the lesion site (Fig. 15B). As it was previously shown that the region caudal to the lesion site shows more dysregulated genes than the area rostral to the lesion site (De Biase et al., 2005) potentially affecting survival and distribution of grafted cells, cell number and distribution and graft volume were quantified rostrally and caudally separately.

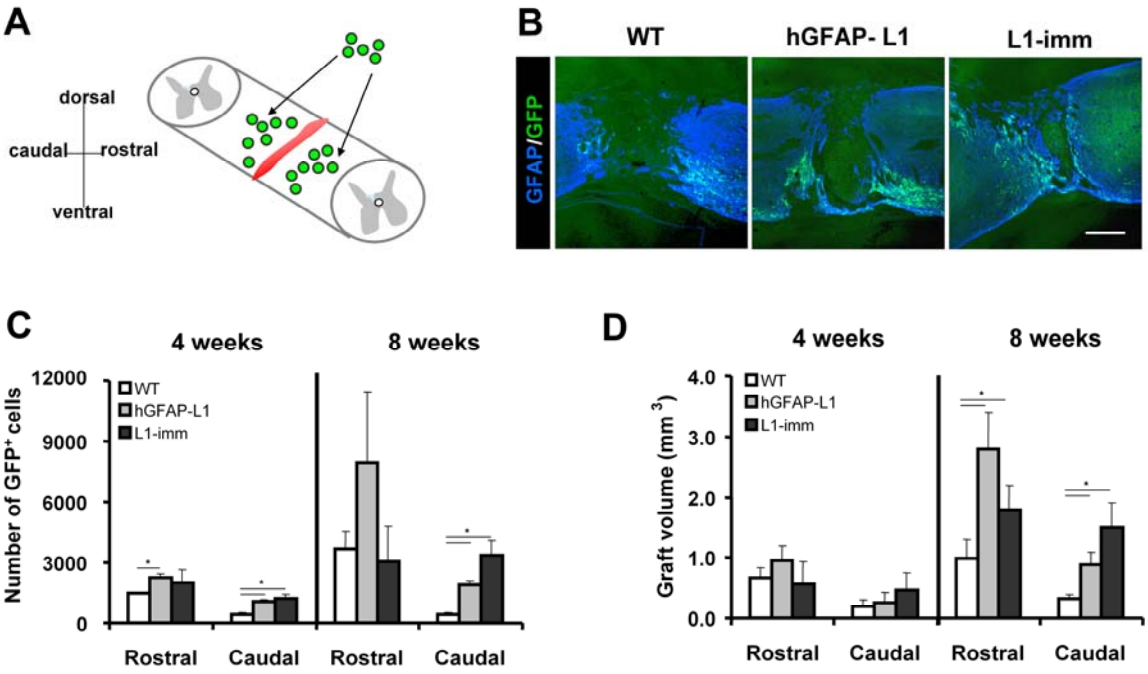


**Figure 15. Analysis of number of grafted cells and graft volume 4 and 8 weeks after transplantation into the compression-injured mouse spinal cord** (A) Schematic drawing illustrating the cell transplantation procedure. WT and hGFAP-L1 NSCs and L1-imm cells expressing enhanced green fluorescent protein (GFP) were injected both rostral and caudal to the lesion site four days after compression-lesioning. (B) Representative immunofluorescence photomicrographs of the location of engrafted cells derived from transplanted WT and hGFAP-L1 NSCs and L1-imm cells eight weeks after transplantation. Scale bar, 300 µm. (C) The numbers of engrafted $GFP^+$ cells rostral and caudal to the lesion-site were determined in serial sections. Mean± s.e.m. is displayed, * $p < 0.05$ (D) The graft volume rostral and caudal to the lesion-site was determined in serial sections. Mean± s.e.m. is displayed, * $p < 0.05$. Student's t-test was applied for statistical analysis.

The number of engrafted $GFP^+$ cells at both rostral and caudal site to lesion area was determined using stereological cell counting per four and eight weeks after transplantation, respectively (Table 1). Analysis of all three transplantation groups revealed poor survival of grafted cells 4 weeks after transplantation. About 3.6±0.3% and 4.1±0.8% of cells survive in hGFAP-L1 and L1-imm group as compared to 2.1±0.2% of cells in the WT group. Interestingly, quantitative comparison revealed that there were significantly higher numbers of engrafted-derived cells caudal to the lesion site in the hGFAP-L1 and the L1-imm group as compared to the WT group, suggesting that cell viability was higher in these groups. Moreover, there is a 2- to 3-fold increase in the cell number between four and eight weeks after transplantation, which implies that graft-derived cells underwent mitotic divisions during this phase. Eight weeks after transplantation, the numbers of grafted-derived cells caudal to lesion site in the hGFAP-L1 and the L1-imm group remained significantly higher than in the WT group (Fig. 15C).

**Table 1 Number of grafted cells and graft volume**

| Time after grafting | | Graft volume ($mm^3$) | | Number of $GFP^+$ cells | |
|---|---|---|---|---|---|
| | | Rostral | Caudal | Rostral | Caudal |
| 4 weeks | **WT** | 0.66±0.18 | 0.19±0.11 | 1456±77 | 398±133 |
| | **hGFAP-L1** | 0.95±0.25 | 0.26±0.17 | 2217±240 | 1076±73 |
| | **L1-imm** | 0.56±0.37 | 0.46 ±0.29 | 2001±651 | 1203±221 |
| 8 weeks | **WT** | 0.99±0.3 | 0.32±0.07 | 3647 ±861 | 427±63 |
| | **hGFAP-L1** | 2.8±0.6 | 0.88±0.21 | 7910±3545 | 1859±212 |
| | **L1-imm** | 1.79±0.4 | 1.5±0.4 | 3067±1704 | 3380±672 |

Graft size was estimated using the Cavalieri principle four and eight weeks after transplantation, respectively (Table 1). Four weeks after SCI, quantitative comparison between the three groups revealed that there were no apparent differences in graft volume, suggesting that graft volume was comparable at this time point. Eight weeks after transplantation, the graft volume had increased in all three groups and was significantly higher in the hGFAP-L1 and L1-imm group when compared to the WT group both rostral and caudal to the lesion site (Fig. 15D, Table 1).

**Table 2 Location of transplanted cells**

| Time after grafting | | Longitudinal ( µm ) | | Horizontal ( µm ) |
|---|---|---|---|---|
| | | Rostral | Caudal | |
| 4 weeks | **WT NSC** | 1028.3±59.2 | 890.0±64.0 | 1456±77 |
| | **hGFAP-L1 NSC** | 1312.7±22.8 | 1276.7±28.0 | 2217±240 |
| | **L1-imm** | 1791.8±398.2 | 1360.1 ±20.1 | 2001±651 |
| 8 weeks | **WT NSC** | 2254.5±314.11 | 874.4±118.1 | 1440 ±146.9 |
| | **hGFAP-L1 NSC** | 3783.1±291.4 | 1809.3±110.4 | 1840±71.8 |
| | **L1-imm** | 3272.1±330.7 | 1622.0±64.3 | 1657.1±84.1 |

Furthermore, the distribution of grafted cells was measured in a rostrocaudal and horizontal direction (Fig. 16A, B). Quantitative comparison among the three groups revealed that concerning the longitudinal distribution there was an increase of the extension of the graft in hGFAP-L1 and L1-imm group at both four and eight weeks after transplantation when compared to the WT group, suggesting that cell migration ability was comparably high in the hGFAP-L1 and the L1-imm group. Moreover, concerning the horizontal distribution, quantitative comparison revealed that there was an increase in the horizontal extension of the graft in the hGFAP-L1 and the L1-imm group when compared to the WT group four weeks but not eight weeks after transplantation, suggesting that grafted cells had reached the limiting boundary of the spinal cord (Table 2).

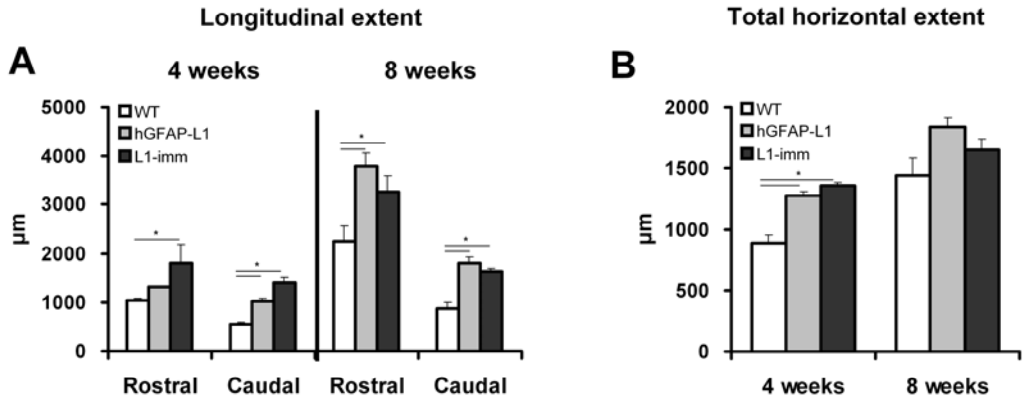


**Figure 16. Characterization of grafted cell distribution at 4 and 8 weeks after SCI.** (A) Location of engrafted area in longitudinal extent at both rostral and caudal to the spinal cord injury site was determined four and eight weeks after cell transplantation (mean ± s.e.m. * $p < 0.01$). (B) Location of engrafted area in horizontal extent was determined four and eight weeks after transplantation (mean ± s.e.m. * $p < 0.01$).

### 5.2 hGFAP-L1 NSCs and L1-imm cells show increased neuronal differentiation and decreased astrocytic differentiation after transplantation into the injured mouse spinal cord.

The differentiation of hGFAP-L1 NSCs, L1-imm cells and WT NSCs grafted into the compression-lesioned spinal cord was monitored to compare the fate of naive NSCs compared with the fate of NSCs ecotopically expressing the neural cell adhesion molecule L1 driven by human GFAP promoter.

