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## DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA  
UNIVERSIDADE DE COIMBRA

Evaluating injury signals and regeneration enhancers following a central nervous system injury

Anabel Rodriguez Simões

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# Evaluating injury signals and regeneration enhancers following a central nervous system injury

Dissertação apresentada à  
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cumprimento dos requisitos necessários  
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Mónica Luísa Ribeiro Mendes de Sousa  
(IBMC-INEB) e co-orientação da  
Professora Doutora Ana Luísa Carvalho  
(Universidade de Coimbra).

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Anabel Rodriguez Simões

2013

## **Júri**

### **Presidente**

Prof. Dra. Emília da Conceição Pedrosa Duarte

Professora Auxiliar do Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

### **Orientadora**

Doutora. Mónica Luísa Ribeiro Mendes de Sousa

Investigadora Principal no Instituto de Biologia Molecular e Celular da Universidade do Porto

### **Co-orientadora**

Prof. Dra. Ana Luísa Monteiro de Carvalho

Professora Auxiliar no Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

### **Arguente**

Doutor Ramiro Daniel Carvalho de Almeida

Investigador Auxiliar no Centro de Neurociências e Biologia Celular/Instituto de Investigação Interdisciplinar



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## List of abbreviations

Arg 1 – arginase 1

ATF – activating transcription factor

ATP – adenosine-5'-triphosphate

BDNF – brain-derived neurotrophic factor

BSA – bovine serum albumine

cAMP – cyclic adenosine monophosphate

cDNA – complementar DNA

cGMP – cyclic guanosine monophosphate

CNS – Central Nervous System

CRMP – collapsin response mediator protein

Csk – c-Src tyrosine kinase

DMEM – dulbecco's modified eagle medium

DNA - Deoxyribonucleic acid

DRG – dorsal root ganglion

DRI – dorsal root injury

ERK – extracellular signal-regulated kinase

FBS – fetal bovine serum

GSK3 $\beta$  – glycogen synthase kinase 3 beta

GTP - guanosine triphosphate

HDAC6 – histone deacetylase 6

HEK 293T - Human Embryonic Kidney 293 cells

Hsp – heat shock protein

IHC - immunohistochemistry

IU – infection units

JNK – c-Jun N-terminal kinases

KIF – kinesin family motor protein

KLC – kinesin light chain

MAG - myelin-associated glycoprotein

MAP – microtubule-associated protein

MEK - mitogen-activated protein kinase

mRNA – messenger RNA

NGF – nerve growth factor

NLS – nuclear localization site

PBS – phosphate buffered saline

Pen/Strep – penicillin/streptomycin

PGP9.5 – protein gene product 9.5

Plk3 – polo-like kinase 3

PNS – Peripheral Nervous System

qPCR - real-time PCR

RAG – regeneration-associated gene

RNA – ribonucleic acid

RockII - Rho-associated, coiled-coil containing protein kinase 2

rRNA – ribosomal RNA

SCI – spinal cord injury

SDS – sodium dodecyl sulfate

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

SNI – sciatic nerve injury

Src - proto-oncogene tyrosine-protein kinase

STAT3 – signal transducer and activator of transcription 3

Syd – Sunday driver

TBS - tris-buffered saline

UTR – untranslated region

WB – western blotting

Wd – Wallerian degeneration





## Resumo

Axónios do sistema nervoso periférico são capazes de regenerar após uma lesão através da ativação de um programa de regeneração com o transporte de sinais de lesão do local de lesão para o corpo celular. Estes sinais de lesão irão então induzir a expressão dos potenciadores de regeneração. Pensa-se que ambos os sinais de lesão induzidos e os potenciadores de regeneração expressos após uma lesão no sistema nervoso central (SNC) são defeituosos. Durante a última década, têm havido esforços intensivos na tentativa de entender a razão pela qual axónios falham a regeneração no SNC adulto de mamíferos, tendo por objetivo encontrar uma estratégia para uma terapia efetiva para promover a regeneração após patologias no sistema nervoso central, como a lesão na espinal medula.

Esta tese focou na caracterização dos sinais de lesão e potenciadores de regeneração que possam ativar a capacidade de regeneração intrínseca celular de neurónios do SNC. Identificámos a glicogénio sintase quinase 3 beta (GSK3 $\beta$ ), a proteína de choque térmico 40 (Hsp40) e a Rho associada à proteína-quinase 2 (RockII) como sinais aumentados após uma lesão na raiz dorsal que podem atuar como potenciais inibidores da regeneração. Quer a GSK3 $\beta$  como a RockII foram já descritas no contexto de regeneração axonal como moléculas que necessitam de ser inibidas de forma a obter níveis ótimos de regeneração axonal. No caso da Hsp40, o seu papel não está tão bem estabelecido e deverá ser posteriormente analisado. Neste contexto, estamos a explorar a sua relevância durante o crescimento axonal pela realização de ensaios de crescimento de neurites após *knockdown* por *shRNA* em neurónios primários.

A segunda parte deste trabalho focou a identificação de potenciadores de regeneração e mecanismos subjacentes ao aumento no transporte axonal em axónios em regeneração. Identificámos a proteína do gene do produto 9.5 (PGP9.5) e a proteína mediadora de resposta de colapso-5 (CRMP-5) como dois possíveis potenciadores da regeneração axonal, com aumento de transporte anterógrado em axónios em regeneração. No entanto, *knockdown* de ambos os candidatos não inibiu o crescimento de neurites *in vitro*. Estudos futuros devem ser efetuados para clarificar os seus papéis durante o crescimento axonal, nomeadamente avaliando o crescimento axonal em neurónios *knockdown* para CRMP-5 e PGP9.5, quando crescidos em condições que mimetizam o ambiente inibitório de uma lesão no SNC tal como, usando como substrato mielina. Em relação ao aumento do transporte axonal em axónios em regeneração

investigámos se este processo se poderia dever a alterações nos motores moleculares ou a alterações nas modificações pós-transducionais da tubulina. Embora tenhamos observado alterações quer na expressão da quinesina, quer na de dineína, os níveis destes motores por si só não foram suficientes para explicar as alterações observadas no transporte axonal. Ainda em relação aos motores, dado que a fosforilação das cadeias leves da quinesina tem sido descrita como sendo responsável pela libertação da quinesina dos microtúbulos, analisámos esta modificação em axónios em regeneração, não tendo sido encontrada nenhuma correlação que possa explicar este aumento no transporte. O envolvimento das modificações pós-transducionais da tubulina também foi investigado. Como a acetilação da tubulina foi descrita como uma modificação da tubulina que aumenta a ligação dos motores, começámos por analisá-la. No entanto, não se observaram nenhuma diferença nos níveis de tubulina acetilada em axónios em regeneração. Vimos no entanto, que os níveis de tubulina tirosinada estavam aumentados em axónios em regeneração, o que sugere um aumento de quantidade de microtúbulos dinâmicos.

Em resumo, este trabalho contribuiu para a identificação de possíveis novos sinais de lesão e potenciadores de regeneração. A sua relevância no contexto da lesão na espinal medula irá futuramente ser estabelecida.

**Palavras-chave:** lesão na espinal medula, lesão condicionada, transporte axonal, sinais de lesão, potenciadores de regeneração

## Abstract

Peripheral nervous system axons are able to regenerate following an injury through the activation of a regeneration program with transport of injury signals from the site of lesion to the cell body. These injury signals will then induce the expression of regeneration enhancers. Both the injury signals induced and the regeneration enhancers expressed after a central nervous system (CNS) injury are thought to be defective. During the past decade, there have been intensive efforts in trying to understand why axons fail to regenerate in the adult mammalian CNS aiming at finding an effective therapeutic strategy to promote regeneration after CNS pathology such as spinal cord injury (SCI).

This thesis has focused on characterizing injury signals and axonal regeneration enhancers that may activate the cell-intrinsic regeneration capacity of CNS neurons. We have identified glycogen synthase kinase 3 beta (GSK3 $\beta$ ), heat shock protein 40 (Hsp40) and Rho-associated, coiled-coil containing protein kinase 2 (RockII) as signals increased after a dorsal root injury that may act as potential regeneration inhibitors. Both GSK3 $\beta$  and RockII were already described in the context of axonal regeneration as molecules that need to be inhibited in order to obtain optimal levels of axonal growth. In the case of Hsp40 its role is not as well established and should be further addressed. In this context, we are further exploiting its relevance during axonal growth by performing neurite outgrowth experiments following its knockdown through shRNA delivery in primary neurons.

The second part of this work focused on the identification of regeneration enhancers and on the mechanisms underlying the increase in axonal transport in regenerating axons. We have identified protein gene product 9.5 (PGP9.5) and collapsin response mediator protein 5 (CRMP-5) as two possible axonal regeneration enhancers with increased anterograde transport in regenerating axons. However knockdown of both candidates did not inhibit neurite outgrowth *in vitro*. Further studies should be performed to clarify their role during neurite outgrowth, namely by assessing the neurite outgrowth in neurons following knockdown for CRMP-5 and PGP9.5, when grown in conditions mimicking the inhibitory environment of a CNS injury i.e., when grown on myelin as a substrate. Regarding the increase of axonal transport in regenerating axons, we investigated whether this could be due to alterations on molecular motors or to alterations in tubulin post-translation modifications. Although we have observed alterations in both kinesin and dynein expression, their levels alone were not

sufficient to underlie the alterations observed in axonal transport. In relation to motors, as kinesin light chain (KLC) phosphorylation has been described as being responsible for releasing kinesin from microtubules, we analyzed this modification in regenerating axons, but no correlation was found that could underlie increased transport. The involvement of the tubulin post-translational modifications was also investigated. As tubulin acetylation was described as a tubulin modification that increases binding of motors, we started by analyzing it. However, no differences were observed in the levels of acetylated tubulin in regenerating and non-regenerating axons. We found however, that the levels of tyrosinated i.e., dynamic microtubules, were increased in regenerating axons.

In summary, this work contributed to identify possible new injury signals and regeneration enhancers. Their relevance in the context of spinal cord injury will be further established in the future.

**Key words:** spinal cord injury, conditioning lesion, axonal transport, injury signals, regeneration enhancers

# Introduction





# **Introduction**

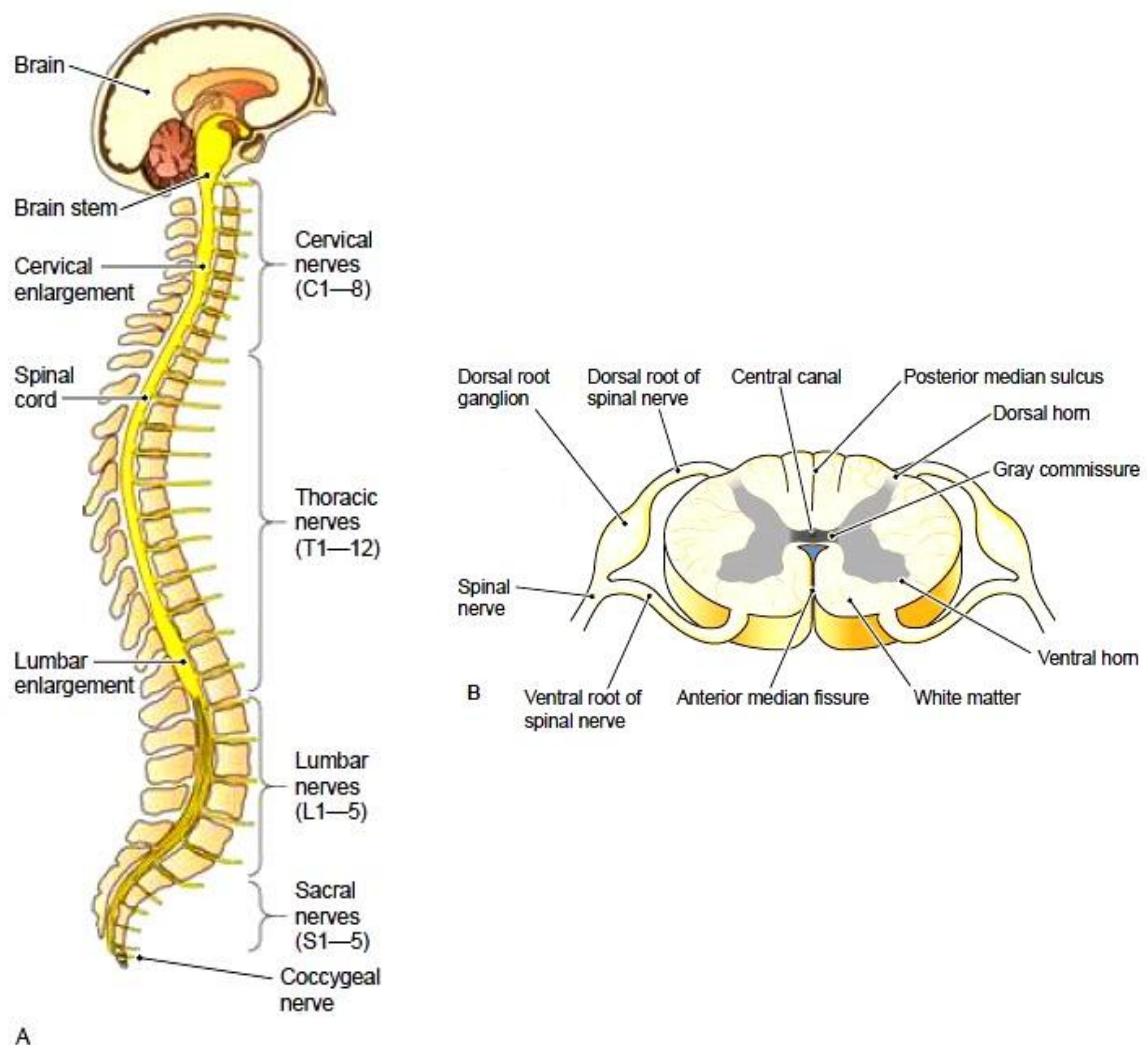
## **1 –The Nervous System**

The Nervous System is the body's collector, storage center and control system of information. As a primary function it collects information, analyzes it and initiates an appropriate response. Due to its complexity it is responsible for the control of all the biological processes in the body, which include awareness, thoughts, movements, speech, memory and sensations. The nervous system is divided into the Peripheral Nervous System (PNS) and the Central Nervous System (CNS). The CNS is constituted by the brain and the spinal cord, and represents the center of command that receives and analyzes inputs from the body and the environment, so that an appropriate response is produced. The PNS is made up by the nerves emerging from the brain (cranial nerves) and from the spinal cord (spinal nerves), and it links the CNS to sensory organs and takes the information from the CNS to the periphery. At the cellular level, the nervous system has two main cells types: neurons and glial cells. Neurons are responsible for the transmission and storage of information, while glial cells confer support, including myelination. Glial cells differ in the CNS and PNS. Besides other differences, while in the PNS Schwann cells that myelinate and support neurons, in the CNS oligodendrocytes are responsible for myelination. The myelin layers wrap around the axons allowing rapid transmission of action potentials.

### **1.1 – Spinal Cord**

The spinal cord is responsible for muscle control, as well as for the control of autonomic functions such as heart rate, bladder and bowel functions. Upon spinal cord injury (SCI) several complications may arise such as motor function loss, inability to regulate blood pressure (that can lead to autonomic dysreflexia), among others. We can observe in figure 1A and 1B that this long tubular structure is divided in peripheral white matter (myelinated axons) and central gray matter (cell bodies and their fibers). The spinal cord receives inputs and projects outputs via nerve fibers in the spinal rootlets and roots, spinal nerves, and their branches. Two types of fibers emerge from the spinal cord, forming the ventral (anterior) and the dorsal (posterior) roots (figure 1B). While the ventral roots consist of efferent (motor) fibers that are constituted by multipolar neurons, the dorsal roots are afferent (sensory) fibers that are constituted by pseudounipolar neurons. While the cell body of the motor neurons is located within the ventral

gray matter, the cell body of the sensory neurons is located outside of the spinal cord in the dorsal root ganglion (DRG). In humans, a total of 31 spinal nerves are present on each side of the cord, and are numbered (as a general rule) in accordance with the number of vertebrae above the nerve. Although there are 7 cervical vertebrae, we can count 8 pairs of cervical nerves, which is due to the fact that the first cervical nerve leaves the vertebral canal above the first cervical vertebrae. So, we can find 12 pairs of thoracic (T) nerves, 5 pairs of lumbar (L) nerves, 5 pairs of sacral (S) nerves and the humans have only 1 pair of coccygeal (Co) nerves (figure 1A).



**Figure 1** – Spinal cord structure. Lateral view (A) and cross section of the spinal cord (B). Adapted from Tyxo, 2006.



The spine in rodents is not simply a scale down version of the human spine. The spinal cord is constituted by 34 segments: 8 cervical, immediately caudal to the skull, 13 thoracic, 6 lumbar, 4 sacral and 3 coccygeal.

## **1.2 – Spinal Cord Injury (SCI)**

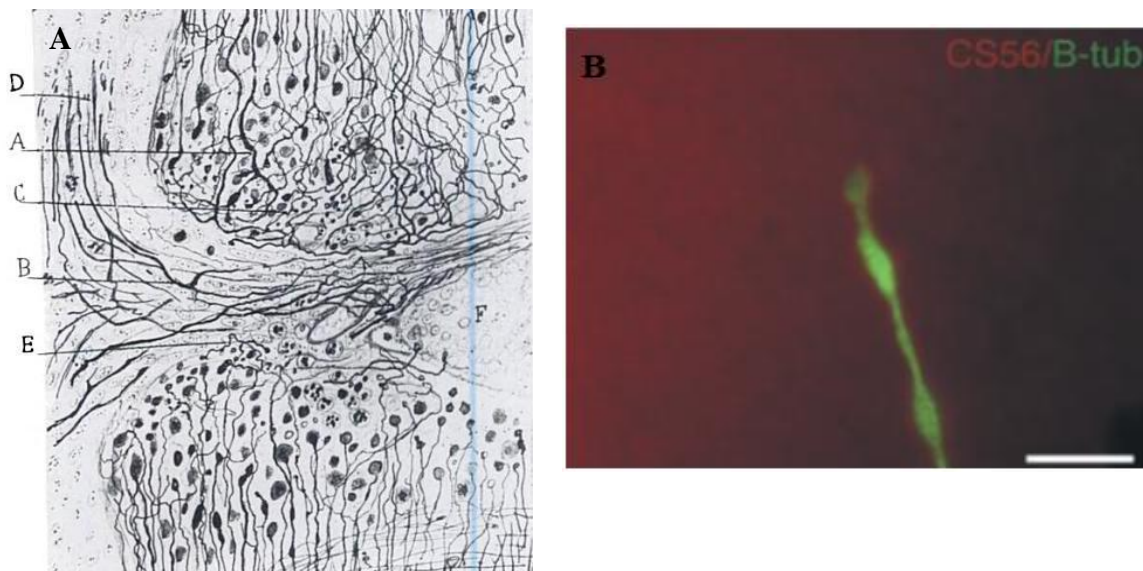
The Alternative Health & Medicine Encyclopedia states that: “If life is a symphony of activities-both voluntary activities, such as walking, talking, reading, or going to class, and involuntary activities, such as breathing, digesting food, pumping blood, and perspiring - then the nervous system is the orchestra that makes it all happen.” SCI is a permanent and devastating neurologic injury usually occurring in the prime of patient’s life. Due to its high cost to society and the lack of treatment, it is important to develop novel approaches to the understanding of the pathophysiology and treatment of SCI.

