Cellular therapy after spinal cord injury using neural progenitor cells

Vroemen, Maurice

tional recovery over weeks to months after the initial injury [32].

The steady progression of tissue damage in the proximity of the injury site exceeding by far the size of the initial damage is typical for SCI in particular and CNS injuries in general. It therefore is generally accepted that there are two separate mechanisms of damage after acute spinal cord injury: the primary injury and secondary injury mechanisms [33, 34]. Although both mechanisms cannot be strictly temporarily separated, the primary and secondary damage events possess distinct pathophysiological characteristics.

3.1 Primary injury mechanisms

The primary injury includes the tissue damage that can be directly attributed to the force of the traumatic insult. The primary injury induces disruption of the axon pathways, cell membrane damage and mechanical damage to the spinal cord vasculature. Particularly in the lower spinal cord, the initial traumatic insult most frequently causes the deformation of the spinal canal, leading to a contusion injury that is followed by an acute compression of the soft spinal cord tissue. Additionally, the mechanical impact can cause the dislocation of bony fragments and disc material, which leads to penetration and laceration of the spinal cord [35]. Sharp insults or direct transection of the spinal cord tissue on the contrary is a less frequent occurring injury type that often takes place after gun shot or knife wounds [19]. At the cervical region of the spinal cord, hyperextension injuries are occurring more frequently than crush injuries because of the greater flexibility of the cervical spinal column. Hyperextension injuries directly cause disruption of axons and typically occur due to falls on the head for instance after diving in shallow water [20].

3.2 Secondary injury mechanisms

The primary damage to the spinal cord tissue elicits the autocatalytic processes that are involved in the secondary damage phase. The secondary damage phase consists of a wide repertoire of vascular events, biochemical disturbances and cellular responses that evolve over minutes to hours after the primary injury, leading to massive additional loss of spinal cord tissue, scar tissue formation and demyelination. Since it predominantly is the loss of white matter that is decisive for the loss of function after SCI, it is hypothesized that therapeutical interventions that prevent secondary damage could be able to improve functional outcome after SCI [14]. It therefore is of obvious interest to better understand the pathophysiology of the secondary injury processes.

3.2.1 Vascular events

Within minutes after the primary injury, vasospasms and the loss of vascular autoregulation cause a reduced perfusion at the injury site [36]. Particularly in the spinal cord grey matter, this leads to a remarkably impaired microcirculation [37]. In combination with the systemic hypotension and the reduced cardiac output typically occurring after SCI [38], the vascular changes cause post traumatic ischemia of the

spinal cord parenchyma in the segments surrounding the injury site [34]. Especially in hemorrhagic regions of the injured spinal cord, the posttraumatic ischemia leads to infarction and necrosis of the spinal cord tissue [39]. Furthermore, damage to the vasculature endothelial lining causes disruption of the blood-brain barrier (BBB), which leads to a progressive edema that spreads to adjacent segments of the injured spinal cord [40].

3.2.2 Biochemical alterations

Directly following the primary injury, synaptic overactivity leads to the uncontrolled release of the amino acid glutamate, the major excitatory neurotransmitter in the adult mammalian CNS [41, 42]. Elevated levels of glutamate cause the activation of the NMDA subclass of glutamate receptors, which have been shown to induce Ca^{2+} influx resulting in a cellular Ca^{2+} overload [43]. A further disturbance of the ionic homeostasis is caused by a reduction of the Na^{+}/K^{+}-ATPase activity in cells at the lesion epicenter [44]. In physiological normal CNS tissue, the intracellular concentration of Na^{+} and Ca^{2+} is kept at a low level, while cell organelles such as the endoplasmatic reticulum and mitochondria function as intracellular Ca^{2+} storage. The increase of intracellular Na^{+} and Ca^{2+}, however, is thought to reverse the normal action of the intracellular Na^{+}/Ca^{2+} exchanger by pumping out Ca^{2+} that is accumulated in the intracellular Ca^{2+} stores in reverse of Na^{+} [44]. It is the increase of cytoplasmic Ca^{2+} levels, which starts a detrimental cascade of events inducing both axon degeneration and cell death [45]. First, increased levels of cytosolic Ca^{2+} concentrations are shown to destabilize the cytoskeleton and the cell membrane, causing impaired axoplasmic transport [46, 47]. Furthermore, high concentrations of cytosolic Ca^{2+} activate a series of cell death inducing catabolic enzymes such as proteinases, cysteine proteases, and phospholipases [48-50]. The state of impaired Ca^{2+} homeostasis is autocatalytic and self-propagating since it can lead to the opening of the mitochondrial permeability transition pore. This is not only detrimental for the cell's ATP production, which further reduces the Na^{+}/K^{+}-ATPase activity, but also leads to release of cytochrome c, which induces apoptotic cell death by caspase 3 activation [51].

After collapse of the oxidative metabolism, incomplete electron transport in the respiratory chain causes the overproduction of free radicals such as super oxide (O^{2-}) [52]. Free radicals are highly reactive oxygen metabolites that possess an extra electron in the outer orbit. The enzyme superoxide dismutase converts the superoxide in the free radical species H_2O_2, which in its turn is degraded into H_2O and O_2 by catalase and gluthatione peroxidase activity. In the presence of free iron that originates from hemoglobin, transferrin or ferretin, H_2O_2 forms the highly reactive hydroxyl radicals (•OH) [53]. The uncontrolled and excessive release of these free radicals causes lipid peroxidation in the cell membranes, which leads to impairment of the phospholipid dependent enzymes, disruption of ionic membrane gradients and eventually dis-

Table 1 *Pathophysiological Events of the Secondary Injury that Occur After Acute Spinal Cord Injury*

