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MODULATING THE EXTRACELLULAR ENVIRONMENT DURING AXONAL REGENERATION FOLLOWING SPINAL CORD INJURY

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“Happiness does not come from doing easy work but from the afterglow of satisfaction that comes after the achievement of a difficult task that demanded our best.”

Theodore Isaac Rubin

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Abstract

In the CNS, axon regeneration fails. This is the main reason why paralysis and loss of sensation is permanent in conditions such as spinal cord injury (SCI). The limited ability for axon regeneration has been attributed to the glial scar at the injury site, composed of inhibitory molecules that comprise axon guidance molecules, chondroitin sulphate proteoglycans (CSPGs) and myelin associated inhibitors (MAIs), and to the lack of neuron-intrinsic growth potential. In order to assess the extracellular environment that modulates neural regeneration post-injury one of the aims of this study was to address the possible involvement of myelin lipids as inhibitory molecules. Here, we used shiverer mice (shi) to assess axonal regeneration following SCI in the presence of MAIs and CSPGs, but in the absence of compact myelin. Although *in vitro* shi neurons displayed a similar intrinsic neurite outgrowth to WT neurons, *in vivo*, shi fibers had increased regenerative ability, suggesting that the WT spinal cord contains additional inhibitors besides MAIs and CSPGS. Our data shows that besides myelin protein, myelin lipids are highly inhibitory for neurite outgrowth and demonstrates that this inhibitory effect is released in the shi spinal cord given its decreased lipid content. Specifically, we identified cholesterol and sphingomyelin as novel MAIs with activity in multiple neuron types. We further demonstrated the inhibitory action of cholesterol and sphingomyelin *in vivo*, by showing that delivery of 2-hydroxypropyl-β-cyclodextrin (HPβCD), a drug that reduces the levels of these lipids, leads to increased axonal regeneration following SCI. The second aim of this study also focused the relevance of the extracellular environment in the modulation of axonal regeneration, but on a different perspective, namely on the study of the capacity to ameliorate the pathophysiology of SCI through the application of a chitosan guidance scaffold seeded with endothelial stem cells and neural stem (NS) cells in an anatomically complete transected SCI model. Accordingly, we examined through the specific labeling of vital ascending and descending fiber tracts, whether this bioengineering approach would support regeneration and consequently result into functional recovery. We observed that the scaffold seeded with NS cells functioned a tissue bridge that was filled by βIII positive axons. Moreover through the lumen of the scaffold our results show the presence of some regenerating axons from sensory and motor tracts, revealing that our strategy allows endogenous axons to regenerate.

Keywords

Spinal cord injury, myelin lipids, axonal regeneration, chitosan scaffold

Resumo

As lesões na medula espinal levam a perdas sensoriais e motoras permanentes. Os danos que ocorrem no sistema nervoso central (SNC), levam a que a regeneração axonal não ocorra, sendo esta falha devida ao diminuído potencial intrínseco de regenerar dos neurónios do SNC e à formação de uma cicatriz glial no local da lesão, que é composta por moléculas inibitórias entre as quais sinais guia axonais, proteoglicanos de sulfato de condroitina (PGSCs) e inibidores associados à mielina (IAM). De modo a estudar o ambiente extracelular que modula a regeneração axonal após lesão, um dos objetivos deste trabalho foi investigar o possível envolvimento dos lípidos da mielina como possíveis moléculas inibitórias. Para isso foi usado o modelo de ratinho shiverer (shi) de forma a aceder à regeneração axonal após lesão na presença de PGSCs e IAMs, mas na ausência de mielina compacta. Apesar de os neurónios shi *in vitro* apresentarem crescimento de neurites semelhante aos controlos (wt), *in vivo* as fibras axonais do shi demonstraram ter maior capacidade regenerativa, sugerindo que a medula espinal dos animais wt contém inibidores adicionais para além da existência de PGSCs e IAMs. Os nossos resultados mostram que para além das proteínas da mielina, os lípidos que compõem esta estrutura são grandemente inibitórios para o crescimento de neurites e que no caso dos ratinhos shi o efeito inibitório não se verifica, uma vez que o componente lipídico da sua medula espinal está diminuído. Especificamente, nós identificámos o colesterol e a esfingomielina como novos IAM com atividade em múltiplos tipos neuronais. Seguidamente demonstrámos que a ação inibitória destes lípidos *in vivo*, é reduzida pela distribuição de 2-hidroxipropil-β-ciclodextrina (HPβCD) que atua como um fármaco, reduzindo os níveis lipídicos no local de lesão e aumentando assim a regeneração axonal. O segundo objetivo deste estudo está também focado na relevância do ambiente extracelular e na modulação da regeneração axonal, mas numa perspetiva diferente, nomeadamente no estudo da capacidade de melhorar a patofisiologia das lesões da espinal medula, através da aplicação de um suporte de quitosano guia preenchido com células estaminais neurais e endoteliais num modelo de lesão completa da medula espinal. Assim através da marcação específica de tractos ascendentes e descendentes da medula espinal, fomos analisar se esta abordagem poderia resultar num suporte efetivo para a regeneração e recuperação funcional. Primariamente observámos que os suportes de quitosano preenchidos com células

estaminais neurais, permitiram gerar uma ponte de ligação entre as duas extremidades da medula espinal e que o biomaterial continha células do tipo neuronal como demonstra a marcação com βIII-tubulina. Além disso, através do lúmen do suporte de quitosano, os nossos resultados mostram a presença de alguns axónios sensoriais e motores, indicando que a nossa estratégia permite o crescimento de tractos endógenos.

Palavras-Chave: Lesão da medula espinal, lípidos da mielina, regeneração axonal, suporte de quitosano

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List of Abbreviations

5HT - 5-hydroxytyptamine

BBB - Blood-brain-barrier

BDA - Biotin dextran amine

BDNF - Brain-derived neurotrophic factor

cAMP - Cyclic adenosine monophosphate

Cdc42 - Cell division cycle 42

CE - Cholesterol esters

Cer - Ceramide

ChABC - Chondroitinase ABC

CNP - Cyclic nucleotide phosphodiesterase

CNS - Central Nervous System

CO - Cholesterol

CSPGs - Chondroitin sulfate proteoglycans

CST - Corticospinal tract

CTB - Cholera toxin-β

DA - Degree of acetylation

DD - Degree of deacetylation

DLK-1 - Dual leucine zipper kinase

DRG - Dorsal root ganglia

ECM - Extracellular matrix

EFA-6 - Exchange factor for Arf6

EGF - Epidermal growth factor

EGFR - Epidermal growth factor receptor

ERK - Extracellular signal-regulated kinase

ES - Embryonic stem cells

FDA - US Food and Drug Administration

FGF-2 - Fibroblast growth factor 2

GalCer - Galactocerebroside

Gb4 - Globotetrahexosylceramide

GDNF - Glial-derived neurotrophic factor

GFs - Growth factors

GM1- Ganglioside

GS - Sulfatide

H&E - Hematoxylin-eosin staining

HDAC5 - Histone deacetylase 5

HPβCD - 2-hydroxypropyl-β-cyclodextrin

HSCs - Haematopoietic stem cells

IGFs - Insulin like growth factors

IL-1α - Interleukin 1α

IL-1β - Interleukin 1 β

IL-6 - Interleukin-6

IN-1 - Monoclonal antibody against Nogo

iPSCs - Induced pluripotent stem cells

JNK - c-Jun amino-terminal kinase

Lac - Lactocerebroside

LIF - Leukemia inhibitory factor

MAG - Myelin-associated glycoprotein

MAIs - Myelin-associated inhibitors

MBP - Myelin basic protein

MCP-1 - Monocyte chemoattractant protein-1

MOG - Myelin oligodendrocyte glycoprotein

MSCs - Mesenchymal stem cells

NGF - Nerve growth factor

NgR - Nogo receptor

Nogo-66 - Nogo 66-amino-acid sequence

NS - Neural stem

NSPCs - Neural stem /progenitor cells

NT-3 - Neurotrophin-3

OEG - Olfactory ensheathing glia

OMgp - Oligodendrocyte myelin glycoprotein

OPC - Oligodendrocyte progenitor cell

PAN - Polyacrylonitrile

PBS - Phosphate buffered saline

PC - Phosphatidylcholine

PE - Phosphatidylethanolamine

PFA - Paraformaldehyde

PGA - Poly(glycolic acid)

PHEMA - Poly(2-hydroxyethyl methacrylate)

PI - Phosphatidylinositol

PKA - Protein kinase-A

PKC - Protein kinase C

PLA - Poly(lactic acid)

PLCL - Poly(lactide-co-caprolactone)

PLGA - Poly(lactic acid-co-glycolic acid)

PLP - Proteolipid protein

PNS - Peripheral Nervous System

PS - Phosphatidylserine

PTEN - Phosphatase and tensin homolog

PTFE - Poly(tetrafluoroethylene)

PVC - Polyvinylchloride

RAGs - Regeneration-associated genes

RGD - Arginine-glycine-aspartic acid

RGMa - Repulsive guidance molecule A

rhFN - Recombinant fragment of human fibronectin

ROCK - Rho associated kinase

SCI - Spinal Cord Injury

SEM - Scanning electron microscopy

Sema3 - Semaphorin 3

Sema4D - Semaphorin 4D

sGAG - Sulphated glycosaminoglycan

sGalCer - Glycosphingolipid sulfatide

SGZ - Subgranular zone

Shi - Shiverer

SOCS3 - Suppressor of cytokine signaling 3

SpH - Sphingomyelin

STAT3 - Signal transducer and activator of transcription 3

SVZ - Subventricular zone

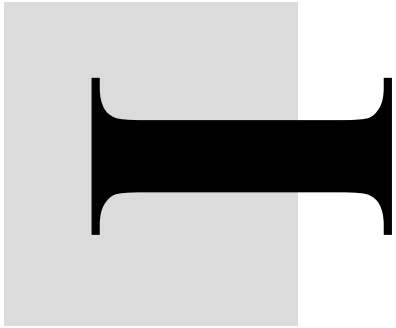
TG - Triglycerides

TNF-α - Tumor necrosis factor-α

Wd - Wallerian degeneration

WT - Wild-type

Introduction



1. Axonal regeneration during evolution

Regeneration is a remarkable phenomenon of biology that allows some species to regrow substantial parts of their body. However, there are many species like *Humans* that have poor regenerative ability. Regeneration in the neuroscience field means re-acquiring nervous system function after injury or disruption, with or without the need to replicate the original structure or its full functionality (Tanaka and Ferretti 2009). In the context of axonal regeneration, this represents a new growth at the tip of a transected axon, and generally suggests propagation of growth over some distance (Yoon and Tuszynski 2012).

During development, axonal elongation occurs both in the Peripheral Nervous System (PNS) and in the Central Nervous System (CNS). In many ways, regeneration can be viewed, at least in part, as a recapitulation of the developmental process, since axons need to regrow towards their targets. Ramon y Cajal described for the first time the axonal regeneration of peripheral nerves, and stated that these nerves have an environment that could guide and fuel regrowth of the injured axons. In relation to CNS axons, they are characterized by abortive regeneration both in the spinal cord and in the cerebral cortex, and have been described as having limited ability to regenerate only in young animals (Ramon y Cajal and May 1928).

Injuries can occur both in PNS and CNS neurons, although the regenerative responses are different. Injured peripheral axons can undergo regeneration, whereas regeneration in CNS neurons is less successful and often, only limited recovery is possible. The consequences of injury are not just a break in communication between healthy neurons, but a cascade of events that can lead to neuronal degeneration and cell death. The ability of injured neurons to generate an effective growth response is modulated by two different types of mechanisms: extrinsic and intrinsic. The extrinsic environment is characterized by the response of the supporting glia and of the immune system after injury, while the intrinsic elements comprise the ability of neurons to express genes that allow their survival and regeneration following injury.

Until now, it was not possible to promote efficient axonal regeneration in the adult human CNS. In the future it will be important to study and understand the entire cellular and molecular environment that will allow improving regeneration of CNS axons.

2. Wallerian degeneration

After nerve injury, axons that are physically separated from the neuronal cell body degenerate. Axonal degeneration happens in the distal part of the nerves and is mediated by calcium influx, which activates axonal proteases leading to disintegration and degeneration of the axolemma and axoplasm of nerve fibers (Fenrich and Gordon 2004). Degenerative events that occur after injury are collectively known as Wallerian degeneration (Wd) (Figure 1). This process was first described in 1850 by Augustus Volney Waller (Waller 1850) and consists in the production of a positive environment that supports axonal regeneration through the clearance of inhibitory debris such as myelin remains.

The nerve fibers of both PNS and CNS are surrounded by glial cells that produce myelin insulation and provide trophic support of mature neurons. Myelin is a multilamellar sheath laid down along selected nerve fibers that facilitate saltatory conduction of stimuli from the nerve-cell body to its target. The myelination process is performed by Schwann cells and oligodendrocytes in the PNS and CNS, respectively. In the PNS, Schwann cells myelinate a single axon, whereas small diameter axons are surrounded by a thin layer of Schwann cell cytoplasm, resulting in a structure called the Remak bundle (Salzer 2008). In the CNS, a single oligodendrocyte is capable of myelinating several axons.

Following PNS injury the myelin sheath breaks down and Wd is initiated with increased permeability in the blood brain barrier (BBB) that leads to an influx of macrophages, and finally, myelin debris clearance (Gaudet, Popovich et al. 2011). Schwann cells play an important role in the fast response to PNS injury, because they have the ability to differentiate in a phagocytic phenotype starting myelin debris removal, even before macrophage recruitment occurs. This dedifferentiation comprises the downregulation of genes related to myelination such as genes involved in cholesterol synthesis and structural proteins: P0, myelin basic protein (MBP) and myelin-associated glycoprotein (MAG) (Jessen and Mirsky 2008). At the same time there is the expression of numerous trophic factors essential for neuronal survival, such as brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), nerve growth factor (NGF) and the production of several cytokines like leukemia inhibitory factor (LIF), tumor necrosis factor-α (TNF-α), interleukin-1α (IL-1α) (Shamash, Reichert et al. 2002) and interleukin-6 (IL-6). Cytokine expression stimulates

Schwann cells to express the monocyte chemoattractant protein-1 (MCP-1), an essential component for macrophage recruitment (Subang and Richardson 2001, Tofaris, Patterson et al. 2002). After macrophage recruitment, Schwann cells start to proliferate and align along the basal lamina forming the bands of Bungner, which provide support to the production of trophic factors and laminin, an adhesion molecule that is part of the extracellular matrix of basal lamina tubes and is essential for axon growth (Chen and Strickland 2003).

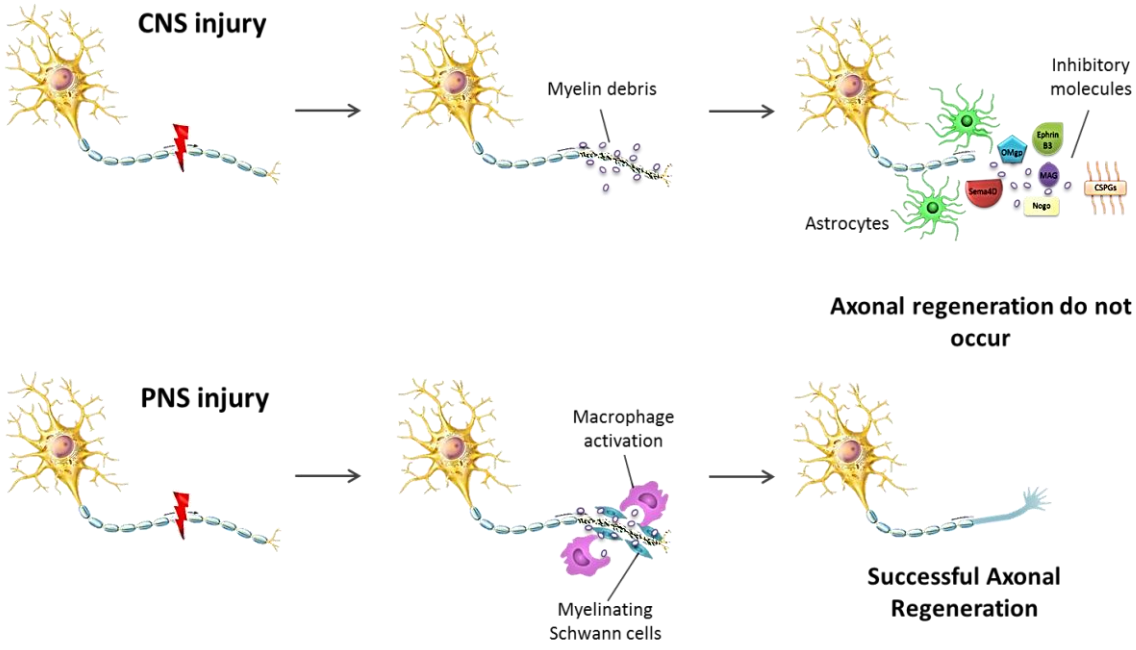


Figure 1 Wallerian degeneration in the CNS and PNS. Upper panel: CNS injury causes the local destruction of axons and glia, followed by a slow Wallerian-type degeneration, which produces an inhibitory environment for the injured axons, impeding regeneration. Lower panel: PNS injury causes local destruction of axons and Schwann cells. Wallerian degeneration of the distal region involves macrophage activation and Schwann cell proliferation, which result in the support of sprouting axons, that regenerate along the Schwann cell-filled basal lamina tubes.