SCI is most commonly caused by trauma, but it can also have other causes like tumors, infection, vascular lesions or iatrogenic procedures. The traumatic event can be either endogenous, as a result for example of an intervertebral disc herniation, or exogenous, frequently caused by fractures or dislocated vertebrae. The initial mechanical trauma to the neural tissue can result from contusion, compression, penetration or maceration of the spinal cord, and can damage both the CNS and PNS (McDonald and Sadowsky, 2002). This type of injury causes the disruption of nerve fibers and local neuronal circuits, interrupting the transfer of information between the brain and the structures below the injury site, often resulting in the loss of sensory and motor function (McDonald and Sadowsky, 2002; He, 2005). Besides sensory and motor impairment, other problems have higher impact on the life of SCI patients. Bladder and bowel control, sexual activity and other autonomic complications appear to be more deleterious on the quality of life of these patients.

The pathology of this type of injury arises from primary and secondary mechanisms. The primary injury can lead to the presence of severed axons, direct mechanical damage to cells, and ruptured blood vessels. The secondary injury is responsible for the expansion of the primary injury and is the result of alterations in local ionic concentrations, reduced spinal cord blood flow, production of free radicals, imbalance of activated metalloproteinases, inflammation, and release of cytotoxic neurotransmitters (Oyinbo, 2011).

## 2 – Axonal regeneration: the PNS success *versus* the CNS failure

Axonal regeneration of peripheral nerves was first described by the Spanish neuroscientist, Santiago Ramón y Cajal. He described it as an environment that could guide and fuel the regrowth of injured axons, not only due to its orienting function but also by its trophic character, since the sprouts that have arrived at the peripheral stump were robust and demonstrated a great capacity for ramification. The difficult regeneration of CNS axons (spinal cord and cerebral cortex axons) (figure 2A) was also described by Cajal. In young animals (cats and dogs of ten to twenty days of age), the terminal clubs of the axons were interrupted inside the white matter (central stumps) (Ramon y Cajal, 1928). After an injury, the terminals of injured axons assumed the form of a “retraction bulb” (figure 2B) (Garrison, 1929).



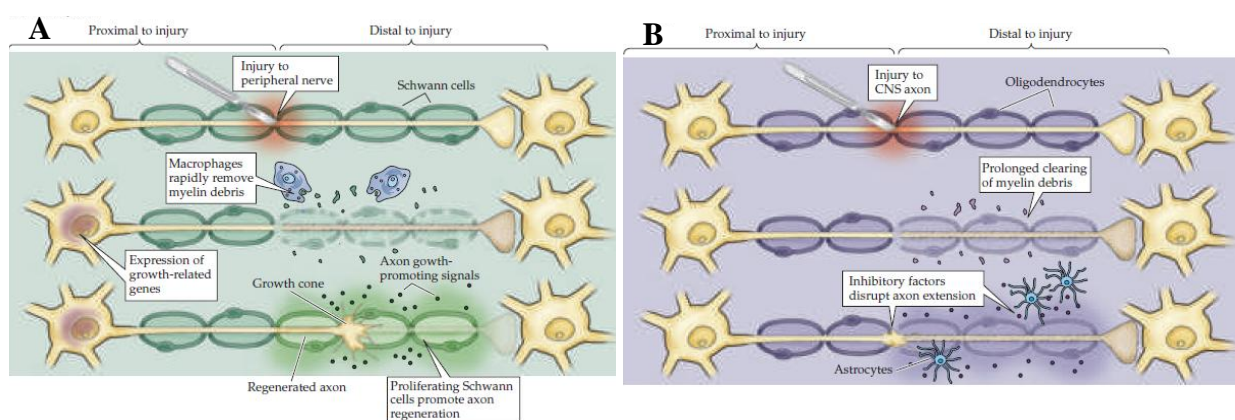
**Figure 2** – Absence of regeneration following spinal cord injury. Illustration of a central nervous system injury, displaying scar tissue and absence of axonal growth (A), by forming "dystrophic" endballs (B). Adapted from Ramon y Cajal, 1928; Tom et al., 2004.

Why are injured axons unable to regenerate after injury in the adult mammalian CNS? This question has been a major challenge within the neuroscience community over the last decades, and the general concept has not changed. Aguayo and colleagues have demonstrated that some injured axons can regrow into a sciatic nerve implant, through the lesion site in the adult CNS. This suggests that inhibitory activity in the lesion site may act as a prevention mechanism for axon regeneration (David and Aguayo, 1981; Abe and Cavalli, 2008).

Recent studies identified some mechanisms that underlie the improved PNS regeneration. As suggested by Aguayo and colleagues, the PNS is able to create an environment that promotes regeneration, while in the CNS there is the accumulation of inhibitory molecules (David and Aguayo, 1981). Also, PNS neurons are able to mount a vigorous response to injury by expressing several regeneration associated genes (RAGs), while CNS neurons fail to do so (Sun and He, 2010). These mechanisms are discussed in more detail below.

## 2.1 – Wallerian degeneration (Wd) and glial scar formation

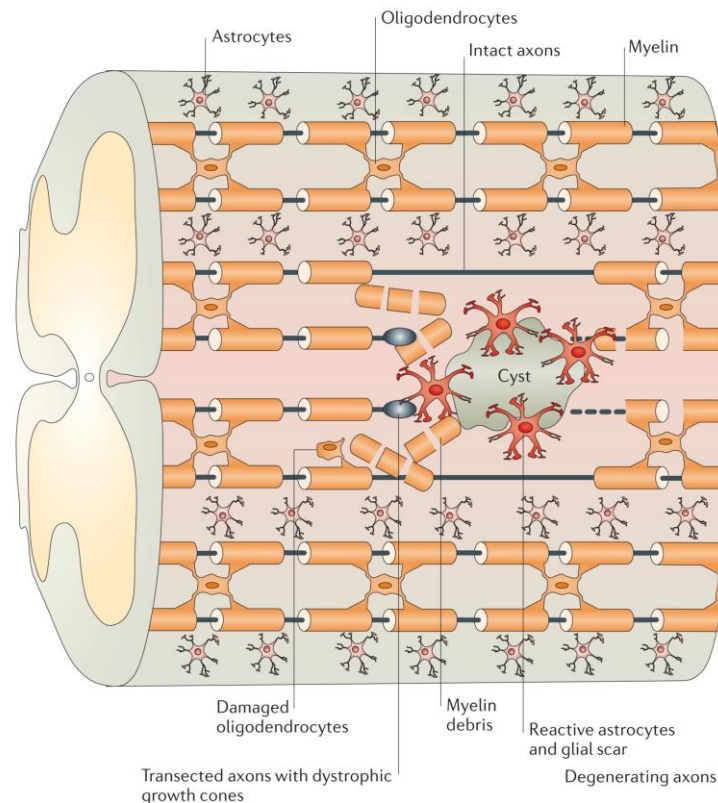
Following nerve injury there is the degeneration of the distal axon stump, followed by the clearance of myelin and cell debris. This process is known as Wallerian degeneration (Wd). To ensure axonal regeneration following injury, Wd is a prerequisite, as myelin-associated inhibitors, including myelin-associated glycoprotein (MAG), oligodendrocyte myelin glycoprotein, Nogo and netrin-1 potently inhibit axonal regeneration (Yiu and He, 2006; Vargas and Barres, 2007). Wd occurs both in PNS and CNS, however the speed and extension of this process is remarkably different in both systems. In the PNS rapid Wd results in an extracellular environment prone to axon regeneration, where the blood-brain-barrier permeability increases, the myelin sheets break down (demyelination) and axon and myelin debris are cleared by Schwann cells and macrophages (figure 3A). As such, the potential inhibitory environment in the PNS is cleared. Contrarily to the PNS, Wd in CNS is much slower, taking months to years, instead of just a few days. This is not only due to a delay in axonal degeneration in the CNS but rather to a failure to clear CNS myelin debris (figure 3B) (Horner and Gage, 2000; Dill et al., 2008).



**Figure 3** - Different responses to injury in the peripheral (A) and central (B) nervous system. Adapted from Purves et al., 2004.

The immune system plays an important role in removing toxic debris from the injury site. While macrophages and Schwann cells in the distal stumps of the injured peripheral nerves are effective in removing the inhibitory proteins associated with myelin, the microglia and oligodendrocytes in the CNS are not (Filbin, 2003). It is also important to consider that inflammation within the CNS is a potential source of cytokines and of other signaling molecules that can lead to the upregulation of inhibitory pathways after injury. Microglia cells from the CNS and activated macrophages from the PNS both respond to trauma, and this inflammatory response may contribute to secondary tissue damage after the primary insult (Fitch and Silver, 2008).

Overall, the different glia and the different immune responses of the CNS and PNS, explain the inefficient Wd in the CNS, culminating in the formation of a glial scar. This scar consists in the recruitment of microglia/macrophages, oligodendrocyte precursors, meningeal cells, astrocytes and fibroblasts, acting as an additional barrier to axon regrowth (figure 4) (Zurn and Bandtlow, 2006; Kimura-Kuroda et al., 2010). The glial scar is more than just a physical barrier, as it accumulates several inhibitory molecules, such as inhibitory extracellular matrix molecules, like tenascin, semaphorin3, chondroitin sulfate proteoglycans and ephrins, that make the biochemical milieu surrounding the injury site inhospitable for regenerating axons, and are upregulated during the inflammatory stages after lesion. It has been recently reported that axons with these dystrophic endings do not lose their ability to regenerate, and they can in fact return to active growth states if the proper environment is provided (Tom et al., 2004).

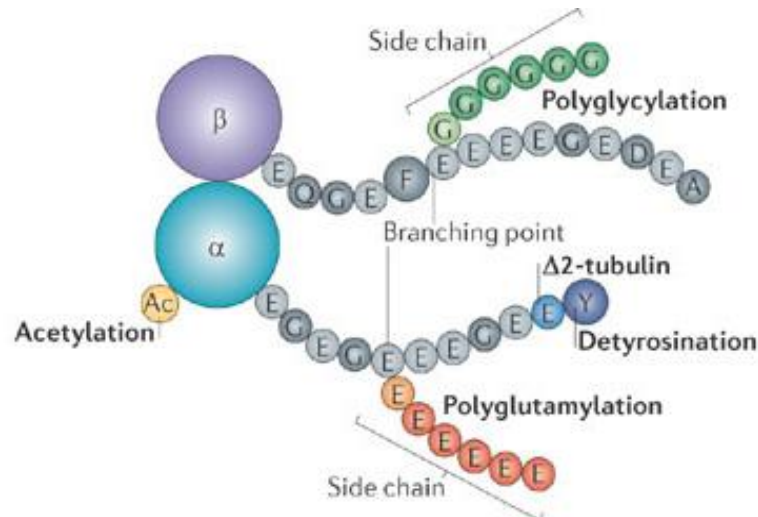


**Figure 4** - Schematic representation of the CNS injury site. Adapted from Yiu and He, 2006

## 2.2 –Cytoskeletal dynamics: polarization and growth of an axon

The axon shaft is mainly composed of microtubules. Microtubules are polymers of tubulin subunits composed of a heterodimer formed of  $\alpha$  and  $\beta$  subunits aligning end-to-end to form protofilaments, which join laterally (protofilaments) to form a hollow tube. Given the highly polarized nature of neurons, and the need to elongate an axon during development and following injury, the cytoskeleton organization is crucial for axonal formation, growth and regeneration.

Several post-translational modifications affect tubulin, like acetylation, tyrosination, polyglutamylation, polyglycation, palmytoylation and phosphorylation (figure 5), and these are believed to underlie the dynamic properties of microtubules (Fukushima et al., 2009; Cho and Cavalli, 2012). For the purpose of my thesis I will only describe the ones that were relevant during my work.



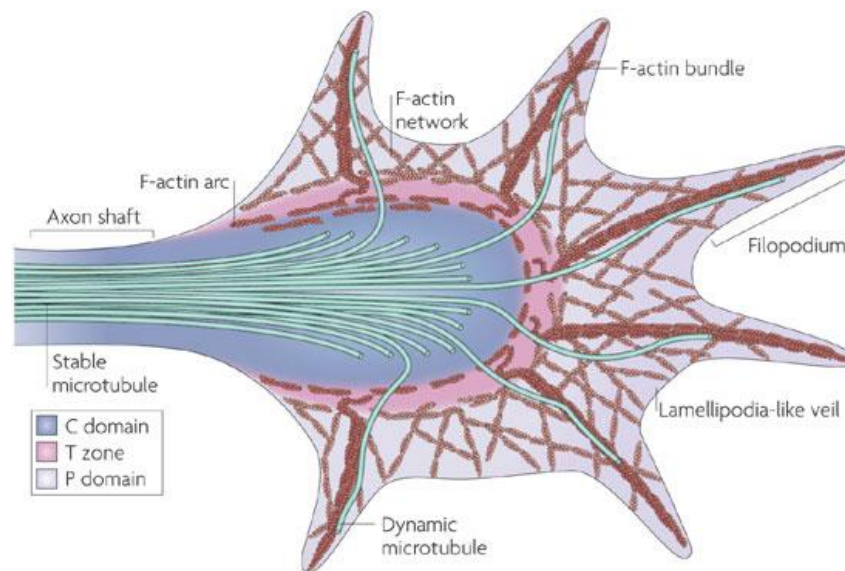
**Figure 5** - Representation of the  $\alpha/\beta$  tubulin dimer and its associated modifications. Both  $\alpha$  and  $\beta$  tubulin can be modified by polyglutamylation and polyglycylation on different Glu residues within carboxy-terminal tails. Acetylation of Lys40 is located at the amino-terminal domain in  $\alpha$  tubulin. Adapted from Janke and Bulinski, 2011.

Acetylation usually exists in higher amounts in stable and mature microtubules, and is correlated with microtubule stability. This modification occurs after microtubule assembly when an acetyl group attaches to  $\alpha$ -tubulin Lys40 (Janke and Kneussel, 2010). This addition is performed by acetylases, and the removal can be performed by deacetylases (Maruta et al., 1986). There are two enzymes responsible for deacetylation: histone deacetylase 6 (HDAC6) and the mammalian homolog of silent information regulator 2/sirtuin type 2 (SIRT2) (Fukushima et al., 2009). Almost all  $\alpha$ -tubulins contain a tyrosine residue at the C-terminus right after translation, which forms a heterodimer with  $\beta$ -tubulin and assembles into microtubules (Gundersen and Jensen, 1987). Tyrosinated tubulin unlike the acetylated one, is present in higher amounts in the growth cone area correlating with microtubule dynamics in this region (Dent and Gertler, 2003). Polyglutamylation, that is present in about half of neuronal  $\alpha$ -tubulin, involves the addition of one to six glutamyl units and takes place on  $\alpha$ 1A- and  $\alpha$ 1B-tubulin, which contain similar amino acid sequences within their C-terminus. Polyglutamylated tubulin has been suggested to be important in the association of microtubules with MAPs and motor proteins (Fukushima et al., 2009).

### 2.2.1 – The growth cone: axonal outgrowth and guidance

The tip of the axon, known as growth cone, is the structure responsible for axonal elongation and guidance. It comprises a plastic structure that is able to respond to environmental cues and is able to determine the direction of the growing axon. The growth

cone extends and retracts membrane protrusions, that assume a form of tapered finger-like projections – *filopodia*- and flat sheet-like protrusions – *lamellipodia* (figure 6) (Lowery and Van Vactor, 2009). Growth cone protrusion occurs by polymerization/depolymerization of actin filaments which direct axonal outgrowth. Although the growth cone direction is not dependent on microtubule polymerization, axonal outgrowth occurs by polymerization/depolymerization of the microtubules (Dent and Gertler, 2003).



**Figure 6** - Growth cone structure. Adapted from Lowery and Van Vactor, 2009

The guidance molecules present in the environment are fundamental for correct pathfinding. These can be divided in chemoattractive and chemorepulsive, depending on their ability to attract or repel growing axons. They do so by binding to specific receptors at the growth cone. Examples of these cues that direct the axons are ephrins (membrane-bound proteins that are primarily repulsive leading to growth cone collapse), netrins (proteins that can act as attractive or repulsive cues, depending on the environment) and semaphorins (transmembrane proteins that act primarily as repulsive cues, although there are some records of an attractive effect) (Song and Poo, 1999; Dent et al., 2003; Nixon and Yuan, 2011). Neurotrophins are one of the most important cues. They are not important for the initial stage of axonal growth, but are essential in later stages for transcription of genes necessary for elongation (Polleux and Snider, 2010).