Vascular events	
	Post traumatic ischemia
	Edema
	Disruption of blood brain barrier
Biochemical alterations	
	Uncontrolled excitatory amino acid release
	Ca2+ influx into cells
	Na+ influx into mitochondria
	Collapse of oxidative metabolism and ATP production
	Cytochrome c release
	Free radical overproduction
	Lipid peroxidation
Cellular Events	
	Invasion of blood bound immune cells
	Microglia activation
	Reactive Astrogliosis
	Wallerian degeneration
	Rupture of terminal clubs resulting in hydrolytic enzyme release
	Apoptosis of glial cells
	Invasion of fibroblast and Schwann cells

ruption of the cell membrane [54]. When a cell membrane is destroyed, the fatty acids that make up the cell membrane are released and form, when peroxidated, highly toxic compounds such as acrolein and 4-hydroxynonenal. These on their turn destroy cell membranes, releasing more fatty acids that undergo peroxidation, resulting in an autocatalytic cell lysis process, which is typical for the secondary damage phase of SCI [55, 56].

3.2.3 Cellular events

The response of the immune system represents the first cellular event that takes place in the secondary damage phase of SCI. Within 6 hrs after the primary injury, neutrophils enter the lesion site and start to remove tissue debris by phagocytosis [57]. Besides the restoration of tissue homeostasis, neutrophils contribute to the progress of the secondary damage phase by releasing proteases and reactive oxygen species. Microglia, the resident immune cells in the CNS, respond quickly to the changes in the microenvironment and become activated [58]. Activated microglia remove degenerated fibers by phagocytes and function as antigen presenting cells to mediate the T-cell response. [59]. Several days after the initial injury, blood born macrophages and lymphocytes infiltrate the injured spinal cord tissue [57]. The infiltration and prolonged activation of immune cells has been shown to have both beneficial and deleterious effects on

the functional outcome after SCI. The release of pro-inflammatory cytokines such as tumor necrosis factor-alpha and inducible nitric oxide synthase, are thought to induce cellular degeneration in the secondary damage phase [60, 61]. In a more chronic phase of the injury, activated immune cells produce growth factors that contribute to neuronal survival and tissue repair [62, 63]. Furthermore, the clearance of myelin and axonal debris by immune cells may promote axonal regeneration considering that adult myelin contains potent axonal growth inhibitors [64].

CNS injury induces astroglial hypertrophy, process extension and moderate cell division. These so called reactive astroglial cells can be detected by the increased production of intermediate filament protein such as glial fibrillary acidic protein (GFAP) and vimentin [65]. Reactive astrocytes seal the injury site by producing a wide variety of extracellular matrix (ECM) proteins. Reactive astrogliosis is often regarded as detrimental to functional outcome since they partly form the scar tissue surrounding the injury site, which is thought to act as a major obstacle for axon regeneration [66, 67]. Nevertheless, reactive atrocytes are essential for wound healing and blood–brain barrier repair. In addition, reactive astrocytes are able to restrict inflammation, protect neurons and oligodendrocytes, and preserve motor functions after mild or moderate SCI [68, 69]. Thus, reactive astroglia have an ambivalent role in the injured spinal cord, secreting factors, which inhibit axonal regeneration, on one hand, and stabilizing the injured tissue during the secondary damage phase on the other hand. Axonal breakdown takes place in the ascending fiber tracts above the lesion and descending fiber tracts below the lesion and is spatially associated with phagocytosis of tissue debris by activated microglia, a process which as also known as Wallerian degeneration [70]. Abortive sprouting can be observed along the proximal part of lesioned CNS axons, which has already been described by Ramon y Cajal [1]. As a result of continuing proximodistal axonal transport, terminal club structures are formed at the distal tip of lesioned axons. After rupture of the terminal club, the hydrolytic enzymes that are enriched in the terminal clubs are released, causing autolysis of the spinal cord tissue [71].

The described vascular events, biochemical alterations and responses of the immune system as part of the secondary damage phase also affect spinal cord cells that initially survived the injury. The programmed cell death machinery becomes activated through death receptors, mitochondrial malfunction or caspases, ultimately leading to loss of glial and neuronal cells [72]. The number of apoptotic cells is the highest close to the lesion epicenter. Apoptosis of oligodendroglia in the proximity of spared axons causes chronic demyelination, which often represents the morphological correlate for the delayed sensory and motor dysfunction occuring after SCI [73].