Four weeks after transplantation, NSC-derived cells were present mainly surrounding the lesion-site. To monitor neuronal differentiation of engrafted cells, immunohistochemical analysis with antibodies against doublecortin (DCX, a microtubule-associated protein that serves as a specific marker for migrating immature neurons) was performed. In mice grafted with $GFP^+$ hGFAP-L1 NSCs and L1-imm cells, a significantly higher fraction of cells showed immunoreactivity with antibodies against DCX than in mice grafted with $GFP^+$ WT NSCs (10.9±2.4% and 8.5±2.6%, respectively vs 2.9±1.2% $DCX^+$ cells of all $GFP^+$ cells) (Fig. 17A).

Eight weeks after transplantation, neuronal differentiation was monitored applying antibodies against the neuronal marker protein β–III tubulin (Tuj1). Mature astrocytes were identified by their expression of glial fibrillary acidic protein (GFAP) and oligodendrocytes by their expression of 2',3'-cyclic nucleotide 3' phosphodiesterase (CNPase). The fraction of grafted ($GFP^+$) cells showing Tuj1 immunoreactivity was enhanced in mice transplanted with hGFAP-L1 NSCs and L1-imm cells (5.7±1.09% and 6.9±1.2%) when compared with mice transplanted with WT NSCs (2.3±0.7%). Furthermore, the $Tuj1^+$ graft-derived neurons extended β-III tubulin-immunoreactive processes with a length of several cell body diameters. The length of main process was measured (Fig. 17C). Quantification of the average neurite length revealed that neurons derived from hGFAP-L1 NSCs and L1-imm cells had significantly longer processes (107.6±12.1 and 119.4±15.2μm) than neurons derived from WT NSCs (63.8±10μm). Furthermore, ectopic expression of L1 led to decreased astroglial differentiation of grafted cells in the hGFAP-L1 and the L1-imm group (58.9±6.5% and 52.8±9.9%, respectively) when compared to the WT group (67.9±9.1%) (Fig. 17B).

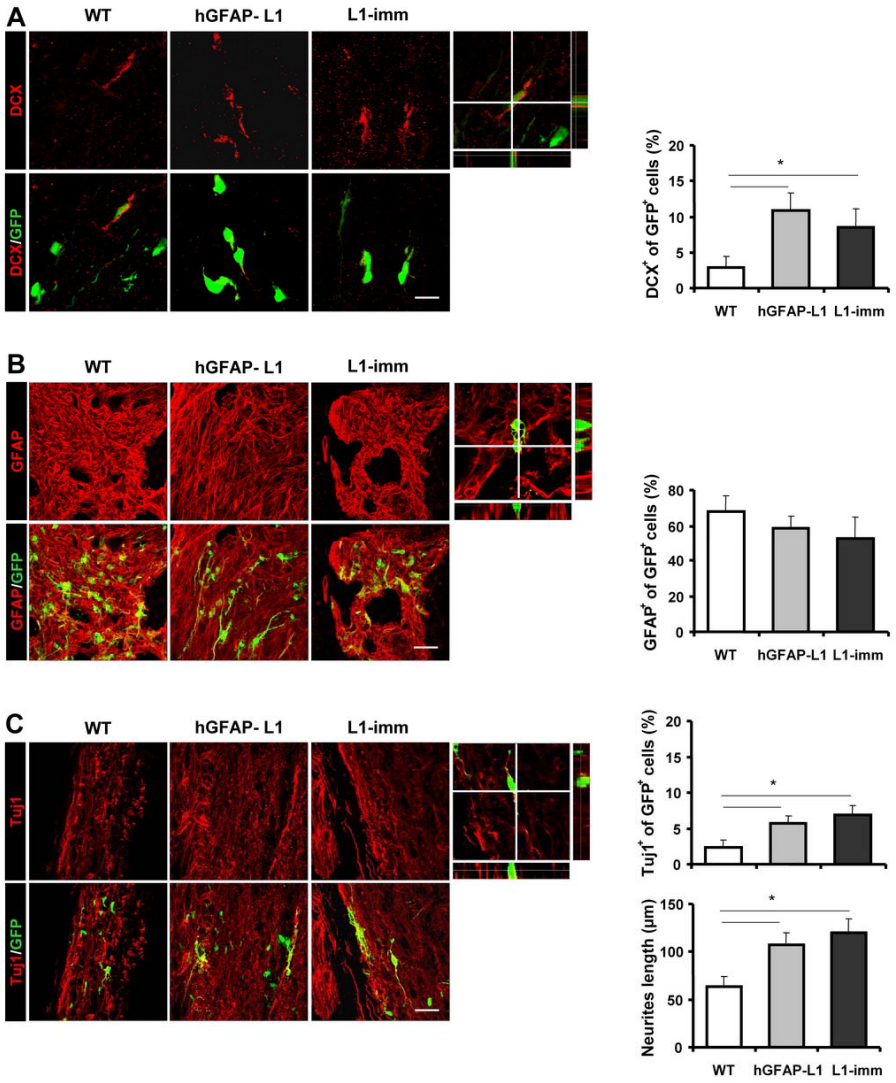


**Figure 17. hGFAP-L1 NSCs and L1-imm cells show increased neuronal differentiation, decreased astrocytic differentiation, and increased length of neurites after transplantation into the injured mouse spinal cord.** (A) Confocal images of WT and hGFAP-L1 NSCs and L1-imm cells (green) immunostained with antibodies against the early neuronal marker doublecortin (DCX, red) four weeks after transplantation into the spinal cord. Scale bar, 40µm. In the upper right, an orthogonal view is shown to confirm the double labeling with DCX and GFP. Percentages of $DCX^+$ cells of $GFP^+$ cells are shown (mean ± s.e.m.).* $p < 0.01$. (B) Engrafted cells (green) immunostained with an antibody against glial fibrillary acidic protein (GFAP, red) eight weeks after transplantation. Scale bar, 40 µm. In the upper right, an orthogonal view is shown to confirm the double labeling with GFAP and GFP. Percentages of $GFAP^+$ cells of $GFP^+$ cells are shown (mean ± s.e.m.). Scale bar, 40 µm. (C) Confocal images of grafted cells (green) immunostained with an antibody against the neuronal marker Tuj1 8 weeks after transplantation into the spinal cord. Scale bar, 40 µm. In the upper right, an orthogonal view is shown to confirm the double labeling with Tuj1 and GFP. Percentages of $Tuj1^+$ cells of $GFP^+$ cells are shown (mean ± s.e.m.). Furthermore, the length of the longest $Tuj1^+$-$GFP^+$ neurite was determined. Mean± s.e.m. are shown * $p < 0.01$.

In all three groups, some of the graft-derived neurons acquired a GABAergic phenotype, which was identified by immunostaining with antibodies against glutamate decarboxylase (GAD) (Fig. 18A). Moreover, immunostaining with antibodies against the vesicular GABA transporter (VGAT) revealed that graft-derived GABAergic nerve endings were observed surrounding some host motoneurons identified by size of their cell somata and nuclei (Fig. 18B). Graft-derived glutamatergic terminals could also be identified by staining with antibodies against VGLUT1/VGLUT2 (to label a maximal number of glutamatergic terminals, antibodies against VGLUT1 and VGLUT2 were used in combination) (Fig. 18C). Oligodendroglial differentiation was rarely detected in all three groups (Fig. 18 D).

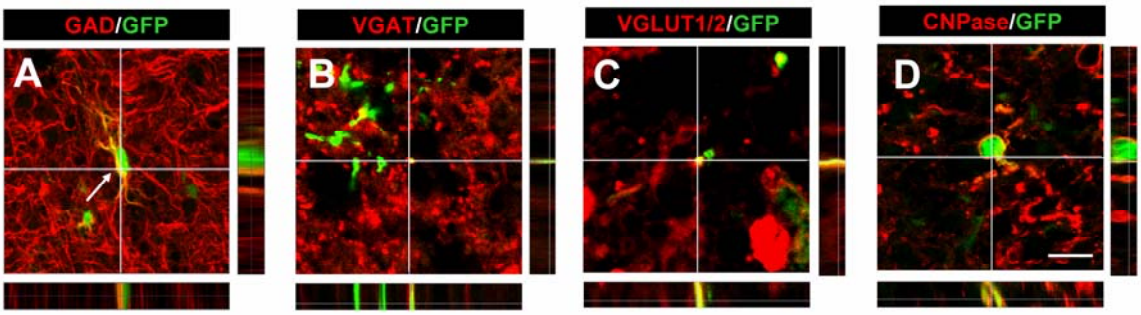


**Figure 18. Differentiation into neuronal subtypes and oligodendrocytic differentiation of grafted cells.** Orthogonal views of confocal images are shown to illustrate evidence of GABAergic (A and B) and glutamatergic (C) neuronal differentiation and oligodendrocytic differentiation (D) of grafted cells. (A) Sections containing grafted ($GFP^+$) cells (green) are stained with antibodies against the GABA-synthesizing enzyme GAD (red). Arrow indicates cell with cytoplasmic GAD immunoreactivity. (B) Sections are stained with antibodies against vesicular GABA transporter (VGAT) showing GABAergic nerve endings (red) of grafted-derived cells (green) on the host motoneurons (motoneurons were identified by size of the cell somata and nuclei). (C) Immunohistochemical staining with antibodies against VGLUT1 and VGLUT2 (red) to identify graft-derived (green) glutamatergic axon terminals. (D) Immunohistochemical analysis with antibodies against CNPase (red) to identify graft-derived (green) oligodendrocytes. Scale bars, 20 µm.

## 5.3 Analysis of host motoneuron soma size and synaptic inputs to host motoneurons after transplantation of NSCs into the compression-lesioned mouse spinal cord

To analyze whether transplanted cells could enhance functional recovery after spinal cord injury by remodeling of cell circuitries, the densities of synaptic terminals around the cell bodies (somata) of host motoneuron were determined eight weeks after SCI.