## 2.3 – Intrinsic mechanisms

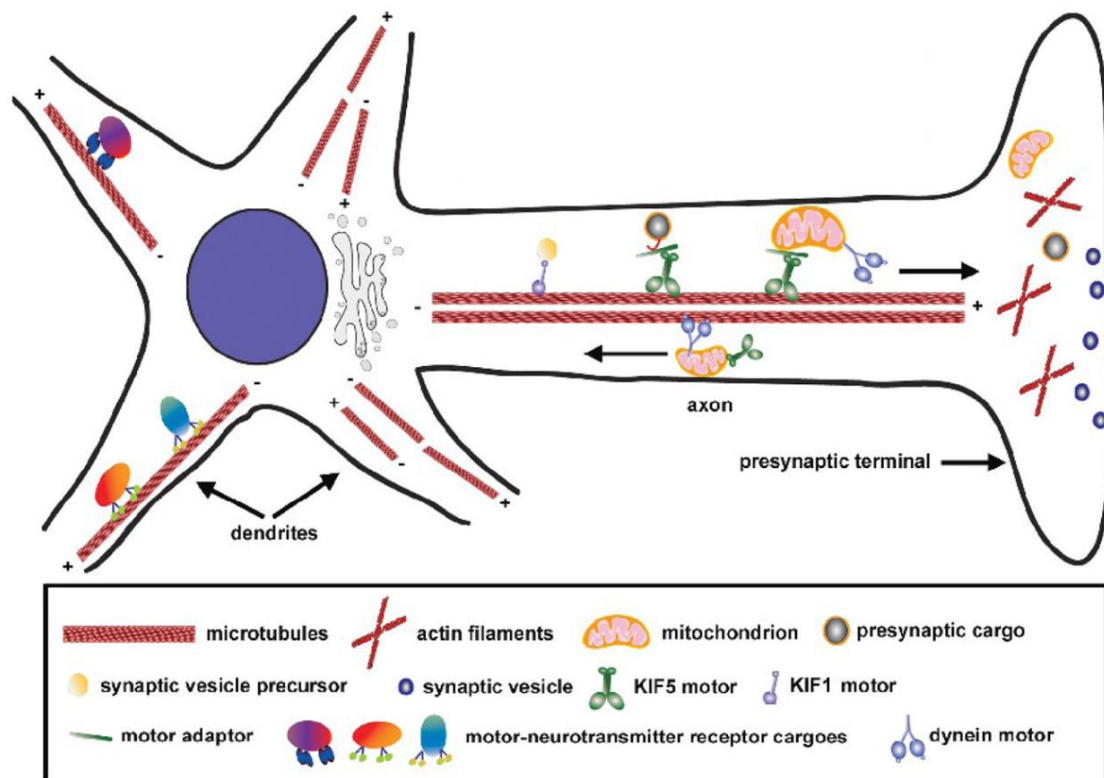
The polarized morphology of neurons leads to challenging problems for intracellular signaling pathways, namely for the communication between the cell body and the axon tip that can be separated by a distance longer than 1 meter. If thinking on pathogenic/injury processes, the ability of the cell body to sense a distant axonal injury, and initiate a proper regenerative response provides a great challenge to neurons (Abe and Cavalli, 2008). Injury to adult peripheral neurons, but not to CNS neurons, reactivates their intrinsic growth capacity promoting regeneration (Abe and Cavalli, 2008). This evidence led to the question: Which are the mechanisms that activate the intrinsic growth capacity of neurons after injury? Recent studies have shown that merely removing extracellular inhibitory cues is not sufficient to promote successful regeneration in the adult CNS (Sun and He, 2010). It is also clear that in adult neurons the molecular machinery required for sustaining axonal growth is not constitutively active and is subjected to strict control mechanisms (Rossi et al., 2007). In the PNS, upon injury, an injury signal is generated in the lesioned axon and relayed to the neuronal soma, so that the transcription program can be initiated for regeneration to occur (Sun and He, 2010). It is known that this transcription changes involve the expression of RAGs, such as growth-associated protein 43, cytoskeleton-associated protein 23 and arginase 1 (Arg1). The existence of injury signals that can trigger the expression of RAGs, together with the transport of the newly synthesized proteins to the growth cone where elongation takes place, are crucial for a successful regeneration.

### 2.3.1 – Axonal transport

Due to the limited protein synthesis in axons, most of the axoplasm constituents are synthesized in the cell body and then transported to their final destination (Grafstein and Forman, 1980; Siegel, 2006). This process also known as “axoplasmic flow”, was first described in 1948 and is mostly done along the microtubules in an adenosine-5'-triphosphate (ATP)-dependent way by cargo binding to molecular motors – kinesin and dynein (Grafstein, 1995). In the axon, microtubules are oriented with their plus ends toward the axonal terminus and the minus end towards the cell body, which is critical for correct axonal transport. **Dynein** travels mainly towards the minus end, mediating the retrograde axonal transport of cargoes and **kinesin** travels mostly towards the plus end which mediates anterograde axonal transport (figure 7) (Guzik and Goldstein, 2004; Lodish et al., 2008). There is an additional type of molecular motor – **myosin** – that contrarily to the other motors, moves along actin filaments



and is responsible for short distance transport (Bridgman, 2004; Chevalier-Larsen and Holzbaaur, 2006).

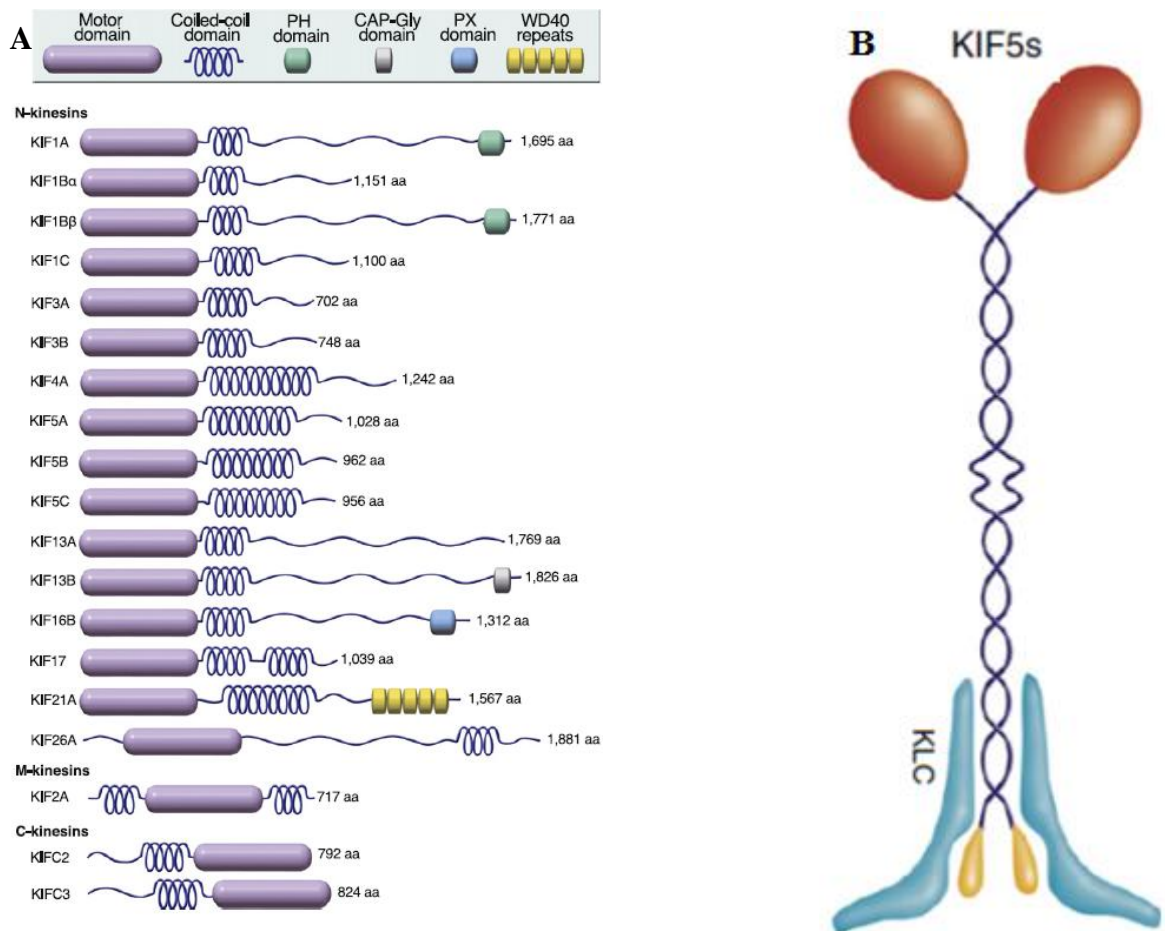


**Figure 7** - Axonal transport. Kinesin (mostly plus-end directed) is responsible for anterograde transport and dynein (travels towards the minus end of microtubules) for retrograde transport. Adapted from Cai and Sheng, 2009.

### 2.3.1.1 – Anterograde transport

Anterograde transport allows for the “slow” or “fast” movement from the cell body towards the axon terminals. Most of the transport in axons is done by the slow transport mechanism, which is divided in *slow component a*, that includes the transport of neurofilaments and microtubules at a rate of 0,2-1 mm/day, and the *slow component b* that is able to transport different polypeptides, like actin and tubulin, and glycolytic enzymes, at a rate of 2-8 mm/day (Orlinger and Lasek, 1984; Grafstein, 1995; Brown, 2003; Siegel, 2006). Regarding the “fast” mechanism, vesicles and membranous organelles are transported at a rate of 50-400 mm/day (Orlinger and Lasek, 1984). The similarities between the motors and kinetics of both slow and fast components of transport, and the different average rates, may be explained by an intermittent behavior of cargoes during transport (Brown, 2003).

The molecular motor responsible for this type of transport is **kinesin**, a superfamily composed of more than 45 members (figure 8A).



**Figure 8** - Kinesin superfamily proteins (KIFs) (A) and the structure of kinesin 1, that has two KIF5s and two kinesin light chains (KLCs) (B). Adapted from Hirokawa et al., 2010.

Kinesin 1, the most abundant in both neuronal and non-neuronal cells, has a rod-shaped conformation which comprises two heavy chains (KIF5s) and two light chains (KLCs) (figure 8B). The motor domain present on KIFs has ATP and microtubule binding motifs, and is the most highly conserved region within this family (Pfister et al., 1989; Siegel, 2006). Binding to the cargo is made through the KLCs that seem to play a role in targeting different types of membrane-bounded organelles. The motile force that is generated requires energy that is provided by ATP hydrolysis through KIFs (Siegel, 2006; Hirokawa et al., 2009).

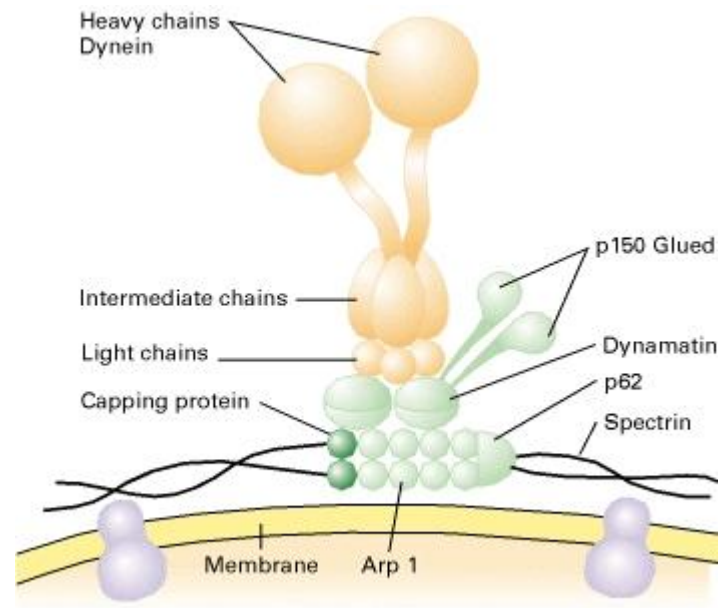
The family has three heavy chain genes expressed in mammals, one that is ubiquitously expressed (KIF5B) and two kinesin heavy chain genes (KIF5A and KIF5C) that are neuron-specific (Miki et al., 2003; Hirokawa et al., 2009).

Anterograde transport is not only important for the maintenance of normal axon physiology, it also plays a critical role during regeneration. When lesion occurs, there has to be a mechanism that transports the necessary proteins for regeneration to happen, like tubulin, actin and neurofilaments (Tashiro and Komiya, 1992; Jacob and McQuarrie, 1996; Siegel, 2006). It is also known that the rate of axonal growth during development and regeneration is very similar to the rate of anterograde transport (Wujek and Lasek, 1983; Siegel, 2006).

### ***2.3.1.2 – Retrograde transport***

Retrograde transport is responsible for carrying material from the axon terminal to the cell body. Some of the moving material arises from material moved through anterograde transport, that has turned around at the axon terminal. Retrograde transport is vital for neuronal survival as it allows to acquire exogenous compounds and to send trophic substances and growth factors to the soma.

Cytoplasmic dynein 1, most commonly known as dynein, is a minus end-directed motor (Paschal et al., 1987; Siegel, 2006). These large multimeric proteins have two or three heavy chains, with a C-terminal that has ATP-binding activity due to the presence of six AAA units (ATPase associated with cellular activities) and a tail domain located at the N-terminal that interacts with intermediate light and light chains forming the cargo-binding complex. The heavy chains are complexed with two intermediate chains, four light intermediate chains and two light chains, and dimerization of the heavy chains is required for motor activity (figure 9) (Gee et al., 1997; Hirokawa et al., 2010). This motor has a particular feature, it is not able to mediate transport by itself (only *in vitro*). Microtubule-binding proteins are required to link to cargoes, like dynactin (the best characterized heterocomplex).



**Figure 9** - Dynein/dynactin complex structure, which comprises two heavy chains, two light intermediate chains, two intermediate chains and two light chains that bind to dynactin. Adapted from Lodish et al., 2008.

Following an axonal injury, in order for regeneration to occur, it is necessary to transport the injury signals that are linked to dynein from the lesion site back to the cell body (Ambron et al., 1992; Schmied and Ambron, 1997; LaMonte et al., 2002; Hanz et al., 2003; Perlson et al., 2005).

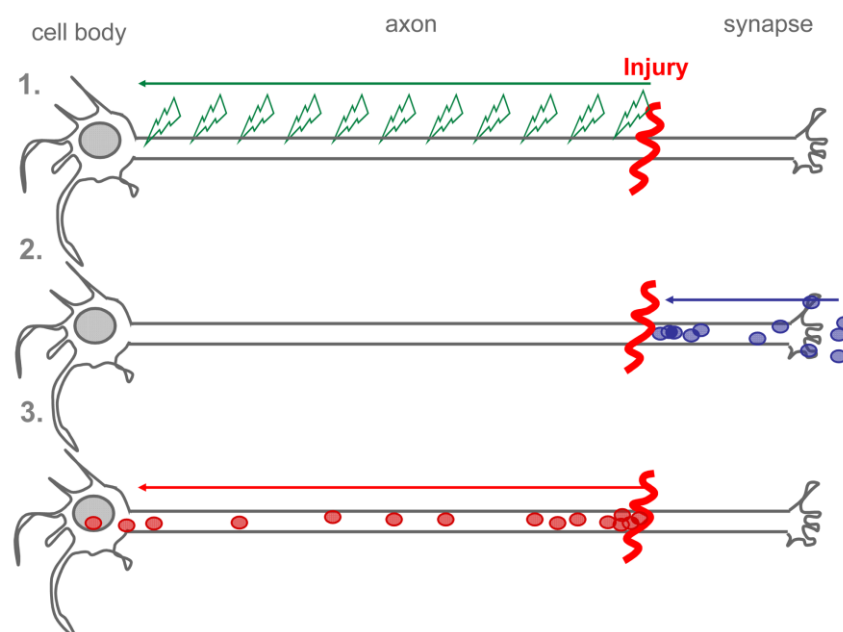
### 2.3.2 – Axonal protein synthesis

The dogma that was established regarding local protein synthesis in axonal growth cones stated that: all proteins destined for the distal part of the axon were synthesized in the cell body and transported to the appropriate sites. However, studies from the past decade have shown that like dendrites, axons have the ability to synthesize new proteins, particularly in developing systems (Alvarez et al., 2000; Willis and Twiss, 2006; Satkauskas and Bagnard, 2007; Farrar and Spencer, 2008). Local protein synthesis in growth cones is important for axon growth and guidance and the process is described in the adult and during nerve regeneration (Zheng et al., 2001; Verma et al., 2005). Following axotomy, successful axonal regeneration starts with the formation of a growth cone. This is achieved by a sequence of complex events, that includes local protein synthesis, since the arrival to the injury site of newly synthesized proteins could take a few days, limiting the possibility of regeneration (Verma et al., 2005; Willis and Twiss, 2006; Gummy et al., 2010).

Extracellular stimuli (attractive and repulsive guidance cues) can affect and alter the activity of the translational machinery, and local protein synthesis seems to control the growth cone's rapid response to these stimuli. Several guidance cues have been shown to take part on the process, like semaphorin 3A, netrin-1, EphB/ephrin B, NGF and BDNF, deciding the response of the growth cone (Campbell and Holt, 2001; Willis and Twiss, 2006; Satkauskas and Bagnard, 2007). Regenerating axonal processes contain ribosomal proteins, translational initiation factors and ribosomal ribonucleic acid (rRNA) which shows the ability to synthesize new proteins. However, those components were only found in PNS adult axons, suggesting a limited or even absent capacity for adult CNS axons to synthesize new proteins (Zheng et al., 2001; Verma et al., 2005; Satkauskas and Bagnard, 2007). Such differences can also account to the limited regeneration ability of CNS axons.

### 2.3.3 – Injury mechanisms

In the early 90s a series of studies were performed that demonstrated the existence of multiple injury signals functioning in a temporal sequence: injury-induced discharge of axonal potentials, interruption of the normal supply of retrogradely transported target-derived factors (negative injury signals) and retrograde injury signals traveling from the injury site back to the cell body (positive injury signals) (figure 10) (Ambron and Walters, 1996; Abe and Cavalli, 2008). These signals are thought to be crucial for the injured neurons to mount a regenerative response.



**Figure 10** - Signaling mechanisms. (1) injury-induced discharge of axonal potentials, (2) negative injury signals and, (3) positive injury signals. Adapted from Abe and Cavalli, 2008.

#### 2.4.3.1 – *Electrical activity*

The diverse types of retrograde signals elicited in axons upon injury, vary from rapid action potential discharges to slower mechanisms dependent on molecular motors. After injury, there is an increase of calcium influx in the axon which triggers depolarization that propagates the response to the soma through activation of voltage-dependent sodium channels (Rishal and Fainzilber, 2010). Local calcium increase after axotomy induces proteolytic activity that is an essential step in the cascade of events that leads to growth cone formation (Erez et al., 2007). This rise in calcium is likely important for cytoskeleton rearrangement. *In vitro* studies showed that electrical stimulation accelerates motor and sensory axon outgrowth and increases cAMP levels in DRG neurons (Al-Majed et al., 2004; Udina et al., 2008). However, in the adult CNS electrical stimulation does not promote regeneration, suggesting that electrical stimulation alone is not sufficient to trigger a complete regenerative response, and a second phase of signaling is required in order to accomplish an effective regeneration (Harvey et al., 2005; Abe and Cavalli, 2008; Rishal and Fainzilber, 2010). A single signaling pathway is unlikely to completely mediate nerve regeneration. Little is known about the signaling mechanisms in the CNS.