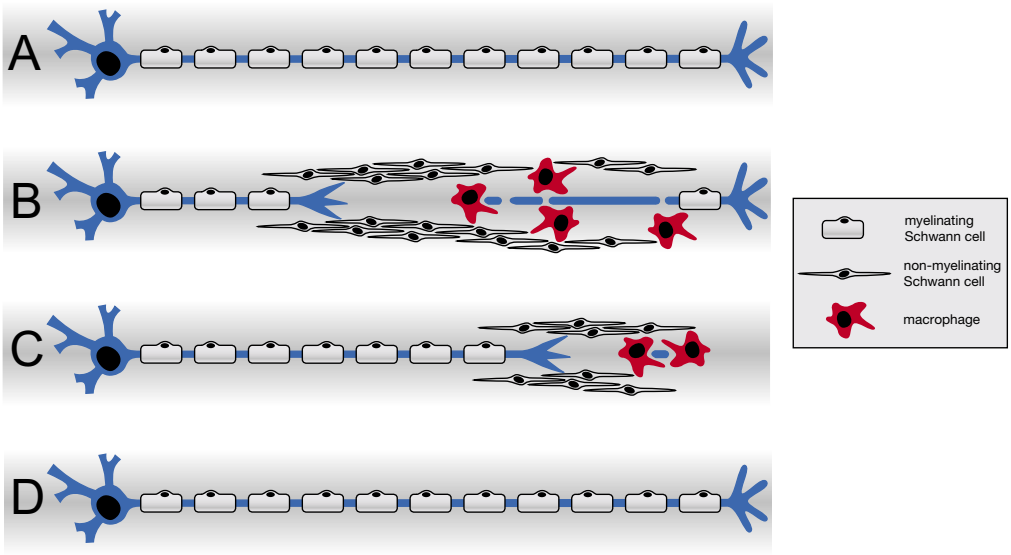

Figure 2: *Axonal regeneration in the peripheral nervous system. (A) Peripheral axons (blue) are either ensheathed or myelinated by Schwann cells (grey). (B) As long as the perineurium is intact, lesioned peripheral axons are able to spontaneously regenerate over considerable distances. Therefore, resident Schwann cells that lose axonal contact start to proliferate and form axon-guiding structures, the so-called bands of Büngner. Schwann cells furthermore, produce neurotrophic factors and guidance molecules that enhance axonal regeneration. Infiltrated macrophages (red) remove the debris that originates from the degenerating distal part of the lesioned axons. (C) Regenerating axons become remyelinated by the resident Schwann cells. (D) Ultimately, the regenerating peripheral axons are capable of reinnervating their target organ.*

4. CAUSES FOR THE POOR REGENERATIVE CAPACITY OF THE CENTRAL NERVOUS SYSTEM

In contrast to the CNS, the peripheral nervous system (PNS) is capable of spontaneous recovery after axonal damage. Schwann cells, which are the resident PNS glial cells, play a crucial role in this regeneration process. When the distal part of a lesioned PNS axon degenerates, the Schwann cells that lose axonal contact start to proliferate and form a cell strand within the basal lamina tube, the so-called bands of Büngner [74]. Furthermore, the Schwann cells in the denervated nerve stump express adhesion molecules and neurotrophic factors essential for axon regeneration. Severed axons sprout into the Schwann cell columns of the distal nerve segment, which ultimately can lead to reinnervation of the denervated target, in most cases the skeletal muscle [75]. Although CNS axons are capable of long distance axonal sprouting into a peripheral nerve graft, injured CNS neurons react differently compared to PNS neurons. However, in particular the inhospitable environment surrounding severed CNS axons determines their inability to regrow [2, 3]. Substantial progress has been made revealing the biological bases of the

regeneration inhibiting properties of the CNS in comparison to the PNS. A number of factors have been identified: the presence of extrinsic axonal growth inhibiting factors, decreased intrinsic regenerative potential of CNS axons, the absence of remyelination and the development of a cystic tissue defect, which all are thought to play a significant role for the poor regenerative capacity after SCI.

4.1 Extrinsic axonal growth inhibiting factors

4.1.1 Glial scar

Glial scar formation represents a major obstacle for axonal regeneration after SCI. The glial scar consists of reactive astrocytes and their ECM proteins. As soon as the surrounding dura mater gets disrupted in more severe lesions, invading fibroblasts will contribute to the glial scar. Subsequently, astrocytes start to up-regulate the expression of proteoglycans, a class of ECM molecules [76]. Proteoglycans have been identified as potent inhibitors of CNS axon extension both *in vitro* and *in vivo* [6, 77-79]. Ultrastucturally, growth cones of sprouting axons are not able to regenerate through the glial scar and form dystrophic endbulbs [66, 67]. Remarkably, cellular sources of proteoglycans in the lesion site also produce axon growth permissive ECM components such as L1 and laminin. [80]. Both *in vitro* and *in vivo*, the balance between inhibitory and permissive ECM components substantially influences the ability of axons to regenerate [80, 81]. Therefore, the obstruction of neurite extension by inhibitory ECM components such as proteoglycans and the growth promoting capacity of permissive ECM components of the glial scar cannot be described as an "all or nothing" mechanism [80]. Moreover, it is now clear that at least certain subpopulations of dystrophic axons are able to return to an active growth state when they are given a proper stimulus, for instance additional neurotrophic factor support[82, 83].

In addition to proteoglycans, several other inhibitors for axonal regeneration are up-regulated in the glial scar. The secreted protein semaphorin 3 is upregulated in invading fibroblasts and acts as a chemorepellent for neuropilin expressing neurons. [84]. Furthermore, *Slit* proteins along with their glypican 1 receptors, which are important inhibitors for axonal elongation during development, are upregulated in reactive astrocytes [85]

The formation of the glial scar starts after disruption of the BBB and is most likely triggered by the influx of molecules, normally absent in the CNS, and the invasion of activated microglia in the injured CNS parenchyma [86]. The search for potential candidate molecules, which may promote reactive gliosis, is ongoing. However, there is clear evidence that both interleukin-1 and transforming growth factor-*beta* (TGF-*beta*) produced by macrophages play a major role in the transformation of normal astrocytes into the reactive phenotype [87, 88]. Furthermore, the secretion of the inflammatory cytokine interferon- *gamma* mediates glial scarring after brain injury [89]. Additionally, signaling between B-ephrins and EphB receptors in response to injury

supports the fibroglial scar formation initiated by meningeal fibroblasts, which invade the injured spinal cord in the adult CNS [90].

4.1.2 Myelin associated inhibitors

Distinct from the already described growth cone dystrophy is the collapse of growth cones where mature regenerating CNS axons encounter mature oligdendrocytes or myelin. Growth cone collapse results in a shrunken growth cone in combination with a stalled forward progress that can restart over time, and has been described best *in vitro* [91]. It is unclear whether growth cone collapse precedes growth cone dystrophy.