Before, the mean areas of the somata of host motoneurons were determined caudal to the lesion-site in the compression-lesioned spinal cord of animals grafted with in hGFAP-L1 NSCs, L1-imm cells, WT NSCs, or sham-injected with PBS. Immunohistochemical analysis revealed that the mean area of the somata of host motoneurons was significantly enhanced in the hGFAP-L1 and the L1-imm groups (1009±71 and 967±65 $\mu m^2$, respectively) were significantly larger than in the WT and PBS group (797±63 and 706±53 $\mu m^2$, respectively). Moreover, quantitative analysis showed a tendency towards an enhanced average host motoneuron soma size in the WT group when compared to the sham-injected (PBS) group (Fig. 19). These findings indicate, that hGFAP-L1 NSCs and L1-imm cell transplantation might have an enhanced direct or indirect neurotrophic effect on host motoneurons caudal to lesion-site when compared to PBS only that is less pronounced in WT NSCs.

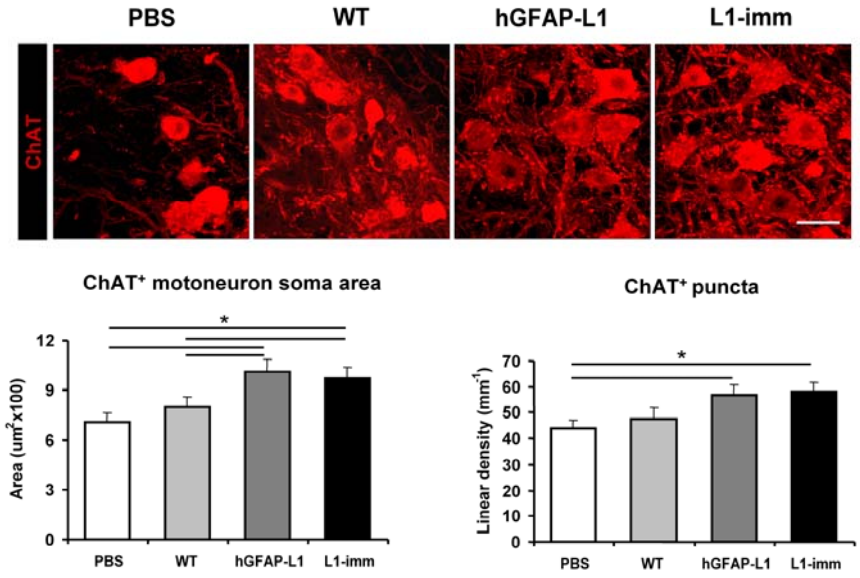


**Figure 19. Analysis of soma size and perisomatic terminals of host motoneurons in sections from mice treated with PBS, WT NSCs, hGFAP-L1 NSCs and L1-imm cells.** $ChAT^+$ Soma area and linear density of $ChAT^+$ boutons (punctae) were determined by measuring one image per cell at the level of the largest cell body cross-sectional area in confocal images. Shown are mean ± s.e.m., * $p < 0.05$. Scale bar, 20 µm.

Furthermore, the linear density (number per unit length) of large perisomatic $ChAT^+$ boutons (puncta), which have been shown to comprise C-type synapses on motoneurons associated with muscarinic type 2 receptors (Davidoff and Irintchev, 1986; Hellstroem et al., 2003), was determined. Coverage of host motoneuron somata by $ChAT^+$ puncta was significantly higher in the hGFAP-L1 and the L1-imm group (56.6±4.1, 58.0±3.6

$mm^{-1}$) when compared to the sham-injected (PBS) group (43.8±3.2 $mm^{-1}$) while the WT groups showed a tendency towards enhanced coverage of host motor neurons by $ChAT^+$ puncta (47.4±4.7 $mm^{-1}$) that was not statistically significant (Fig. 19).

### 5.4 Mice transplanted with hGFAP-L1 NSCs and L1-imm cells show enhanced numbers of $TH^+$ axons in the compression-injured spinal cord distal to the lesion-site

Monoaminergic descending pathways, located in the ventral and lateral columns of the spinal cord originating from the reticular formation, raphe nuclei, locus coeruleus, pretectal area, and the substantia nigra (Kiehn et al., 1992, Shapiro, 1997) modulate the excitability of spinal cord circuitries capable of initiating and controlling rhythmic coordinated movements (Fouad and Pearson, 2004). To assess whether transplantation of NSCs expressing L1 under the human GFAP promoter could enhance monoaminergic reinnervation of the spinal cord caudal to the lesion-site improve, the numbers of catecholaminergic (tyrosine hydroxylase-positive ($TH^+$)) and serotonergic ($5\text{-}HT^+$) axons projecting beyond an arbitrarily selected border 250 μm caudal to the lesion-site in spaced serial parasagittal sections eight weeks after transplantation. The number of $TH^+$ fibers eight weeks after transplantation were increased in the hGFAP-L1 and L1-imm group (11.2±1.1 and 11.5±1.2, respectively) when compared to the WT and PBS group (7.6±1.2 and 5.7±1.0, respectively) (Fig. 20A, B). Although it is not clear whether the increase in fiber numbers was attributable to regrowth of axons across the lesion scar or sprouting of spared axons caudal to the lesion, this observation indicates an enhanced regenerative response of catecholaminergic fibers in the hGFAP-L1 and L1-imm group. Moreover, a tendency towards enhanced numbers of serotonergic ($5\text{-}HT^+$) axons in the hGFAP-L1 and L1-imm group, that was statistically not significant, was detected when compared to the WT and PBS group (Fig. 20C).

After injury, the proximal segments of the cortical spinal tract (CST) axons, which are required for voluntary movement, retract from the lesion site (Houle and Jin, 2001). This ‘dying back’ allows detection of treatment-related effects such as enhanced axonal regrowth towards the lesion site or reduced retraction. CST axons were labeled with CaM-kinaseII (CaMKII) and the distance between the tips of the labelled axons and the GFAP positive rostral border of the lesion site was measured (picture not shown). No significant differences in this distance were detected among the four experimental groups (Fig. 20D).

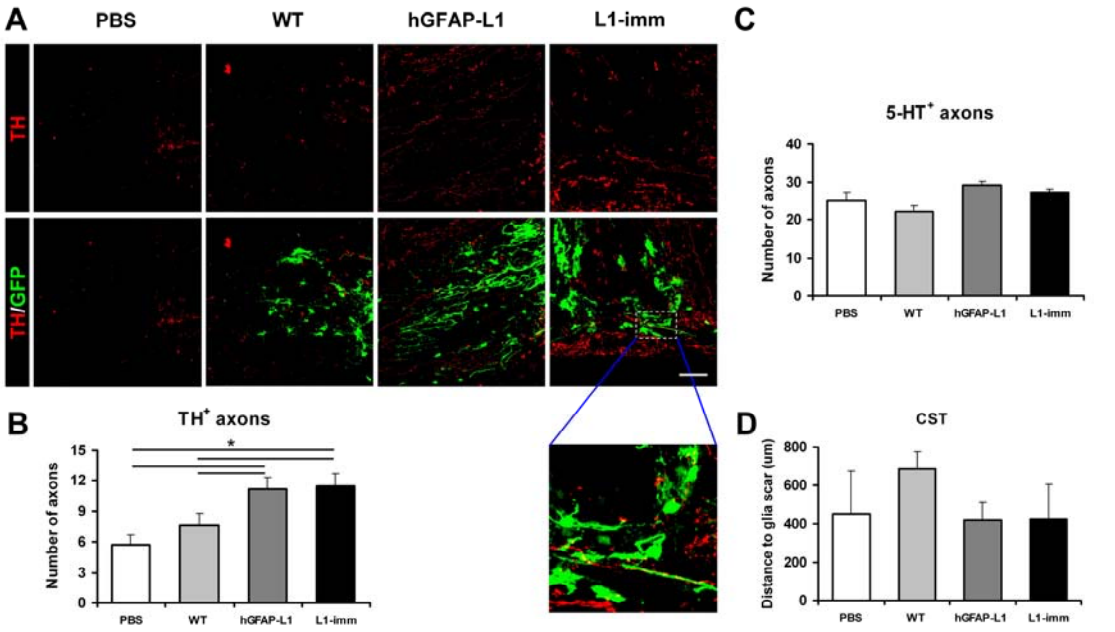


**Figure 20. Enhanced numbers of catecholaminergic TH$^+$ axons distal to the lesion-site in mice transplanted with hGFAP-L1 NSCs and L1-imm cells.** Catecholaminergic axons identified by immunohistochemical staining with antibodies against tyrosine hydroxylase (TH). (A) Confocal image showing host TH$^+$ axons (red) associated with graft-derived (GFP$^+$) processes (green). Scale bar, 25 µm. (B) Mean numbers of TH$^+$ fibers crossing an arbitrary border 250 µm caudal to the lesion-site eight weeks after transplantation are shown (mean ± s.e.m.). * $p < 0.05$. (C) Mean numbers of serotonergic (5-HT$^+$) fibers crossing an arbitrary border 250 µm caudal to the lesion-site eight weeks after transplantation are shown (mean ± s.e.m.). (D) CST axons were labeled with CaM-kinaseII (CaMKII) and the distance between the tips of the labelled axons and the GFAP positive rostral border of the lesion site was measured eight weeks after transplantation. Mean distances ± s.e.m. are shown.

## 5.5 The host tissue shows a decreased microgial reaction after syngeneic transplantation of NSCs into the lesioned spinal cord, but no difference in the glial reaction.

Activation of microglia and astrogliosis occur in reaction to various lesions in the central nervous system. As NSCs have been shown to have an anti-inflammatory effect when transplanted into the traumatized brain (Park et al., 2002; Lee et al., 2007) and spinal cord (Teng et al., 2002; Yang et al., 2008), microglial reaction and reactive gliosis was monitored in mice transplanted with hGFAP-L1 NSCs, L1-imm cells, and WT NSCs or sham-injected into the compression-lesioned spinal cord. The microglial marker protein Iba1 was used to efficiently monitor microglial activation in the spinal cord six weeks after transplantation. In region-matched sections, the activated microglia was significantly reduced in the hGFAP-L1, L1-imm and WT group when compared to PBS-injected mice. This indicates that syngeneic transplantation of NSCs into the

injured spinal cord did not provoke an immunorejection but could reduce microglial reaction in the injured spinal cord tissue (Fig. 21A, B). No significant differences were detected between the L1 expressing (hGFAP-L1 and L1-imm) and the WT group indicating that L1 has no effect on the microglial reaction.