Wd is slow in CNS injured neurons, given the failure to clear myelin debris. Compared to Schwann cells, oligodendrocytes are more sensitive to axonal injury, undergoing apoptosis, and leading to an accumulation of myelin growth inhibitors such as Nogo, MAG and oligodendrocyte myelin glycoprotein (OMgp). Moreover, the BBB is maintained following CNS injury, limiting the infiltration of circulating macrophages. Besides, resident CNS microglia (immune system phagocytic cells) display limited ability to remove myelin when compared to infiltrating macrophages (Fitch and Silver 2008). Microglia also produces cytokines, but because of the reduced blood flow at the injury site, the immune response is slowed and, in turn, inflammation is prolonged (Fenrich and Gordon 2004). The failure of myelin debris clearance together with

astrocyte activation (Sun and Jakobs 2012) leads to the formation of a dense scar tissue surrounding the injured area, that is called glial scar. The formation of a mechanical obstructive glial scar was thought to explain the failure of regeneration after CNS injury. However, the glial scar is far more than just a physical barrier, involving complex cellular and molecular interactions to prevent successful regeneration (Fitch and Silver 2008).

3. Intrinsic ability to regenerate

During development, through a cell intrinsic program composed of multiple pathways, neurons display a robust elongation capacity. After the establishment of connections, adult CNS neurons have a limited regenerative ability as the consequence of decreased calcium changes induced by injury, no increase in histone acetylation, lack of robust synthesis of regeneration-associated genes (RAGs), limited local protein synthesis, and presence of inhibitors of axon regrowth (phosphatase and tensin homolog (PTEN), suppressor of cytokine signaling 3 (SOCS3), and exchange factor for Arf6 (EFA-6)) (Mar, Bonni et al. 2014).

In the PNS, following injury, neurons have the capability to reactivate the intrinsic growth expression of regeneration-related transcription factors and growth-associated genes (Figure 2). However, following injury to adult CNS neurons, the intrinsic growth machinery fails and regeneration does not occur. One of the reasons for this failure is the lack of injury signals in adult neurons that could enhance the expression of RAGs, crucial for the regenerative process (Liu, Tedeschi et al. 2011, Yang and Yang 2012).

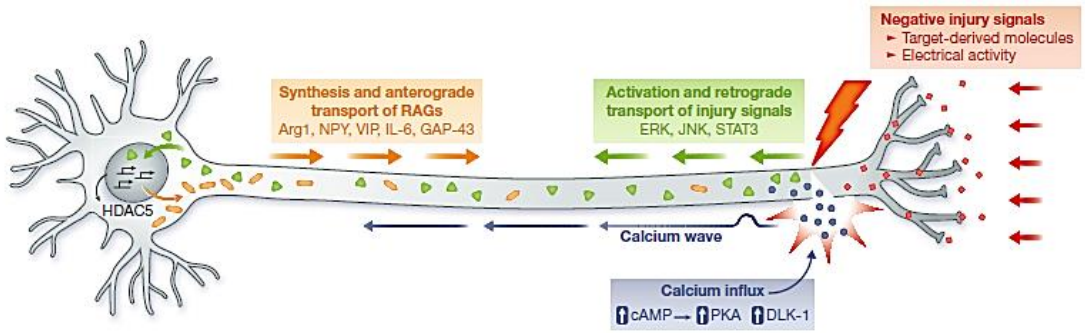


Figure 2 Cell intrinsic mechanisms of axonal regeneration in the PNS. Adapted from (Mar, Bonni et al. 2014).

In the PNS, the neuronal cell body senses axonal damage through calcium influx into the axoplasm that is one of the first signals elicited by injury and through the depolarization triggered by the inversion of the calcium/sodium flux that travels along the axon to the cell body. Axonal regeneration in the PNS is regulated by axonal calcium waves, which activate calcium-dependent enzymes including adenylate cyclase, leading to increase cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) levels, and also to the signaling via the downstream dual leucine zipper kinase (DLK-1), promoting growth cone formation, local protein synthesis, and resealing of the axonal membrane. The calcium wave back-propagates to the cell body causing protein kinase C (PKC) activation followed by nuclear export of histone deacetylase 5 (HDAC5), thereby increasing histone acetylation and activating the proregenerative transcription program. Following the calcium-dependent early phase, retrograde transport of injury signals including extracellular signal-regulated kinase (ERK), c-Jun amino-terminal kinase (JNK), and signal transducer and activator of transcription 3 (STAT3) occurs.

4. Glial Scar

Scar tissue formation and its components are important players in the immediate response to CNS injury. The glial scar integrating components are the reactive astrocytes that seal the injury area, microglia/macrophages that elicit the inflammatory reaction essential to subsequent repair, extracellular matrix molecules like chondroitin sulfate proteoglycans (CSPGs) and myelin debris composed of several regeneration inhibitors (Fitch and Silver 2008). We can divide the injury process in the CNS in two phases: the acute phase that is the degeneration of neuronal connections, and the secondary injury where neurons are exposed to a microenvironment with toxic factors, imbalance of amino acids and ions, reactive oxygen species, free radicals and overwhelming inflammation (Figure 3). In the acute phase, astrocytes demarcate the lesion area and separate the injury tissue from the surrounding healthy tissue. Through the injury site, astrocytes also provide trophic factor support for the surviving neurons, by the production of glucose, insulin like growth factors (IGFs), nerve growth factors, BDNF and neurotrophin-3 (NT-3). Besides sealing the site of injury, the glia scar can fill in the lesion area, creating a scaffold for the vascularization network (Rolls, Shechter et al. 2009).

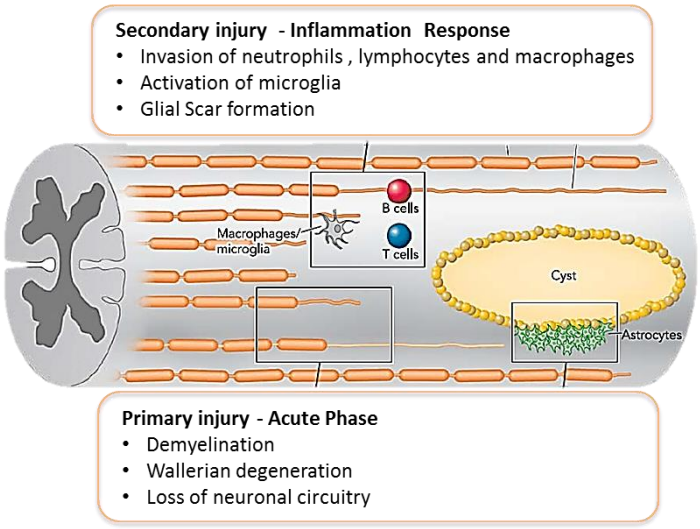


Figure 3 Schematic drawing showing a CNS injury and the subsequent injury mechanisms. Adapted from (Obermair, Schroter et al. 2008).

The immune response is a key event in the secondary injury cascade. The immune cells that first arrive at the site of injury are neutrophils, followed by lymphocytes and macrophages, in that order. These cells secrete pro-inflammatory cytokines, including interleukin-1β (IL-1β), IL-6, and TNF-α, free radicals and growth factors, which can affect cells in positive and negative ways (Oyinbo 2011). The immune control of the lesion area is essential, because overactivation of the inflammatory response can exacerbate injury and damage healthy tissue. The glial scar presence after injury can be seen as part of the process of immune regulation and repair, but equally as a physical and biological barrier to axonal regeneration.

4.1 Glial scar inhibitors

Dorsal root ganglia (DRG) neurons have axons in the CNS and PNS, but can only regenerate their peripheral processes. These considerations suggest that interactions with different environments may lead to different regenerative responses. All the events described previously, lead to the development of the glial scar milieu that is composed by inhibitory molecules that form a hostile environment for axon growth and repair (Silver and Miller 2004).

4.2 Myelin associated inhibitors (MAIs)

Myelination is an essential CNS developmental process. However, myelin proteins components were shown to be inhibitory for axonal regeneration in the adult mammalian CNS.

CNS myelin was first proposed as a major source of axonal growth inhibition, unlike PNS myelin. To allow the identification of individual inhibitory molecules, an antibody termed IN-1 was developed against the inhibitory fraction of myelin, and was found to be a supportive molecule for axon growth (Caroni and Schwab 1988). Subsequent studies allowed the discovery of Nogo as the antigen recognized by the IN-1 antibody (Chen, Huber et al. 2000). Nogo exists in three isoforms (A, B and C) and is a member of the reticulon family of membrane proteins. Nogo-A is the isoform with the highest expression in oligodendrocytes, having a unique amino (N) terminus (amino-Nogo) and 66-amino-acid sequence (Nogo-66) that are the inhibitory domains of axonal regeneration (McKerracher and Winton 2002). Besides Nogo, other myelin-inhibitors were previously described, namely MAG that was the first myelin inhibitor of neurite outgrowth characterized *in vitro* (McKerracher, David et al. 1994). This transmembrane protein with five immunoglobulin-like domains in the extracellular region, in contrast to Nogo, is expressed both in CNS and PNS glial cells where it is associated to myelin formation and maintenance (Filbin 2003). This bi-functionality results from a specific switch in the development of young neurons (Turnley and Bartlett 1998). OMgp, contrary to its name, is expressed not only by oligodendrocytes, but also in neurons of both CNS and PNS. OMgp is a glycosilphosphatylinositol (GPI)-linked protein, localized next to the node of Ranvier and responsible for the inhibition by the myelin fraction originally termed "arretin". This protein might act to prevent collateral sprouting and similarly to MAG and Nogo can induce growth cone collapse and inhibit neurite outgrowth (Filbin 2003, Yiu and He 2006).

In biomolecular terms, myelin is a bimolecular lipid leaflet sandwiched between two layers of protein (Figure 4). From its components, MBP maintains the correct structure of myelin, interacting with lipids in the myelin membrane. Myelin sheaths are comprised of 80% of lipids and 20% of proteins distributed according to charge, lipo- or hydro-philicity, and relative molecular weight. Regarding myelin lipid components, the three major classes of lipids are cholesterol, glycosphingolipids and phospholipids (Podbielska, Levery et al. 2011). With a contribution of 4-7% of myelin lipid, the

glycosphingolipid sulfatide (sGalCer) has recently been reported to have a strong inhibition effect in neurite outgrowth retinal ganglion cells (Winzeler, Mandemakers et al. 2011). Sulfatide is an amphipatic glycolipid that has a polar sulfated galactosyl head group facing out from the plasma membrane and that is anchored in the membrane bilayer by ceramide-containing N-acyl-linked long-chain fatty acids.

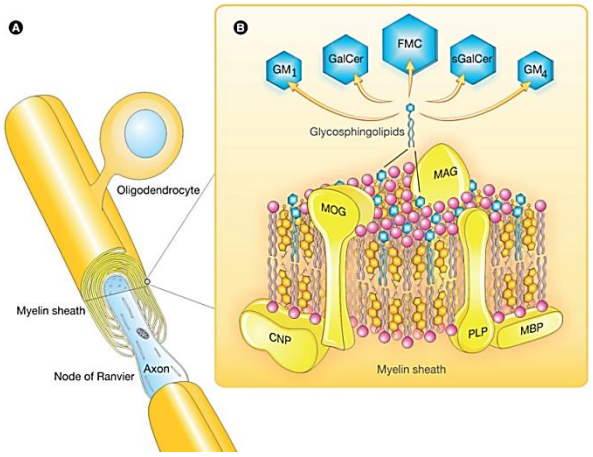


Figure 4 CNS myelin components. (A) Architecture of myelin sheath in an oligodendrocyte. (B) Molecular composition of CNS myelin. Proteins (proteolipid protein (PLP), myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), myelin-associated glycoprotein (MAG) and cyclic nucleotide phosphodiesterase (CNP) are marked in yellow and the comprising lipids are indicated as follows: cholesterol in orange, phospholipids in pink and glycosphingolipids in blue. Adapted from (Podbielska, Levery et al. 2011)

The identification of sulfatide as a novel myelin-associated axon growth inhibitor, demonstrates that specific lipids can powerfully inhibit axonal growth. Knowledge of the biochemical and physicochemical properties of myelin lipids, and particularly of their composition, organization, structure and accessibility, is central to understanding how these lipids can contribute to axon regenerative failure in the CNS.

4.3 Inhibition mediated by CSPGs

CSPGs are inhibitory extracellular matrix (ECM) molecules (aggrecan, brevican, neurocan, versican, phosphacan and proteoglycan NG2) produced by hypertrophic astrocytes which turn reactive after glial scar formation (Volpato, Fuhrmann et al. 2013). These molecules are defined by protein cores which have covalently linked sulphated glycosaminoglycan (sGAG) chains. Proteoglycans like CSPGs are extensively described as inhibitory to axonal regeneration, and after CNS injury are rapidly upregulated by reactive astrocytes. The turning behavior suggests that these molecules serve as a mechanical and biological barrier through the inhibition of neurite-promoting components of the extracellular matrix, particularly laminin, fibronectin and

neural cell adhesion molecules. Currently it has been demonstrated that the enzymatic removal of sGAG chains from the protein core of CSPGs, with chondroitinase ABC (ChABC) leads to the elimination of the neurite outgrowth inhibitory action of these molecules (Silver and Miller 2004, Yiu and He 2006, Giger, Hollis et al. 2010).

4.4 Canonical Axon Guidance Molecules

In addition to the inhibitory role of myelin associated proteins and CSPGs, several other molecules are known to contribute to the growth-retarding effects of the glial scar (Figure 5). The canonical axon guidance molecules are implicated in signaling pathways that regulate adult nervous system plasticity following injury (Harel and Strittmatter 2006). Ephrin is one of these molecules that can be expressed by reactive astrocytes and meningeal fibroblasts, contributing to the regulation of these two cell types during scar formation. Ephrin-B3 is specifically expressed on mature myelinating oligodendrocytes, playing a role in myelin-dependent inhibition of axonal growth. During early neuronal development, semaphorins like ephrins have axon repulsive functions that are mediated through plexin receptors and neuropilin co-receptors (Liu, Cafferty et al. 2006). The molecules semaphorin 3 (Sema3) and semaphorin 4D (Sema4D) are widely associated to the blocking of axonal regeneration in the lesion site. Sema3 is increased in fibroblasts that infiltrate the lesion, thus preventing the penetration of regenerating neurites (Pasterkamp, Giger et al. 1999). Sema4D is a transmembrane protein present in oligodendrocytes, which is capable of inhibiting axonal growth near to the injury site (Silver and Miller 2004; Giger, Hollis et al. 2010).

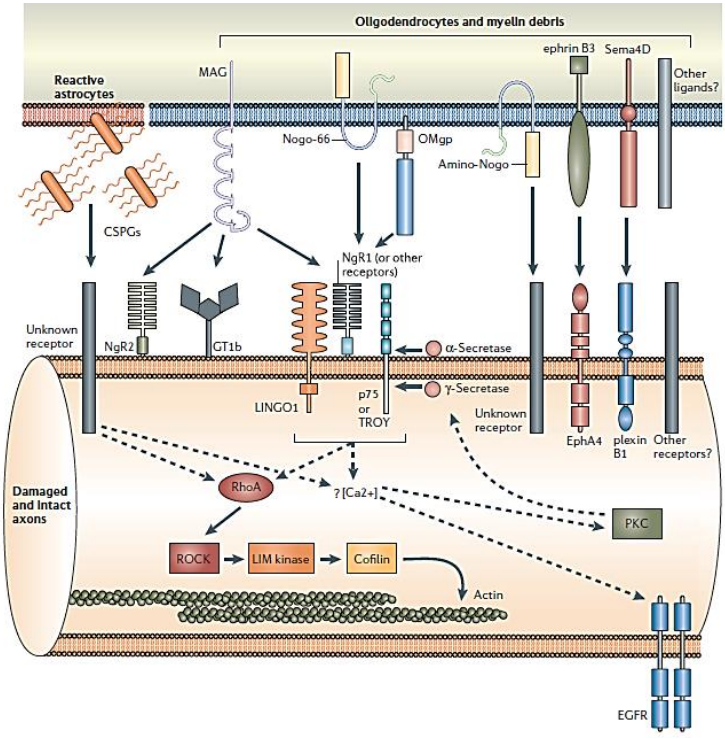


Figure 5 Glial inhibitors and intracellular signalling mechanisms. Adapted from (Yiu and He 2006).