Three different classes of myelin-associated molecules have been described causing growth cone collapse and inhibition of neurite outgrowth: Nogo, Myelin-Associated Glycoprotein (MAG) and Oligodendrocyte Myelin Glycoprotein (OMGP). Nogo exists in three isoforms, Nogo-A, Nogo-B and Nogo-C and is mostly associated with the endoplasmatic reticulum of oligodendrocytes, however, a proportion can be detected on the cell surface. The three isoforms share the inhibitory Nogo-66 domain, Nogo-A has an additional inhibitory domain, amino-Nogo, at the N-terminus [92-94]. MAG, a member of the immunoglobulin superfamily, is a sialic acid-binding protein and can be found in both CNS and PNS myelin [95, 96]. OMGP is a glycosyl phosphatidylinositol-linked protein not only expressed by oligodendrocytes, but also in by Schwann cells, their counterparts in the PNS [97]. Interestingly, all three classes of myelin inhibitors seem to act through the neuronal expressed Nogo-receptor (*Ngr*) [98-100]. *Ngr* uses p75 low affinity NGF receptor (p75-LNGR) as a co-receptor to activate the small GTPase *Rho* and its signal transduction pathway to inhibit neurite outgrowth [101, 102]. The role of myelin inhibitors in growth cone collapse and inhibition of neurite outgrowth has been impressively demonstrated *in vitro*, the exact role of myelin inhibition *in vivo* remains unclear. Inhibition of Nogo using specific neutralizing antibodies elicit an axonal response and improved functional outcome after SCI [103, 104], presumably through the activation of collateral sprouting of uninjured axons [105]. The inhibition of *Ngr* signaling after SCI through specific antibodies promotes local sprouting, however, long range axon regeneration is minimal [7, 103]. Animals lacking either specific or all three Nogo variants allow only limited axonal regeneration at best [106-108]. Furthermore, adult sensory neurons regrow in both intact and degenerating white matter tracts of adult mice [79, 109]. There is strong evidence suggesting that the geometrical organization of myelin is biologically relevant *in vivo*, which is based on the finding that white matter supports parallel axonal sprouting but inhibits nonparallel sprouting against the orientation of the white matter tracts [110]. Therefore, it is likely that myelin associated inhibitory molecules could be essential for oriented and fascicled long distance axon growth [111]. Myelin-associated inhibition is considered to be crucial to maintain the topography of highly specific nervous

system projections, which is supported by studies inducing aberrant sprouting of local circuitry in the adult intact cerebellum after neutralizing Nogo [112]. In other words, neutralizing myelin based inhibitors as a repair strategy for SCI could induce aberrant and dysfunctional sprouting of non-injured systems.

4.1.3 Other inhibitors

Besides myelin-associated inhibitors, there are indications that at least some of the chemoattractant and repulsive effectors that play a role during development also are present in the adult spinal cord. The highly conserved laminin-related molecule netrin system with its receptors DCC and Unc5 can function both as an attractant and repellent for growing axons, depending on the cAMP or cGMP level within the growth cone [113]. In the injured rat spinal cord, Netrin-1 is expressed throughout grey and white matter by oligodendrocyte precursors still undergoing division [114]. Furthermore, grafts of netrin-1 over-expressing fibroblasts reduce axonal growth after adult spinal cord injury, which suggest a role for endogenous netrin-1 as an inhibitor of intra-spinal neuron derived axon regeneration [115].

4.2 Intrinsic regeneration limiting factors

4.2.1 Regeneration-associated genes

Besides the above described extrinsic factors that limit the regenerative capacity of CNS axons, differences in the intrinsic state of the lesioned neurons contributes significantly to the poor regenerative capacity of the diseased CNS. Neurons react upon axotomy by up-regulating the expression of regeneration-associated genes (RAG), including growth-associated proteins such as GAP-43 and CAP-23 and adhesion molecules like L1, N-CAM. Most changes in RAG expression occur in response to axotomy of CNS neurons and are qualitatively and quantitatively different from those that occur in the PNS. In general, up-regulation of RAG is weaker and more transient or even absent in CNS neurons compared to PNS neurons [116, 117]. The expression level of RAG appears to correlate with the regenerative capacity of an axotomized neuron [118]. Under certain conditions, the over-expression of multiple RAG is sufficient to induce axonal regeneration, even in a CNS environment [119].

4.2.2 Trophic support

The differential expression of growth factors and their receptors after axotomy represents another significant difference between PNS and CNS neurons. In contrast to PNS neurons, CNS neurons fail to induce sufficient expression of trophic factors such as BDNF and NT-4/5 after axotomy, which results in significant atrophy of fiber tracts such as the rubrospinal and the corticospinal tract [120]. After administration of BDNF and NT-3 in rodent models of SCI, structural and functional recovery has been reported [5, 121]. Functional improvements are most likely attributable to the reduced atrophy of axons and stimulation of injury-induced plasticity of surviving axons, since long-distance

tract regeneration was not observed[5, 8, 121, 122]. These findings support the notion that the administration of neurotrophic factors is a promising strategy to induce axonal and functional recovery after SCI.

4.3 Demyelination

Loss of oligodendrocytes through programmed cell death contributes significantly to functional deficits observed after SCI. The resulting demyelination slows down or even blocks completely appropriate nerve conduction in uninjured axons. In contrast to the PNS, the CNS exhibits only a limited capacity to remyelinate affected fiber tracts [123]. Replacement of oligodendroglia and remyelination has been achieved by transplanting Schwann cells, olfactory ensheathing cells or oligodendrocyte precursor cells [124-126]. Either protection of intrinsic oligodendrocytes or their appropriate replacement to maintain myelination will result in significant improvement in functional outcome after spinal cord injury.