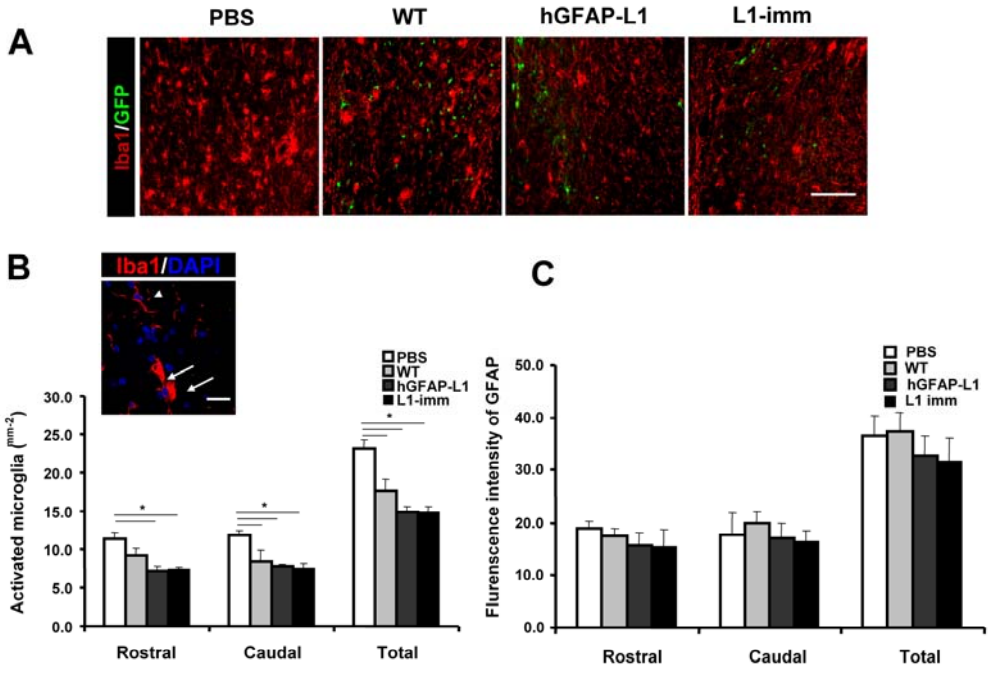


**Figure 21. The host tissue shows a decreased microgial reaction after syngeneic transplantation of NSCs into the lesioned spinal cord, but no difference in the glial reaction.** (A) Confocal images of the compression-lesioned mouse spinal cord grafted with WT NSCs, hGFAP-L1 NSCs, and L1-imm cells (green) and matched regions in the spinal cord of the PBS group immunostained with antibodies against the microglial marker protein Iba-1 (red). Scale bar, 100 µm. (B) The profile density of activated microglia identified by the morphology of $Iba1^+$ immunoreactive cells (red) was determined. In the upper left, representative confocal images of host quiescent, ramified (arrowhead) and activated microglia (arrow) are indicated. Nuclei were counterstained with DAPI (blue). Mean values ± s.e.m. of profile density of activated microglia eight weeks after spinal cord injury (*$p < 0.01$, Tukey's one-way ANOVA was performed for statistical analysis). Scale bar, 100 µm. (C) Mean expression of GFAP in the host tissue, is shown (± s.e.m.).

To evaluate whether cell transplantation could modulate reactive astrogliosis, which is an impediment to neuronal recovery and axonal regeneration, astrocytes were stained with antibodies against GFAP and the immunoflurenscence intensity was measured eight weeks after transplantation. No significant differences in the mean fluorenscence intensity of GFAP immunostaining were determined between the four experimental groups (Fig 21C) indicating that transplanted cells did not influence reactive gliosis.

### 5.6 hGFAP-L1 NSCs and L1-imm cells promote functional recovery after spinal cord injury

Finally, the capacity of NSCs ectopically expressing L1 under the control of human GFAP promoter and L1 expressing radial glia-like cells to promote functional recovery after spinal cord injury was analysed.

Measuring the parameters described in the Materials and Methods section, functional recovery was monitored in the hGFAP-L1, L1-imm, WT, and PBS group before and one, three, and six weeks after compression-injury.

The recovery of ground locomotion was first evaluated using the Basso, Beattie, Bresnahan (BBB) rating scale (Basso et al., 1995), modified for mice (Joshi and Fehlings, 2002). The preoperative values were similar in the four groups. Spinal cord compression caused severe disabilities, the degree of impairment found one week after injury was also similar in the four groups (Fig. 22A). Between one and six weeks, the walking abilities improved significantly more in mice transplanted with hGFAP-L1 NSCs and L1-imm cells when compared with mice gradted with WT NSCs and PBS-treated mice as revealed by analysis of the absolute BBB score values (Fig. 22A) and recovery indices, estimating the gain of function after the first week as a fraction of the functional loss induced by the surgery (data not shown). There was a tendency that transplantation of WT NSCs promoted recovery of hindlimb movement compared with vehicle injection (vehicle) in BBB score at 6 week after injury.

In addition to the BBB score, the plantar stepping ability of the animals was analysed using a novel parameter, the foot-stepping angle (Apostolova et al., 2006). This analysis revealed, in agreement with the BBB scores, an enhanced recovery in the hGFAP-L1 and L1-imm group when compared with the WT and PBS group three and six weeks after transplantation (Fig. 22B).

The rump-height index was determined as a measure of the ability to support body weight during ground locomotion, an ability requiring activity and coordination of muscles working at different joints in both extremities thus (in contrast to the foot-stepping angle) measuring complex motor functions. No differences in the outcome after transplantation into the compression-lesioned spinal cord were found among the four groups (Fig. 22C).

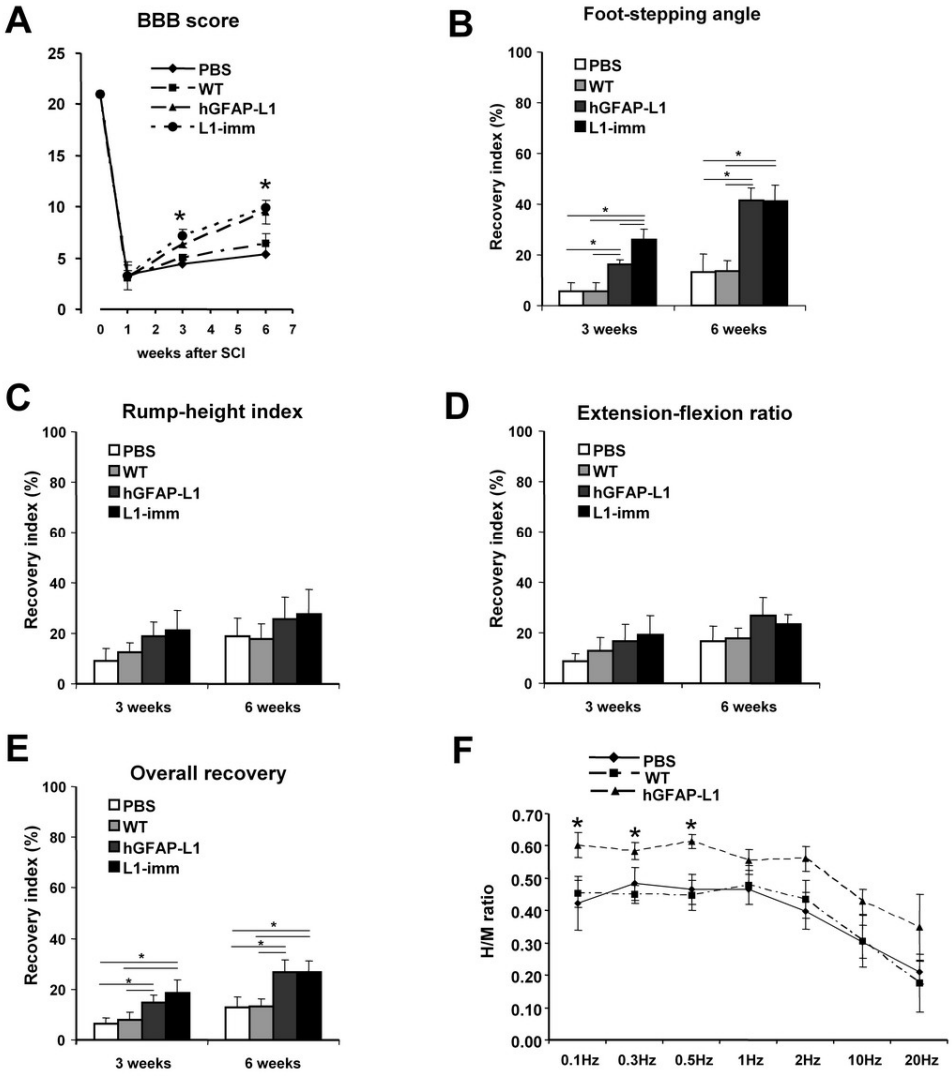


**Figure 22. hGFAP-L1 NSCs and L1-imm cells enhance functional recovery in mice after spinal cord injury.** Time course and degree of functional recovery after spinal cord injury in mice transplanted with WT NSCs, hGFAP-L1 NSCs, L1-imm cells, or sham-injected with PBS is shown. Mean values ± s.e.m. of open-field locomotion (BBB) scores (A), recovery indices for foot-stepping angles (FSA) (B), RHI (rump-height indices) (C), extension–flexion ratios (EFR) (D) and overall recovery indices (E) before injury (0) and one, three, and six weeks after injury are displayed. Numbers of mice studied per group are 11 for PBS, WT, and hGFAP-L1 groups, and 8 mice for L1-imm group. (F) Rate depression of the H-reflex at different time-points after spinal cord injury in mice treated with WT NSCs, hGFAP-L1 NSCs, L1-imm cells, or sham-injected with PBS. The alterations of M- and H-responses were measured in plantar muscle during repetitive stimulation of the sciatic nerve with electronic pulses at frequencies ranging between 0.1 and 20 Hz six weeks after spinal cord injury. The H/M ratios in both the hGFAP-L1 and L1-imm group are significantly higher than in the WT and PBS group at low stimulation frequencies ranging from 0.1 to 0.5Hz. Shown are mean values ± s.e.m. of H/M ratios at different stimulation frequencies. (*$p < 0.05$, one-way ANOVA for repeated measurements with Tukey's *post hoc* test were performed for statistical analysis; $n = 5$ mice per group).