4.5 Intracellular signaling by inhibitors of regeneration

The interaction between myelin associated inhibitors and their receptors in the axon surface leads to the initiation of a specific inhibitory signaling cascade in neurons. This pathway involves Rho A and its effector, Rho associated kinase (ROCK). Rho family of small GTPase proteins such as Rho, Rac1 and cell division cycle 42 (Cdc42) .(Feltri, Suter et al. 2008) are implicated as key regulators of cytoskeleton dynamics (Sit and Manser 2011) and myelin inhibitory effects. Rho functions are regulated by shuttling between an active (GTP-bound) and inactive (GDP-bound) state. MAIs exercise their inhibitory effects via a common, tripartite neuronal receptor complex that comprises the Nogo receptor (NgR), p75NTR and Lingo-1. Interactions of these receptors with Rho after MAI binding, originates Rho activation and initiation of the signaling pathway (Alabed, Grados-Munro et al. 2006).

The downstream effector ROCK exists in two isoforms, ROCK I that is expressed in nonneuronal tissues like liver, stomach, spleen, kidney, and testis, and ROCK II which is preferentially expressed in brain and muscle. Activation of the signaling cascade is made through the interaction of ROCK with the active Rho GTP domain (Kubo, Yamaguchi et al. 2008). This pathway is intimately related to the regulation of growth cone morphology, by regulation of target proteins such as myosin chains, cofilin and collapsin-response mediator proteins (McKerracher, Ferraro et al. 2012).

5. Spinal cord injury pathophysiology and recovery

Spinal cord injury (SCI) is an overwhelming condition that originates an acute traumatic lesion of neural elements in the spinal canal, resulting in permanent deficits of motor, sensory and autonomic function. The incidence of this pathology worldwide is estimated in 2.5 milion people and more than 130,000 new injuries are reported each year (Adams and Cavanagh 2004). This condition leads to a huge impairment in the quality of life for affected individuals and their families, as well in the economic burden caused by the continuous medical care.

Restorative therapies of motor and sensory function are the most requested by patients with this type of injury. Concerning quadriplegic patients, the priorities are the recovery of arm and hand function, whereas paraplegics consider recovery of sexual function as the most important, when compared to bladder/bowel function, chronic pain, trunk stability and walking movement recovery (Anderson 2004). Understanding the complex pathophysiology mechanisms of SCI is a major research challenge. Until now

we can divide these mechanisms in primary and secondary phases. Firstly the initial mechanical injury can result from contusion, compression, penetration or laceration of the neural tissue of the spinal cord, which results in direct death of cells and formation of a cyst that may interrupt descending and ascending axonal tracts (Thuret, Moon et al. 2006).

After the initial insult, secondary processes are taking place, being responsible for the expansion of the injury site and limiting restorative events. Features of secondary injury include: (1) loss of regulation of local and systemic blood pressure due to the breakdown of the BBB; (2) inflammatory responses with the release of inflammatory mediators, migration of peripheral inflammatory cells, penetration of serum proteins and activation of microglia; (3) chemical change or ionic alterations, including increased intracellular calcium, extracellular potassium, and sodium permeability; (4) cellular dysfunction including apoptosis, neurotransmitter accumulation, production of free radicals and lipid peroxidation, as well imbalance of activated metalloproteinases; (5) anatomic and functional change including edema, ischemia, necrosis and demyelination of spinal cord tissue (Varma, Das et al. 2013).

In contrast to these destructive events, there is some spontaneous repair after SCI by limited axon sprouting. "Sprouting" means that new growth can arise from a transected or an intact axon, anywhere along the length of the axon (Yoon and Tuszynski 2012). However, this mechanism is blocked by physical and molecular factors, and few axons can regenerate and achieve their original target. Instead, some of the injured corticospinal axons can sprout and together with long descending propriospinal tracts can increase connectivity with lumbar motor neurons (Weidner, Ner et al. 2001, Thuret, Moon et al. 2006).

5.1 Axonal tracts of the spinal cord

The spinal cord is composed by a region termed the grey matter that contains neuronal cell bodies, unmyelinated axons and capillaries, whereas the white matter mostly contains myelinated axons and glial cells. The anterior and posterior grey horns divide the white matter on each side into three broad areas called columns: (1) anterior (ventral) white columns, (2) posterior (dorsal) white columns, and (3) lateral white columns. Each column contains distinct bundles of axons, which may extend for long distances up or down in the spinal cord, until they reach the target, these are called tracts. Two main tracts exist, an ascending sensory tract where axons conduct nerve

impulses towards the brain and a descending motor tract that consists of axons that carry nerve impulses from the brain. Sensory and motor tracts of the spinal cord are continuous with sensory and motor tracts in the brain.

For the study of regeneration and complete recovery following SCI, several animal lesion models can be used. If the lesion is incomplete, some axons have the ability to regenerate along lateral or through the opposite side of the lesion cavity.

Through the search of specific axonal tracts that may regenerate after a SCI, in the future we will be able to understand which are the mechanisms that lead to functional recovery. Ongoing studies have used several axonal tracts with different functions and regeneration abilities, namely the dorsal column fibers, the raphespinal tract, the rubrospinal fibers as well as the corticospinal tract (CST).

The dorsal column fibers originate in the dorsal root ganglia which have a peripheral branch in the PNS and a central ascending branch in the dorsal white matter of the spinal cord. These are ascending sensory fibers that can be labeled by a tracer injection like cholera toxin-β (CTB) in the sciatic nerve. A motor descending tract is the raphespinal tract that initiates in the brainstem raphe nuclei and descends through the spinal cord as disperse bundles of axons. These are serotonergic fibers with high ability to regenerate, which can be labeled by immunohistochemistry against serotonin (5-hydroxytyptamine) (5HT). Other motor fibers are the Rubrospinal fibers that unlike raphespinal tract are driven by a descending tract that initiates in red nuclei of the midbrain through the dorsal part of the lateral column. These fibers can be seen with a tracer injection in the brainstem. Lastly the corticospinal tract is the most important tract for voluntary motor function. This tract begins in the sensory motor cortex and in rodents it descends mainly in the dorsal white matter, with some fibers in the lateral white matter. In humans, the lateral tract is the major corticospinal tract. It can be labeled by a tracer injection of biotin dextran amine (BDA) in layer 5 of the sensory motor cortex (Tuszynski and Steward 2012).

6. New strategies to study and restore function following SCI

Present strategies that have been developed for the treatment of SCI are directly related with minimizing further complications secondary to the injury and increasing physical recovery through rehabilitation.

6.1 Overcome inhibition in the complex environment of the lesion

As mentioned before, the deep alterations that occur following SCI may lead to the overproduction of extracellular matrix components like CSPGs. Enzymatic degradation of inhibitory CSPGs by ChABC is described as being effective in the promotion of CNS regeneration of both ascending sensory and descending motor axons after dorsal column crush (Bradbury, Moon et al. 2002). Moreover the intrathecal delivery of ChABC also ameliorates bladder and hindlimb function. Through the delivery of ChABC within peripheral nerve grafts the regrowth of CNS axons in the spinal cord cavity was demonstrated (Chau, Shum et al. 2004).

Inhibitory molecules of axon regeneration are mostly derived from myelin elements. Nogo is the myelin-inhibitor which has been demonstrated to have greatest potential for SCI therapy. An antibody produced against Nogo-A which is highly expressed in oligodendrocytes, leads to enhancement of axonal regeneration and compensatory sprouting of uninjured tracts. This antibody currently undergoes a Phase I clinical trial in human subjects with acute SCI (Zorner and Schwab 2010). Antibodies targeting the NgR complex are also being tested because of their ability to bind all the three myelin associated-molecules (Nogo, MAG and OMgp) (Klussmann and Martin-Villalba 2005). The signaling cascade of inhibitory effects of myelin is mediated by the small GTPase Rho A with its main effector being ROCK.

Inactivation of Rho through an enzyme from *Clostridium botulinum*, the C3 transferase, blocks Rho signaling and promotes axonal regeneration (Winton, Dubreuil et al. 2002, Auer, Schweigreiter et al. 2012). Cethrin® is a recombinant variant of C3 transferase that is being tested on an ongoing clinical trial with SCI patients (Fehlings, Theodore et al. 2011). Fasudil is the only accepted clinical drug for the inactivation of ROCK and together with Y-27632, another ROCK inhibitor, it improves axonal regrowth and functional recovery in rodents with SCI (Kubo, Yamaguchi et al. 2008).

Besides promoting axonal growth, the maintenance of a myelin sheath in the surviving fibers and in newly regenerated axons is essential for the axonal conduction.

For that, therapy with a potassium channel blocker (4-aminopyridine) has been tested in clinical trials in patients with chronic SCI. However this drug has only provided improvements in spasticity (Thuret, Moon et al. 2006).

6.2 Enhancing the intrinsic regeneration machinery

It is now known that just the removal of inhibitory cues present in the glial scar, might be not enough to reach a long range regrowth. Exogenous administration of growth factors (GFs) like NT3, NGF and BDNF has been proposed as one potential therapeutic treatment for SCI. These GFs are being studied in rats with SCI, revealing the ability to induce growth of axons in several tracts and improve recovery of forelimb function (Blesch and Tuszynski 2003). However, the individual delivery of GFs can result in only partial recovery. To overtake this issue, the combinatorial strategies of two GFs or GF with transplanted cells is being taken into consideration (Thuret, Moon et al. 2006). A priming therapy with neurotrophins like NT3 increases cAMP in the cell body and PKA providing a mechanism to surpass neurite outgrowth inhibition. Increased levels of cAMP can be obtained by treatment with dibutyryl-cAMP or with the phosphodiesterase inhibitor Rolipram, resulting in axonal regeneration and functional recovery when delivered after SCI (Nikulina, Tidwell et al. 2004). Treatment with direct cAMP delivery in zebrafish shows the capacity of this molecule for boosting the neuronal intrinsic ability to regenerate (Bhatt, Otto et al. 2004).

7. Providing bridges into the lesion site

7.1 Cell therapy

As referred to before, SCI leads to the formation of a scar in the lesion cavity. Current strategies to surpass the negative processes that result from this scar event have focused on stem cell therapy. Stem cells have three fundamental properties: highly proliferative potential, assymmetric divisions leading to self-renewal, and ability to differentiate into multiple cell types (Sahni and Kessler 2010). Cell transplantation following injury can ameliorate some secondary injury mechanisms by bridging the lesion cavity and promoting a permissive environment for axon regeneration.

Therapeutic use of stem cells includes regulation of gliosis and scar formation, cell replacement, remyelination of spared axons, restoration of neuronal circuits, secretion of neurotrophic factors and anti-inflammatory cytokines to promote neovascularization and a favorable behavior for axon elongation (Figure 6) (Mothe and Tator 2012).

Peripheral nerve grafts which were described by Aguayo's group, were the first cell therapy method, showing regeneration of CNS axons (Richardson, McGuinness et al. 1980). Later it was shown that grafted fetal spinal cord supported the regrowth of host axons (Bregman and Reier 1986). For transplantation purposes, there are several cell types under investigation, which include Schwann cells, olfactory ensheathing glia (OEG), activated macrophages, embryonic stem cells (ES), mesenchymal stem cells (MSCs), haematopoietic stem cells (HSCs), neural stem/progenitor cells (NSPCs), genetically modified neurotrophin-expressing fibroblasts and induced pluripotent stem cells (iPSCs).

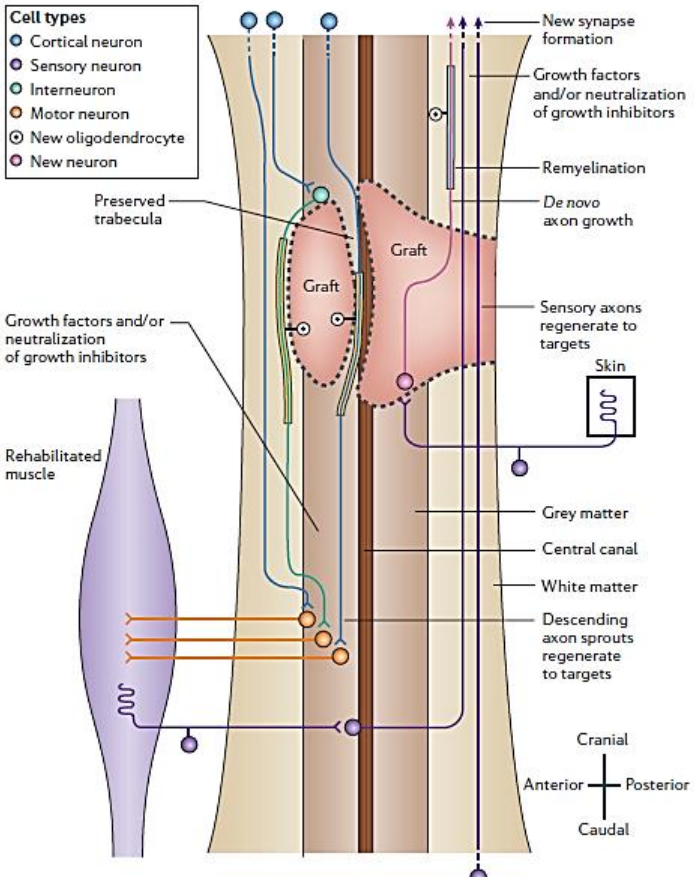


Figure 6 Cell transplantation and anatomy of spinal cord circuits. Schematic showing a sagittal view through the injured cervical spinal cord after a hypothetical combination of potential therapies. Grafts provide remyelinating cells. Inhibitory molecules in the scar regions and in intact spinal cord are neutralized using antibodies, peptides or enzymes. Grafted neurons allow the formation of new relay circuits or the regeneration of injured axons back to their original targets. Furthermore, rehabilitation may allow correct synapses to be stabilized and reverses muscle atrophy. Adapted from(Thuret, Moon et al. 2006)

Schwann cells play several roles following PNS injury including an effective clearance of debris, trophic support of regenerating axons and remyelination of spared axons. Schwann cells have been used as injected suspensions after contusion injury (Cheng, Liao et al. 2004), implanted inside of nerve guidance conduits involved in a matrigel matrix after complete transection (Xu, Guenard et al. 1995), genetically modified to secrete increased amounts of NGF and BDNF (Hurtado, Moon et al. 2006), and the most promising approaches are related with combined therapies as with cAMP which allows the neurons to extend into otherwise inhibitory substrates and results in the functional recovery after T8 contusion injury (Pearse, Pereira et al. 2004).

OEG can be derived from embryonic and adult olfactory bulb or mucosa and after injection into the lateral cervical hemisection these cells can lead to improvements in respiratory function, myelination and prevent loss of neural tissue (Li, Decherchi et al. 2003, Sasaki, Lankford et al. 2004). However OEG do not promote CNS axon regeneration and functional recovery under all circumstances (Thuret, Moon et al. 2006). Regarding the extracellular environment of the lesion cavity, fibroblasts are capable of secreting extracellular molecules, such as collagen and glycoproteins. These cells are easy to obtain and expand in culture, making them attractive for use as genetically modified cells. The Tuszynski lab has been performing studies with transplanted fibroblasts overexpressing neurotrophins for the treatment of SCI, and the results demonstrate an increase in functional recovery when transplanting the genetically modified cells (Tobias, Shumsky et al. 2003). Additional strategies that include intrinsic activation or depletion of macrophages show that by modulation of this cell type, hindlimb functional recovery and tissue survival can be achieved (Popovich, Guan et al. 1999, Popovich, Guan et al. 2002).

In 2009 the US Food and Drug Administration (FDA) approved the first human ES cells that were driven to differentiate into oligodendrocyte progenitor cells (OPCs). This strategy has previously been shown to result in remyelination of spared axons and improved recovery when transplanted into rats with SCI. However, the clinical trial was discontinued due to funding challenges. The adult bone marrow contains several different stem cell populations, including MSCs and HSCs. HSC transplant after compression by SCI, was shown to promote functional recovery. These cells are commonly harvested from the peripheral blood following cytokine administration, and they can be autologously derived (Koshizuka, Okada et al. 2004). MSCs are attractive given their immunomodulatory properties and the expression of neurotrophic factors

that are beneficial for repair. These cells can be obtained from a variety of tissues like the Wharton jelly of the umbilical cord, adipose tissue and adult muscle. Pre-differentiated adipose-derived MSCs transplanted into rats with SCI resulted in some functional recovery due to the wrapping of demyelinated fibers by grafted cells (Arboleda, Forostyak et al. 2011). The umbilical cord HSCs and MSCs are attractive because this tissue is readily accessible and the cells, besides being in low numbers, can be rapidly expanded and the tissue can be frozen and used later (Park, Lee et al. 2011).

NSPCs are multipotent stem cells that give rise to the cells of the nervous system. These cells are often cultured as free-floating neurospheres that are 3D aggregates comprising progenitors cells, a small percentage of stem cells, and small numbers of more differentiated cell types. These cells can be differentiated into neurons, oligodendrocytes, and astrocytes. The later represents the major downback of these cells when they are transplanted into the lesion cavity of the SCI. In recent studies, however it was demonstrated that grafted neuronal stem cells in combination with a cocktail of growth factors can reach connectivity and axonal regeneration for long distances into and beyond the lesion cavity, leading to functional recovery following SCI (Lu, Wang et al. 2012).

In to 2012, Shinya Yamanaka and Sir John Gurdon received the Nobel Prize in Physiology and Medicine given the development of iPSCs and the interest of these cells in therapies such as SCI has tremendously increased (Nakamura and Okano 2013). Human iPSC-derived neurospheres have been recently grafted in the spinal cord lesion cavity of mice and synaptic connections between host and grafted cells was observed, as well as expression of neurotrophic factors, improved locomotor recovery and increased myelination (Nori, Okada et al. 2011).