#### 2.4.3.2 – *Negative injury signals*

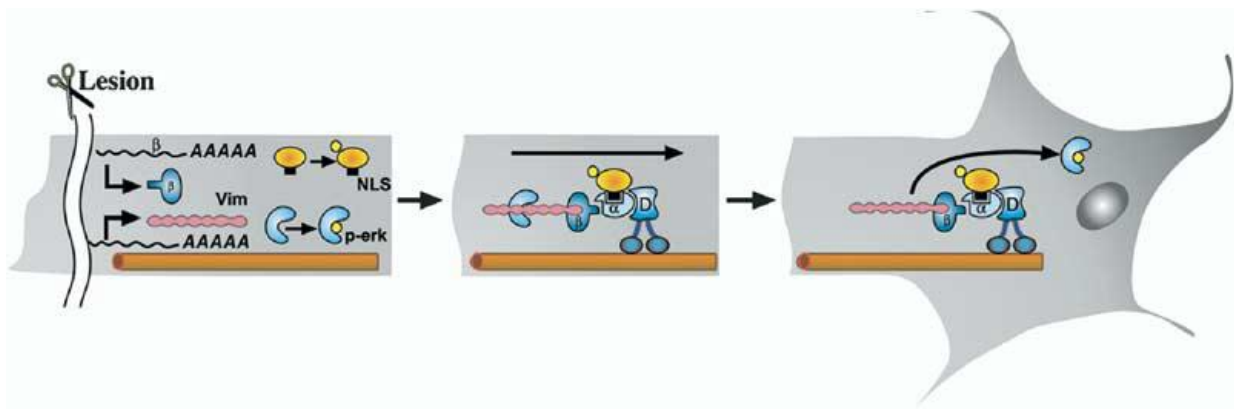
The loss of negative cues is another important mechanism to sense injury but still little is known about this. Under normal conditions, axons elongate until they find their targets, from which neurons receive several target-derived factors through retrograde transport that repress the elongation process converting the growth cone into a presynaptic terminal. When injury occurs, there is disconnection from the targets and interruption of the normal supply of target-derived signals that will relieve the repression of axon elongation genes. These negative injury signals when absent can trigger a regenerative response and neurotrophins were shown to be decreased in DRG neurons following injury (Raivich et al., 1991). But although they represent ideal candidates, their role as negative signals following injury has not yet been established. Some putative negative injury signals have been identified, like the transforming growth factor beta/SMAD2/SMAD3 pathway, where SMAD2 is downregulated following PNS injury suggesting a gene transcription dependence to restrict axonal growth ability in healthy neurons and relieve of this inhibition upon injury. But regarding the regeneration ability of adult CNS neurons, the contribution of this pathway is still not established. Besides SMAD2, activity transcription factor 2 (ATF-2) also seems to repress neuronal growth capacity (Martin-Villalba et al., 1998; Abe and Cavalli, 2008). Despite these evidences, still no clear negative injury

signals were identified and further studies are needed to the development of new methods to improve axon regeneration.

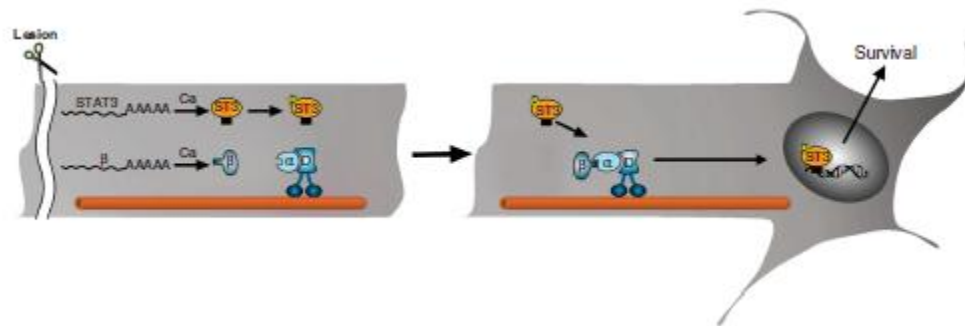
### 2.3.3.3 – Positive injury signals

The positive injury signals identified so far share a common requirement: microtubule-dependent retrograde transport. Previous studies have shown that injury signals from axons are transported retrogradely to the cell body in a nuclear localization signal (NLS)-dependent manner (Ambron and Walters, 1996). Several functionally distinct proteins were described to participate in this type of signaling, like cytokines (leukemia inhibitory factor, interleukin-6 and ciliary neurotrophic factor) and their downstream effectors (JAK-STAT pathway). Studies in PNS neurons suggested that injury increases the local translation of  $\beta$ -importin. The synthesis of  $\beta$ -importin allows for the formation of importin- $\alpha/\beta$  heterodimers which bind to NLS and link those proteins to the retrograde transport machinery through dynein binding (Hanz et al., 2003).

Until now the study of rodent injured sciatic nerves led to the identification of several injury signals that are locally activated and retrogradely transported to the cell body: the extracellular signal regulated kinase (ERK) (figure 11) (Hanz et al., 2003; Perlson et al., 2005), c-Jun N-terminal kinases (JNK) (Cavalli et al., 2005; Lindwall and Kanje, 2005) and signal transducer and activator of transcription 3 (STAT3) (figure 12) (Ben-Yaakov et al., 2012).



**Figure 11 - Retrograde transport of pERK following PNS injury.** In the axoplasm, following nerve injury, MAP kinases are phosphorylated and vimentin binds phosphorylated ERKs, linking them to the retrograde transport machinery. Adapted from Perlson et al., 2005

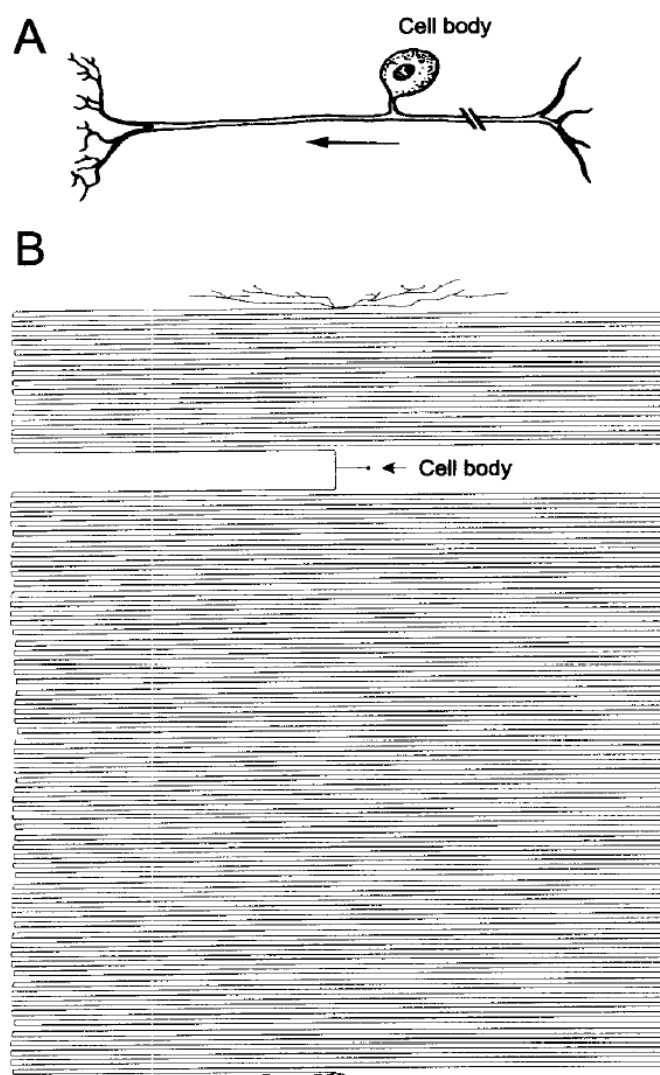


**Figure 12 - Retrograde transport of pSTAT3 following PNS injury.** In lesioned sensory axons there is the induction of local synthesis of importin $\beta$ 1 and STAT3, being the last one phosphorylated and then linked with the importins-dynein complex that enables retrograde transport to the cell body. Adapted from Ben-Yaakov et al., 2012



### 3 – Dorsal Root Ganglion neurons: *a model of study*

DRG contain most of the body's primary sensory neurons. These are pseudounipolar neurons (figure 13B). They receive the sensory information from the periphery through their long peripheral axons, and transmit it to the CNS through a central axon that reaches the dorso-lateral region of the spinal cord via the sensory roots of each segment (Purves et al., 2004). They are large, roughly round cells with a diameter ranging from 20 to 150  $\mu\text{m}$  in humans. Most of the adult DRGs are separated from each other by one or two layers of satellite cells (Devor, 1999; Sapunar et al., 2012).

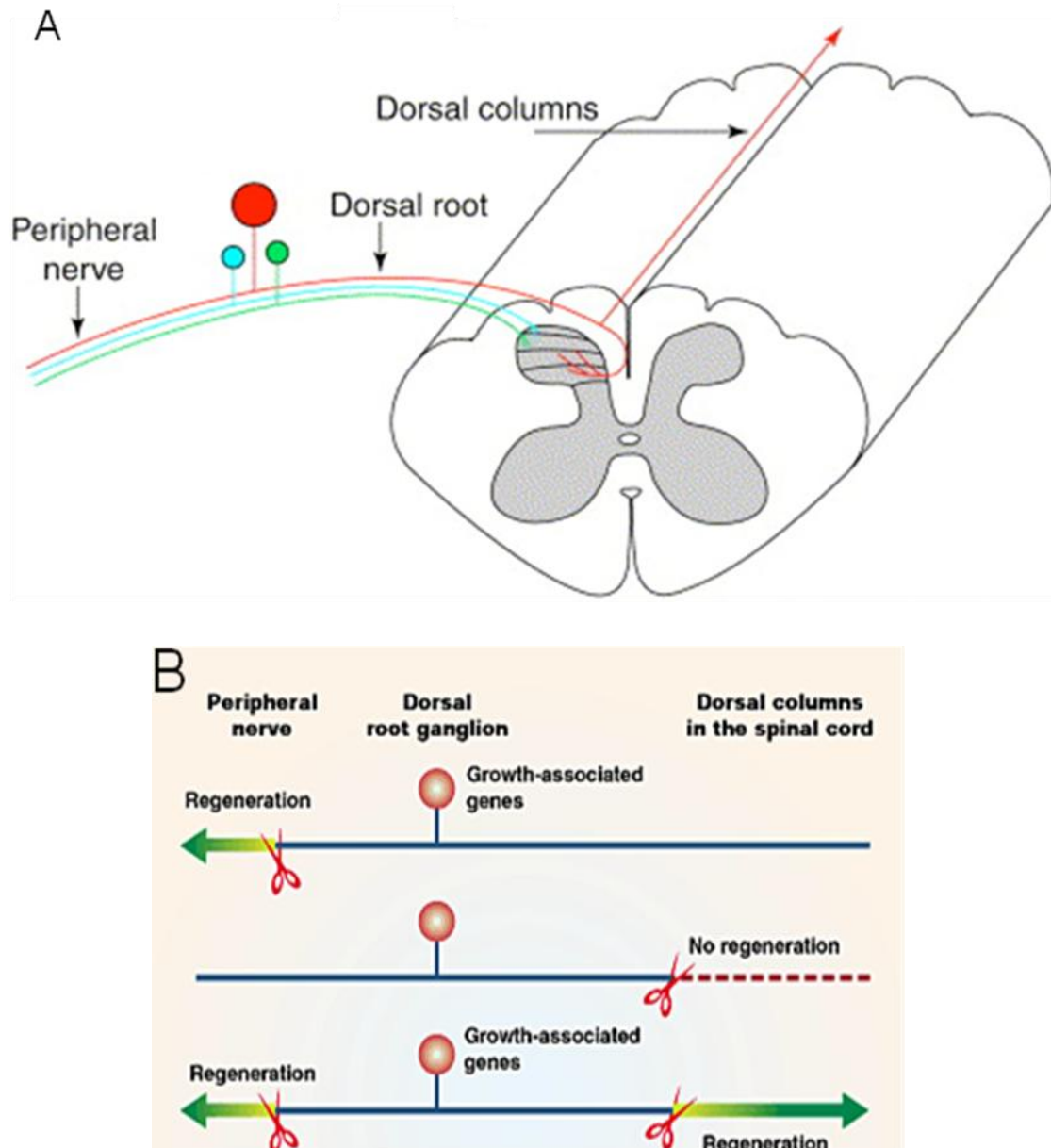


**Figure 13** – Pseudounipolar DRG neuron with the cell soma (cell body), the dorsal root axon branch (left) and the peripheral nerve axon branch (right) (A). Actual size relations of a typical DRG neuron, where the drawing of the cell body, T-stem, dorsal root and peripheral nerve axons are in their real proportions (B) Adapted from Devor, 1999.

These cells are the few neuronal types without dendrites or afferent synapses, but the cell bodies have one peripheral branch that innervates peripheral targets (like the skin, muscle, viscera, etc) and a central branch that travels through the dorsal root and enters the spinal cord originating the dorsal column fibers.

DRG neurons provide a useful system to examine central regeneration because they do not die after peripheral or central axonal injury. Besides, when injuring the peripheral but not the central axon, their intrinsic growth state will increase. A peripheral injury to the DRG neuron is known as a conditioning lesion, since it is able to condition DRG neurons to a regenerative state both *in vitro* and *in vivo*. By inducing a peripheral nerve-conditioning lesion one week before performing a central injury, is promoted in the spinal cord, regeneration of dorsal column fibers past the SCI site (figure 14) (Neumann and Woolf, 1999).

Not only these axons show higher ability to elongate, but also they become insensitive to the inhibitory cues normally present in the glial scar. As such, the conditioning injury model is a good model to study the gain of regeneration capacity through the modulation of intrinsic mechanisms in neurons. Several molecules have been identified as being important for the conditioning effect, such as cAMP and RAGs, like neuropeptide Y, vasoactive intestinal peptide, ATF-3, Arg1 and others (Ylera et al., 2009). Nevertheless, none of them was shown to reproduce the conditioning effect on its own. This suggests that this robust effect may be mediated by innumerable pathways (Richardson and Issa, 1984; Richardson and Verge, 1986; Schreyer and Skene, 1993; Chong et al., 1994; Hu-Tsai et al., 1994; Oudega et al., 1994; Edström et al., 1996; Coggeshall et al., 1997; Smith and Skene, 1997).



**Figure 14 – Primary sensory neuron organization.** Axons emerging from the cell body bifurcate to send one branch to the periphery and another towards the CNS in the dorsal root (A). "**Conditioning lesion**" paradigm. After injury the sensory axons in the adult spinal cord do not regenerate, while PNS injury results in regenerative response. Regeneration of the central branch can be greatly enhanced by a previous injury to the peripheral branch (B). Adapted from Bradbury et al., 2000 and Woolf, 2001.



## Research goals

In this thesis the main goal was to understand mechanisms controlling axonal regeneration. To do so, the following issues were addressed:

- ✓ The existence of injury signals has been described in PNS axons. Such signals have been shown to promote axonal regeneration following injury. In this context, previous studies in our lab have focused on identifying the nature of putative signals following CNS injury. To do so, axoplasm of peripheral and central axons of DRG neurons was analyzed following injury by Kinex antibody microarray. In this work and based on the previous screening performed, we analyzed by western blot and immunohistochemistry injury signaling differences between the PNS and CNS (Chapter 3).
  
- ✓ The conditioning injury paradigm was used to identify novel mechanisms that might increase the intrinsic ability of neurons to regenerate. Previous work in the lab has shown that a conditioning injury is able to increase axonal transport of putative regeneration enhancers. Here, we aimed at validating two of these molecules as regeneration enhancers and at identifying the molecular mechanisms underlying such alterations (Chapter 4).



# Methods







## Materials and methods

### Animals

Wistar rats or B6 mice were handled according to European Union and National rules. All animals were maintained under a 12h light/dark cycle and fed with regular rodent's chow and tap water *ad libitum*. In order to perform the lesions described below, animals approximately 8 weeks old were used. Naïve animals were used as controls.

### Surgical procedures

**Sciatic nerve injury (bilateral transection):** wistar rats were anesthetized with a mixture of 75mg/kg of ketamine and 0,5mg/kg of medetomidine. B6 mice were anesthetized with a mixture 75mg/kg of ketamine and 1mg/kg of medetomidine. The skin was shaved, the sciatic nerve was exposed at mid-thigh and a crush was performed using Pean forceps, twice during 15 seconds. The animals were sutured and analgesia was performed for 48h following injury, with butorphenol, 1mg/kg twice a day. Animals were allowed to recover for 1 day or 1 week. L4-5 DRGs of wistar rats were then collected to either dry ice or formalin. DRG, dorsal root and sciatic nerve were collected to dry ice.

**Dorsal root injury:** wistar rats were anesthetized with a mixture of ketamine and medetomidine as described above. The skin was shaved, the spinal cord exposed at L2 vertebrae. The *dura matter* was removed. Under a microscope, a crush was performed on the L4, L5 and L6 dorsal roots using forceps twice during 15 seconds. Analgesia was performed 48h following injury, with butorphenol, 1mg/kg twice a day. Animals were allowed to recover 1 day. L4-5 DRGs were then collected to either dry ice or formalin.

**Spinal cord injury (hemisection):** animals were anesthetized with ketamine/medetomidine as mentioned above. Spinal cord hemisection at the dorsal thoracic (T7) level was performed with a microscissor. The postoperative treatment consisted of subcutaneous injection of butorphenol 1mg/kg twice a day for 72h (analgesia); daily subcutaneous injection of 3mL of Duphalyte for 72h (fluid therapy) and manual voiding of the bladder twice a day were performed during the time of the experiment. Wet food was placed in the cage floor and water with antibiotic (0,016% baytril) was supplied in long nipple bottles.

Animals were allowed to recover for 1 week. For conditioning animals, sciatic nerve injury was performed one week prior to SCI. Spinal cord was collected.