Furthermore, the extension–flexion ratio measuring voluntary movements without body weight support was determined. Also for this parameter, no differences in the outcome after spinal cord injury were found among the four groups (Fig. 22D).

However, numbers of correct steps made by the animals during inclined ladder climbing, reduced to almost 0 in all groups 1 week after injury, did not improve with time in all treated mice (data not shown). The ladder-climbing test allows estimation of the ability to perform precise movements requiring a higher degree of supraspinal control compared with the types of movement assessed by the previous tests.

From the values of the parameters shown in Figure 22A-D, on an individual animal basis, overall recovery indices for group mean values were calculated. The overall recovery indices were significantly better in the hGFAP-L1 and L1-imm group when compared with the WT PBS group three and six weeks after transplantation (Fig. 22E).

Recovery of motor functions in mice is associated with alteration in the H-reflex (Lee et al., 2008). To further confirm the functional recovery in the hGFAP-L1 and L1-imm group, an electrophysiological H/M reflex recording method was applied. Since the promotion of functional recovery was similar in the hGFAP-L1 and the L1-imm group only mice transplanted with hGFAP-L1 NSCs, WT NSCs and mice sham-injected with PBS were analysed six weeks after transplantation. The H/M ratios in the hGFAP-L1 group were significantly higher than in the WT and PBS group at low frequencies (0.1–0.5 Hz, Fig. 22F) and showed a tendency towards enhanced H/M ratios at higher frequencies ranging from 1-20 Hz.

Thus, hGFAP-L1 NSCs and L1-imm cells enhance functional recovery after transplantation into the compression-lesioned spinal cord but the recovery is restricted to involuntary movements with no detection of significant functional recovery in higher motor function.

# V Discussion

Spinal cord injury is a devastating neurological disorder. Thus, more research efforts on combinational approaches are needed to facilitate regenerative effects. Neural stem cells or fate-restricted neuronal or glial progenitor cells are promising candidates for the treatment of spinal cord injury. Neural stem cells or fate-restricted neuronal or glial progenitor cells comprise a population of neural restricted cells without contaminating non-neural cells. They can be expanded *in vitro* for a limited amount of time. Moreover, they have the capacity to differentiate into neurons or glial cells after transplantation into the spinal cord (injured or intact) (Herrera et al., 2001; Han et al., 2002; Han et al., 2004; Yan et al., 2004). Another advantage of neural stem cell transplantation when compared to transplantation is that no tumour formation was reported after transplantation of neural stem cells.

However, grafted neural stem cells differentiate mainly into astrocytes with only a minor fraction differentiating into neurons or oligodendrocytes. Therefore, one aim of this study was to improve neuronal differentiation of neural stem cells. Furthermore, the predominant astrocytic differentiation could be taken advantage of to deliver therapeutic genes with beneficial properties for regeneration.

## 1 The neural cell adhesion molecule L1 and the human GFAP promoter

The neural cell adhesion molecule L1 was an ideal candidate for an exogenous therapeutic gene. It plays an important role in the development and regeneration of the central and peripheral nervous systems and improves cell survival, neurite outgrowth and neuronal differentiation (Brummendorf et al., 1998; Castellani et al., 2000; Zhang et al., 2000; Dihné et al., 2003).

To selectively induce the expression of the target gene in astrocytes, the human GFAP promoter was used. This promoter is well-characterized to be highly selective in driving the expression of a gene of interest in radial glia cells (mouse radial glial cells do not contain detectable protein levels of GFAP [Sancho-Tello et al., 1995, Gotz et al., 2002; Noctor et al., 2002; Malatesta et al., 2003]) and GFAP-positive cells of different mammalian species (Kordower et al. 1997; Nolte et al., 2001; Malatesta et al., 2003).

In the present study, neural stem cells overexpressing L1 under the influence of the human GFAP promoter (hGFAP-L1 NSCs) were derived from hGFAP-L1 transgenic mice which were generated by microinjection as described (Diedrich et al 2008). Thus, in the present study, the genetic modification was not introduced by traditional methods like viral transduction, lipofection, or electroporation because on the one hand until recently, stable transfection of neural stem cells – a prerequisite for reliable transplantation experiments with genetically modified cells – was not possible. On the other hand, upon transfection or infection, stem cells may not remain pluripotential or may be fundamentally altered. The use of transgenic mice to control expression of the gene of interest partially avoids these problems (Gao et al., 1994; Carpenter et al 1997). After derivation of neural stem cells from transgenic animals and wild type littermates, the properties of these cells were analyzed *in vitro*.

In the normal nervous system L1 expression is temporally and spatially regulated. It is detected from embryonic day 10 onwards in the central nervous system on unmyelinated axons of postmitotic neurons throughout adulthood (Rathjen and Schachner, 1984; Fushiki and Schachner, 1986). In our study, L1 was ectopically expressed in radial glia and astrocytes. As predicted, L1 was ectopically expressed in radial glia cells and GFAP positive astrocytes under the influence of the human GFAP promoter. *In vitro*, these L1-expressing radial glia cells accounted for around 5% of undifferentiated hGFAP-L1 NSC proliferating under the influence of FGF-2 and EGF. This demonstrated the heterogeneity of cultured neural stem cells (Suslov et al., 2002) and the necessity to establish a method to enhance the fraction of L1 expressing stem cells. By applying immunoisolation with antibodies against L1, L1 expressing radial glia-like cells (L1-imm cells) could be successfully isolated from cultured hGFAP-L1 NSCs *in vitro*. This one-step immunoisolation method could also be used to purify other neural stem cell-derived cell types based on either endogenous surface markers or exogenous surface molecules introduced by genetic modification. Especially, this efficient isolation method could be very useful to purify embryonic stem cell-derived cells to ensure the absence of undesired non-neural cells and other cells that may lead to tumor formation or other unwanted effects in therapeutic stem cell transplantation (Jüngling et al., 2003). As alternative methods, magnetic-activated cell sorting (MACS) (David et al., 2005; Fong et al., 2009) and fluorescence-activated cell sorting (FACS) (Uchida et al., 2000; Pruszak et al., 2007; Fong et al., 2009) have been used to separate

particular antigen-labelled cells from a heterogeneous population of stem cells to obtain both enrichment and decontamination.

Interestingly, neurosphere-derived cells immunoisolated from hGFAP-L1 NSCs proliferated after replating indicating the isolated population of L1-expressing radial glia like cells could proliferate and be expanded in cell culture. In addition, immunoisolated cells were able to generate neurospheres *in vitro,* supporting the presence of proliferating progenitor cells with neurosphere forming capacity among these immunoisolated cells. Moreover, the ability to generate progeny belonging to the three major neural lineages (neurons, astrocytes and oligodendrocytes) also indicate the multipotency of the stem cells derived from the transgenic animals and L1-expressing radial glia cells.

The proliferation, differentiation, and migratory ability of hGFAP-L1 NSCs and L1-imm cells were analyzed *in vitro*. Based on the results in the present study, over-expression of L1 under the influence of the human GFAP promoter in neural stem cells not only increased neuronal differentiation and cell migration but also reduced proliferation and decreased astroglial differentiation when compared to wild type neural stem cells (WT NSCs). The differences may alternatively be attributed to ectopic L1 expression in radial glia and astrocytes. In the present study, L1 expressed on the surface of radial glia cells and astrocytes possibly modified neural precursor cell proliferation, differentiation, and migration by heterophilic and homophilic mechanisms as described in a previous study (Dihné et al., 2003). Another possibility is that ectopic expression of L1 on radial glia inhibits astrocytic proliferation (Chen et al., 2007) consequently leading to an enhanced fractions of neurons as oligodendrocytic differentiation is rare with the differentiation protocol used in the present study. In L1 deficient mice, the number of hippocampal neurons is reduced demonstrating the importance of L1 in neuronal differentiation (Demyanenko et al., 1999).

It is also important to note that the beneficial effects of L1-expressing astrocytes could be demonstrated *in vitro* by co-culture of L1-positive astrocytes with wild type neural stem/progenitor cells. L1 expressed on the cell surface of viable astrocytes not only increased the neuronal differentiation and suppressed the astrocytic differentiation of co-cultured wild type neural stem/precursor cells, but also promoted neurite outgrowth of neurons differentiated from these neural stem/progenitor cells. This demonstrates that, to some extent, the predominant astrocytic differentiation of neural stem/progenitor cells

can be usefully applied to elicit beneficial effects with ectopically expressed genes. Since the human GFAP promoter can successfully drive expression of the target gene in astrocytes and radial glia cells, in principle, the use of the human GFAP promoter might be applied to allow the expression of other versatile target genes favorable for regeneration in neurodegenerative diseases.

## 2 Enhanced functional recovery in mice transplanted with hGFAP-L1 NSCs and L1-imm cells after spinal cord compression-injury

In the present study, neural stem cells overexpressing L1 under the influence of the human GFAP promoter were transplanted into a mouse model for spinal cord injury to evaluate their regenerative potential. It could be shown that treatment of spinal cord injury with hGFAP-L1 NSCs and L1-imm cells allowed for better locomotor recovery of the hindlimbs when compared to treatment with WT NSCs or sham-injection with PBS.

### 2.1 Spinal cord injury models

Three classes of injury models are commonly used in rodents: transection, compression, and contusion.