8. Bioengineered scaffolds

For SCI patients, the main clinical therapy available is functional training that helps in motor rehabilitation. Secondary injury mechanisms lead to loss of tissue and axonal connectivity, impeding the re-growth of ascending and descending axons that carry signals between the spinal cord and the brain. Therefore, therapeutic strategies to overcome the unfavorable nature of the scarring increase the number of regenerating axons and guide them to their appropriate targets are still urgently needed.

Bioengineered scaffolds are a feasible strategy to reconstruct the spinal cord tissue architecture, giving structural and/or chemical support for axonal regrowth, and

concomitantly providing a matrix for the delivery of cells and bioactive substances (Subramanian, Krishnan et al. 2009). Scaffold design must consider biocompatibility with the host tissue to avoid adverse immune reactions, sterilizability, controlled porosity, permeability (that allows cell infiltration), and mechanical properties, such as elastic moduli similar to the spinal cord to minimize damage at points of contact and, additionally, support for cell attachment, growth and differentiation (Figure 7) (Kubinova and Sykova 2012).

The use of single and multi-channeled nerve guidance tubes is seen as a solution that offers mechanical support, corrects orientation of the fibers and promotes adequate environment to regenerated axons. The regenerative capacity of these scaffolds is strictly related with the dimensions, material of construction and luminal components (Fig.11).

The choice of material for use in nerve guidance tubes is dependent of the regeneration strategy and must be distinguished between using non-degradable (normally bioinert) grafts or biodegradable (normally biointeractive) grafts.

Non-degradable materials are usually synthetic and can be modified by controlled and uniform synthesis techniques, to achieve desired material properties. Current materials that have been used to fabricate nerve guidance tubes include silicone, polyacrylonitrile/polyvinylchloride (PAN=PVC), poly(tetrafluoroethylene) (PTFE), and poly(2-hydroxyethyl methacrylate) (PHEMA) (Straley, Foo et al. 2010). PHEMA is the most studied non-degradable material in nerve guidance tubes and a scaffold of this polymer has been reported to support axon ingrowth when used for SCI treatment (Woerly, Petrov et al. 1999). However, these materials can induce inflammation and often there is the need of a second surgery to remove them.

Biodegradable materials can be composed by natural or synthetic materials, but the majority come from natural sources. Natural polymers in comparison with the synthetic ones, present the advantage of containing specific signals for cell adhesion, allowing cell infiltration (Saracino, Cigognini et al. 2013). However the undefined composition and difficulties to purify these polymers may lead to low reproducibility. Some common degradable materials that have been studied for SCI strategies comprise collagen, chitosan, hyalurnic acid, alginate, agarose, matrigel and methylcellulose (Wang, Zhai et al. 2011). For the construction of nerve guidance tubes only collagen and chitosan are being used. The synthetic degradable polymers include poly(glycolic acid) (PGA), poly(lactic acid) (PLA), poly(lactic acid-co-glycolic acid) (PLGA), and poly(lactide-co-

caprolactone) (PLCL) (Straley, Foo et al. 2010). In SCI research, to provide a suitable environment for cell attachment, growth and differentiation, the inner part of the scaffold can be filled with a gel such as fibrin leading to tissue repair (Subramanian, Krishnan et al. 2009, King, Hewazy et al. 2010).

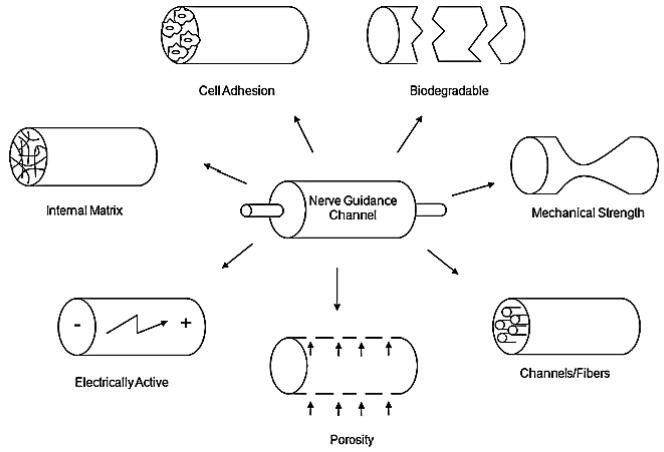


Figure 7 Design elements for the construction of nerve guidance tubes. Properties of promissing nerve conduits include (clockwise from top left):design that focus on cell adhesion; biodegradability or a porous channel wall, or both; address their mechanical strength to avoid channel collapse and obstruction of nerve regeneration; the incorporation of intraluminal channels to mimic the fascicular structure of the nerve; electrically charged materials in the ECM can generate electric fields that act as signals to promote and control growth, remodeling, and protein adsorption; inclusion of an internal matrix, attempting both to increase the bioactivity of nerve guidance channels and to prevent their collapse; Adapted from (Karin S. Straley 2010).

Chitosan, collagen, agarose and alginate have been extensively investigated in a variety of tissue engineering applications, including in spinal cord repair: agarose scaffolds support motor axon regeneration into the lesion site (Gao, Lu et al. 2013), collagen scaffolds comprising BDNF and epidermal growth factor receptor (EGFR) provide effective bridging and positive signals for neuronal regeneration (Han, Jin et al. 2010), alginate-based scaffolds were shown to promote axonal elongation (Prang, Muller et al. 2006) and chitosan was employed to favor neuroprotection and physiological recovery after SCI (Cho, Shi et al. 2010).

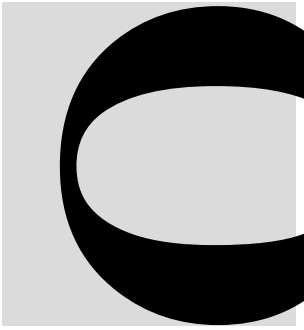
Chitosan is a natural polysaccharide produced from the N-deacetylation of chitin, the primary polysaccharide component of crustacean shells (Kim, Tator et al. 2011). It is an attractive material for channel design given its capacity to exhibit tunable properties through the variation in the acetyl group content. The degree of deacetylation (DD) of chitosan is related with its degradation rate, if DD=100% it is non-degradable and if DD=70% it is fully degradable. The cationic nature of chitosan is responsible for interactions with anionic compounds present in the ECM, adhesion neuronal properties

and growth factors. Using chitosan has some issues associated with low mechanical strength and its insolubility in many common solvents. To solve these problems new techniques are being applied including forming gels at high pH and deacetylation of chitin tubes from hollowed crab tendons (Yamaguchi, Itoh et al. 2003).

Collagen and agarose are temperature-responsive polymers that form gels at certain temperatures. Collagen forms a gelatinous structure at physiological temperatures and like agarose it makes *in situ* gelification possible (Wang, Zhai et al. 2011). Collagen is abundant in connective tissue and is a component of the glial scar. The nerve guidance tubes are typically formed through the addition of chemical crosslinking agents, which allow strength and degradation control (Cholas, Hsu et al. 2012). Like chitosan, the major drawback of collagen is the purification in an inexpensive and reproducible manner.

Agarose is a water soluble biomaterial that can fill the lesion defect by adopting its shape and minimizing gap formation between tissue and scaffold. These scaffolds do not evoke an immune or inflammatory response and neurotrophic factors be coming incorporated in these scaffolds that elicit a robust axonal growth (Stokols and Tuszynski 2006).

Objectives



9. Objectives

The inherent complexity of the adult CNS as well as its amazing plasticity and adaptiveness make regeneration experiments a promise to reach new therapies for the functional recovery after spinal cord injury.

In order to assess the extracellular environment that modulates neural regeneration post injury one of the aims of this study was to address the possible involvement of spinal cord lipids as inhibitory molecules. For that we defined the following objectives:

- Given that lipids account for approximately 75% of myelin dry weight, we asked whether myelin lipids would contribute to the inhibitory properties of myelin;
- Characterize the specific lipids underlying this effect;
- Elucidating the molecular mechanism that contributes to this inhibitory effect in axonal regeneration;
- To further confirm the inhibitory role of these lipids *in vivo* using 2-hydroxypropyl-β-cyclodextrin (HPβCD) as a therapeutic approach to rescue the inhibitory lipids present in the lesion site of the injured spinal cord.

The second aim of this study also focused the relevance of the extracellular environment in the modulation of axonal regeneration, but on a different perspective, namely on the study of the capacity to ameliorate the pathophysiology of spinal cord injury (SCI) through the application of a chitosan guidance scaffold seeded with endothelial cells and neurospheres in an anatomically complete transected SCI model. For that we defined the following objectives:

- Bridge a spinal cord injury gap using a chitosan scaffold containing endothelia and neural stem cells;
- Evaluate the capacity of the therapy in the promotion of axonal regeneration following SCI.

Chapter 1

Myelin lipids as modulators of axonal regeneration following spinal cord injury

10. Theoretical Background

In the Central Nervous System (CNS), axon regeneration fails. This is the main reason why paralysis and loss of sensation is permanent in conditions such as spinal cord injury (SCI). The limited ability for axon regeneration has been attributed to the glial scar at the injury site, composed of inhibitory molecules that comprise axon guidance molecules, proteoglycans and myelin associated inhibitors (MAIs), and to the lack of neuron-intrinsic growth potential.

Studies from different groups using triple knockout mice for myelin-associated glycoprotein (MAG), Nogo and oligodendrocyte myelin glycoprotein (OMgp) produced conflicting results ranging from limited (Lee, Geoffroy et al. 2010) to extensive (Cafferty, Duffy et al. 2010) regeneration abilities. Although the field has largely concentrated on MAIs, other inhibitors play important roles *in vivo*: blocking chondroitin sulfate proteoglycans (CSPGs) or repulsive guidance molecule A (RGMa) or deleting ephrinB3 increases axonal regeneration following SCI (Bradbury, Moon et al. 2002, Hata, Fujitani et al. 2006, Duffy, Wang et al. 2012). As such, the wide variety of inhibitors present in the spinal cord milieu is thought to underlie the absence of a robust axonal regeneration when blocking either single or a limited combination of inhibitors. Despite the structural differences, several MAIs share receptors and an inhibitory mechanism dependent on RhoA/ROCK activation (Yiu and He 2006), as demonstrated mainly through the use of RhoA/ROCK inhibitors (Kubo and Yamashita 2007).

To further explore the importance of myelin in the inhibition of axonal regeneration, we used shiverer (shi) mice which lack myelin basic protein (MBP), a key player in myelin compaction in the CNS (Boggs 2006). In the shi CNS, the absence of MBP results in a complete lack of compact myelin (Rosenbluth 1980) which is accompanied by a severe phenotype comprising shivering, convulsions and early death.

Previous results from our group have shown that following complete spinal cord transection, wild-type (WT) and shi mice displayed an equivalent glial scar and that all axonal regeneration inhibitors were present in shi spinal cords. Interestingly, following injury, MAG was the only for which decreased levels were found and besides that increased levels of the inhibitors Nogo, OMgp and Ephrin-B3 were present in the shi spinal cord.

Although the shi spinal cord contains all the canonical axonal regeneration inhibitors, we asked whether the absence of compact myelin could have an impact in axonal regeneration *in vivo*. Following dorsal hemisection, and in contrast to WT axons, shi dorsal column tract axons entered the lesion site (Figure 8 A) and regenerated for longer distances (Figure 8 B and C). In the raphespinal tract, following complete spinal cord transection, shi 5-hydroxytryptamine (5HT)-positive fibers found caudally to the injury site were more frequent and were capable of regenerating for longer distances (Figure 8 D and E).

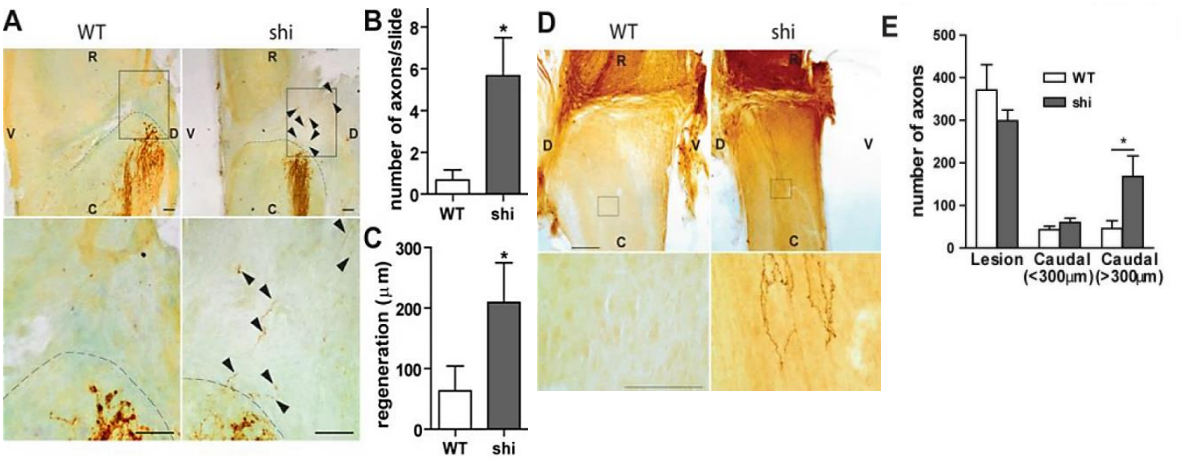


Figure 8 Shi mice present increased axonal regeneration after SCI. (A) Cholera toxin-β **(**CTB**)** immunohistochemistry in sagittal spinal cord sections of WT and shi littermates (n=6 mice/genotype), 4 weeks following dorsal hemisection. Arrowheads highlight regenerating CTB positive dorsal column axons in shi spinal cords. R: rostral; C:caudal; D: dorsal; V: ventral. Lower panels are higher magnifications of the selected boxed regions. Scale bar 100μm. (B) Number of CTB positive dorsal column axons able to regenerate through the glial scar in WT and shi animals (n=6 mice/genotype). *p<0.05. (C) Length of the longest regenerating CTB positive dorsal column axon in WT and shi animals (n=6 mice/genotype). *p<0.05. (D) 5HT immunohistochemistry in shi and WT spinal cords 5 weeks following complete spinal cord transection (WT, n=5; shi, n=7) showing regenerating raphespinal fibers. Lower panels are higher magnifications of the selected boxed regions.(E) Quantification of regenerating raphespinal fibers (WT, n =5; shi, n=7).

Despite the presence of canonical MAIs and of axonal abnormalities generally related to decreased regeneration capacity, our group has shown that CNS axons in shi mice have an increased ability to regenerate through the spinal cord glial scar (Figure 8). Here we aimed at defining the reasons underlying the increased axonal regeneration in shiverer mice. For that a combination of *in vivo* and *in vitro* approaches were designed. In summary, we show that besides MAIs, myelin lipids, specifically cholesterol and sphyngomyelin are novel inhibitors of axonal regeneration.

11. Materials and Methods

11.1 Animals

Mice were handled according to European Union and National rules. Wild type (WT) and shiverer (shi), (Jackson laboratories) littermates of either sex, on a Swiss Webster: C3HeB/Fe5 background were obtained from heterozygous breeding pairs. For drug delivery studies, 8 weeks-old C57BL/6 and NMRI mice of either sex were used.

11.2 Spinal cord injury

Animals were deeply anesthetized with a medetomidine/ketamine mixture (medetomidine 1mg/Kg; Ketamine 75mg/Kg) via intraperitoneal injection. In order to compensate for the diuresis caused by anesthesia, and for the blood loss, a fluid supplement of 0.5ml of 5% glucose saline solution was subcutaneously injected. The skin was shaved along the vertebral column and one incision was performed through the thoracic vertebras 6 to 11 (T6-T11). Under a microscope, the vertebral column was exposed and a laminectomy was performed at the T8 level. A 30-gauss needle was used to break the dura mater followed by a complete transection or dorsal hemisection using a micro feather ophthalmic scalpel. The muscle and skin were sutured and the animals were allowed to recover. Analgesia was performed twice a day for 72h following injury with subcutaneous injection of Buprenorphine (0.08mg/kg) and fluid therapy was also done with 0.5ml of Duphalyte®. Manual voidance of the bladder was performed twice a day during the period of the experiment.

11.3 Injection of Cholera toxin B in the sciatic nerve

Animals with dorsal hemisection were allowed to recover for 4 weeks. Four days before sacrifice, 2µl of 1% cholera toxin B (CTB; List Biologicals) were injected in the left sciatic nerve. Animals were anesthetized with 5% isofluorane in a closed chamber for the initial induction, followed by delivery of 2-3% of isofluorane using a mask, which was maintained through the surgery procedure. In order to expose the sciatic nerve, the skin was shaved along the femur and the fascia linking the two muscles was ripped with a scissor, leading to the sciatic nerve exposure. Two tight knots were placed below the sciatic notch, used to stabilize the nerve, followed by cholera toxin B injection with a 10µl syringe (Hamilton, USA). The muscle and skin were sutured and

the animals were allowed to recover. Analgesia was performed twice a day for 48h following injury with subcutaneous injection of Buprenorphine (0.08mg/kg).