## ***1 – Injury signals in the CNS***

### **Preparation of protein extracts**

L4-5 DRGs were homogenized in 200 $\mu$ L of PBS (phosphate buffered saline) containing 0,3% triton X-100, protease inhibitors and 1mM orthovanadate. The samples were then sonicated, centrifuged 10 min at 15000g, at 4°C and the supernatant collected. Protein concentration was quantified by the Lowry method (Bio-Rad DC Protein Assay) and the measurements were carried out according to the manufacturer's instructions (Bio-Rad, Hercules, CA).

### **Western Blotting**

The protein lysates (25-50 $\mu$ g/lane) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10 or 12% acrylamide gel and transferred to nitrocellulose membranes (Amersham). Membranes were blocked for 1h at room temperature with blocking buffer (5% nonfat dried milk (Sigma) in tris-buffered saline (TBS) with 0,1% Tween 20) and incubated overnight at 4°C in 5% bovine serum albumin (BSA) in TBS with 0,1% Tween 20 with the primary antibodies described on Table I. After washing, membranes were incubated with the correspondent secondary antibodies (anti-rabbit HRP (Jackson Immunoresearch, 1:5000), anti-mouse HRP (Thermo Scientific, 1:5000) and anti-goat/sheep HRP (Binding Site, 1:20000)) for 1h at room temperature. Proteins were detected using a chemiluminescent substrate, (Pierce ECL Western Blotting Substrate (Thermo Scientific)). For each experiment representative Western blots are shown. Densitometry was performed with Quantity One software (Bio-Rad).

**Table I** - Antibodies used for western blotting (WB) and immunohistochemistry (IHC) assays

Antibody (source)	Company	Catalog number	WB Dilution used	IHC Dilution used	Antigen retrieval
$\beta$ -actin (mouse)	Sigma-Aldrich	A5441	1:5000	-	-
GSK3 $\alpha/\beta$ (mouse)	SCB	sc-7291	1:2000	-	-
GSK3 $\beta$ (mouse)	Cell Signaling	9315	-	1:100	citrate
Csk (mouse)	SCB	sc-166560	1:1000	1:50	citrate
Src (rabbit)	SAB	Ab-529	1:1000	1:200	citrate
Hsp40 (goat)	SCB	sc-1801	1:1000	1:50	citrate
MKK6 (rabbit)	Enzo Life Sciences	ADI-KAP-MA014	1:1000	1:200	citrate
Caspase 4 (rabbit)	Enzo Life Sciences	ADI-AAP-104	1:1000	-	-
Caspase 4 (rabbit)	Thermo Scientific	9990-0003	-	1:100	TE
Plk3 (rabbit)	ABGENT	APB044b	1:500	-	-
ROCK-II (rabbit)	Millipore	07-443	1:1000	1:100	citrate

**Paraffin inclusion:** The DRGs were fixed in formalin at 4°C. Formalin was replaced with alcohol 50%, and the tissue was dehydrated in graded alcohol following 10-16h incubation. Finally the tissue was cleared in toluene for 30 minutes, and then incubated in liquid paraffin overnight. The tissue was then embedded in paraffin and 4 $\mu$ m sections were performed in a microtome.

**Immunohistochemistry:** paraffin sections were deparaffinized, rehydrated through ethanol and for each antibody, the correct antigen retrieval was performed (Table I). Slides were then blocked with 5%FBS (fetal bovine serum) in TBS-tween for 1h at room temperature and then incubated overnight at 4°C with primary antibody. The appropriate secondary antibodies conjugated with alexa 488 were used (Invitrogen, 1:1000) and mounted with VECTASHIELD Mounting Medium with DAPI (Vector laboratories). Tissue sections without primary antibody were used as negative controls. 10x magnification pictures were obtained with Zeiss Axio Imager Z1. The percentage of positive DRG neurons was then determined using imageJ.

## ***2 – Axonal transport following conditioning injury***

**Kinesin immunoprecipitation:** Spinal cords of animals with or without conditioning injury were homogenized with PBS containing 0,3% triton X-100, protease inhibitors (GE Healthcare) and 1mM orthovanadate. The homogenate was centrifuged 10 min, 15000g at 4°C and the supernatant collected. Protein concentration was determined as described above. For immunoprecipitation, 500µg of protein extract was pre-cleared with protein G beads (GE Healthcare) by incubation for 1h at 4°C. The pre-cleared extracts were incubated with 10µg of antibody against kinesin heavy chain (Millipore) overnight at 4°C. Then, immunocomplexes were captured by incubating the extracts with protein G beads for 2h at room temperature. Beads were then washed 3 times and the immunocomplexes were disassembled from the beads with SDS (sodium dodecyl sulfate) sample loading buffer (50mM Tris-HCl pH 6.8, 2%SDS, 10 % glycerol, 1% β-mercaptoethanol, 12,5mM EDTA and 0,02% Bromophenol Blue). As negative control, immunoprecipitation was performed on spinal cord extracts using mouse IgG (Santa Cruz Biotechnology). The immunoprecipitation product and 25µg of input protein extract were separated on SDS gel and immunoblotted. Immunoblot against rabbit anti-14-3-3 (#8312, Cell Signaling, 1:1000) and rabbit anti-PGP9.5 (#7863-0504, AbD Serotec, 1:1000) were performed as described above.

### **Cell culture and silencing assay**

**Virus production:** HEK 293T (Human Embryonic Kidney 293T) cells were plated in 10cm dishes until 90% confluence. Cells were then transfected with a mixture of DNA (deoxyribonucleic acid): 6µg CRMP-5 shRNA plasmid (Sigma Aldrich), or PGP9.5 shRNA plasmid (Sigma Aldrich) or pLKO control plasmid (Sigma Aldrich) with 3µg pPAX packaging plasmid and 3µg VSVG envelope plasmid (a kind gift from Dr. João Relva) using polyjet (SignaGen Laboratories) at a ratio of 12µL polyjet to 15µg of plasmid DNA. The cells were incubated overnight. On the next day, the medium was replaced with DMEM (dulbecco's modified eagle medium) supplemented with glutamax, 15% FBS and 1% Penicillin/Streptomycin (Pen/Strep) and incubated for 48h. The supernatant with the produced virus was collected, filtered, aliquoted and stored at -80°C.

**Virus titration:** HEK 293T cells were plated on a 6-well plate so that the confluence at the time of transduction would be ~25-50% confluent. The cells were then infected with the produced virus (dilutions used: 1:1000, 1:10000 and no virus). The infection medium was replaced on the next day with fresh medium (DMEM supplemented with 10% FBS and 1%

Pen/Strep. At the third day, selection of the infected cells started with puromycin (0,5µg/mL-Millipore). Selection was performed for 4 days with medium exchange every two days. During selection, puromycin-resistant colonies formed (no live cells were found on the control well). The cells were then washed with PBS and the number of colonies counted to assess the number of infection units (IU).

**Primary cultures of dorsal root ganglia neurons:** DRG were collected aseptically from the vertebral column of 8 weeks old Wistar rats to DMEM/F12 (Sigma) supplemented with 1% Pen/Strep and 10% FBS.

In the flow cabinet, DRG were digested with 0,125% of collagenase IV-S (Sigma) for 2h at 37°C. After washing 3 times, a single cell suspension was obtained by trituration with fire-polished Pasteur pipettes of decreasing diameters until no clumps of cells were observed. Neurons were isolated placing the cell suspension on top of a 15% BSA cushion and centrifuged for 10 minutes at 1000g; interphase and supernatant were discarded. Cells were resuspended in DMEM/F12 supplemented with 50ng/mL of NGF (Millipore), 1% Pen/Strep, 1x B-27 (Invitrogen) and L-glutamine (2mM).

For coating, cover slides were incubated with Poly-L-Lysine at 20µg/mL in PBS for 1h at 37°C. Cover slides were washed and air dried until no solution was visible. Then laminin at 5µg/mL in PBS was added for 30 minutes at 37°C.

Cells were plated on coated cover slides and maintained in an incubator with 5% CO<sub>2</sub> at 37°C.

**Silencing assay:** 24h after performing DRG neuron cell culture, the cells were infected with lentivirus for empty vector (control), shRNA for PGP9.5 or shRNA for CRMP5 (5000 IU/well). Following 16h, infection was terminated and the medium was changed. The cells were allowed to recover for 24h and then were treated with puromycin (5µg/mL) for 2 days. After treatment cells were either replated for neurite outgrowth assay or collected to assess knock down efficiency.

To assess knockdown efficiency, cells were collected using Qiazol reagent (Qiagen), total RNA was extracted using the RNeasy lipid tissue kit (Qiagen) and cDNA was synthesized by reverse transcriptase with SuperscriptII (Invitrogen) using 1µg of RNA. qRT-PCR was performed using iQ supermix (Bio-Rad) and specific primers for each of the genes under analysis designed with Beacon designer.

For neurite outgrowth assays, cells were replated in DMEM:F12 with Pen/Strep, L-glutamine, B27, NGF and puromycin (5µg/mL), following trypsin treatment (3 min at 37°C). 12h after replating the cells were washed and fixed with 4% paraformaldehyde (PFA) for 15min. Cells were then permeabilized with 0.2% Triton X-100, blocked in blocking buffer (5% fetal bovine serum, 0.4% Tween-20 in PBS) for 1h at room temperature and incubated with the primary antibody anti-β-tubulin III (1:2000, Promega) in blocking buffer overnight at 4°C. The cells were later incubated with anti-mouse Alexa Fluor 488 IgG (1:1000, Invitrogen) in blocking buffer for 1h at room temperature and finally mounted with Vectashield with DAPI. Neurite outgrowth measurement was carried out from 10x magnified fields using Neuron J plugin for Image J, a public domain JAVA image-processing program. At least 100 cells were counted per condition.

**Western blot analysis:** dorsal roots of animals 1 day and 1 week following sciatic nerve injury were collected, homogenized and protein concentration was determined as described above. Naïve animals were used as control. Proteins were separated in SDS-PAGE and immunoblotted against kinesin (MAB1614, Millipore, 1:500), dynein (MAB1618, Millipore, 1:250), acetylated tubulin (cat no. T7451, Sigma, 1:5000), α-tubulin (cat no. T6199, Sigma, (1:1000), HDAC6 (#7612, Cell Signaling, 1:500), tyrosinated tubulin (MCA77G, Arium, 1:2000), anti-polyglutamylated tubulin (GT335, AdipoGen, 1:4000), HPRT (sc-20975, Santa Cruz Biotechnology, 1:1000), and desphosphorylated KLC (kindly given by Dr. Scott Brady from the University of Illinois, Chicago; 1:500).

**Real-time PCR (polymerase chain reaction):** L4-5 DRG of animals 1 day and 1 week following sciatic nerve injury were collected. Naïve animals were used as controls, total RNA was extracted using the RNeasy lipid tissue kit (Qiagen) and cDNA (complementar DNA) was synthesized by reverse transcriptase with SuperscriptII (Invitrogen) using 1µg of RNA. qRT-PCR was performed using iQ supermix (Bio-Rad) and specific primers for each of the genes under analysis: HPRT (sense: 5'-ATGGACTGATTATGGACAGGACTG-3' and antisense: 5'-GCAGGTCAGCAAAGAACTTATAGC-3'), kif5b (sense: 5'-GGAACAGGAGCGGCTAAGG-3' and antisense: GCCACCGTCTCTTCCAACC) and dynein (sense: 5'-CTGGTGGTCGTGGGCTACTATG-3' and antisense: 5'-GTTGTGGTCCATGTCATCCTTCTG-3') designed with Beacon designer.

### ***Statistical analysis***

The results are presented as mean  $\pm$  SEM of the indicated number of experiments. Statistical significance was determined using the student *t*-test and one-way ANOVA test for multiple comparisons, followed by the posthoc Tukey-Kramer test (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ).





# Results & discussion





## **Results and discussion**

### **1 – Injury signals elicited following injury**

Injuries to each of the DRG branches elicit different regeneration abilities; while an injury to the peripheral branch increases the intrinsic ability of DRG neurons to regenerate, an injury to the central branch (dorsal root or spinal cord injury) does not have a similar effect (Smith and Skene, 1997). The injury signals described in the literature were identified following PNS injury, being the sciatic nerve injury the most commonly used model. Using the crush-ligation paradigm where a ligation is performed proximal to the cell body to trap the retrogradely transported injury signals, data from our lab showed, ERK, JNK and STAT3 are activated in a similar fashion both after a peripheral and a central lesion. Overall this data suggest that canonical injury signals are not responsible for the robust increase in the regeneration ability triggered by a peripheral injury. So, which signals differ between sciatic nerve and dorsal root injury that explain the different responses? To answer this question a screening was performed to identify possible differences between the two injury types. To do so, axoplasm of sciatic nerve and dorsal root was collected 8h following injury (either sciatic nerve or dorsal root injury) and dynein immunoprecipitation was performed. The product of the immunoprecipitation was analyzed by Kinex antibody microarray (Kinexus) that tracks the differential binding of dye-labeled proteins in lysates, providing insights into differences in protein expression, phosphorylation and protein-protein interactions (Bioinformatics, 2012). From the final analysis that induced a validation of the array data by western blot (also performed as a service by Kinexus), 8 candidates were validated in the axoplasm as being differentially regulated following sciatic nerve injury when compared to dorsal root injury (Table II). This data was available when I joined the group to perform the work related to this thesis. We went on to further test which of these proteins was differentially transported to the cell bodies. Western blot and immunohistochemistry for the chosen candidates were performed in DRG following, either sciatic nerve or dorsal root injury. With this procedure we aimed at further validating the candidates as injury signals.

**Table II-** Axoplasm proteins differentially regulated as assessed by Kinexus analysis after dorsal root injury (DRI) when compared to sciatic nerve injury (SNI)

Candidates	Kinexus array data	WB data
	dorsal root/ sciatic nerve (z-ratio)	dorsal root/sciatic nerve (fold change)
Heat shock protein 40 (Hsp40)	1,40	1,4
Polo-like kinase 3 (Plk3)	1,36	2,2
Rho-associated, coiled-coil containing protein kinase 2 (RockII)	1,18	1,3
Mitogen-activated protein kinase 6 (MEK6)	1,11	3,0
c-Src tyrosine kinase (Csk)	1,05	1,9
Glycogen synthase kinase 3 beta (GSK3 $\beta$ )	1,04	3,2
Proto-oncogene tyrosine-protein kinase (Src)	-1,18	0,6
Caspase 4	-1,20	0,7

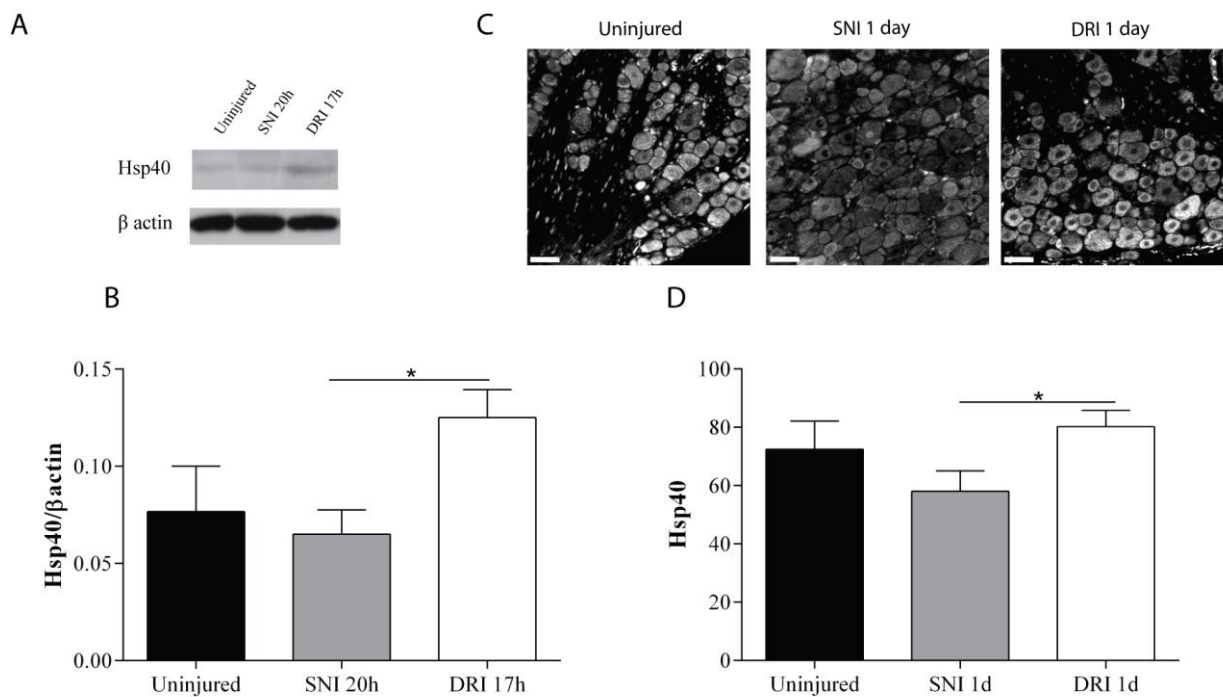
**z-ratio:** ratio between the z-scores (transformation approach that corrects data internally within a single hybridization; hybridization values for individual proteins are expressed as a unit of standard deviation from the normalized mean) (Cheadle et al., 2003)

The quantitative analysis that Kinexus send us, gave us a row with the z-ratio, the ratio between the z-scores of the two samples (dorsal root and sciatic nerve) and another row with the western blot analysis were each sample is normalized for PGP9.5, and a ratio between dorsal root and sciatic nerve was created, given us a % change from the control sample.

Of the 8 candidates tested, only GSK3 $\beta$ , Hsp40 and RockII were validated as being significantly increased in the DRG both by western blot and by immunohistochemistry after dorsal root injury when compared to sciatic nerve injury. Details are provided below.