Transection involves opening the dura and cutting some or all of the spinal cord with a sharp instrument. Transection injuries, when performed correctly, are amenable to clear anatomical demonstrations of regeneration, because the experimenter controls the exact location and extent of the lesion (but see Steward et al., 2003, for a discussion of the unintentional sparing of axons). Most human spinal cord injuries, however, are quite different from the transection model. Most humans suffer an acute bruise to the spinal cord followed by a period of spinal cord compression. Many researchers thus use the compression and contusion injury models (Rosenzweig et al., 2004).

Compression injuries are induced by squeezing the spinal cord with a modified aneurysm clip or forceps (Rivlin et al., 1978; Fehlings et al., 1995; Rosenzweig et al., 2004), or by placing a weight onto the exposed spinal cord (Holtz et al., 1989; Martin et al., 1992; Rosenzweig et al., 2004).

Contusion injuries (Young, 2002) are induced by hitting the exposed spinal cord, most often with a dropped weight (Gruner 1992), or a solenoid-driven device that displaces

the spinal cord by a preselected amount (Somerson et al., 1987; Behrmann et al., 1992; Beattie, 1992) The contusion and compression injury models seem to be the best available mimics of human injury, but they do not offer precise control over the exact location and extent of the lesion ( Kwon et al., 2002; Rosenzweig et al., 2004).

In our present study, a mouse spinal cord compression device was used to elicit compression injury (Curtis et al., 1993; Steward et al., 2003). Compression force (degree of closure of the forceps) and duration were precisely controlled by an electromagnetic device. The spinal cord was maximally compressed (100%, according to the operational definition of Curtis et al., 1993) for 1 s by a time-controlled current flow through the electromagnetic device. This compression could offer relatively precise control over the exact location and extent of the lesion (Apostolova et al., 2006; Jakovcevski et al., 2007). Moreover, to normalize the injured baseline, in the present study, the "severely" injured mice with individual foot-step angles of more than 150° were selected for transplantation experiments.

### 2.2 Enhanced functional recovery in mice transplanted with hGFAP-L1 NSCs and L1-imm cells after spinal cord compression-injury

The improved overall outcome after spinal cord injury in the hGFAP-L1 and L1-imm group reflected improvement of motor functions controlled at different levels of supraspinal control. Plantar stepping, evaluated by both the BBB score and the foot-stepping angle, requires low levels of supraspinal control only. We found evidence that plantar stepping was improved (measured by BBB score and foot-stepping angle) in mice transplanted with hGFAP-L1 NSCs and L1-imm cells transplanted mice when compared to mice transplanted with WT NSCs or sham-injected with PBS. This functional recovery correlated with increased catecholaminergic innervation of the distal spinal cord by tyrosin hydroxylase-expressing axons. The fact that locomotor activity was significantly better three weeks after transplantation in L1-imm cells versus hGFAP-L1 NSCs points to the possibility that L1 expressing radial glia-like cells, serving as multipotent neural stem cells and a scaffold for migration during normal development of the central nervous system, exerted their beneficial effects at early stages of integration and suggests an early protective effect on the spinal cord following contusive injury (Malatesta et al., 2003; Rakic et al., 2003; Hasegawa et al., 2005). Interestingly, in a previous study, implanted immortalized RG3.6 radial glial cells migrated widely into the contusion site and the spared white matter. Behavioral analysis

indicated higher BBB scores in rats injected with RG3.6 cells than rats injected with fibroblasts or sham-injected with medium as early as 1 week after injury (Hasegawa et al., 2005). The combined observations from different groups indicate that transplanted of radial glia could probably promote early functional recovery following spinal cord injury.

Determining the BBB score as a measure of functional recovery, some groups reported modest improvements by observing plantar stepping ability which requires low levels of supraspinal control only. For example, implantation of a multicomponent polymer scaffold together with neural stem cells in an adult rat hemisection model of SCI increased the efficacy of cellular transplantation strategies and promoted long-term improvement in function beginning two weeks after transplantation when compared to transplantation of neural stem cells only and sham-injected controls (Teng et al., 2002). In a transplantation approach using neurally differentiated mouse embryonic stem cells into the rat spinal cord nine days after traumatic injury, gait analysis demonstrated that transplanted rats showed hindlimb weight support and partial hindlimb coordination 3 weeks after transplantation compared with sham-operated controls or control rats transplanted with adult mouse neocortical cells (McDonald et al., 1999).

Other groups demonstrated enhanced functional recovery without any obvious replacement of neural cells. Transplantation approaches using haematopoietic stem cells (Koshizuka et al., 2004; Koda et al., 2005), bone marrow stromal cells (Wu et al., 2003), and activated macrophages (Rapalino et al., 1998; Bomstein Y et al., 2003) have been shown to lead to partial recovery in paraplegic rodents. The mechanism here appears to be indirect, such as providing trophic support, modulating the inflammatory response, or providing a substrate for axonal growth (Barnabé-Heider et al., 2008).

Thus, direct comparison of these studies is complicated because of the varying degree of characterization of the transplanted cells, different injury models and severity, and transplantation at different time points after the injury.

In the present study, to fully explore the recovery of locomotor function after cell transplantation in injured spinal cord, we adapted some novel approaches for evaluation of motor function. Except for the BBB score and plantar stepping, no parameters of functional locomotor recovery were improved in the hGFAP-L1 and L1-imm versus the WT and PBS group. The rump-height index, a parameter estimating the abilities for coordinated and rhythmic activation of muscles working at different joints, and the

ability to initiate and perform voluntary movements without body weight support estimated by the flexion-extension ratio, were also not improved in the hGFAP-L1 and L1-imm group. Thus, we assume that transplantation of hGFAP-L1 NSCs and L1-imm cells into the compression-injured mouse spinal cord has an impact on local adaptive responses in the injured spinal cord rather than the recovery of voluntary movements during the time span the animals were observed. This is further corroborated by the fact, that no improvement in the regrowth of the corticospinal tract identified by immunohistochemical staining with antibodies against CaMKII was observed.

In the present study, a new electrophysiological H/M reflex recording method was applied to evaluate the effects of cellular transplantation on functional recovery. The H-(Hoffmann) reflex is an electrically elicited analog of the spinal stretch reflex providing information about the functional properties of Ia afferents and homonymous alpha-motoneurons under physiological and pathological conditions (Gozariu et al., 1998; Pierrot-Deseilligny et al., 2000; Lee et al., 2008). This is an objective measure of reflex hyperexcitability in muine transplantation paradigmns in spinal cord-injured mice. Here we show that in mice showing better functional recovery there is also an enhanced H-reflex response. This is consistent with the results that spontaneous functional recovery after compression injury of the spinal cord in C57BL/6J mice is associated with a progressively increasing excitability of the plantar H-reflex (Lee et al., 2005; Lee et al., 2008). Moreover, enhanced H-reflex responses at frequencies between 0.1 and 5 Hz were also observed in mice deficient in the extracellular matrix glycoprotein tenascin-R and the adhesion molecule close homolog of L1, mice previously shown to have better motor recovery after spinal cord injury than wild-type littermates (Lee et al., 2008). These results indicate that better functional outcome of compression spinal cord injury in mice is associated with alterations of the monosynaptic reflex pathway which facilitate motoneuron recruitment.

## 3 Possible mechanisms of enhanced recovery

### 3.1 Engrafted neural stem cells

#### 3.1.1 Survival and Migration

The quantification of cell survival after transplantation into the injured spinal cord is an important parameter to assess beneficial effects of genetic manipulation of stem cells. Comparison revealed significantly higher numbers of grafted-derived cells caudal to the

lesion-site in the hGFAP-L1 and the L1-imm group when compared to the WT group at both, 4 weeks and 8 weeks after transplantation, suggesting that cell viability was higher in the hGFAP-L1 and the L1-imm transplantation group. Previous studies showed that the region caudal to the lesion-site displayed far more altered transcripts than the rostral region after severe spinal cord injury. Functional classification of these altered transcripts revealed that apoptosis-related genes were upregulated and metabolism-related genes were downregulated caudal to lesion-site when compared to the rostral segments of the spinal cord. Such altered transcripts may be attributed to differences in blood flow or metabolic responses potentially impairing survival and distribution of grafted cells, regeneration and functional recovery by creating an unfavourable environment (De Biase et al., 2005). The higher numbers of grafted-derived cells caudal to lesion site in the hGFAP-L1 and L1-imm group point to the possibility that L1 exerts beneficial effects on survival of transplanted cells especially under unfavorable conditions caudal to the lesion site. This goes in line with previous studies showing that L1 could enhance the survival of neurons both as soluble and substrate-bound molecule *in vitro* (Chen et al., 1999).

Migration of the progeny of engrafted hGFAP-L1 NSCs and L1-imm cells was enhanced when compared to WT NSCs. This might be due to the ectopic expression of L1 in radial glia and astrocytes. It is known that L1 involves the migration. Local regulation of L1 expression is important for growth cone motility, one of the processes requiring a dynamic regulation of adhesion (Kamiguchi and Lemmon, 2000). L1 also binds to ezrin, another linker protein of the membrane cytoskeleton, at a site overlapping that for AP-2 binding (Dickson et al., 2002). This interaction seems to occur predominantly during migration and axon growth suggesting functional importance in early stages of development (Mintz et al., 2003). Significant migration of transplanted cells is a prerequisite for efficient integration and reconstruction of functional circuits (Martinez-Serrano et al., 1997). Typically, spinal cord injury leads to immediate cell death. The loss of spinal cord tissue is not confined to the lesion center but can spread progressively up to one to one and one-half segmental level of the spinal cord or sometimes even more. Previous studies have shown the expression of representative neurotrophic factors and neuregulins (NRGs) in neural stem cell during the proliferation and differentiation phase (Lu et al., 2003; Yan et al., 2004; Yan et al., 2007). Thus, these combined results indicated that tropic transcripts expressed by cultured NSCs (both from human and mouse) might be able to protect neurons against

damage and rescue of host cells to counteract neuronal degeneration. It is reasonable to anticipate that wide distribution of cells supports integration and interaction with degenerating or dying host neural cells such that they do not degenerate, thus ensuring enhanced locomotor recovery.