4.4 Cyst formation

Necrosis and apoptosis of spinal cord parenchyma results in a fluid filled lesion cavity forming at the lesion epicenter. Subsequently, a disturbed cerebrospinal fluid circulation along the central canal often supports the development of a fluid filled cavity – so called syringomyelia. It is evident that cystic lesion cavities represent a major obstacle for the regeneration of severed axons. In the PNS, the resident Schwann cells react upon the injury by proliferation and cells migrate into the lesion site, providing a substrate for regenerating axons [75]. Although some proliferation of astrocytes can be observed after SCI, the extent of cell renewal, even after the application of growth factors, does not allow to replace the cystic lesion cavity [4]. Therefore, strategies need to be developed, which provide regrowth conducive substrates either through cell transplantation approaches or through implantation of appropriate acellular matrices.

5. INDUCING RECOVERY AFTER SPINAL CORD INJURY

Recovery after spinal cord injury can be described on both structural and functional levels. Structural recovery can be observed in terms of tissue repair, axonal growth and elongation, remyelination and synapse formation and is measured using descriptive tests that determine the integrity of the injured system. Functional recovery describes improved function of the injured subject after therapeutical intervention. Functional recovery can be determined using electrophysiology and specific behavioral tests that describe the ability of the injured system to perform a certain task. Although the induction of structural recovery is the most compelling approach to induce functional recovery, it is not the only means that has the potential to improve the outcome after SCI.

5.1 Prevention of secondary damage

The disruption of ascending and descending axon pathways in the white matter represents the main structural

correlate for functional impairment caused by spinal cord trauma. Of note, a small percentage of spared axon projections is sufficient to maintain a large degree of function [127]. Pathophysiological changes during the secondary injury phase are responsible to a significant extent for the ultimate white matter damage occuring over time after the initial injury. Therefore, therapeutic interventions need to be introduced before the cascade of secondary damage events starts, in order to promote white matter sparing and improved functional outcome. The inhibition of earlier described effectors of secondary damage (for instance free radical cellular damage, cytochrome C release and Na^{+} and Ca^{2+} influx-related cell death) has been shown to induce tissue sparing and improvement of functional outcome after SCI in animal models [9, 128, 129]. Most interventions to reduce secondary damage require either pre- or immediate post-injury application in order to be effective, which of course is problematic to realize in a clinical setting [130]. Moreover, the benefits of the standard administration of a high dose methylprednisolone immediately after the spinal trauma in order to reduce secondary damage are still under dispute [15]. No doubt, the investigation of approaches to attenuate sequelae of secondary injury events will be an important research topic in the future. However, the translation into clinically relevant strategies continues to be a challenging task.

5.2 Induction of injury-induced plasticity

The success of a therapeutic intervention aiming for functional recovery depends on the ability to reestablish a critical number of connections between supraspinal and spinal neurons. The fact that 55% of the SCI patients have neurological incomplete lesions and that even a majority of the complete SCI patients exhibit spared rims of white matter at the lesion epicenter suggests that enhancing the injury-induced plasticity of spared axons represents a promising mechanism to induce functional recovery after SCI [23]. Indeed, there have been several animal studies in which the enhancement of compensatory collateral sprouting after incomplete SCI can be induced by the administration of neurotrophic factors, GAP-43 up-regulation or the neutralization of myelin-associated inhibitors [13, 131, 132]. The efficacy of strategies enhancing the intrinsic plasticity remains to be determined. However, this approach can only be applied for structural incomplete SCI. After denervation in complete SCI, only therapies that induce regeneration of severed axons and subsequently target reinnervation will be able to promote functional regeneration.

5.3 Induction of axonal regeneration

Axonal growth can represent either plasticity or regeneration. Therefore, it is important to strictly define the possible axonal growth phenomena. First, the most restricted form is reactive synaptogenesis, which depicts local ingrowth of otherwise non-injured afferents that terminate in the

close proximity of the denervated sites. Furthermore, new connections can be established by ectopic ingrowth of sprouts from non-injured axons that originally project at remote locations, which is defined axonal sprouting. Alternatively when the axonal growth originates from the amputated axon itself, it is referred to as regenerative sprouting. Axonal regeneration finally describes regenerative sprouting that leads to reconnection of lesioned axon with their original targets [133]. The ultimate goal of SCI research is to induce axonal regeneration of disrupted fiber tracts, leading to regain of function, which further is referred to as functional axonal regeneration.

It is of utmost importance to keep in mind that if functional recovery is observed, this not necessarily has to be the direct correlate of the elicited structural recovery. Since the majority of experimental SCI models employed represent incomplete injuries, a limited degree of regain of function after SCI most frequently can be explained by compensation of spared connections. It therefore is very challenging to determine whether the observed functional recovery can be attributed to true axonal regeneration or by plasticity phenomena within spared axon projections. Before functional axonal regeneration can be claimed, each individual aspect of structural recovery, including cell survival, axon growth, synapse formation and remyelination must be described for the injured connection. Furthermore, it must be proven that the observed functional recovery can be attributed to the observed morphological changes, for instance by specifically targeting regenerates with pharmaceutical (e.g. by neurotransmitter antagonists) or surgical interventions (e.g. by retransection) to reverse functional improvement. Numerous studies report regenerative sprouting in combination with behavioral recovery [4-13]. Although these studies provide valuable insights into the regenerative mechanisms that play a role after SCI, most studies only provide data on a limited series of tests. The true significance of the described regeneration therefore remains subject of further investigations.