11.4 Tissue preparation

The animals were perfused with phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA), post fixed at 4°C for 48h and later cryopreserved in 30% sucrose until 50µm sagittal cryosections (cryostact; Leica) were performed.

11.5 Analysis of regeneration of dorsal column fibers

For detection of labeled regenerating neurons, 50µm sagittal free floating spinal cord sections were immunostained for anti-CTB (1:30,000; List Biologicals). Briefly, endogenous peroxidase was inhibited with H_2O_2, and sections were then blocked in blocking buffer (5% fetal bovine serum, 0.3% Triton-X100 in 0.1M phosphate buffer) for 1h at room temperature and incubated overnight at 4°C with anti-CTB antibody, followed by incubation in biotinylated horse anti-goat IgG (1:200; Vector) in blocking buffer. Antigen detection was performed with extravidin peroxidase (1:1000; Sigma) in 0.1% Triton-X100 TBS for 1h and development was done with diaminobenzidine (DAB, 5mg/10ml; Sigma). Sections were mounted with chrome-potassium sulphate-gelatin solution; air dried overnight and stained with 1% toluidine blue. From each animal, the section displaying the highest number of regenerating axons was selected. Axonal regeneration was quantified by counting the number of CTB labeled axons within the glial scar and by measuring the length of the longest fiber found rostrally to the injury border. All quantifications of axonal regeneration were performed with the researcher blinded for genotype and experimental group.

11.6 Neuron cultures and inhibition assays

Primary cultures of dorsal root ganglia (DRG) neurons from P6 mice were performed as described (Fleming, Mar et al. 2009). Glass coverslips were coated with poly-L-lysine (20µg/mL) and laminin (0.5µg/mL) followed by either crude membranes (0.1µg protein), myelin (0.5µg protein), myelin protein fraction (0.5µg), myelin basic protein (MBP,0.9µg) or total protein from spinal cord extracts (3µg). To test the effect of lipids, the myelin lipid fraction (corresponding to 0.5µg of protein) and lipids extracted from the spinal cord of either WT or shi mice (corresponding to 2 µg of protein) were used. Coverslips were coated with poly-L-lysine followed by lipids in

chloroform/methanol/water (2:1:0.1) and laminin (0.5μg/mL). The amount of individual lipids present in 2μg of WT spinal cord lysate was determined by HPTLC and referred to as 1x (cholesterol (CO)= 120ng, phosphatidylinositol (PI)= 2ng, galactocerebroside (GalCer)= 200ng, sulfatide (GS)= 120ng, sphingomyelin (SpH)= 50ng, globotetrahexosylceramide (Gb4)= 24ng, phosphatidylethanolamine (PE)= 40ng, lactocerebroside (Lac)= 20ng and ganglioside (GM1)= 0.6ng). In the analysis of single lipids, 1x, 10-fold less (0.1x) or 10-fold more (10x) were used per coverslip. Gb4, PE, SpH and Lac were from Matreya LLC and CO, GalCer, GS, GM1 and PI were from Sigma Aldrich. GalCer, GS and PI were dissolved in chloroform/methanol (2:1), and CO, PE, SpH, Gb4, GM1 and Lac in chloroform/methanol (1:2). For each condition, 5,000 cells/coverslip were plated in triplicate and immunostained 12h later against βIII-tubulin (1:2.000; Promega). In at least 100 neurons/condition, the longest neurite was traced using NeuronJ. Similar experiments were performed with cortical and hippocampal neurons isolated from E17.5 WT and shi embryos as described (Dent, Callaway et al. 1999, Kaech and Banker 2006), respectively. For cortical neurons, 50,000 cells/coverslip were maintained for 48h and for hippocampal neurons, 16,500 cells/coverslip were maintained for 72 h.

11.7 Rho A inactivation assay

WT DRG neurons were plated onto coverslips coated with laminin, myelin, solvent, CO or SpH, as described above. Thirty minutes before plating, cells were incubated with 1μg/mL of C3 transferase (Cytoskeleton, Inc.), a Rho inhibitor that was maintained during the entire experiment. 12h later, cells were fixed and neurite outgrowth was evaluated.

11.8 HPβCD treatment

To assess axonal regeneration of dorsal column fibers, C57BL/6 mice (n=24) were subjected to SCI dorsal hemisection as described above. At the time of injury, osmotic minipumps (Alzet 2006) were placed subcutaneously with a tube allowing perfusion of the injury site at a rate of 0.15μl/hr with either 27μg/g/day of HPβCD (Sigma) in aCSF (128mM NaCl, 2.5mM KCl, 0.95mM $CaCl_2$, 1.9mM $MgCl_2$; n=12), or vehicle (aCSF; n=12). Besides delivery at the injury site, 4mg/g of HPβCD were injected subcutaneously twice a week. Five weeks following injury, the SCI site was collected and lipids were extracted and quantified as described above (n=6, HPβCD-

treated mice; n=6, aCSF-treated mice). In n=6 HPβCD-treated mice and n=6 aCSF-treated mice, dorsal column fibers were labeled with CT-B, and axonal regeneration was assessed as previously described.

11.9 Data analysis

Data is shown as mean±SEM. Statistical significance was determined by Student's t test or Tukey's test (One-way ANOVA).

12. Results

12.1 Myelin lipids inhibit neurite outgrowth

Despite the alterations described in shi axons (Brady, Witt et al. 1999, Kirkpatrick, Witt et al. 2001, Andrews, White et al. 2006, Hellal, Hurtado et al. 2011) that are generally related to decreased regeneration capacity (Hellal, Hurtado et al. 2011), neurite outgrowth of WT and shi DRG and cortical neurons was compared and no differences were found (Figure 9 A). This data suggests that the intrinsic growth capacity of shi neurons does not underlie their increased regeneration *in vivo*. To evaluate whether the shi spinal cord environment might be less inhibitory than that of WT mice, and as myelin cannot be isolated from the shi CNS, we tested the effect of crude membranes prepared from spinal cords of both strains. Although DRG neurons were inhibited in the presence of both WT and shi membranes, the inhibition produced by shi membranes was lower (Figure 9 B), suggesting that the shi spinal cord environment presents less inhibitory cues to axonal growth. The decreased inhibitory environment of the shi spinal cord was unrelated to the lack of MBP as no effect on neurite outgrowth was produced when WT DRG neurons were plated on MBP (Figure 9 C). To further confirm that the decreased inhibitory environment of the shi spinal cord was unrelated to a protein component, WT DRG neurons were grown on top of protein extract from either WT or shi spinal cords. Both extracts were equally inhibitory (Figure 9 D).

Given that lipids account for approximately 75% of myelin dry weight, we asked whether myelin lipids would contribute to the inhibitory properties of myelin. When WT DRG neurons were grown on myelin protein or myelin lipids as substrates, a decreased neurite outgrowth was observed for both, demonstrating that myelin lipids also play a role as axonal regeneration inhibitors (Figure 9 C). To further demonstrate that the different lipid content of the shi spinal cord creates a more permissive environment to axonal growth, WT DRG neurons were grown on spinal cord lipids extracted from either WT or shi mice. In contrast to lipids from WT spinal cords, lipids from shi spinal cords did not inhibit neurite outgrowth (Figure 9 E and F). These data demonstrate that neurite outgrowth of WT DRG neurons can be modulated by exposure to different lipid milieus.

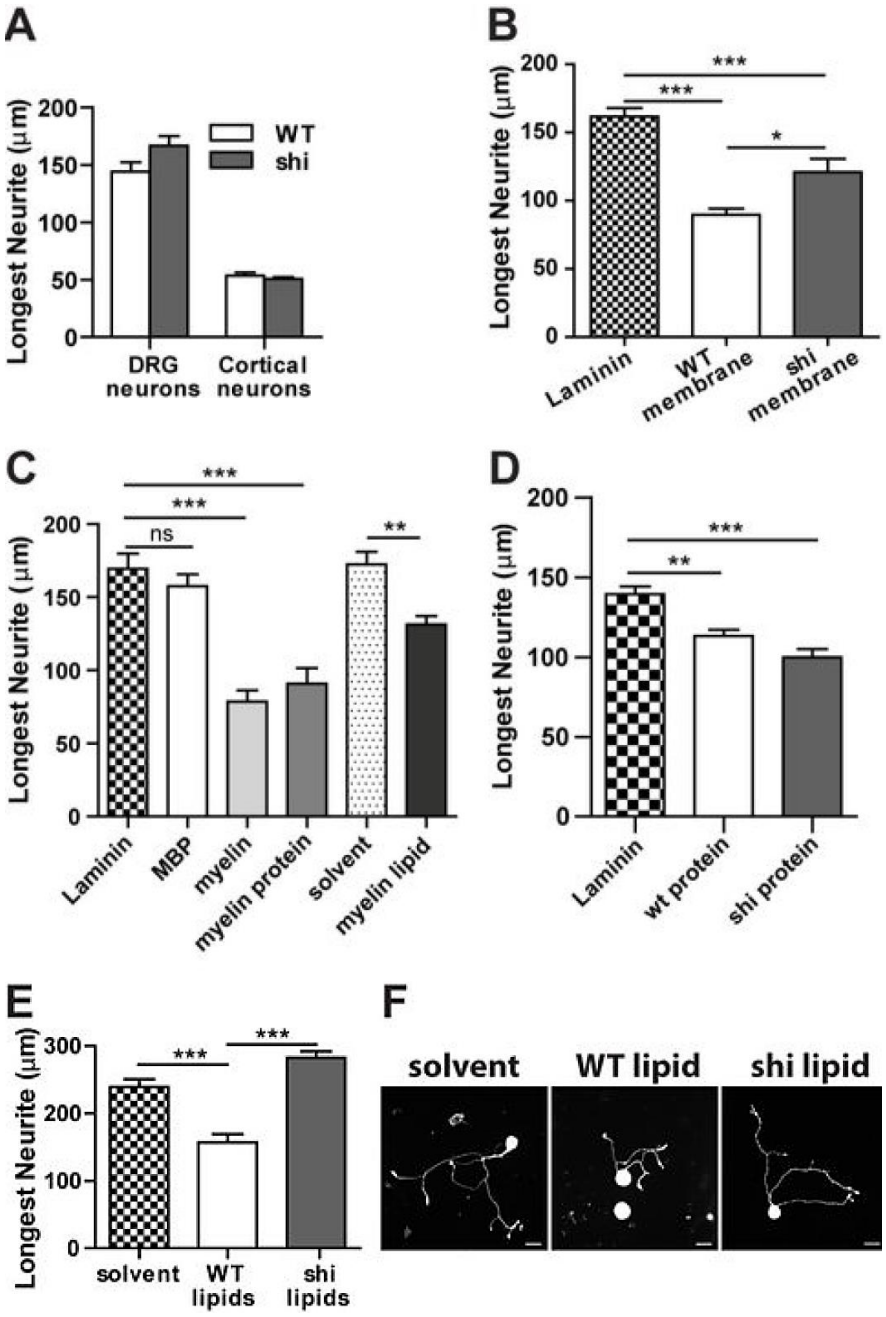


Figure 9 Myelin lipids inhibit neurite outgrowth. (A) Neurite outgrowth of DRG and cortical neurons from WT and shi littermates. (B) Neurite outgrowth of WT DRG neurons grown on 0.1µg of protein from WT and shi crude membranes. (C) Neurite outgrowth of WT DRG neurons grown on 0.9µg MBP, 0.5µg WT myelin, 0.5µg WT myelin protein or myelin lipids corresponding to 0.5µg of WT myelin. (D) Neurite outgrowth of DRG neurons grown on 3µg of total protein from either WT or shi spinal cords. (E) Neurite outgrowth of WT DRG neurons grown on lipids extracted from either WT or shi spinal cords. (F) Representative βIII-tubulin immunocytochemistry of (E), scale bar 25µm. For all neurite outgrowth experiments, n=3. *p<0.05,**p<0.01, ***p<0.001 and ns: non statistical.

12.2 Cholesterol and sphingomyelin inhibit axonal growth through a Rho-dependent mechanism

To identify the lipids that might underlie the increased axonal regeneration in the shi spinal cord, our group compared the lipid composition of WT and shi spinal cords. Among others, the most abundant myelin lipids were analyzed, namely: cholesterol (CO), ceramide (Cer), phospholipids (phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS) and sphingomyelin (SpH), GM1 ganglioside (GM1) and galactocerebroside (GalCer) (Norton and Poduslo 1973). In the shi spinal cord a decrease of most lipids (namely CO, sulfatide (GS), GalCer, SpH, PE, globotetrahexosylceramide (Gb4), lactocerebroside (Lac) and GM1), an increase of cholesterol esters (CE) and PI, and normal amounts of Cer, PE, PS, PC and triglycerides (TG) were observed. From all the lipids with abnormal concentrations in the shi spinal cord, only CO and SpH inhibited DRG neurite outgrowth in a dose-response manner (Figure 10 A and B). The effect of both CO and SpH was not restricted to DRG neurons as these lipids also produced a dose-dependent inhibitory effect in neurite outgrowth of cortical (Figure 10 C) and hippocampal neurons (Figure 10 D). Interestingly, when hippocampal neurons were grown on CE, that shares the same backbone structure as CO, the esterification of CO reduced its ability to inhibit neurite outgrowth (Figure 10 E), suggesting a specific inhibitory effect of unesterified CO.

The RhoA/ROCK pathway is the major mediator of myelin inhibition (Yiu and He 2006), and the inhibitory effect of myelin can be reverted by the Rho inhibitor C3 transferase (Winton, Dubreuil et al. 2002). To assess whether myelin lipids share similar pathways to block axonal regeneration than those described for myelin proteins, DRG neurons were grown on the inhibitory substrates myelin, CO and SpH and in the presence of C3 transferase. As expected, C3 was able to overcome myelin inhibition (Figure 10 F). Inhibition by both CO and SpH was also relieved by C3 treatment (Figure 10 F) suggesting that lipid inhibition of axonal growth occurs, at least in part, through a Rho dependent mechanism.

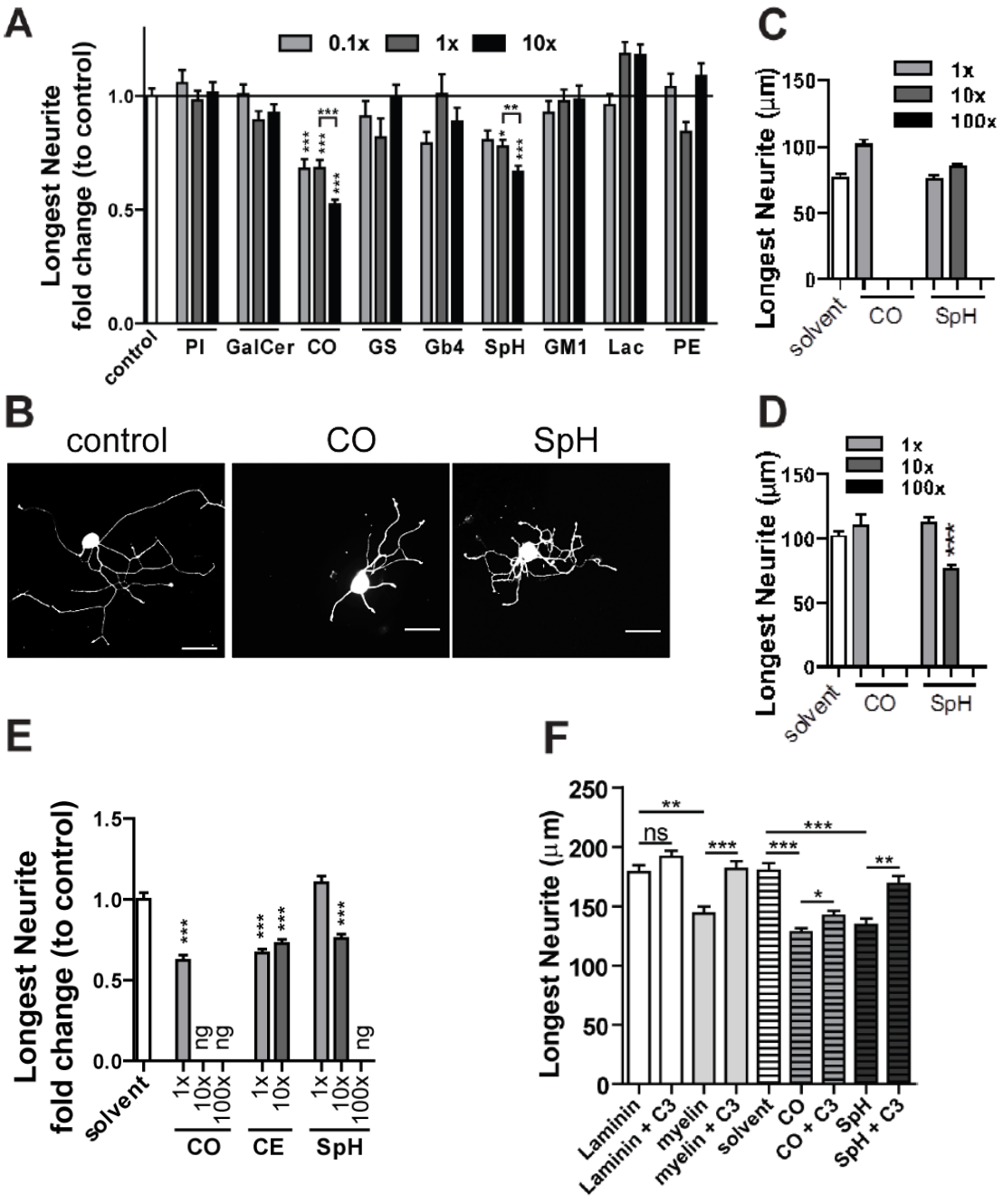


Figure 10 Cholesterol and sphingomyelin inhibit neurite outgrowth through a Rho dependent mechanism. (A) Effect of individual lipids in DRG neurite outgrowth. (B) Representative βIII-tubulin immunocytochemistry of DRG neurons grown on top of solvent (control), CO and SpH coated coverslides. Scale bar 50μm. (C) and (D) Neurite outgrowth of cortical and hippocampal neurons plated on CO and SpH (n = 3) . (E) Neurite outgrowth of hippocampal neurons plated on CO, CE and SpH (n = 3). (F) Neurite outgrowth of DRG neurons plated on top of myelin, CO or SpH in the presence or absence of the Rho inhibitor C3 transferase (n = 3). *p<0.05,**p<0.01, ***p<0.001 and ns: non statistical.