### 3.1.2 Differentiation and fate choices

It is critical for neural stem cell replacement therapy to generate the appropriate types of neurons or glia before or after grafting. Neuronal differentiation of NSC grafts in the injured adult spinal cord has been documented in the literature (McDonald et al., 1999; Ogawa et al., 2002; Teng et al., 2002; Cummings et al., 2005) but the majority graft-derived differentiated cells are astrocytes, possibly due to the lack of essential inductive signals for neuronal differentiation in the adult spinal cord. It is necessary to tailor the differentiation of neural stem cells to enhance the generation of neurons. Currently, different methods have been introduced to that measure. Advances in molecular biology (for example, viral vectors) have facilitated the manipulation of these cells to express molecules of interest. For instance, introduction of the MASH1 gene, a proneuronal basic helix-loop-helix transcription factor, into mouse embryonic stem cells led to differentiation of neuronal precursors that could be applicable for transplantation to spinal cord injury (Hamada et al., 2006). Transduction with neurogenin-2, a member of the basic helix-loop-helix family of transcription factors which is involved in the determination and differentiation of multiple neural lineages during development, enhanced the positive effects of engrafted stem cells, including suppressed astrocytic differentiation of engrafted cells, increased amounts of neurons and myelin in the injured area, and recovery of hindlimb locomotor function, as determined by functional magnetic resonance imaging (Hofstetter et al., 2005). In the present study ectopic expression of the neural cell adhesion molecule L1, a molecule involved in neuronal differentiation, neuronal migration, neurite outgrowth, myelination, axon guidance and fasciculation (Brummendorf et al., 1998; Castellani et al., 2000; Zhang et al., 2000; Dihné et al., 2003) led to a substantial increase in neuronal differentiation after transplantation of hGFAP-L1 NSCs and L1-imm cells into the compression-injured spinal cord when compared to WT NSCs. Potential mechanisms of this enhanced neuronal differentiation have been discussed above and include heterophilic and homophilic interactions with not fate-determined neural stem cells which have been analyzed in a previous study (Dihné et al., 2003). Although there is an increase in

neuronal differentiation after transplantation of hGFAP-L1 NSCs and L1-imm cells, the yield is still low. Introducing some other techniques, for example, preparation and propagation of neural stem cells in monolayer cultures instead of floating neurosphere cultures might enhance neuronal differentiation (Yan et al., 2007) thus further improving the potential of transplanted neural stem cells.

The oligodendrocytic differentiation was not affected by L1 expression in neural stem cells indicating that L1 specifically influenced the fate decisions of the neuronal and astrocytic lineage only although oligodendrocytic differentiation was low in all groups.

### 3.1.3 Neurotransmitter identification and close nerve contacts of graft with host cells

One of the ultimate achievements which transplantation of NSCs could possibly attain is to functionally replace neurons that were lost as a result of spinal cord injury by integrating into the host tissue participating in neuronal circuitries. In the present study, some of the differentiated neurons derived from transplanted hGFAP-L1 NSCs, L1-imm cells, and WT NSCs acquired a GABAergic phenotype. Furthermore, graft-derived GABAergic nerve terminals were detected surrounding host motoneurons. Graft-derived glutamatergic terminals could also be identified. This demonstrated that neurons derived from transplanted NSCs did not only differentiate into neurons but also displayed neurotransmitter subtypes and formed close anatomical nerve contacts with host spinal cord neurons in the injured spinal cord. Recently, Yan and his colleagues defined the neuronal phenotypes of engrafted neurons and their synaptic connections with host motor neurons with extensive immunohistochemical analysis (Yan et al., 2007). Other groups could demonstrate the integration of grafted cells, for example, graft- derived neurons were, in some cases, found to form synapses with host motor neurons at the injury site by electron microscopy (Ogawa et al., 2002). The partial anatomical integration of grafted cells with host neurons via the establishment of contacts observed here has important theoretical and practical implications.

To further elucidate the effects of transplanted neural stem cells ectopically expressing L1, the intrinsic regeneration after cell transplantation was determined.

## 3.2 Intrinsic regeneration after cell transplantation

### 3.2.1 Analysis of motoneuron soma size and synaptic inputs to motoneuron somata

L1 is expressed by motoneurons in the embryonic and postnatal spinal cord (Nishimune et al., 2005). L1 is also essential for maintenance of normal synaptic plasticity (Luthi et al., 1996; Saghatelyan et al., 2004). Therefore, it appears reasonable that the universal L1 expression in radial glia and astrocytes derived from hGFAP-L1 NSCs can influence synaptic input to motoneurons. Evaluation of the linear density of perisomatic puncta in the immunohistochmical staining of cholinergic axons provided evidence supporting this idea. The numbers of cholinergic synaptic boutons surrounding the soma of host motoneurons were higher in mice transplanted with hGFAP-L1 NSCs and L1-imm cells when compared to WT NSCs and PBS. The origin and function of these synapses are not precisely known. A recent study has shown that these cholinergic (muscarinergic) synapses modulate several ionic conductances responsible for repetitive discharges of motoneurons and significantly contribute to modulate motor behavior (Chevallier et al., 2006). In the compression-lesioned spinal cord, this difference in synaptic inputs might be interpreted as an indication for the degree of preservation or reestablishment of the modulatory cholinergic input to motoneuron somata. This notion is supported by recent electrophysiological data showing that, in the intact spinal cord, the cholinergic perisomatic synapses regulate motoneuron excitability during locomotion (Miles et al., 2007).

In lesioned mice, spinal cord compression-injury led to alterations of the soma size of motoneurons possibly resulting from cell loss with subsequent transformation of medium cells to larger cells (Bose et al., 2005). Analysis revealed that the mean area of host motoneuron somata caudal to the lesion in the hGFAP-L1 and the L1-imm group was larger than in the WT and PBS groups. This demonstrates that hGFAP-L1 NSCs and L1-imm cells show better neurotrophic effects on host motoneurons caudal to lesion-site than WT NSCs and PBS after transplantation. As described, L1 is expressed by motoneurons in the embryonic and postnatal spinal cord (Nishimune et al., 2005). L1 might exert its neurotrophic actions by heterophilic interactions (Brummendorf et al., 1998; Hortsch, 2000). One candidate is the FGF receptors which are highly expressed by motoneurons (Gouin et al., 1996; Peters et al., 1992). It has been shown that the FGFRs tyrosine kinase is important in L1-mediated neurite outgrowth (Saffell et al., 1997). Thus, it is possible that binding of L1 triggers signaling through these receptors.

Another potential coreceptor is suggested by a report of crosstalk between semaphorin 3A and L1 signaling pathways during development of the corticospinal tract *in vivo* (Castellani et al., 2000). Furthermore, semaphorins have been reported to trigger neuronal death responses (Bagnard et al., 2001; Gagliardini and Fankhauser, 1999; Shirvan et al., 1999). Another possibility is that L1 expressed by motoneurons interacts with exogenous L1 in a homophilic way, which then triggers downstream signaling cascasdes (Loers et al., 2005; Nishimune et al., 2005).

A number of trophic molecules that have been shown to be expressed by NSCs in culture are likely to also play a role in the neurotrophic process. These protein signals are expressed and released in amounts and gradients that allow trophic interactions with the host tissue. Based on previous studies, these proteins might include glial cell line derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF), potent trophic factors for motor neurons, neuregulins (NRGs) 1, 2, and 3, and angiogenic factors such as fibroblast growth factor and vascular endothelial growth factor (VEGF) (Henderson, 1994; Beck, 1995; Azzouz, 2004). Use of neurotrophic factors as a potential treatment in spinal injury has been studied for many years (von Meyenburg et al., 1998; Blits et al., 2000; Takami et al., 2002). Previous studies have shown that neural stem cells constitutively secrete neurotrophic factors and promote extensive host axonal growth after spinal cord injury and exert trophic effects (Lu et al., 2003; Yan et al., 2004; Yan et al., 2007). In spinal cord injury, neuronal cell atrophy can occur not only as a result of the physical trauma of the injury itself, but also due to the oxidative and inflammatory reaction that subsequently occurs. The introduction of neurotrophic factors into the lesion site could increase neuronal survival, decrease dying back of neuronal axons, and induce sprouting of new axons to allow formation of alternative circuitries (Barnabé-Heider et al., 2008).

### 3.2.2 Enhanced catecholaminergic reinnervation of the distal spinal cord by $TH^+$ axons in mice transplanted with hGFAP-L1 NSCs and L1-imm cells

The spinal cord does not have spontaneous activity like the brain, neural activity below the injury level is primarily determined by limb movements and sensory stimuli transduced by the moving limb. The lack of neural activity is expected to negatively influence most of the cellular mechanisms important for regeneration (Belegu et al., 2007).

Promotion of host axonal regeneration is regarded as one of the key components of a successful treatment strategy for spinal cord injury. Trophic support by engrafted NSCs induces local sprouting of different nerve fiber systems (McDonald et al 1998). As L1 enhances neurite outgrowth (Brummendorf et al., 1998) as well as axon guidance, fasciculation, and regeneration (Castellani et al., 2000; Zhang et al., 2000) and because we could also show that L1 expressed by astrocytes enhanced the prolongation neurites derived from cocultured NSCs-derived cells *in vitro*, it appeared reasonable to analyze the reinnervation of the distal spinal cord in mice after transplantation of NSCs. In our study, it could be shown that catecholaminergic ($TH^+$) axons (which are involved in the modulation of locomotor function as described above) were increased in mice treated with hGFAP-L1 NSCs or L1-imm cells when compared to mice treated with WT NSCs and control mice sham-injected with PBS. Catecholaminergic fibers provide diffuse innervations, and it can be expected that axonal regrowth, would be beneficial even if not specifically targeted, in contrast to other systems that require precise reestablishment of synaptic connections (Edgerton et al., 2004; Fouad and Pearson, 2004; Lee et al., 2006; Jakovcevski I et al., 2007). Interestingly, a previous *in vitro* experiment showed that substrate-bound L1 enhanced outgrowth of tyrosine hydroxylase-immunoreactive neurites (Poltorak et al., 1992). Whereas, there was only a tendency that the number of serotonergic ($5\text{-}HT^+$) axons were increased in the hGFAP-L1 and L1-imm group when compared to the WT and PBS group. Serotonergic neurons are powerful modulators of intrinsic circuitries capable of initiating and controlling rhythmic and coordinated movements (Edgerton et al., 2004; Fouad and Pearson, 2004). In contrast, no differences were observed between the experimental groups in the regeneration of the corticospinal tract (CST) axons, which are required for high level of voluntary movement. This goes in line with the behavioral results as discussed above. But when L1 was expressd by embryonic stem cells (Chen et al., 2005) or an adeno-associated viral (AAV) vector (Chen et al., 2007) in the injured spinal cord, transplantation could enhance regrowth of the CST in mice after spinal cord injury to some extent. Moreover, combined neural stem cell transplantation and neutralization of ciliary neurotrophic factor could reduce astrocytic differentiation and promote regeneration of CST fibers in spinal cord injury (Ishii et al., 2006). Functional recovery following traumatic spinal cord injury mediated by a unique polymer scaffold transplanted together with neural stem cells was also shown to induce regrowth of the CST (Teng et al., 2002).