**Hsp40**, also known as DNAJ, is a member of the heat shock protein family responsible for the response to stress and heat shock. This cytosolic protein is widely expressed in all cell types, including neurons, and its response when heat shock occurs, involves increase of the expression levels and translocation from the cytosol to the nucleus (Manzerra and Brown, 1996; Chen et al., 2005; Setler et al., 2010). In order to facilitate protein folding, this co-chaperon binds to Hsp70, regulating Hsp70 ATPase activity. Hsp40 can also bind to a misfolded substrate and is able to initiate refolding and prevent aggregation with or without Hsp70 (Hartl and Martin, 1995; Jana et al., 2000). High levels of the protein seem to be

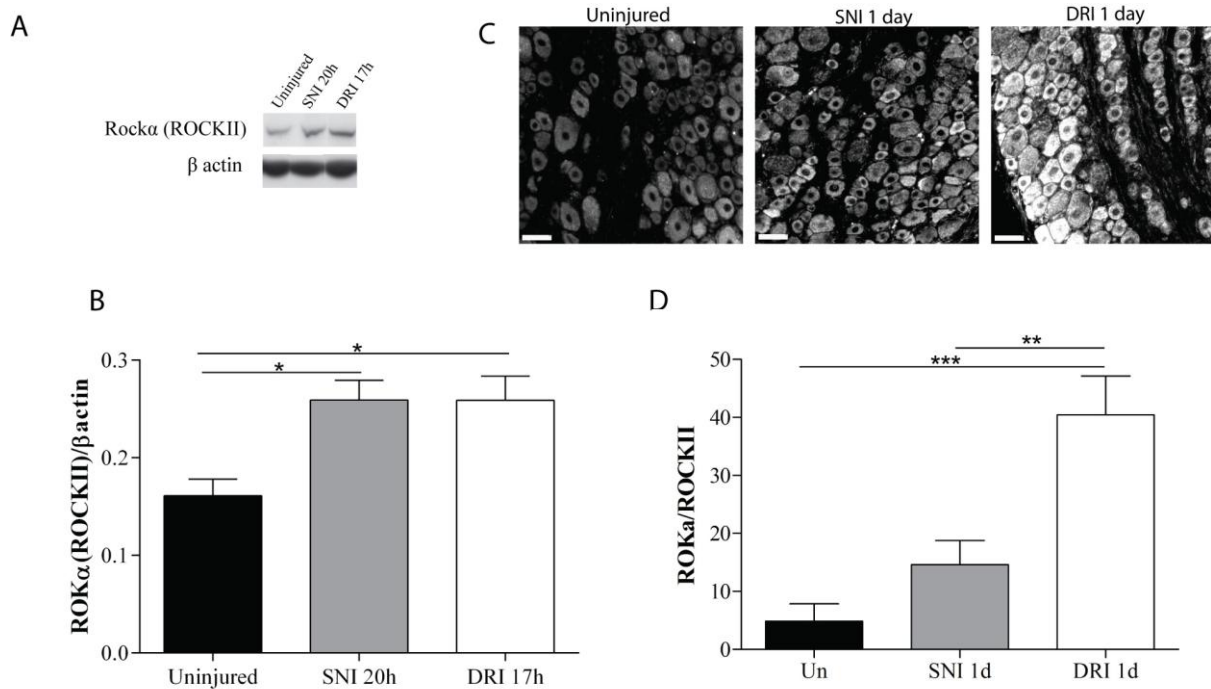
involved on the prevention of neurodegenerative disorders and on the activation of the ubiquitin-proteasome system due to its ubiquitin-interacting motif (Howarth et al., 2007). We were able to confirm that a dorsal root injury increases the levels of Hsp40, in the DRG by both western blot (figure 15 A and B) and immunohistochemistry (figure 15 C and D). These results suggest that Hsp40 may be acting as a regeneration inhibitor, i.e., that increased levels following dorsal root injury might contribute to decreased axonal regeneration. The role of Hsp40 in axonal growth should be further addressed. In this respect we are currently testing neurite outgrowth in DRG neurons following Hsp40 silencing by shRNA.



**Figure 15 – Hsp40 is differentially regulated following dorsal root injury.** (A) western blot of Hsp40 on DRGs from animals either uninjured, 1day following sciatic nerve injury (SNI 20h) or 1day after dorsal root injury (DRI 17h). Representative images are shown; (B) quantification of A. (C) representative images of Hsp40 immunostaining of DRGs either uninjured, 1 day after sciatic nerve injury or 1 day after dorsal root injury; (D) quantification of C. Scale bar, 77μm. \*p<0,05.

**RockII** is a protein of the family Rho-associated coiled-coil-containing protein kinases mainly found in the brain and heart tissue (Leung et al., 1995; Schmandke et al., 2007). ROCKs are known to mediate the control of the actin cytoskeleton by Rho family GTPases in response to extracellular targets (Nagumo et al., 2001; Yamazaki et al., 2002; Amano et al., 2003). Also, the upstream activator of Rock, Rhoa, has been shown to be involved on growth cone collapse through activation by myelin-based inhibitory signals, leading to cofilin phosphorylation that

stabilizes the growth cone of damaged axons and restricts regeneration outgrowth (Yiu and He, 2006). Pharmacological inhibitors of ROCK are able to enhance neurite outgrowth and increase regeneration in CNS neurons (Alabed et al., 2006; Lingor et al., 2007). By western blot (figure 16 A and B) we were not able to see an increase in RockII after dorsal root injury, when compared to sciatic nerve injury. However, by immunohistochemistry (figure 16 C and D), a significant increase of RockII in DRG neurons was found 1 day after dorsal root injury when compared to sciatic nerve injury. We did not observe significant differences by western blot probably because this technique analyses all cell types that compose the DRG, whereas by immunohistochemistry we specifically analyzed the DRG neurons. Overall our data suggests that increase in RockII following dorsal root injury may contribute to lack of axonal regeneration which is in accordance with the fact that inhibition of Rock increases axonal growth following injury (Alabed et al., 2006; Lingor et al., 2007).

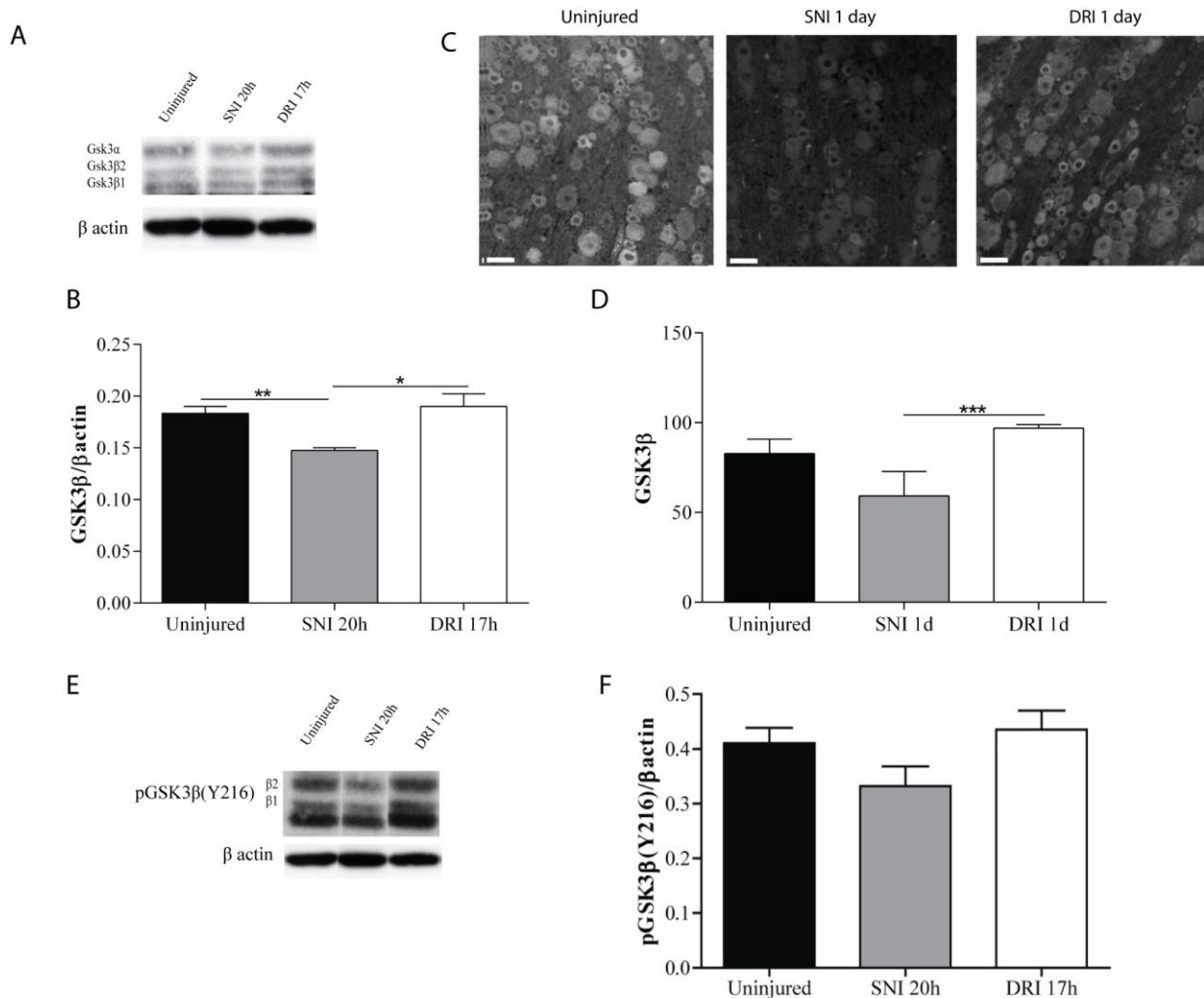


**Figure 16 - RockII is differentially regulated following dorsal root injury.** (A) western blot of RockII on DRGs from animals either uninjured, 1 day following sciatic nerve injury (SNI 20h) or 1 day after dorsal root injury (DRI 17h). Representative images are shown; (B) quantification of A. (C) representative images of the RockII immunostaining of DRGs either uninjured, 1 day after sciatic nerve injury or 1 day after dorsal root injury; (D) quantification of (C). Scale bar, 77μm. \*p<0,05; \*\*p<0,01; \*\*\*p<0,001.

GSK3β is a serine/threonine kinase known to have an important role in axonal regeneration by local regulation of the cytoskeletal dynamics of the growth cone (Polleux and

Snider, 2010). By western blot analysis we observed that following sciatic nerve injury, total GSK3 $\beta$  present in DRG was decreased (figure 17 A and B) when compared to DRG neurons collected from animals with dorsal root injury. A similar finding was observed by immunohistochemistry (figure 17 C and D). Previous results from our lab have demonstrated that GSK3 $\beta$  has a reduced activity in DRG neurons when regeneration occurs (Liz MA et al; submitted). This was seen in animal models with neuronal deletion of GSK3 $\beta$  where increased neurite outgrowth and increased microtubule growth speed was observed in vitro and increased axonal regeneration following SCI was detected in vivo. GSK3 $\beta$  has 2 phosphorylation sites that regulate its activity. Phosphorylation of residue tyrosine 216 promotes GSK3 $\beta$  activity, while serine 9 phosphorylation inhibits it (Haar et al., 2001). Decreased levels of GSK3 $\beta$  phosphorylated at tyrosine 216 were observed following sciatic nerve injury when compared to sciatic nerve injury (figure 17 E and F), suggesting a decrease in kinase activity. This data is in accordance with additional unpublished data from our lab, showing that in regenerating axons, GSK3 $\beta$  activity is reduced by decreased phosphorylation of tyrosine 216. Recent advances on the behavior of GSK3 $\beta$  during axonal regeneration, have shown that the efficient axonal elongation only occurs when partial inhibition of GSK3 $\beta$  in the growth cone is observed. The control of axonal growth seems to be related to the downstream regulation of GSK3 $\beta$  substrates, CRMP-2, MAP1B, and cytoplasmic linker protein-associated protein (CLASPs), that require partial inhibition of GSK3 $\beta$  to promote axonal growth and regeneration (Alabed et al., 2010; Hur and Zhou, 2010).

In summary, decrease in GSK3 $\beta$  activity following sciatic nerve injury might function as signal leading to increased axonal regeneration.



**Figure 17 – GSK3β is differentially regulated following sciatic nerve injury.** (A) western blot of GSK3β on DRGs from animals either uninjured, 1day following sciatic nerve injury (SNI 20h) or 1day after dorsal root injury (DRI 17h). Representative images are shown; (B) quantification of A; (C) Representative images of GSK3β immunostaining of DRGs either uninjured, 1 day after sciatic nerve injury or 1 day after dorsal root injury; (D) quantification of C. Scale bar, 77μm. (E) western blot of pGSK3β(Y216) on DRG from animals either uninjured, 1 day following sciatic nerve injury (SNI 20h) or 1 day after dorsal root injury (DRI 17h); (F) quantification of (E). \*p<0,05; \*\*p<0,01

For the remaining axoplasm proteins differentially regulated as assessed by Kinexus analysis (Plk3, MEK6, Csk, Src and caspase-4), similar studies were performed by western blot analysis and immunohistochemistry on DRG to the ones described for Hsp40, RockII and GSK3β. We were however unable to find differences in their levels in DRG from animals after either sciatic nerve injury or dorsal root injury. Although these data do not rule out their importance in controlling regeneration, future studies in our group will mainly focus those where differences have been found namely Hsp40, RockII and GSK3β.

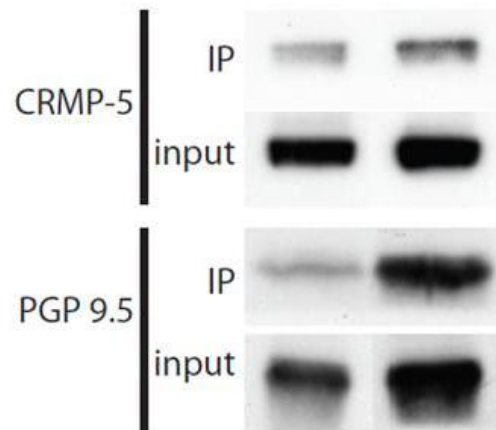


## **2 – Regulation of axonal transport following conditioning injury**

To dissect the mechanisms underlying the increase in the regeneration capacity produced by the conditioning model, we aimed at identifying regeneration enhancers synthesized in the DRG and anterogradely transported to the SCI site following a priming lesion. To do so, prior to my arrival to the Nerve Regeneration Group, the proteins newly synthesized following conditioning injury and anterogradely transported to the spinal cord injury site were tracked through the injection of radiolabeled amino acids in the DRG followed by collection of spinal cord segments for 2D analysis combined to mass spectrometry. Increase in radiolabeled proteins transported to the spinal cord of animals with a conditioning injury was found, suggesting that a conditioning injury increases protein expression and/or transport to the injury site. Many of the proteins identified were glycolytic enzymes but other interesting candidates arised such as collapsin response mediator protein-5 (CRMP-5) and protein gene product 9.5 (PGP9.5).

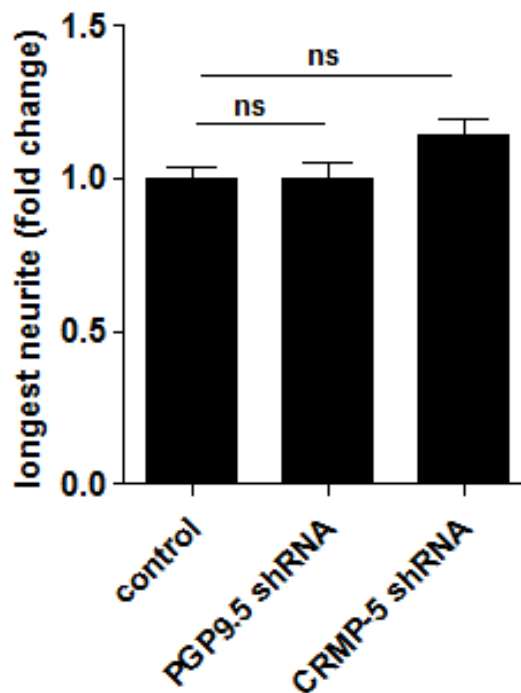
### **2.1 – Evaluate the role of PGP9.5 and CRMP-5 as possible new regeneration enhancers**

To validate the importance of PGP9.5 and CRMP-5 in axonal regeneration, we started by confirming that these proteins are anterogradely transported following conditioning injury. Kinesin immunoprecipitation was performed on spinal cord samples of animals with SCI with or without a previous conditioning lesion. The amount of PGP9.5 and CRMP-5 bound to kinesin was assessed by western blot. For both PGP9.5 and CRMP-5, increased levels (2,8-fold and 1,3-fold, respectively) were found bound to kinesin (figure 18) after conditioning injury, suggesting increased anterograde transport.



**Figure 18 – CRMP-5 and PGP9.5 are differentially regulated following conditioning lesion.** Spinal cords from animals with SCI or conditioning lesion were collected and kinesin immunoprecipitation was performed using 500 $\mu$ g of protein extract and western blot against CRMP-5 and PGP9.5 was performed.

To further validate CRMP5 and PGP9.5 as regeneration enhancers, their expression in DRG neurons was silenced by transduction using lentivirus expressing specific shRNAs. qPCR analysis showed that the knock down efficiency was 60% for PGP9.5 and 95% for CRMP-5. However, in both cases no alterations in neurite outgrowth was observed (figure 19), suggesting that PGP9.5 and CRMP-5 are probably not pivotal for axonal growth.



**Figure 19 – Silencing PGP9.5 or CRMP-5 did not produce alterations in neurite outgrowth.** DRG neuron cultures were infected with lentiviruses expressing either PGP9.5 or CRMP-5 shRNA. DRG neurons were maintained for 3 days and then replated. Neurite outgrowth was determined by measuring the longest neurite after immunostaining with  $\beta$ III-tubulin.

PGP 9.5 is a neuron and neuro-endocrine specific member of the ubiquitin hydrolase family of proteins highly present in all differentiated neurons throughout the CNS and PNS at all stages of development (Schofield et al., 1995; Kalbermatten et al., 2008). During regeneration, there is a striking increase of PGP9.5 expression (Yin et al., 2010). Whether this protein is really essential for axonal growth to occur should be further investigated.

CRMP-5 is a cytosolic phosphoprotein highly expressed in the developing brain, but decreased in adulthood. Its involvement on the regulation of microtubule polymerization, actin binding and endocytosis make it a player during neuronal differentiation and axonal growth (Fukada et al., 2000; Arimura and Kaibuchi, 2007). CRMP-5 has been described as having an inhibitory function in neurite outgrowth, since it inhibits tubulin polymerization by forming a complex with tubulin heterodimers and MAP2, locking the microtubular structure (Brot et al., 2010). In our setup, CRMP-5 appears as a possible regeneration enhancer. This hypothesis should be further assessed in the future. It is possible that an effect in neurite outgrowth, either

inhibitory or stimulatory, might only be observed when neurons are grown in a non-permissive environment such as myelin.