12.3 Reduction of lipid levels in the spinal cord injury site through 2-hydroxypropyl-β- cyclodextrin delivery promotes axonal regeneration of dorsal column axons

To further confirm the inhibitory role of CO and SpH *in vivo*, WT mice with SCI were treated with 2-hydroxypropyl-β-cyclodextrin (HPβCD), a drug capable of reducing the levels of CO and sphingolipids, as already demonstrated in models of Niemann-Pick type C (Abi-Mosleh, Infante et al. 2009, Liu, Turley et al. 2009, Aqul, Liu et al. 2011) and Alzheimer's disease (Yao, Ho et al. 2012). When comparing the changes in lipid content induced by SCI in WT mice, SpH and CO remained unchanged (as well as PC, Cer, PE, Gb4, SpH and GalCer), whereas CE, PE, PI and GS, PS increased at the SCI site. At the SCI site and following HPβCD delivery, our group observed decreased levels of inhibitory lipids namely CO (28% decrease), CE (26% decrease) and SpH (21% decrease) and also of others, although PC levels were not affected (Figure 11 A). Of note, in the uninjured spinal cord, administration of HPβCD did not decrease the levels of either inhibitory lipids or of the other lipids analysed (Figure 11 B). In HPβCD-treated mice, improved axonal regeneration of dorsal column fibers was obtained, with a 2.5-fold increased number of axons being able to enter the lesion site (Figure 11 C and D), and a 2-fold increased length of regenerating axons (Figure 11 C and E). Combined, this study identified CO and SpH as new myelin lipids that inhibit axonal regeneration through the glial scar and determined that HPβCD may be used to reduce the levels of inhibitory lipids in the injury site and improve axonal regeneration.

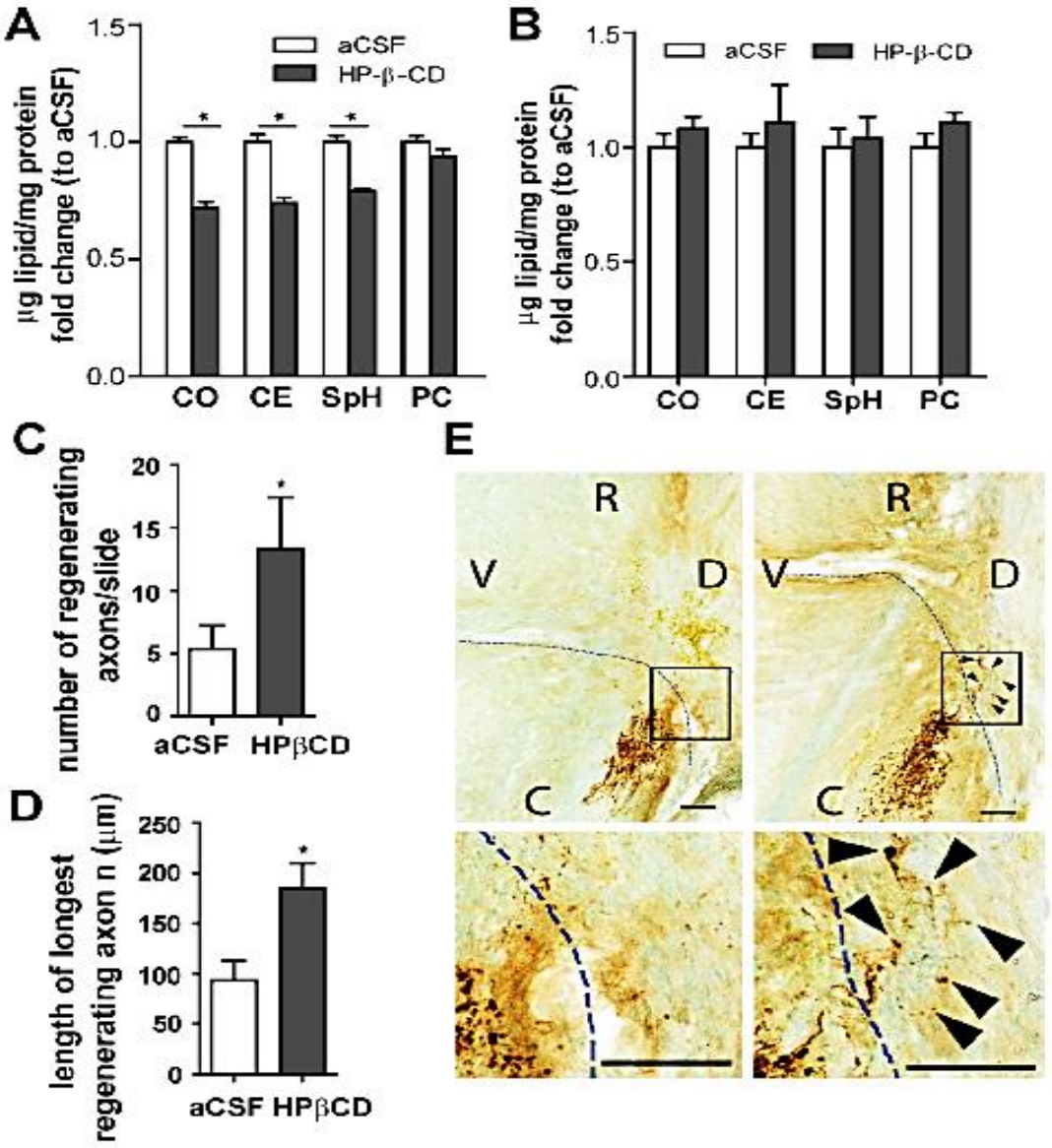


Figure 11 2-hydroxypropyl-β-cyclodextrin delivery promotes axonal regeneration following SCI. Quantification of CO, CE, SpH and PC in the spinal cord injury site (A) and in uninjured spinal cord (B) from HPβCD and aCSF treated mice, 5 weeks following dorsal hemisection (n=6 mice/condition). Number of CT-B positive dorsal column axons able to regenerate through the glial scar (C), and the length of the longest regenerating axon (D) in HPβCD and aCSF-treated mice. (E) CT-B immunohistochemistry in sagittal spinal cord sections of aCSF and HPβCD-treated animals, 5 weeks following dorsal hemisection. Arrowheads highlight regenerating CT-B positive dorsal column axons in HPβCD-treated spinal cords. Scale bar: 100µm. R: rostral; C:caudal; D: dorsal; V: ventral. Lower panels are higher magnifications of the selected boxed regions in the upper panels. *p<0.05.

13. Discussion

Lipids represent 75% of myelin dry weight. From these, CO is the most abundant, accounting for 28% of the myelin lipid content, whereas SpH accounts for 4%. Although CO is an essential component of the plasma membrane crucial for axonal growth, CO accumulation in the mammalian brain is a risk factor for neurodegenerative diseases including Alzheimer's disease (Puglielli, Tanzi et al. 2003) and Niemann-Pick (Aqul, Liu et al. 2011). In the scenario of injury, with the presence of myelin debris within the injury site, myelin-derived CO is presented to regenerating axons in a different form from glial-derived CO. Whereas glial-derived CO can stimulate axon growth by providing lipoproteins as a source of both CO and apolipoprotein E to regenerating axons, free CO i.e., without the context of a lipoprotein particle, as is the case of myelin-derived CO, fails to enhance axon extension (Hayashi, Campenot et al. 2004). In the case of SpH, its accumulation, which is the hallmark of Niemann- Pick disease, leads to changes in plasma membrane that similarly to CO, also culminate in neurodegeneration (Ledesma, Prinetti et al. 2011). As such, this work suggest that following CNS injury, when growing axons need to elongate through an environment filled with myelin debris, exposure to free CO and to SpH contributes to axonal growth inhibition. This mechanism may also underlie, at least in part, the increased axonal regeneration in the PNS. Despite that peripheral and central myelin have similar CO and SpH content, as dedifferentiated Schwann cells and invading macrophages are able to phagocyte and clear myelin debris following PNS injury (Stoll, Griffin et al. 1989), regenerating axons are probably not exposed to a lipid-rich environment, in contrast to the CNS where such clearance is not as effective.

As mentioned above, accumulation of unesterified CO and SpH is the hallmark of Niemann-Pick disease, which culminates in neurodegeneration (Aqul, Liu et al. 2011, Ledesma, Prinetti et al. 2011). Interestingly, cultured neurons from mice lacking Niemann-Pick type C1 protein (npc1 knockout mice) displayed increased rate of growth cone collapse that was mediated by ROCK activation and reverted by ROCK inhibition (Qin, Liao et al. 2010). In npc1 mice, administration of HPβCD delays the onset of clinical symptoms by reducing the buildup of CO and sphingolipids within the nervous tissue (Davidson, Ali et al. 2009, Aqul, Liu et al. 2011). HPβCD is a well-tolerated FDA-approved drug used in animal models and in human clinical trials, known for its ability to form inclusion complexes, remove cholesterol from membranes and increase

cholesterol trafficking (Davidson, Ali et al. 2009, Aqul, Liu et al. 2011, Matsuo, Togawa et al. 2013). Our group showed that following HPβCD delivery, decreased levels of inhibitory lipids, namely CO, CE and SpH are specifically obtained in the SCI site whereas uninjured spinal cord segments have unaltered lipid composition. Upon spinal cord injury, HPβCD may exert a generalized sequestering effect allowing the removal of lipids from the injury site, and thus relieve the inhibitory level within the glial scar. Although HPβCD did not seem to have a clearly defined lipid specificity, we did not observe any lipid changes in the treated un-injured spinal cord, indicating that this non-toxic agent does not lead to lipid dysregulation in normal tissues. In addition, HPβCD treatment led to increased axonal regeneration, supporting that HPβCD delivery should be considered as a therapeutic option in the context of SCI.

At the molecular level, inhibition induced by CO and SpH could be reverted by the Rho inhibitor C3 transferase. These data support that both myelin proteins and lipids signal inhibition through similar pathways involving Rho activation. CO and SpH may act as ligands to induce receptor mediated activation of the Rho signaling cascade, or alternatively they can augment receptor activation leading to increased Rho activity. Whether CO and SpH engage the same receptors (e.g. Nogo receptor and PirB) as several structurally unrelated myelin proteins is to be unraveled. Specific binding sites for CO have been identified in G protein-coupled receptors (Cherezov, Rosenbaum et al. 2007, Liu, Chun et al. 2012). As such, CO and/or SpH at the extracellular matrix in which axons are growing may interact and engage other receptors eliciting a signaling cascade that impairs axonal growth. Sulfatide has also been identified as a lipid that specifically inhibits neurite outgrowth of retinal ganglion cells through a Rho-mediated mechanism (Winzeler, Mandemakers et al. 2011), although the pathway by which this occurs remains poorly understood. The next challenge will be to further characterize the pathways by which lipids mediate the repression of neurite outgrowth (Figure 12).

In summary our work shows that myelin lipids namely CO and SpH, are important modulators of axonal regeneration that should be considered as critical targets in strategies aiming at improving axonal growth following injury. Moreover, we provide the initial data supporting that the FDA-approved HPβCD delivery could be tested in the context of SCI.

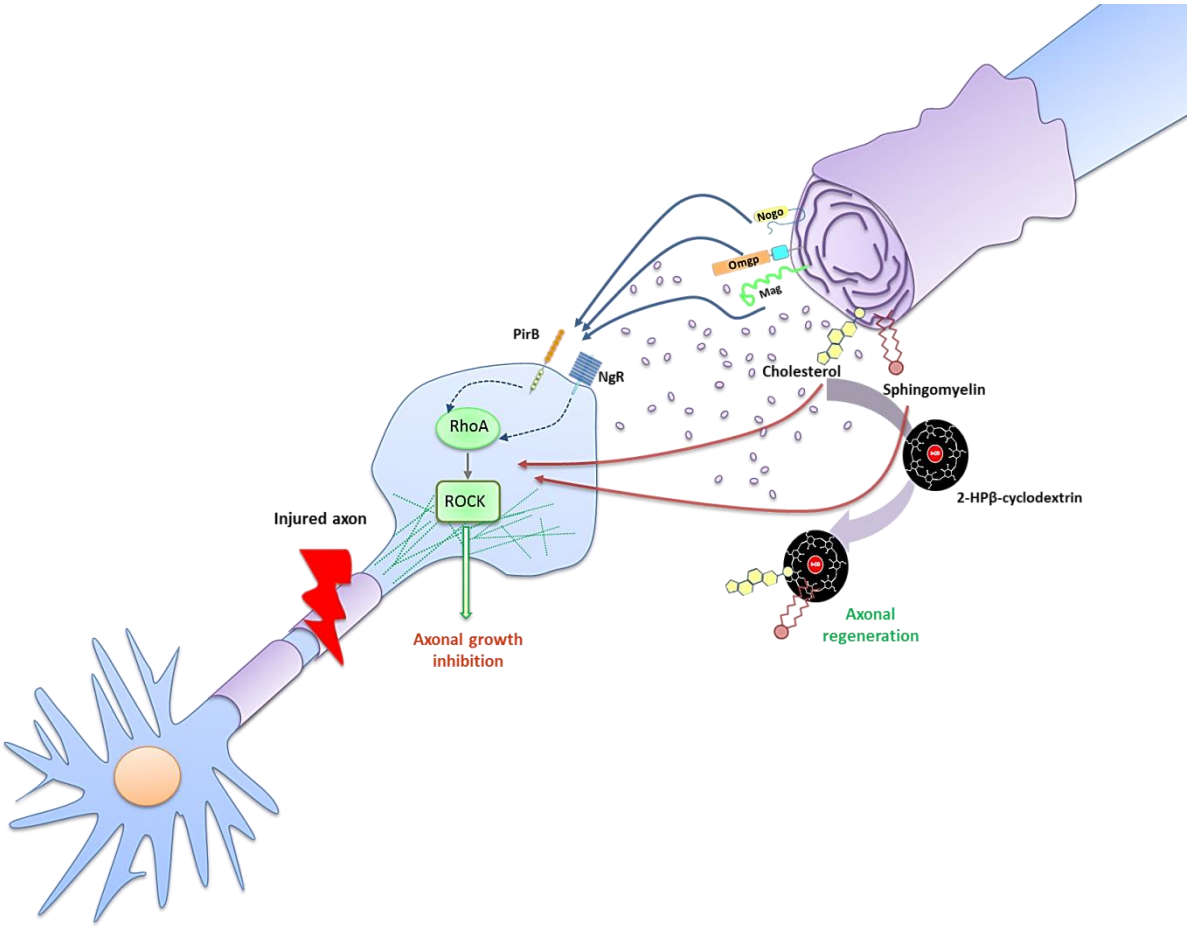


Figure 12 Myelin lipids act as modulators of CNS axon growth following injury. Lipids represent 75% of myelin dry weight, and similarly to myelin proteins they play a role as axonal regeneration inhibitors. Specifically cholesterol and sphingomyelin can block axonal regeneration through RhoA/ROCK pathway. Upon injury these myelin derived molecules lead to axon growth inhibition. However when we deliver HPβCD the sequestering effect of myelin lipids such as cholesterol and sphingomyelin leads to increased axonal regeneration due to the relieve of the inhibitory environment in the injury site. CNS, central nervous system; OMgp, Oligodendrocyte myelin glycoprotein; MAG, myelin-associated glycoprotein; ROCK, Rho-associated protein kinase; PirB, paired immunoglobulin-like receptor B; Ngr, nogo receptor; HPβCD, 2-hydroxypropyl-β-cyclodextrin.