The development of a therapeutic intervention that enables regeneration of lesioned axons over long distances is often considered to be an essential aspect to induce functional axonal regeneration. Nevertheless, it remains to be determined in which degree long distance axonal regeneration is required in order to induce substantial functional recovery. It is possible that only bridging the lesion site is sufficient to form a connective relay with spared axonal fibers below the lesion. The functional recovery that can be observed after injury-induced plasticity indicates that the wiring of the adult spinal cord could be more plastic then expected[134]. In this respect, it is an interesting observation that the axonal reconnection in cold-blooded species that are capable of axonal regeneration after SCI predominantly occurs by regeneration of cells that are closely rostral to the injury site[135]. Significant axonal regeneration of lesioned CNS axons over long distances within the CNS has never been shown.

5.4 Cellular therapy as a multi-facet tool to enable functional axonal regeneration

In order to achieve the challenging goal of functional axonal regeneration, injured neurons must survive, extend axons through an adversive environment, find appropriate target neurons and ultimately form functionally relevant synapses. It is evident that a multi-facet therapeutic intervention is needed in order to accomplish these complex tasks. Moreover, if the regenerative failure of injured CNS axons is perceived as an imbalance between the present stimulators and inhibitors of axonal regeneration, a combined therapy that both neutralizes inhibition and provides additional growth stimulation is most likely to be effective. Since the CNS is not able to intrinsically replace lost spinal cord parenchyma sufficiently, cellular replacement is considered to be a crucial prerequisite in a putative therapeutical approach. The regenerative properties of the PNS can be be transferred to a limited extent to the CNS by the transplantation of Schwann cells and olfactory ensheathing cells. Schwann cells induce axonal regeneration by providing a growth supportive substrate and by supplying trophic support [136]. Furthermore, Schwann cells are able to remyelinate CNS axons to restore proper nerve conduction. However, Schwann cells do not facilitate axonal regrowth beyond the graft to reenter the caudal host spinal cord [137]. Alternatively, olfactory ensheathing cells (OEC) are shown to be interesting candidates for the use in a cellular therapy approach after SCI. OEC represent a “hybrid” between CNS (astroglia) and PNS glial cells (Schwann cells) that promote reentry of olfactory nerve endings from the PNS into the CNS throughout life. The olfactory system is unique in terms of its constant renewal of adult neuronal cells [138]. OEC can be isolated from adult olfactory nerves. When OEC are grafted into the lesioned spinal cord, tissue repair, axonal regeneration, remyelination and functional recovery can be observed to a limited degree. Whether the observed functional recovery can be attributed to axonal regeneration of lesioned axons has not been shown yet [139, 140].

Neural stem cells (NSC) as a source for cell-based therapies have the important advantage to give rise to all three major cell types of the central nervous system (CNS): neurons, astrocytes and oligodendrocytes. Therefore, they can replace degenerated spinal cord parenchyma in an organotypcially appropriate manner. NSC have been isolated from both the developing and adult CNS and can be expanded in culture using epidermal growth factor and fibroblast growth factor as mitogens [141, 142]. Embryonic derived NSC have been shown to promote partial functional recovery following spinal cord injury [12, 143]. Since ethical and logistic concerns restrict the large-scale use of embryonic derived stem cells, NSC derived from adult neural tissue are preferable. Furthermore, adult NSC could be isolated from the patient’s own

neural tissue, thus avoiding the need of life long immunosuppression in transplanted subjects to avoid graft rejection. The potential of adult derived NSC to induce axonal regeneration remains to be determined.

6. INTRODUCTION TO THE USED SPINAL CORD INJURY MODELS

6.1 Animal models of spinal cord injury

In animal models of SCI, both structural and functional regeneration can be investigated. Unfortunately, the conclusions obtained from animal models of SCI can be transferred to the human situation only to a limited extent. On both the structural and functional level, the human spinal cord differs significantly with the animal situation. Already the sheer difference in size means that axons need to regenerate over tens of centimeters in humans instead of only a few centimeters in animals, particularly in rodents. Furthermore, the cytoarchitecture of the human spinal cord is significantly different when compared to the rat, which is the most frequently used laboratory animal in SCI research. In this respect, the most prominent example is the significance of the rubrospinal and corticospinal tract, which are the major descending axon pathways that project to the spinal cord. During phylogenetic development, the ability to make precise movements became more and more advantageous, which required increasingly complex motor control systems. Therefore, the red nucleus in the brain stem and its rubrospinal tract that represents the major somatic motor system in phylogenetic more primitive vertebrates was copied to the motor cortex and its corticospinal tract in phylogenetically more advanced mammals. The additional space for projection neurons and supraspinal input in the cortex as compared to the brainstem is paralleled with a much more refined somatopy of the motor cortex when compared to the red nucleus, which has lead to an increased capacity to perform complicated motor tasks. Additionally, the rubrospinal tract consists of considerably less descending fibers in humans than in rats [144]. As a consequence, the rubropsinal tract has only very limited significance for the motor abilities of humans, whereas in rats, the rubrospinal tract is crucial for skilled locomotion [145]. It therefore is not surprising that the comparison of function between humans and animal models of SCI is problematic. This is further explained by the fact that humans are the only obligatory bipedal animals. To a great extent, the unique gait of humans is controlled by supraspinal control [146]. This is in contrast to the locomotion of four-legged animals in which the motor activity of stepping and gait is predominantly controlled by specialized reflex circuits, known as the central pattern generators (CPGs) [147]. Since interneurons only are lost at the injured segment, reflexes remain intact above and below the lesion. Therefore, it is not surprising that even after a severe SCI, locomotion is preserved significantly in four-legged animals as long as the CPG is not affected directly. There are significant differences in the control of locomotion between humans and animals, e.g. the role of