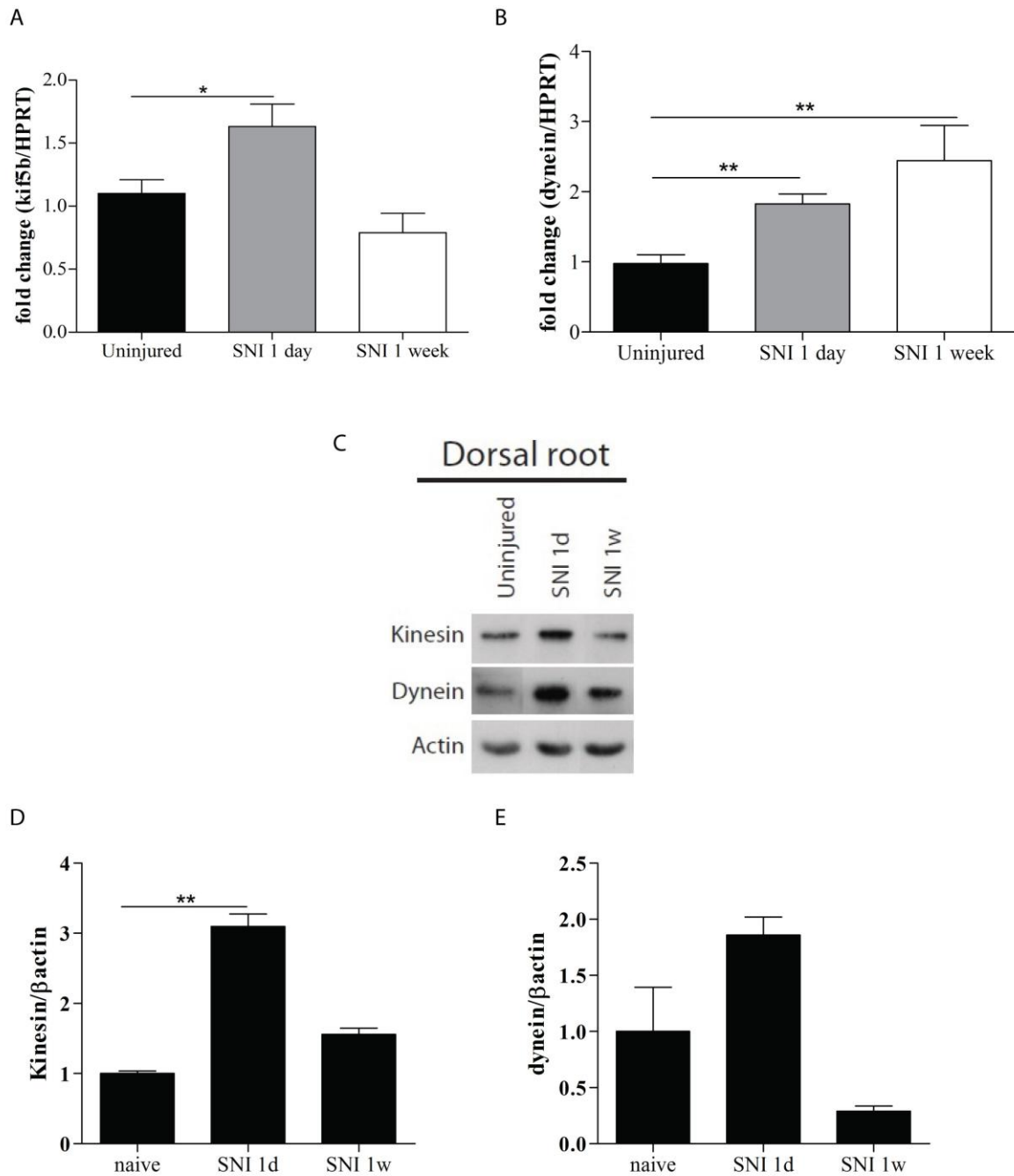
In summary, we show that following conditioning lesion binding of CRMP-5 and PGP9.5 to kinesin is increased. Additional data from our lab further supports a generalized increase in transport following conditioning lesion. Increased transport of mitochondria and synaptic vesicles was detected in the dorsal root after injury to the peripheral root. The mechanism underlying this effect was evaluated as is described next.

## **2.2 – Evaluation of the mechanism leading to increased axonal transport following a conditioning lesion**

The mechanisms that lead to increased axonal transport following conditioning injury are still unclear. Two main hypotheses can be proposed: alterations in molecular motors (kinesin and dynein) or alterations in the railroad, namely tubulin post-translational modifications.

We started by checking if the molecular motors kinesin and dynein were altered following a conditioning lesion. To assess this hypothesis, we started by evaluating their expression by qPCR in DRG. Kinesin expression was increased 1 day after sciatic nerve injury, but that increase was not sustained overtime (figure 20A). On the other hand, dynein showed increased expression in the DRG at both 1 day and 1 week after sciatic nerve injury (figure 20A). It is known that when injury occurs, kinesin allows the transport of several proteins to promote regeneration (Tashiro and Komiya, 1992; Jacob and McQuarrie, 1996; Siegel, 2006). Our results show that there is a transient increase in the expression of kinesin, which is consistent with the need of transporting proteins necessary for axonal growth. The accumulation of dynein at the lesion site has been previously reported as increasing with time (Li et al., 2000). This data is consistent with our results.

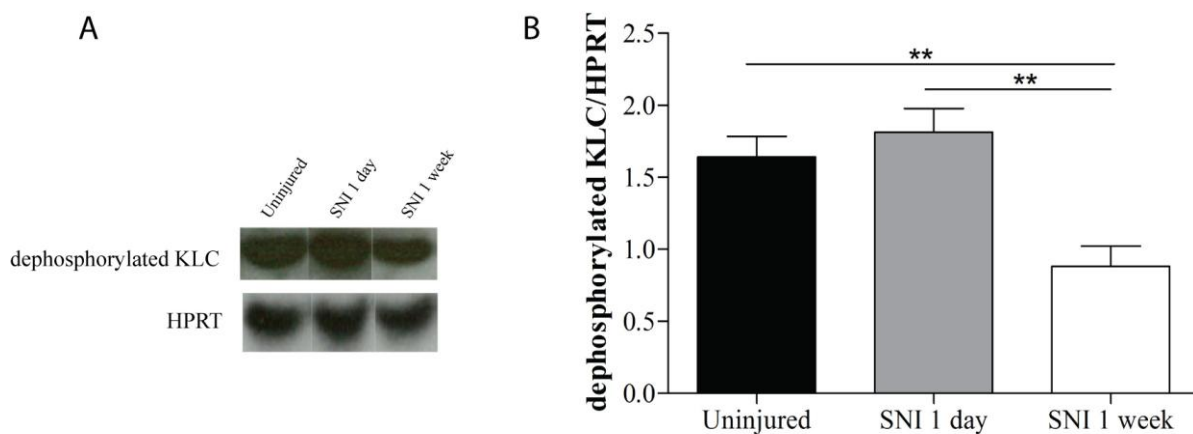
Levels of motors were additionally evaluated by western blot on the dorsal root of DRG. There was a peak of both kinesin (figure 20 C and D) and dynein (figure 20 C and E) 1 day after SNI. In summary, an early increase in motors was found. However, this increase in motors *per se* is not sufficient to explain the robust alterations in axonal transport, sustained overtime, observed in our lab (Mar FM, unpublished).



**Figure 20 – Kinesin and dynein are differentially regulated in the central branch following a conditioning lesion.** L4 and L5 DRGs from animals either uninjured, 1 day or 1 week following sciatic nerve injury were collected. RNA was extracted and cDNA was synthesized by reverse transcriptase using 1 µg of RNA. qPCR was performed for kif5 (A) or dynein (B); dorsal root of animals 1 day and 1 week following sciatic nerve injury were collected and kinesin and dynein western blot was performed (C); quantification of kinesin in C (D); quantification of dynein in C (E).

Kinesin has 2 light chains that although not essential for motor function, play a role in cargo-binding and motor regulation. Phosphorylation of KLCs regulates kinesin-mediated axonal transport by detachment of kinesin from cargo and inhibition of transport (Morfini et al.,

2002). This mechanism could underlie the increase in axonal transport observed after conditioning lesion. As such, dorsal roots collected from animals 1 day and 1 week following sciatic nerve injury were examined by western blot to assess desphosphorylation of KLCs. Our results show that desphosphorylated KLC is decreased in dorsal roots collected 1 week after sciatic nerve injury (figure 21 A and B). Given these results, a mechanism through which increased transport might result from decreased phosphorylated KLC levels was ruled out.



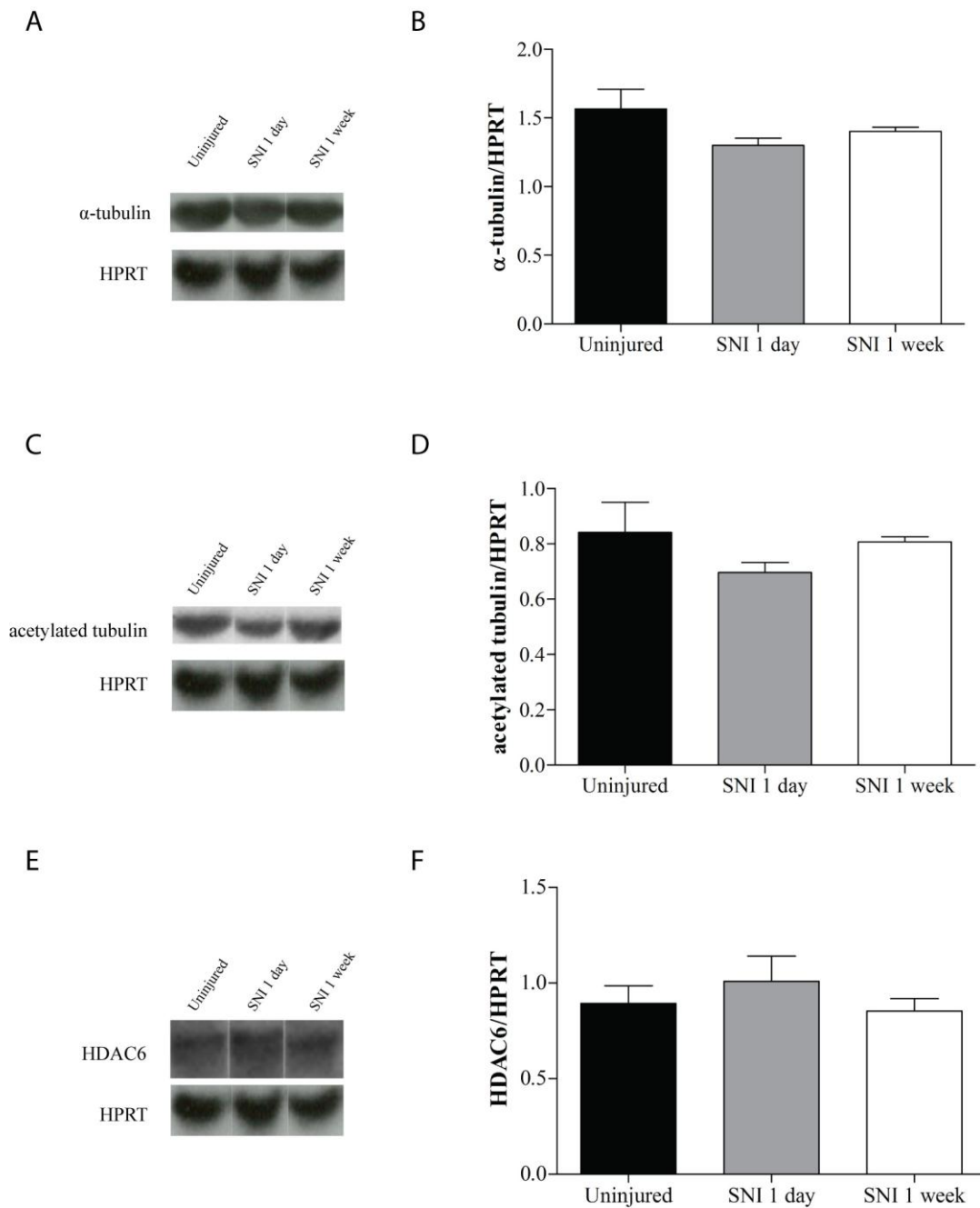
**Figure 21 – Kinesin light chain desphosphorylation decreased following sciatic nerve injury.** (A) western blot of dephosphorylated KLC on dorsal roots from animals either uninjured, 1 day and 1 week following sciatic nerve injury. Representative images are shown; (B) quantification of A. \*\* $p < 0,01$

In summary, the levels of motors in the central branch are altered by a conditioning injury in the peripheral branch, with increase being observed specially at early time points post-lesion. Nevertheless, these early alterations in the levels of motors are not sufficient to underlie the robust increase in axonal transport overtime that is seen after conditioning lesion. As such, tubulin modifications were subsequently assessed.

Tubulin is one of the cytoskeleton constituents that in mature neurons acts as a “railway” for cargo transport. Binding of motors to tubulin can be modulated by tubulin modifications (Reed et al., 2006; Lakämper and Meyhöfer, 2006; Ikegami et al., 2007; Chen et al., 2010). To assess whether a conditioning lesion alters microtubule composition in the central branch, dorsal roots of animals 1 day and 1 week following sciatic nerve injury were collected and western blots were performed for some of the post-translational modifications of tubulin.

The total  $\alpha$ - tubulin levels in the dorsal root had no differences between uninjured animals and animals with a sciatic nerve lesion (figure 22 A and B).

Inhibition of histone deacetylases have been shown to have a beneficial effect in animal models of neurodegenerative disorders, since they exert neuroprotection by increasing the  $\alpha$ -tubulin acetylation levels and consequently improving the axonal transport (d'Ydewalle et al., 2011; Simões-Pires et al., 2013). Also, HDAC6 has been implied in regulating the traffic of intracellular cargoes either by binding to molecular motors, like kinesin, or by altering the acetylation state of the microtubules (Chen et al., 2010; d'Ydewalle et al., 2012). A mechanism involving HDAC and tubulin acetylation was thought as a possible action for explain the increase in axonal transport. However, we did not observe any increase on acetylated tubulin upon injury and the levels of HDAC6 also remained unaltered (figure 22 E and F). As such, increase in acetylation and concomitant decrease of deacetylation is not the mechanism by which the increase in transport is obtained after conditioning lesion, and other modifications might influence this alteration.

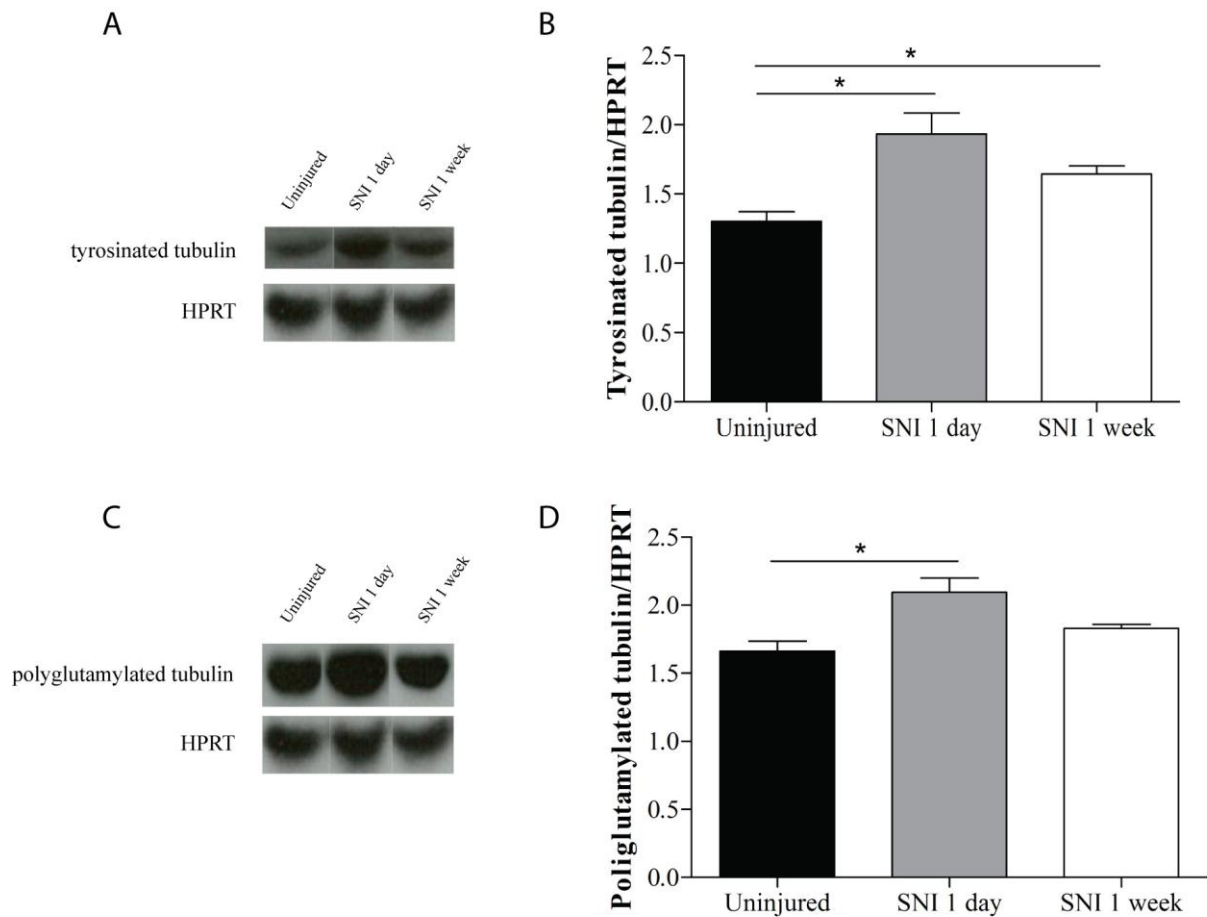


**Figure 22 –  $\alpha$ -tubulin and tubulin acetylation/deacetylation are not differentially regulated following sciatic nerve injury.** (A) Western blot of  $\alpha$ -tubulin on dorsal roots from animals either uninjured, 1 day and 1 week following sciatic nerve injury (SNI). Representative images are shown; (B) Quantification of A.; (C) Western blot of acetylated tubulin on dorsal roots from animals either uninjured, 1 day and 1 week following sciatic nerve injury (SNI). Representative images are shown; (D) Quantification of A.; (E) Western blot of HDAC6 on dorsal roots from animals either uninjured, 1 day and 1 week following sciatic nerve injury. Representative images are shown; (F) Quantification of A. \* $p < 0,05$



We continued to test other tubulin modifications, and the next was tyrosinated tubulin which is present in highly dynamic structures. Our results show a significant increase of tyrosinated tubulin on dorsal roots collected 1 day after sciatic nerve injury (figure 23 A and B), which suggest an increase of microtubule dynamics in the central branch after peripheral injury. This is in agreement with an increase of tyrosinated  $\alpha$ -tubulin described in the peripheral branch after a sciatic nerve injury (i.e., in regenerating axons) (Mullins et al., 1994).