Chapter 2

Chitosan scaffold-mediated cell-based therapy for spinal cord injury recovery

14. Theoretical Background

Several approaches have been developed to generate a more permissive environment in the spinal cord injury (SCI) site to enhance the growth of central axons and promote functional recovery. Strategies available to promote recovery following SCI include limitation of inflammation, blockage of inhibitory cues, prevention of secondary cell death, enhancement of plasticity of spared circuits (Raineteau 2008) and stem cell therapies (Willerth and Sakiyama-Elbert 2008). Bioengineering approaches are a great promise for the ability to reconstruct the spinal cord tissue architecture, mimicking the natural organization and orientation of axons, providing a platform for regeneration and preventing the infiltration of scar tissue and cyst formation in the injury site (Gao, Lu et al. 2013).

Recent progress in stem cell engineering revealed that stem cells transplanted within the injured site may lead to restoration of neuronal circuits, bridging of lesion cavities, remyelination of spared axons, promotion of tissue regeneration and neovascularization giving rise to a more permissive environment for functional reestablishment (Du, Xiong et al. 2011, Mothe and Tator 2012). Moreover, cell-based therapy has been proven to be an effective way to restore function after SCI in diverse experiments (Rossi and Keirstead 2009).

Replacement of the lost cells in the injured CNS could be undertaken by two types of strategies: stimulation of endogenous stem cells (Obermair, Schroter et al. 2008) and exogenous stem cell transplantation (Nori, Okada et al. 2011). Neuro Stem/Progenitor Cells (NSPCs) have been an attractive cell source for the treatment of SCI given their capacity to induce remyelination, support cells by releasing growth factors and neurotrophins, namely nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glial-derived neurotrophic factor (GDNF) (Rossi and Keirstead 2009) and restore disrupted connectivity at lesion sites (Lu, Kadoya et al. 2014). NSPCs are multipotent stem cells that self-renew allowing for expansion in culture, and differentiate into lineage-specific neural precursor cells, which can give rise to neurons, astrocytes, and oligodendrocytes through asymmetric cell division (Ryan Salewski 2013). NSPCs can be derived from various regions along the neuroaxis during adult life time, from the subventricular zone (SVZ) (Kim, Cooke et al. 2012) of the lateral ventricles, or the subgranular zone (SGZ) (Palmer, Takahashi et al. 1997), or from the ependymal lining of the spinal cord central canal (Weiss, Dunne et al. 1996).

NSPC can also be generated from pluripotent stem cells, including embryonic stem (ES) cells or induced pluripotent stem cells (iPSCs).

In this study we used an immortalized mouse neural stem (NS) cell line. This cell line was obtained from pluripotent mouse ES cells cutivated with fibroblast growth factor 2 (FGF-2) and epidermal growth factor (EGF), which were demonstrated by (Conti, Pollard et al. 2005) to be sufficient for the derivation of pure cultures of NS cells with continuous expansion by symmetrical division in an undifferentiated state. *In vitro* after prolonged expansion and *in vivo* after transplantation into de adult brain, these cells remain able to differentiate efficiently into neuronal precursors (Conti, Pollard et al. 2005, Glaser, Pollard et al. 2007). NS cells were maintained in culture via propagation of floating cell clusters termed neurospheres that are supposed to provide the niche environment for neural stem cell maintenance, survival and proliferation (Glaser, Pollard et al. 2007). In this work, to provide a suitable environment for cell attachment, growth and differentiation, neurospheres of NS cells were incorporated with a fibrin gel. Fibrin matrices have been widely used in neural regeneration approaches (Lu, Wang et al. 2012) due to the promotion of cellular migration and sprouting of neural fibers into the lesion site, without eliciting an increased inflammatory response, and delaying the accumulation of reactive astrocytes at the lesion (Johnson, Parker et al. 2010).

Angiogenesis plays a central role following SCI, was it leads to repair of the blood-brain-barrier (BBB) and affects neurogenesis directly via contacting with NSPCs (Rauch, Hynes et al. 2009). To create a more permissive environment in our model, an immortalized human pulmonary microvascular endothelial cell line was used, which was generated by cotransfection with plasmids encoding human telomerase to extend their life time, maintaining characteristics of the primary human microvascular endothelial cells (Unger, Krump-Konvalinkova et al. 2002). The vascular microenvironment is composed by endothelial cells, soluble trophic factors and extracellular matrix that is responsible for the interactions with NSPCs. Metabolically, this relation between CNS and blood vessels, provides the intake of nutrients and the secretion of the metabolic waste of NSPCs, ensuring the self-renewal, proliferation, differentiation, and migration of neuronal cells (Shen, Goderie et al. 2004, Yang, Bi et al. 2011). To allow the incorporation of the human endothelial stem cell line a multi-channelled chitosan nerve guidance tube with a degree of acetylation (DA) of 4% was used (Figure 13 C). This was demonstrated by (Amaral, Unger et al. 2009) to be the

most effective to promote cell adhesion but with low density of adherent inflammatory cells (Barbosa, Amaral et al. 2010). Chitosan scaffolds with lower degree of acetylation were described by (Vasconcelos, Fonseca et al. 2013) to be ideal for transplantation as they promote the adhesion of lower numbers of inflammatory cells, being the macrophages type 2 which are anti-inflammatory cell types, the predominant phenotypic profile. Regarding the immune response to biomaterials, the foreign-body-reaction comprise the response of macrophages and foreign body giant cells that act at the end-stage response of the inflammatory and wound healing responses following implantation.

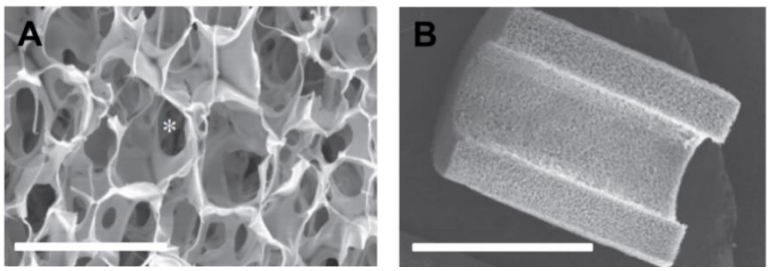


Figure 13 Structural design of a 3D porous chitosan scaffold.
(A) Scanning electron microscopy (SEM) micrographs of a longitudinal cross section of porous scaffold, showing the presence of interconnecting pores (*) in the thin polymeric walls (B) The transversal cross section of the chitosan cylindrical scaffold in lower magnification. Scale bars = 100µm and 3mm respectively. Adapted from (Amaral, Neiva et al. 2013).

The choice of a chitosan scaffold was related to its good biocompatibility and degradability, low toxicity, cost and its versatility to be processed into flexible tubular (Figure 13 B) and porous structures (Figure 13 A) (Kim, Tator et al. 2011). The biodegradation of chitosan occurs through the action of lysozyme that is upregulated after SCI in the cerebrospinal fluid (Tomihata and Ikada 1997, Kim, Tator et al. 2011). The multi-channelled design is seen as a solution that offers mechanical support, correct orientation of the fibers and promotes adequate environment to regenerated axons (Stokols and Tuszynski 2004). In addition, intramedullary implantation of a chitosan tube may provide a means of organizing the lesion site where grafted NS cells could exit in matching linear trajectories and incorporation of the host axons could be oriented along specific routes, which might result in the establishment of reciprocal synapses formed between separated segments of the spinal cord after injury (Lu, Kadoya et al. 2014). Recent *in vitro* and *in vivo* studies have demonstrated high biocompatibility of chitosan with NS cells, namely NSPCs (Nomura, Zahir et al. 2008, Bozkurt, Mothe et al. 2010, Yang, Mo et al. 2010).

Besides, a recombinant fragment of human fibronectin (rhFN) was added by covalently binding to chitosan amines. Fibronectin is an extracellular matrix (ECM) protein with integrin binding domains that allows adhesion of cells to the scaffold. The rhFN achieves higher surface densities of cell-binding domains due to its spatial conformation and due to the presence of an arginine-glycine-aspartic acid (RGD) sequence that confers specificity towards α5β1 integrin, leading to higher cell activity (Amaral, Neiva et al. 2013).

Restoring locomotor function is one of the main goals of spinal cord injury therapies. Hind limb motor function was accessed by INEB (under the supervision of Prof Ana Pêgo), and whereas control animals (empty scaffold) recovered only marginally (score at 9 weeks ≈ 5), whereas locomotor recovery for animals with scaffolds seeded with NS cells and endothelial stem cells, reached the level of own weight support (score at 9 weeks ≈ 8).

With the advantage of using a chitosan guide scaffold seeded with human endothelial cells and filled with a fibrin gel with mouse neural stem cells to bridge a spinal cord injury gap, our aim in this study was to evaluate neuronal regeneration through the scaffold in the lesion site. In order to identify regenerating axons into the host spinal cord we performed an *in vivo* experiment where we traced for the corticospinal tract and the dorsal column fibers.

15. Materials and Methods

15.1 Animals

Rats were handled according to European Union and National rules. For scaffold implantation the NIH nude rats (Charles River) were used. These animals are T-cell deficient and show depleted cell populations in thymus-dependent areas of peripheral lymphoid organs (Hougen 1991).

15.2 Spinal cord injury and scaffold implantation

A total of fourteen adult female nude rats were subjects of this study. Animals were deeply anesthetized with medetomidine/ketamine (Medetomidine 0.5mg/Kg; Ketamine 75mg/Kg). Laminectomy was performed at T6-T8 and the spinal cord was exposed. A 30-gauss needle was used to break the dura mater and a block of spinal cord was excised under microscopic guidance to produce a 3 mm-long complete transection cavity. Scaffolds were then implanted into the lesion cavity using microforceps. Upon implantation of the scaffold, a fibrin gel comprised by thrombin (2 NiH units/ml), $CaCl_2$ (2.5mM), fibrinogen (10mg/ml) and TBS 1x (pH7.4), was placed for correct attachment between the spinal cord segments and the scaffold. The muscle and skin were sutured and the animals were allowed to recover. Analgesia was performed twice a day for 72h following injury with Buprenorphine (0.04mg/kg) and fluid therapy was also achieved with 1ml of Duphalyte®. Manual voidance of the bladder was performed twice a day during the three months of the experimental time.

15.3 Injection of Cholera toxin B in the sciatic nerve

The uptake of the non-toxic β fragment of cholera toxin (CTB) is mediated through specific receptors present in neuronal membranes which results in axonal transport in a retrograde manner when injected in the sciatic nerve. For tracing of dorsal column fibers, animals with the implanted scaffold in the spinal cord were injected in the left sciatic nerve with 2µl of 1% CTB (List Biologicals), 4 days before sacrifice. Animals were anesthetized with 5% isofluorane in a closed chamber for the initial induction, followed by delivery of 2-3% of isofluorane using a mask, which was maintained through the surgery procedure. In order to expose the sciatic nerve, the skin was shaved along the femur, and the fascia linking the two muscles was ripped with a scissor, leading to sciatic nerve exposure. Two tight knots were placed below the sciatic

notch, used to stabilize the nerve, followed by cholera toxin B injection with a 10µl syringe (Hamilton, USA). The muscle and skin were sutured and the animals were allowed to recover. Analgesia was performed twice a day for 48h following injury with subcutaneous injection of Buprenorphine (0.04mg/kg).

15.4 Injection of biotinylated dextran amine in the brain

Animals were anesthetized with medetomidine/ketamine (medetomidine 0.5mg/Kg; ketamine 75mg/Kg) and after that, the head was immobilized on a stereotaxic apparatus and a skin incision was performed along the sagittal suture for skull exposer. Then, 10% solution of biotinylated dextran amine (BDA, 10.000 MW; Molecular Probes, Eugene, OR) in PBS was injected through a fine needle (33G) that was connected to a 10µl syringe (Hamilton,USA), into 4 points of the right cortex (0.3µl/min per injection); stereotaxic coordinates: 1 and 2 mm posterior to the bregma, 3 and 4 mm lateral to the midline, at a depth of 1.5 mm from the cortical surface, to anterogradly label the corticospinal tract originating from this area (Zhang, Xiong et al. 2010). The syringe remained in place for 2 min before and after initiation of the injection. Analgesia was performed with Buprenorphine (0.04mg/kg) for 48h and animals were allowed to survive for 2 weeks before sacrifice.

15.5 Tissue preparation

The animals were perfused with 40.0ml phosphate buffered saline (PBS) followed by 100ml of 4% paraformaldehyde (PFA), post fixed at 4°C for 72h and later cryopreserved in 30% sucrose solution, after which they were frozen and kept at -20°C until 16µm sagittal cryosections (cryostact Leica) were performed. For histochemical analysis, slices were processed with hematoxylin-eosin staining (H&E).

15.6 Tracing of dorsal column fibers

Consecutive spinal cord transversal and sagittal sections (16µm) were sequentially collected for immunofluorescence with anti-Cholera toxin subunit β (CTB,1:10,000; List Biologicals). Section autofluorescence was quenched by incubation in 0.1% sodium borohydride in TE, pH9 for 5 min and then 50 mM NH_4Cl in PBS for 15 min. Sections were then blocked in blocking buffer (5% fetal bovine serum, 0.3% Triton-X100 in 0.1M phosphate buffer) for 1hr at room temperature and incubated overnight at 4°C with anti-CTB antibody (1:10,000; List Biologicals), followed by

incubation in biotinylated horse anti-goat IgG (1:200;Vector) in blocking buffer. Antigen detection was performed with streptavidin Alexa fluor 568-conjugated donkey anti-goat (1:1000; Invitrogen) in 0.1% Triton-X100 TBS for 1h. The labeling with anti-βIII tubulin (1:500; Promega) was performed after 1h at room temperature, followed by incubation with Alexa fluor 488-conjugated donkey anti-mouse (1:1000; Invitrogen). Sections were mounted with vectashield with DAPI (Vector).

15.7 Labeling of the corticospinal tract

To detect biotynilated dextran amine (BDA) labeled fibers, slides were allowed to defrost for 10 min at room temperature followed by incubation in 0.1% sodium borohydride in TE, pH9 for 5 min and then 50mM NH_4Cl in PBS for 15min. Sections were then blocked in blocking buffer (5% fetal bovine serum, 0.3% Triton-X100 in 0.1M phosphate buffer) for 1h at room temperature and incubated overnight at 4°C with anti-βIII tubulin (1:500; Promega). Antigen detection was performed with Alexa fluor 488-conjugated donkey anti-mouse (1:1000; Invitrogen), followed by washing 3x with 0.1% Triton-X100 TBS and by incubation in streptavidin Alexa fluor 568-conjugated donkey anti-goat (1:1000; Invitrogen) for 2h. Sections were mounted with vectashield containing DAPI (Vector).

16. Results

16.1 General characterization of the implanted scaffold

In our study we used a severe model of spinal cord injury, created by performing a 3mm-long complete transection cavity (Figure 14 A) that was filled with a chitosan tube containing endothelial stem cells and NS cells combined with a fibrin matrix. Animals were allowed to regenerate their processes twelve weeks after surgery and later, histology and tract tracing were performed to assess the presence of axons either from host origin or originating from seeded NS cells in the lumen of the chitosan scaffold. The existence of motor and sensory axons from the host spinal cord was also evaluated. In Figure 14 B a schematic representation of a longitudinal section of a spinal cord with scaffold is presented, to aid the understanding of the experimental setup. H&E staining shows almost absence of scar formation, which is correlated with a high degree of tissue preservation at the implantation site of the scaffold (Figure 14 C and D). However in some cases (Figure 14 D) it was possible to visualize cyst formation in the rostral region of the spinal cord. Cyst formation adjacent to the lesion is related to tissue necrosis, which represents a physical barrier to any attempt of severed or endogenous axons to grow above the injury area. In this experiment we did not perform a control group with empty scaffold, as this had been done previously at INEB.

Here we found high densities of β-III tubulin (specific neuronal marker- positive axons) along the scaffold lumen (Figure 14 E). These axons could have been originated from transplanted NS cells, which can differentiate into neurons and/or from host neurons sprouting into the scaffold as is described in more detail below.