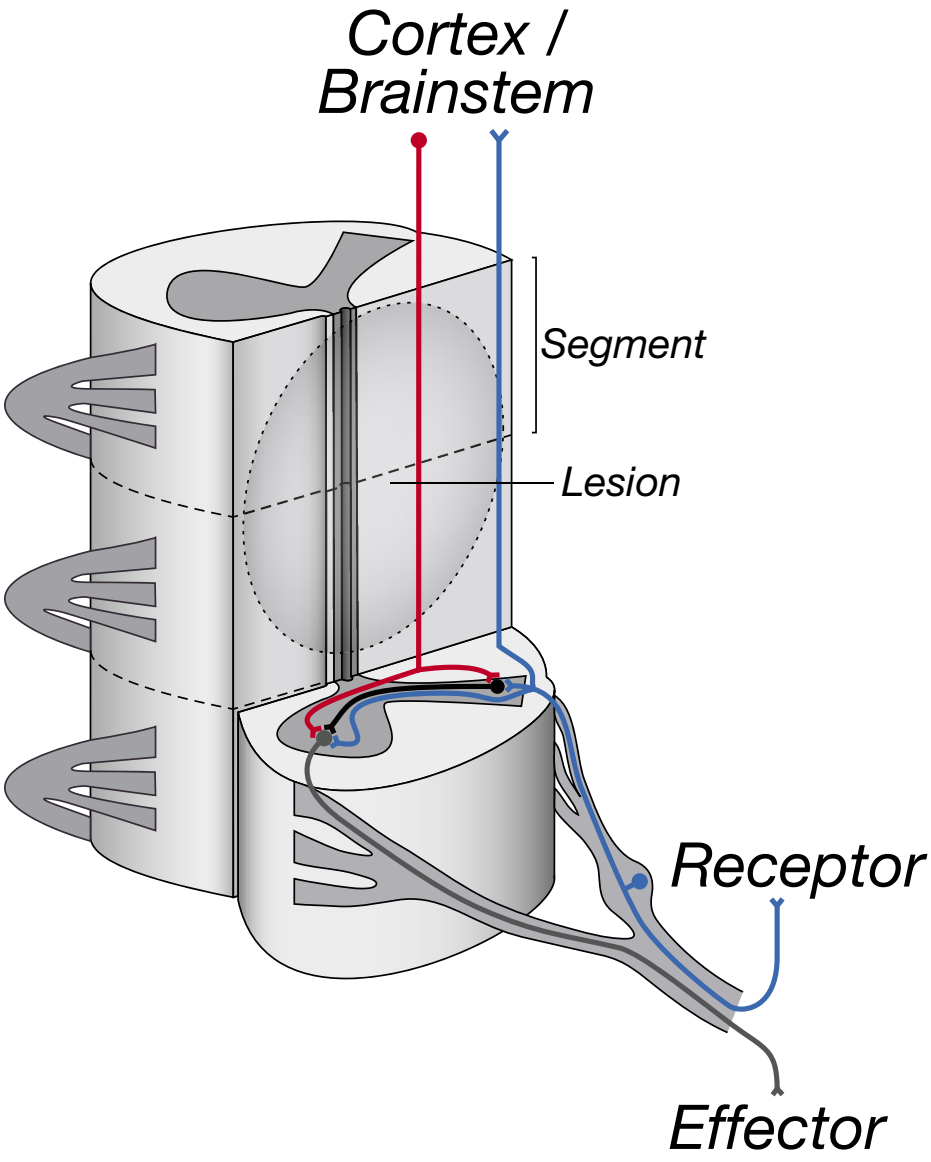

Figure 3: *Schematic graph showing the consequences of SCI on spinal cord projections. Decisive for the functional outcome after SCI is the damage to the long axonal connections in the spinal cord white matter. Mechanical and functional disruption of descending and ascending connections prevents voluntary motor and conscious sensory control, leading to the compartmentalization of the body in a functional and a dysfunctional part. Interneurons only are lost in the injured segments, leaving spinal reflexes intact above and below the injured segments. The blue projection represents the sensory afferent, black the interneuron and grey the lower motor neuron. The corticospinal tract projection is schematized in red.*

the CPG in humans has yet to be clarified [148]. Nevertheless, assessment of locomotor ability is the most frequently employed functional parameter in animal models of SCI [149, 150]. It therefore is important to evaluate whether observed functional recovery of locomotion in SCI animals can be explained by hyperactive reflex responses rather than by reestablishment of functional connections.

The outcome parameters of interest determine the choice for the appropriate SCI animal model. SCI models ideal for the investigation of axonal regeneration may be less suitable for the assessment of function and vice versa. In transection models of SCI for instance, the selective disruption of fiber tracts enables the reliable assessment of the regenerative response of the injured axons. The functional outcome of transection injuries however is less clinically relevant since most SCI represent contusion injuries. Experimental contusion injuries on the other hand are the most relevant in terms of their proximity to pathomechanisms in human SCI [151], but are less reproducible when compared to transection models. Furthermore, the diffuse axonal damage in contusion injuries makes it almost impossible to recognize functional axonal regeneration, since the eventually observed structural and functional recovery cannot be reliably attributed to the regeneration of specific fiber tracts. Alternatively, many alternative models of SCI have been described, including chemical agent induced and aspiration injuries [152-154].

6.2 Cervical dorsal column transection using a tungsten wire knife device

In this thesis, a lesion model for SCI was used in which the cervical dorsal column of rats is specifically transected using a tungsten wire knife device. The stereotactic guidance of the wire knife device allows reproducible transection of the dorsal columns without causing unnecessary damage to the spinal cord grey matter. Unlike suggested by the terminology “wire knife”, the tungsten wire induces a rather blunt transection, causing the formation of a restricted cystic cavity at the lesion site. Because of the minimal disruption of the meninges and surrounding vasculature, a wire knife lesion causes very little vascular disruption (hemorrhage, ischemia) and major invasion of meningeal fibroblasts into the lesion is avoided. The preservation of meningeal sheats allows injecting cell suspension grafts with almost no leaking of cells out of the graft site.