The observations in the present study highlight that in the lesioned mouse spinal cord hGFAP-L1 NSCs and L1-imm cells favor axonal regrowth and remodeling of cell connectivity leading to better functional outcome without reaching higher levels of voluntary movement within the observed time span.

### 3.2.3 Neural stem cells reduce the microglial reaction

In the injured spinal cord, microglial reaction occurs in response to signals from damaged tissue. The etiology of the microglial reaction is currently not well understood but it plays a significant pathophysiological role in SCI. These microglial cells together with other inflammatory cells might have both harmful and beneficial properties concerning recovery of the injured spinal cord (Teng et al., 2002; Hasegawa et al., 2005; Belegu et al., 2007; Yang et al., 2008). In the present study, hGFAP-L1 NSCs, L1-imm cells and WT NSCs could alleviate the activation of microglia in the lesioned spinal cord when compared to PBS-treated mice. Anti-inflammatory effects of NSCs after transplantation into the traumatized brain have previously been described (Park KI et al., 2002; Lee JP et al., 2007) and spinal cord (Teng et al., 2002; Yang et al., 2008).

In summary, it is apparent that transplantation alone of neural stem/progenitor cells after SCI will not lead to optimal recovery; combination strategies will be necessary to further enhance functional recovery. The observations in the present study encourage the use of genetically engineered neural stem/progenitor cells to integrate into the lesioned host tissue, repopulate neuron-depleted spinal cord regions, and enhance neuroprotection and axonal regrowth in an effort to therapeutically ameliorate neurodegeneration.

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# VII Appendix

## 1 Abbreviations

| | |
|---|---|
| Amp | ampicillin |
| AMPA | α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid |
| ATP | adenosine triphosphate |
| BBB | blood brain barrier |
| BDNF | brain-derived neurotrophic factor |
| bFGF | basic fibroblast growth factor, fibroblast growth factor 2 |
| BMSCs | bone marrow stromal cells |
| bp | base pairs |
| BrdU | bromodeoxyuridine |
| BSA | bovine serum albumine |
| cAMP | 3':5'-cyclic monophosphate |
| ChAT | choline acetyltransferase |
| CGRP | calcitonin-gene-related peptide |
| CNPase | 2', 3'-cyclic nucleotide 3'-phosphodiesterase |
| CNS | central nervous system |
| CSF | cerebrospinal fluid |
| CSPGs | chondroitin sulfate proteoglycans |
| dATP | 2'-desoxyadenosinetriphosphate |
| dCTP | 2'-desoxycytidinetriphosphate |
| DCX | doublecortin |
| DMEM | Dulbeccos modified eagle medium |
| dGTP | 2'-desoxyguanosinetriphosphate |
| DAPI | 4',6'-diamidino-2-phenylindole |
| DMSO | dimethylsulfoxide |
| DNA | deoxyribonucleic acid |
| dNTP | 2'-desoxyribonucleotide-5'-triphosphate |
| EDTA | ethylendiamintetraacetic acid |
| EGF | epidermal growth factor |
| EGFP | enhanced green fluorescent protein |
| ESC | embryonic stem cell |
| GABA | gamma-amino-N-butyric acid |

| | |
|---|---|
| GAD | glutamic acid decarboxylase |
| GAPDH | glyceraldehyde-3-phosphate dehydrogenase |
| GDNF | glia-derived neurotrophic factor |
| GFAP | glial fibrillary acidic protein |
| GM-CSF | granulocyte-macrophage colony-stimulating factor |
| HSCs | haematopoietic stem cells |
| HEPES | 2-(4-(2-Hydroxyethyl)-piperzino)-ethansulfonic acid |
| HSAS | L1 deficiency syndrome: hydrocephalus as a result of stenosis of aqueduct of Sylvius |
| IG | immunoglobulin |
| IL-1-beta | interleukin-1-beta |
| KSPGs | keratan sulfate proteoglycans |
| MOG | myelin oligodendrocyte glycoprotein |
| NCAM | neural cell adhesion molecules |
| NGF | nerve growth factor |
| NSC | neural stem cell |
| NPC | neural progenitor cells |
| NT3 | neurotrophin 3 |
| NT4 | neurotrophin 4 |
| NT5 | neurotrophin 5 |
| NRG | neuregulins |
| MAG | myelin associated glycoprotein |
| MASA | L1 deficiency syndrome: mental retardation, aphasia, shuffling gait, adducted thumbs, |
| MP | methylprednisolone sodium succinate |
| MBP | myelin basic protein |
| mRNA | messenger ribonucleic acid |
| NMDA | N-methyl-D-aspartate |
| NT-3 | neurotrophin-3 |
| OMgp | oligodendrocyte myelin glycoprotein |

| | |
|---|---|
| PBS | phosphate-buffered saline |
| PCR | polymerase chain reaction |
| PDGF | platelet-derived growth factor |
| PLL | poly-L-lysine |
| PNS | peripheral nervous system |
| P/S | penicillin/streptomycin |
| RT | room temperature |
| SCI | spinal cord injury |
| s.e.m. | standard error of the mean |
| TAE | tris-acetate-EDTA |
| TAG1 | transient axonal glycoprotein |
| TGF-beta | transforming growth factor-beta |
| TNF-alpha | tumor necrosis factor-alpha |
| TH | tyrosin hydroxylase |
| $T_m$ | melting temperature |
| TM | transmembrane segment |
| Tris | tris(-hydroxymethyl)-aminomethane |
| VGAT | antivesicular GABA transporter |
| VGLUT1/2 | vesicular glutamate transporter |
| v/v | volume per volume |
| Vol. | volume |
| w/v | weight per volume |
| XLSP | X-linked spastic paraplegia |
| ZMNH | Zentrum für Molekulare Neurobiologie Hamburg |

## 2 List of original articles

**Conference (Poster Abstract)**

[1] **Jinchong Xu**, Yifang Cui, Gunnar Hargus, Christian Bernreuther and Melitta Schachner "Neural stem cells engineered to express L1 under the control of the human GFAP promoter promote functional recovery after transplantation in a mouse spinal cord injury model" the 48th Annual Meeting of the American Society for Cell Biology, December 13th-17th, San Francisco, CA, USA, 2008.

**Articles**

[2] Yang Li, Xiaochun Chi, Xiaoxia Li and **Jinchong Xu**. "Multipotency of human neural stem cells from fetal striatum." Neuroreport. 2008 Sep 23 (Epub ahead of print).

[3] Gunnar Hargus, Yifang Cui, Janinne Sylvie Schmid, **Jinchong Xu**, Glatzel Markus, Melitta Schachner and Christian Bernreuther "Tenascin-R promotes neuronal differentiation of embryonic stem cells and recruitment of host-derived neural precursor cells after excitotoxic lesion of the mouse striatum." Stem Cells 26(8):1973-84, 2008.

[4] Feng Lan*, **Jinchong Xu***, Xiaoyan Zhang, Vincent Wai-Sun Wong, Xiaoxia Li, Aili Lu, Wenjing Lu, Li Shen and Lingsong Li, "Hepatocyte growth factor promotes proliferation and migration in immortalized progenitor cells." Neuroreport 19(7):765-9, 2008. *Equally contributed to this work

[5] Christian Bernreuther, Marcel Dihné, Verena Johann, Johannes Schiefer, Yifang Cui, Gunnar Hargus, Janinne Sylvie Schmid, **Jinchong Xu**, Christoph M. Kosinski, and Melitta Schachner "Neural Cell Adhesion Molecule L1-Transfected Embryonic Stem Cells Promote Functional Recovery after Excitotoxic Lesion of the Mouse Striatum." Journal of Neuroscience 26:11532-9, 2006.

[6] Xiaoxia Li, **Jinchong Xu**, Yun Bai, Xuan Wang, Xin Dai, Yinan Liu, Jun Zhang, Junhua Zou, Li Shen and Lingsong Li. "Isolation and identification a new neural progenitor cell population from human fetal striatum." Biochemical and Biophysical Research Communications 326 (2):425-34, 2005.

[7] Yajun Wang, Yun Bai, Xiaoxia Li, Qikuan Hu,Changsheng Lin, Zhifeng Xiao, Yinan Liu, **Jinchong Xu**, Li Shen and Linsong Li, "Fetal Human Neural Progenitors can be the Target for Tumor Transformation." Neuroreport, 15(12):1907-12, 2004.

## 3 Curriculum vitae

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**EDUCATION**

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## 5 Certification

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Department of Neuroscience

February 13, 2009

As a native English speaker I confirm that the PhD thesis of Jinchong Xu titled "Murine neural stem cells engineered to express neural adhesion molecule L1 under the control of the human GFAP promoter promote functional recovery after transplantation in a mouse spinal cord injury model in Mus musculus (L1., 1758) " is written in correct English grammar and comprehensible style.

Sincerely,
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