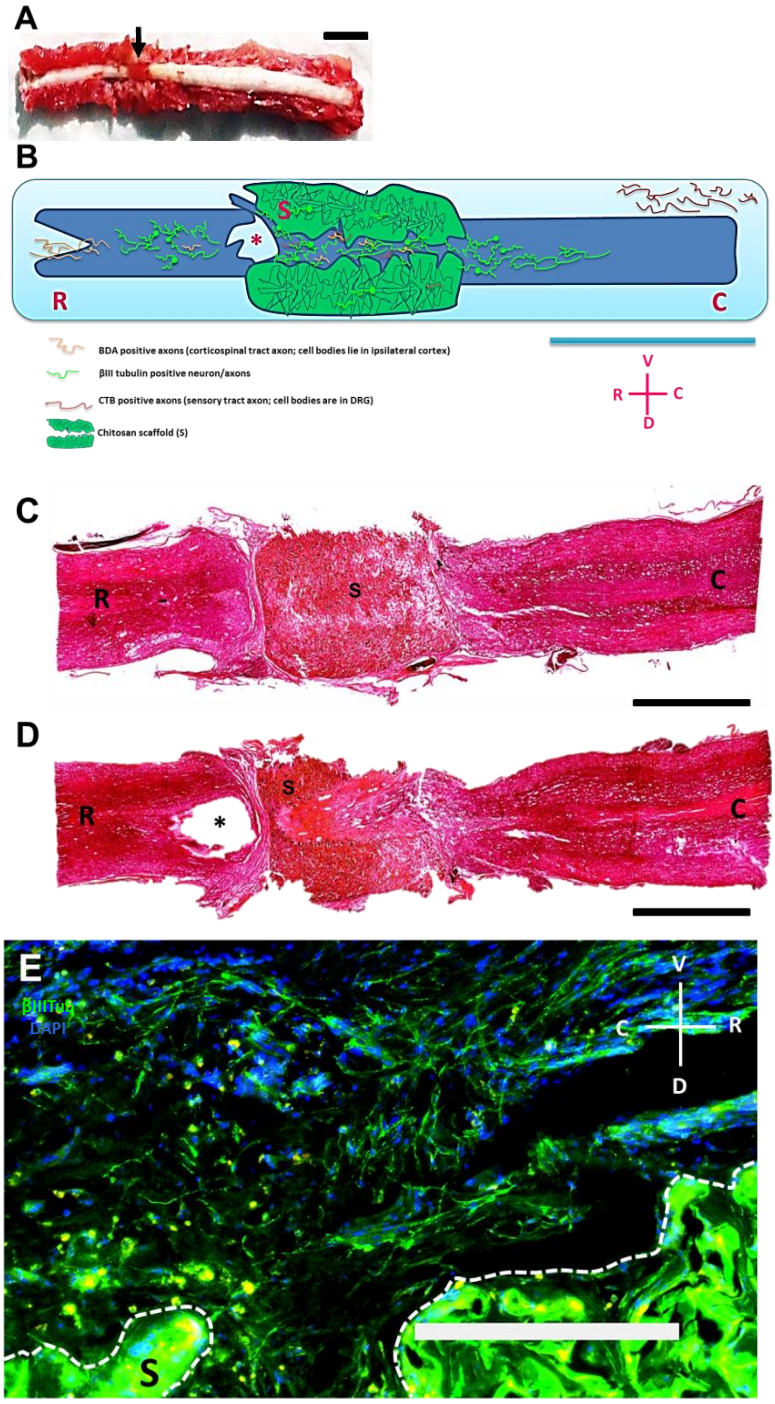


Figure 14 General overview of the architecture of the spinal cord integrating the chitosan scaffold. (A) Dorsal aspect of a spinal cord where a chitosan scaffold was implanted. The complete thoracic transection site is highlighted by an arrow. Scale bar = 3mm. (B) Diagram illustrating the spinal cord with implanted scaffold. Rostral corticospinal tract with BDA labeled motor axons and caudal dorsal column fibers labeled with CTB are depicted. The scaffold lumen is represented with chitosan scaffold containing βIII tubulin positive cells and regenerating endogenous axons. (C and D) Hematoxylin-eosin (H&E) staining of (V) ventral and (D) dorsal slides of the chitosan scaffold. (E) Immunofluorescence against βIII-tubulin reveals high density of axons, which infiltrate the lumen of the tube. The scaffold surrounded by a white dashed line has a high level of autofluorescence. Scale bar = 300µm. (*) Cystic structure; (S) Scaffold; (V, D, R, C) Ventral, dorsal, rostral and caudal spinal cord section orientation, respectively.

16.2 Assessment of regeneration of cortical spinal tract axons

We addressed whether motor corticospinal tract (CST) axons regenerated through the scaffold, using BDA labeling. CST axons descend through the spinal cord in three different tracts: a dorsal tract in the ventral part of the dorsal column, a dorsolateral tract and a ventral tract (Tuszynski and Steward 2012). In this case the injection was performed in the primary motor cortex, targeting the CST axons arbored in the ventral horn (Figures 15 A and a), BDA-positive CST axons were tightly bundled along the white matter of spinal cord in the rostral region before entering the scaffold (Figure 15 B). BDA positive fibers were found through the scaffold lumen (Figures 14 C and c), however the caudal part of the lumen site was completely devoid of CST axons, suggesting an inability of these axons to surpass the scaffold lumen and reach the caudal host spinal cord, even in more permissive conditions, such as those generated by scaffold implantation.

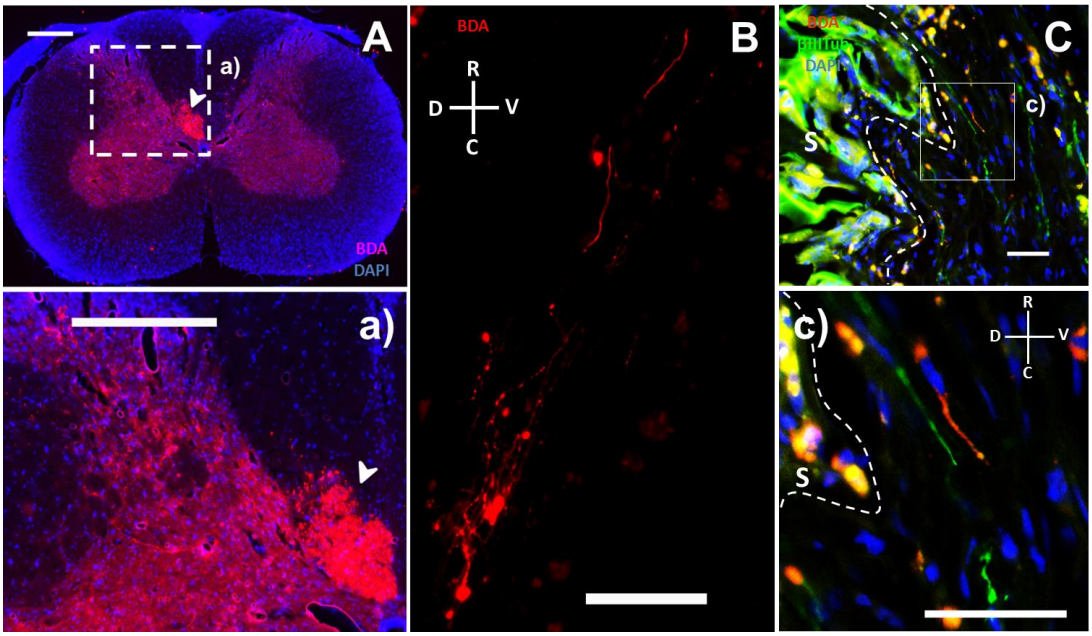


Figure 15 BDA tracing of the corticospinal tract in a rat with a SCI and implantation of a scaffold seeded with NS cells and endothelial cells.
(A) Cross section of the spinal cord from an animal where the corticospinal tract was traced. BDA positive axons were found in the ventral zone (arrow), higher magnification in box a) The arrow highlights the corticospinal tract. Scale bars = 200µm. Longitudinal sections (B) and higher magnification in box b) shows BDA-positive axons in the rostral region of spinal cord, before reaching the scaffold. Scale bars = 50µm. (C) Representative image of the spinal cord tissue bridge containing corticospinal tract fibers passing through the scaffold lumen. The scaffold is surrounded by the dashed line and presents autofluorescence. c) Higher magnification. Scale bars = 50µm. (S) Scaffold; (V, D, R, C) Ventral, dorsal, rostral and caudal spinal cord section orientation, respectively.

16.3 Assessment of dorsal column fiber regeneration

To test the hypothesis that the three dimensional organization of the regeneration milieu would guide sensory regenerating axons, we performed an immunofluorescence against CTB to trace de dorsal column axons (Figure 16 A). CTB labeled positive axons were present in the porous and channeled structure of the three-dimensional chitosan scaffold (Figure 16 B) revealing that endogenous neurons do regenerate through the scaffold bridge. In addition, the CTB positive dorsal column fibers were also found in the scaffold lumen (Figure 16 C and c).

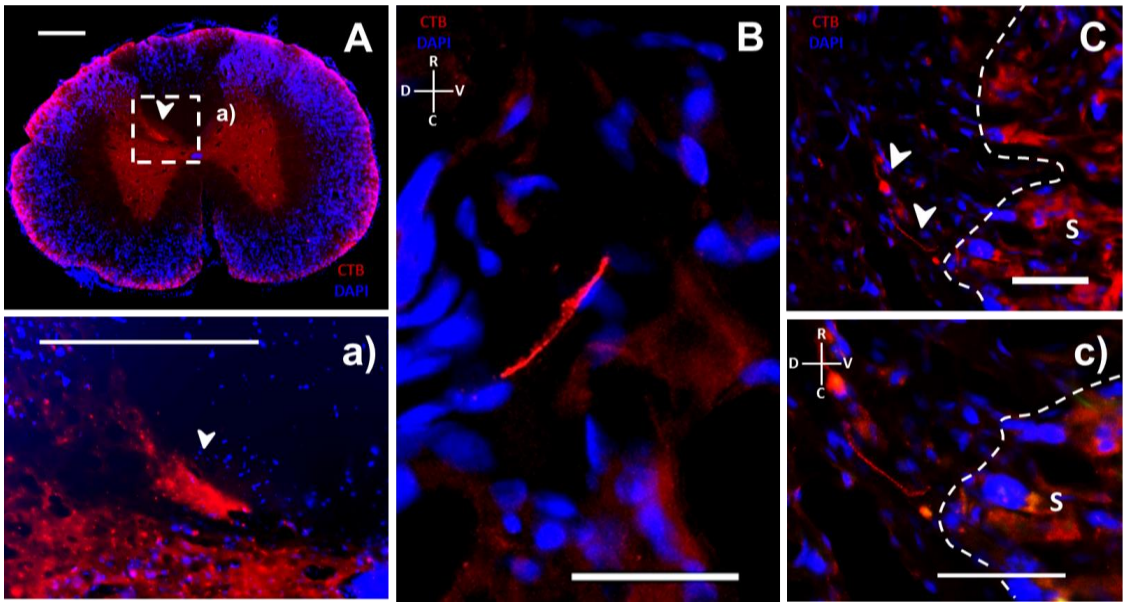


Figure 16 Tracing of dorsal column fibers with CTB in a rat with implanted scaffold. (A) The dorsal column tract (highlighted with a window) was visible after CTB injection and anti-CTB immunofluorescence; a) higher magnification of (A). Scale bars = 300µm. (B) shows CTB-positive sensory axons passing through the scaffold and in the lumen of the scaffold. Scale bar = 50µm. (C) The scaffold is surrounded by a dashed white line and presents high auto-fluorescence. (S) Scaffold; (V, D, R, C) Ventral, dorsal, rostral and caudal spinal cord section orientation, respectively.

17. Discussion

Traumatic injury in the CNS is highly debilitating, requiring urgent regenerative therapies with the potential of being translated into the clinics. Contributing to regeneration failure is the emerging glial scar that contains myelin debris derived from apoptotic death of oligodendrocytes, reactive astrocytes, the disruption of the blood vessels resulting in the loss of trophic factors and the formation of cyst necrotic areas.

Many strategies have been attempted to minimize the extent of injury. However to be effective, these must be administered rapidly following the initial insult, avoiding the undesirable secondary injury mechanisms. One of the most common strategies is the exogenous transplantation of stem cells, for replacing the dead and damaged tissue to re-establish functional connections and promote recovery (Nori, Okada et al. 2011, Park, Lee et al. 2011, van Gorp, Leerink et al. 2013). The hostile environment found by these cells leads to poor survival, proliferation and differentiation in the adequate phenotypic types, namely neurons.

Another challenge is the random organization of the environment, which could result in inappropriate connections between the newly differentiated neurons and endogenous axons. To overcome these problems, the use of 3D channel scaffolds has been demonstrated to increase regeneration by providing linear trajectories to neurons to reach correct targets (Stokols and Tuszynski 2006), supplemented with guidance molecules (Gao, Lu et al. 2013) and functionalization with adhesive extracellular matrix proteins (Cholas, Hsu et al. 2012) to support cell survival and differentiation. In this context, the strategy used was the implantation of a chitosan porous scaffold tube, that besides filling the lesion gap, ablates glial scar formation and leads to a reduction of the inflammatory response, given its biocompatibility with the spinal cord, as already demonstrated by others (Cho, Shi et al. 2010, Kim, Tator et al. 2011). Moreover, chitosan biodegradation *in vivo* is a consequence of the increase in cerebrospinal fluid flow after SCI (Kim, Tator et al. 2011). Of note, our results show that degradation of the scaffold does not occur after the three months of the experimental period.

The use of co-transplantation of NS cells and endothelial stem cells that has been performed here, has been previously described by (Rauch, Hynes et al. 2009) to increase the number of blood vessels, by active modulation of angiogenic factors. Furthermore in our study the use of endothelial stem cells seeded in the porous structure of the chitosan scaffold probably results in the proliferation and differentiation of seeded NS cells into

neurons given the specific stimulation by endothelial factors (Shen, Goderie et al. 2004).

Scaffolds described in the literature and used in the spinal cord are composed of collagen (Cholas, Hsu et al. 2012), agarose (Gao, Lu et al. 2013), fibrin (Johnson, Parker et al. 2010), and laminin (Nomura, Zahir et al. 2008). In this work we used a fibrin matrix that was shown previously by (Johnson, Parker et al. 2010) to support the survival of NS cells to allow innervation by host neurons to improve neural fiber sprouting and to delay the appearance of reactive astrocytes. Our results corroborate these findings. An indication supporting host axonal migration through the scaffold channel is the presence of ascending sensory and descending motor axons in the lumen of the scaffold tube, which were labeled with CTB or with BDA, respectively. Other authors have reported motor functional recovery after implantation of a scaffold following spinal cord injury (Nomura, Zahir et al. 2008, Li, Yang et al. 2009), but the presence of BDA positive axons in the caudal region of the scaffolds were only reported when an incomplete SCI model was performed (Teng, Lavik et al. 2002).

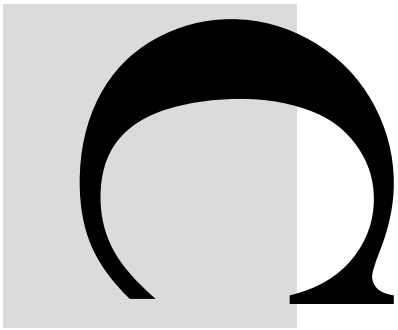
In relation to sensory axons, we also did not observe CTB positive axons after the lumen of the scaffold, in this case the rostral region of the spinal cord. The presence of cystic structures in the rostral region of the spinal cord, as we here document, can be related to the failure of CTB-positive axons to regenerate through this location.

These results show that a chitosan channeled scaffold can mechanically block the invasion of scar tissue, providing a continuous tissue bridge suitable for the reextension of injured axons and support the survival of transplanted cells in an improved microenvironment. It should however be noted that CTB-positive and BDA-positive axons were present in small numbers, certainly much lower than βIII-tubulin-positive axons found within the scaffold. Whether these βIII positive axons are from seeded NS cells or from other endogenous tracts, should be the subject of future research.

The next step in the use of our approach would be to evaluate the presence of bridging and the formation of synapses between the host axons and the transplanted NS cells, understand which are the physical inhibitory barriers presents inside the scaffold and in its boundaries, namely myelin associated inhibitors, chondroitin sulfate proteoglycans and inflammatory products. Additionally future work should be related to elicit axonal regeneration not only in the lumen of the scaffold but also outside its the limits, giving rise to long axonal regeneration. For that, delivery of growth factors,

namely BDNF or NT-3 or enzymes that allow degradation of inhibitory cues like chondroitinase ABC or addition of molecules such as Nogo neutralizing antibodies, or blockers of the post-receptor components Rho-A and ROCK may further induce long-distance axonal regeneration and compensatory sprouting.

Conclusions



SCI is a CNS injury leading to disabilities that range from cognitive impairment to loss of sensation and partial to complete paralysis. The inhibitory extracellular environment that develops in response to secondary injury mechanisms hinders axon growth thereby limiting restoration of function. The lack of functionality is mainly due to an imbalance of local axon growth-promoting and growth-inhibitory mechanisms.

Here we addressed two different approaches to overcome this functional axonal disability to regenerate through the lesion area, targeting extracellular environmental modulators. Firstly we identified new important modulators of axonal regeneration, namely the inhibitory lipid component of myelin, showing that both cholesterol (CO) and sphingomyelin (SpH) inhibit axonal regeneration *in vitro* and *in vivo*. Furthermore we show that delivery of 2-hydroxypropyl-β-cyclodextrin (HPβCD) promotes axonal regeneration following SCI, derived from the fact that this drug is capable of scavenging CO and SpH in the lesion cavity. Secondly the implantation in a spinal cord gap of a chitosan porous scaffold in combination with transplantation of NS cells and endothelial cells successfully lead to regeneration of sensory and motor axons through the injury site, with increased functional recovery. This strategy has the advantage of reducing the molecular inhibitory cues found in the glial scar, such as myelin debris. However in our study, besides the regenerating axons reach the host spinal cord, bridging of the rostral and caudal regions of the spinal cord did not occur. This is consistent with the multi-challenged inhibitory nature of the CNS lesion and suggests that further factors need to be added to our strategy to further improve long distance axon regeneration.

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