The employed dorsal column transection model is specifically developed to completely lesion the crossed dorsal component of the CST, which represents approximately 95% of all CST axons [155, 156]. The CST plays an important role in the control of fine coordinated movement through its terminations in the ventral horn [157]. However, permanent functional deficits in fine motor tasks such as forelimb reaching are only found when a cervical dorsal column transection injury is combined with a ventral transection of the uncrossed ventral component of the CST [31]. Thus, the isolated transection of the dorsal CST, as used

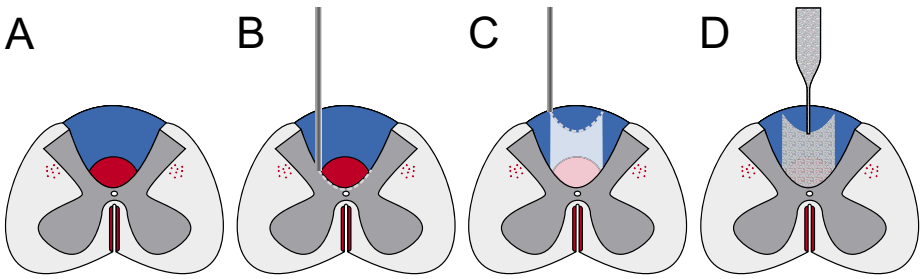

Figure 4: *Schematic representation of the used cervical dorsal column transection model. (A) In rats, the corticospinal tract axons (red) project in the spinal cord through a crossed dorsal component that contains 95% of the axons and an ipsilateral ventral component containing less than 5% of all CST axons. Additionally, there exists a minor dorsolateral component of the CST, containing less than 2% of CST axons. Strikingly, the main part of crossed portion of the CST fibers in humans is located in the dorsolateral component of the CST. The other fibers in the dorsal column (Blue) represent ascending collaterals of the primary sensory afferents and ascending projections of spinal neurons projecting to other segments to the spinal cord. (B) In the used SCI model, a small dural incision is made and the wire knife device is stereotactically lowered into the spinal cord parenchyma. At the correct depth, the tungsten wire is extruded, forming a wire arch below the dorsal CST. (C) Subsequently, the wire knife device with the extruded wire is raised until the tip of the wire is visible, transecting a large part of the dorsal columns, while leaving the dura intact. (D) Directly following the lesion, cellular grafts can be injected into the lesion defect through a pulled glass capillary.*

here, only allows to determine morphological changes, in particular regeneration of corticospinal axons[31]. To a limited extend, ascending proprioceptive axon projections in the dorsal columns will be transected as well. Obvious behavioral alterations have not been observed. The precise functional impact of proprioceptive axon disruption has yet to be determined.

6.3 Contusive spinal cord injury using the Infinitive Horizon Impactor device

In addition, a contusion injury model was used in which a blunt spinal cord trauma in adult rats is induced at thoracal level by employing the computer-controlled Infinite Horizon (IH) spinal cord injury device (Precision Systems & Instrumentation, Lexington, USA). The IH impactor allows the execution of a defined force on the exposed surface of the spinal cord that leads to a reproducible and well-defined contusion injury [158]. Unlike the used wire-knife lesion model, a severe contusion injury at thoracal level induces lasting behavioral deficits and thus allows the assessment of behavioral data such as the locomotor ability [150]. Furthermore, the more clinical relevant nature of the contusion injury allows the pathomorphological comparison between the animal model and injured patients. Reproducibility is often problematic with contusion injury models. However, since the IH impactor is equipped with a force-feedback impounder and uses a defined force rather than displacement to define the severity of the contusion injury, highly

reproducible injuries can be induced using this device [158].

7. AIM OF THIS THESIS

The aim of this thesis was to investigate the capacity of grafts of adult derived neural progenitor cells (NPC) to induce structural regeneration and contact-mediated axon guidance in the injured spinal cord. Therefore, the properties of NPC grafts were carefully studied in small animal models of SCI. Furthermore, the isolation of autologous cell material and the ability to genetically modify NPC using viral vectors was investigated. Finally, non-invasive imaging in small animal models of SCI was investigated in order to facilitate future studies in which the potential of NPC grafts to induce functional regeneration in the injured spinal cord is tested.

In *Chapter 2*, we describe the isolation of NPC, survival, differentiation and tissue replacement capacity after transplantation into the acutely lesioned spinal cord. After having determined that NPC grafts require a supporting matrix to replace cystic lesion defects, we developed a co-transplantation protocol using NPC and syngenic skin fibroblasts, which is described in *chapter 3*. Subsequently, Schwann cells, which not only replace cystic lesion defects, but also have intrinsic regeneration promoting capabilities, were studied. In *chapter 4a*, we describe a fast and efficient method to purify adult Schwann cell from peripheral nerve homogenates for autologous cell therapy. Co-transplantation of NPC with Schwann cells is described in *chapter 4b*. The overexpression of ectopic genes using *ex vivo* gene therapy has been shown to represent a promising tool to augment the regenerative potential of grafted cells in a cellular therapy approach, e.g. by overexpressing growth factors. Experiments investigating the applicability of *ex vivo* gene therapy using adult derived NPC are described in *chapter 5*. In order to enable the monitoring of NPC induced regeneration in future studies, non-invasive imaging techniques need to be developed that allow *in vivo* imaging of neuropathological changes in small animal models of SCI. Therefore, high-resolution magnetic resonance imaging of spinal cord injured rats was studied in *chapter 6*. Finally, in *chapter 7*, the presented studies are discussed in respect to their potential to promote functional axonal regeneration after SCI.

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