Polyglutamylation is a modification that plays an important role in neural development and is a positive signal that increases the binding affinity of MAPs, including molecular motors (particularly KIF1, KIF5 and cytoplasmic dynein) (Boucher et al., 1994; Bonnet et al., 2001; Ikegami et al., 2006; Fukushima et al., 2009). We observed an early increase in glutamylated tubulin on the dorsal root 1 week after sciatic nerve injury, that was not sustained over time (figure 23 C and D). As such, this modification may induce an early increase in the binding of motors that however cannot underlie the sustained increase in axonal transport following a conditioning lesion, observed by our group.



**Figure 23 - Tubulin tyrosination and polyglutamylation are increased following sciatic nerve injury.** (A) western blot of tyrosinated tubulin on dorsal roots from animals either uninjured, 1 day and 1 week following sciatic nerve injury (SNI). Representative images are shown; (B) quantification of A.; (C) western blot of polyglutamylated tubulin on dorsal roots from animals either uninjured, 1 day and 1 week following sciatic nerve injury (SNI). Representative images are shown; (D) quantification of A. \* $p < 0,05$

Overall, the mechanism responsible for the increase in axonal transport following conditioning lesion remains elusive and could not be related to alterations in motors nor to increased tubulin acetylation.

# Concluding remarks

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## Concluding remarks

The first part of the work aimed at the identification of the injury signals that might explain the different responses to injury of the peripheral and central branch of DRG neurons. By comparison between sciatic nerve and dorsal root injury, I have identified and validated three injury signals that are increased following dorsal root injury, GSK3 $\beta$ , Hsp40 and RockII. Regarding GSK3 $\beta$  and Rock II, these have been established in different paradigms as molecules that should be inhibited in the settings of injury and our work further supports this hypothesis. We know that Hsp40 is also a good candidate as a regeneration inhibitor, but further *in vitro* and *in vivo* studies must be performed in order to consolidate this hypothesis.

The second part of the work focused on the identification of regeneration enhancers and of the mechanisms underlying the increase of axonal transport following conditioning injury. From all the candidates identified using the conditioning injury model, we tested PGP9.5 and CRMP-5, nevertheless, none of these candidates showed to promote regeneration *in vitro*. To clarify their role during neurite outgrowth, knockdown of both molecules should be performed in an inhibitory environment mimicking a CNS injury (for instance in the presence of myelin). The increase of axonal transport in regenerating axons could not be explained by the early alterations observed in both molecular motors, kinesin and dynein, as these were not sustained over time. Also, tubulin acetylation was shown to have no relation to the increase of axonal transport, but tyrosinated tubulin levels were increased in regenerating axons and further studies should be performed to investigate the role of this modification in the context of axonal transport.



# References







## References

Abe N, Cavalli V (2008) Nerve injury signaling. *Current opinion in neurobiology* 18:276–283 Available at: <http://www.sciencedirect.com/science/article/pii/S0959438808000573> [Accessed March 7, 2013].

Alabed YZ, Grados-Munro E, Ferraro GB, Hsieh SH-K, Fournier AE (2006) Neuronal responses to myelin are mediated by rho kinase. *Journal of Neurochemistry* 96:1616–1625 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/16441511>.

Alabed YZ, Pool M, Ong Tone S, Sutherland C, Fournier AE (2010) GSK3 beta regulates myelin-dependent axon outgrowth inhibition through CRMP4. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 30:5635–5643 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/20410116> [Accessed March 6, 2013].

Al-Majed AA, Tam SL, Gordon T (2004) Electrical stimulation accelerates and enhances expression of regeneration-associated genes in regenerating rat femoral motoneurons. *Cellular and Molecular Neurobiology* 24:379–402 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/15206821>.

Alvarez J, Giuditta A, Koenig E (2000) Protein synthesis in axons and terminals: significance for maintenance, plasticity and regulation of phenotype. With a critique of slow transport theory. *Progress in Neurobiology* 62:1–62 Available at: <http://linkinghub.elsevier.com/retrieve/pii/S0301008299000623>.

Amano M, Kaneko T, Maeda A, Nakayama M, Ito M, Yamauchi T, Goto H, Fukata Y, Oshiro N, Shinohara A, Iwamatsu A, Kaibuchi K (2003) Identification of Tau and MAP2 as novel substrates of Rho-kinase and myosin phosphatase. *Journal of Neurochemistry* 87:780–790 Available at: <http://doi.wiley.com/10.1046/j.1471-4159.2003.02054.x>.

Ambros R, Walters E (1996) Priming events and retrograde injury signals. *Molecular Neurobiology* 13:61–79 Available at: <http://dx.doi.org/10.1007/BF02740752>.

Ambros RT, Schmied R, Huang CC, Smedman M (1992) A signal sequence mediates the retrograde transport of proteins from the axon periphery to the cell body and then into the

nucleus. *Journal of Neuroscience* 12:2813–2818 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/1377237>.

Arimura N, Kaibuchi K (2007) Neuronal polarity: from extracellular signals to intracellular mechanisms. *Nature Reviews Neuroscience* 8:194–205 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/17311006>.

Ben-Yaakov K, Dagan SY, Segal-Ruder Y, Shalem O, Vuppalanchi D, Willis DE, Yudin D, Rishal I, Rother F, Bader M, Blesch A, Pilpel Y, Twiss JL, Fainzilber M (2012) Axonal transcription factors signal retrogradely in lesioned peripheral nerve. *The EMBO journal* 31:1350–1363 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22246183> [Accessed March 23, 2012].

Bioinformatics K (2012) Kinex™ Antibody Microarray Services. Available at: [http://www.kinexus.ca/ourServices/microarrays/antibody\\_microarrays/details/](http://www.kinexus.ca/ourServices/microarrays/antibody_microarrays/details/) [Accessed July 26, 2013].

Bonnet C, Boucher D, Lazereg S, Pedrotti B, Islam K, Denoulet P, Larcher JC (2001) Differential Binding Regulation of Microtubule-associated Proteins MAP1A, MAP1B, and MAP2 by Tubulin Polyglutamylation. *Journal of Biological Chemistry* 276 :12839–12848 Available at: <http://www.jbc.org/content/276/16/12839.abstract>.

Boucher D, Larcher J-C, Gros F, Denoulet P (1994) Polyglutamylation of Tubulin as a Progressive Regulator of in Vitro Interactions between the Microtubule-Associated Protein Tau and Tubulin. *Biochemistry* 33:12471–12477 Available at: <http://dx.doi.org/10.1021/bi00207a014>.

Bradbury EJ, McMahon SB, Ramer MS (2000) Keeping in touch: sensory neurone regeneration in the CNS. *Trends in Pharmacological Sciences* 21:389–394 Available at: <http://www.sciencedirect.com/science/article/pii/S0165614700015364>.

Bridgman PC (2004) Myosin-dependent transport in neurons. *Journal of Neurobiology* 58:164–174 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/14704949>.

Brot S, Rogemond V, Perrot V, Chounlamountri N, Auger C, Honnorat J, Moradi-Améli M (2010) CRMP5 interacts with tubulin to inhibit neurite outgrowth, thereby modulating the

function of CRMP2. *Journal of Neuroscience* 30:10639–10654 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/20702696>.

Brown A (2003) Axonal transport of membranous and nonmembranous cargoes: a unified perspective. *The Journal of Cell Biology* 160:817–821 Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2173776&tool=pmcentrez&rendertype=abstract>.

Campbell DS, Holt CE (2001) Chemotropic Responses of Retinal Growth Cones Mediated by Rapid Local Protein Synthesis and Degradation. *Neuron* 32:1013–1026 Available at: <http://linkinghub.elsevier.com/retrieve/pii/S0896627301005517>.

Cavalli V, Kujala P, Klumperman J, Goldstein LSB (2005) Sunday Driver links axonal transport to damage signaling. *The Journal of cell biology* 168:775–787 Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2171809&tool=pmcentrez&rendertype=abstract> [Accessed April 1, 2012].

Cheadle C, Vawter MP, Freed WJ, Becker KG (2003) Analysis of Microarray Data Using Z Score Transformation. *The Journal of molecular diagnostics JMD* 5:73–81 Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1907322&tool=pmcentrez&rendertype=abstract>.

Chen S, Bawa D, Besshoh S, Gurd JW, Brown IR (2005) Association of heat shock proteins and neuronal membrane components with lipid rafts from the rat brain. *Journal of Neuroscience Research* 81:522–529.

Chen S, Owens GC, Makarenkova H, Edelman DB (2010) HDAC6 regulates mitochondrial transport in hippocampal neurons. *PloS one* 5:e10848 Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2877100&tool=pmcentrez&rendertype=abstract> [Accessed March 6, 2013].

Chevalier-Larsen E, Holzbaur ELF (2006) Axonal transport and neurodegenerative disease. *Biochimica et Biophysica Acta* 1762:1094–1108 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/16730956>.

- Cho Y, Cavalli V (2012) HDAC5 is a novel injury-regulated tubulin deacetylase controlling axon regeneration. *The EMBO journal* 31:3063–3078 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22692128> [Accessed March 6, 2013].
- Chong MS, Reynolds ML, Irwin N, Coggeshall RE, Emson PC, Benowitz LI, Woolf CJ (1994) GAP-43 expression in primary sensory neurons following central axotomy. *Journal of Neuroscience* 14:4375–4384 Available at: <http://www.jneurosci.org/cgi/content/abstract/14/7/4375>.
- Coggeshall RE, Lekan HA, Doubell TP, Allchorne A, Woolf CJ (1997) Central changes in primary afferent fibers following peripheral nerve lesions. *Neuroscience* 77:1115–1122 Available at: [http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=pubmed&cmd=Retrieve&dopt=AbstractPlus&list\\_uids=9130791](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=pubmed&cmd=Retrieve&dopt=AbstractPlus&list_uids=9130791).
- d'Ydewalle C, Bogaert E, Van Den Bosch L (2012) HDAC6 at the Intersection of Neuroprotection and Neurodegeneration. *Traffic (Copenhagen, Denmark)* 13:771–779 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22372633> [Accessed March 6, 2013].
- d'Ydewalle C, Krishnan J, Chiheb DM, Van Damme P, Irobi J, Kozikowski AP, Berghe P Vanden, Timmerman V, Robberecht W, Van Den Bosch L (2011) HDAC6 inhibitors reverse axonal loss in a mouse model of mutant HSPB1-induced Charcot-Marie-Tooth disease. *Nature Medicine* 17:968–974 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/21785432>.
- David S, Aguayo AJ (1981) Axonal elongation into peripheral nervous system “bridges” after central nervous system injury in adult rats. *Science* 214:931–933 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/6171034>.
- Dent EW, Gertler FB (2003) Cytoskeletal dynamics and transport in growth cone motility and axon guidance. *Neuron* 40:209–227 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/14556705>.
- Dent EW, Gupton SL, Gertler FB (2011) The growth cone cytoskeleton in axon outgrowth and guidance. *Cold Spring Harbor perspectives in biology* 3:a001800 Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3039926&tool=pmcentrez&rendertype=abstract>.

- Dent EW, Tang F, Kalil K (2003) Axon Guidance by Growth Cones and Branches: Common Cytoskeletal and Signaling Mechanisms. *The Neuroscientist* 9:343–353 Available at: <http://nro.sagepub.com/cgi/content/long/9/5/343> [Accessed March 9, 2013].
- Devor M (1999) Unexplained peculiarities of the dorsal root ganglion. *Pain Suppl* 6:S27–35 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/10491970>.
- Dill J, Wang H, Zhou F, Li S (2008) Inactivation of glycogen synthase kinase 3 promotes axonal growth and recovery in the CNS. *Journal of Neuroscience* 28:8914–8928 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/18768685>.
- Edström A, Ekström PA, Tonge D (1996) Axonal outgrowth and neuronal apoptosis in cultured adult mouse dorsal root ganglion preparations: effects of neurotrophins, of inhibition of neurotrophin actions and of prior axotomy. *Neuroscience* 75:1165–1174 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/8938749>.
- Erez H, Malkinson G, Prager-Khoutorsky M, De Zeeuw CI, Hoogenraad CC, Spira ME (2007) Formation of microtubule-based traps controls the sorting and concentration of vesicles to restricted sites of regenerating neurons after axotomy. *The Journal of cell biology* 176:497–507 Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2063984&tool=pmcentrez&rendertype=abstract> [Accessed July 13, 2012].
- Eschbach J, Dupuis L (2011) Cytoplasmic dynein in neurodegeneration. *Pharmacology therapeutics* 130:348–363 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/21420428>.
- Farrar NR, Spencer GE (2008) Pursuing a “turning point” in growth cone research. *Developmental Biology* 318:102–111 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/18436201>.
- Filbin MT (2003) Myelin-associated inhibitors of axonal regeneration in the adult mammalian CNS. *Nature Reviews Neuroscience* 4:703–713 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/12951563>.
- Fitch MT, Silver J (2008) CNS injury, glial scars, and inflammation: Inhibitory extracellular matrices and regeneration failure. *Experimental Neurology* 209:294–301 Available at:

<http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2268907&tool=pmcentrez&rendertype=abstract>.

Fukada M, Watakabe I, Yuasa-Kawada J, Kawachi H, Kuroiwa A, Matsuda Y, Noda M (2000) Molecular characterization of CRMP5, a novel member of the collapsin response mediator protein family. *The Journal of Biological Chemistry* 275:37957–37965 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/10956643>.

Fukushima N, Furuta D, Hidaka Y, Moriyama R, Tsujiuchi T (2009) Post-translational modifications of tubulin in the nervous system. *Journal of neurochemistry* 109:683–693 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/19250341> [Accessed March 6, 2013].

Garrison FH (1929) Ramón y Cajal. *Bulletin of the New York Academy of Medicine* 5:482–508.

Gee MA, Heuser JE, Vallee RB (1997) An extended microtubule-binding structure within the dynein motor domain. *Nature* 390:636–639 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/9403697>.

Grafstein B (1995) *The Axon: Structure, Function and Pathophysiology* (Stephen Waxman, Jeffery Kocsis PS, ed)., First. Oxford University Press, USA.

Grafstein, Forman (1980) Intracellular transport in neurons. *Physiol Rev* 60:1167–1283.

Gumy LF, Tan CL, Fawcett JW (2010) The role of local protein synthesis and degradation in axon regeneration. *Experimental Neurology* 223:28–37 Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2864402&tool=pmcentrez&rendertype=abstract>.

Gundersen HJ, Jensen EB (1987) The efficiency of systematic sampling in stereology and its prediction. *Journal of Microscopy* 147:229–263 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/3430576>.

Guzik BW, Goldstein LSB (2004) Microtubule-dependent transport in neurons: steps towards an understanding of regulation, function and dysfunction. *Current Opinion in Cell Biology* 16:443–450 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/15261678>.

Haar E ter, Coll JT, Austen DA, Hsiao H-M, Swenson L, Jain J (2001) Structure of GSK3 $\beta$  reveals a primed phosphorylation mechanism. *Nature Structural Biology* 8:593–596.

Hanz S, Perlson E, Willis D, Zheng J-Q, Massarwa R, Huerta JJ, Koltzenburg M, Kohler M, van-Minnen J, Twiss JL, Fainzilber M (2003) Axoplasmic Importins Enable Retrograde Injury Signaling in Lesioned Nerve. *Neuron* 40:1095–1104 Available at: <http://linkinghub.elsevier.com/retrieve/pii/S0896627303007700>.

Hartl FU, Martin J (1995) Molecular chaperones in cellular protein folding. *Current Opinion in Structural Biology* 5:689–692 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/8637592>.

Harvey PJ, Grochmal J, Tetzlaff W, Gordon T, Bennett DJ (2005) An investigation into the potential for activity-dependent regeneration of the rubrospinal tract after spinal cord injury. *European Journal of Neuroscience* 22:3025–3035 Available at: [http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list\\_uids=16367769](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16367769).

He Z (2005) Inhibition of axonal regeneration. *Advanced Studies in Medicine* 5:409–412 Available at: [http://www.jhasim.com/files/articlefiles/pdf/XASIM\\_Issue\\_5\\_4D\\_p409\\_412.pdf](http://www.jhasim.com/files/articlefiles/pdf/XASIM_Issue_5_4D_p409_412.pdf).

Hirokawa N, Niwa S, Tanaka Y (2010) Molecular motors in neurons: transport mechanisms and roles in brain function, development, and disease. *Neuron* 68:610–638 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/21092854> [Accessed March 9, 2012].

Hirokawa N, Noda Y, Tanaka Y, Niwa S (2009) Kinesin superfamily motor proteins and intracellular transport. *Nature Reviews Molecular Cell Biology* 10:682–696 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/19773780>.

Horner PJ, Gage FH (2000) Regenerating the damaged central nervous system. *Nature* 407:963–970 Available at: <http://ntp.neuroscience.wisc.edu/neuro670/reqreading/RegeneratingTheNervousSystem.pdf>.

Howarth JL, Kelly S, Keasey MP, Glover CPJ, Lee Y-B, Mitrophanous K, Chapple JP, Gallo JM, Cheetham ME, Uney JB (2007) Hsp40 molecules that target to the ubiquitin-proteasome system decrease inclusion formation in models of polyglutamine disease. *Molecular therapy the journal of the American Society of Gene Therapy* 15:1100–1105 Available at: <http://discovery.ucl.ac.uk/113973/>.

- Hur E-M, Zhou F-Q (2010) GSK3 signalling in neural development. *Nature Reviews Neuroscience* 11:539–551 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/20648061>.
- Hu-Tsai M, Winter J, Emson PC, Woolf CJ (1994) Neurite outgrowth and GAP-43 mRNA expression in cultured adult rat dorsal root ganglion neurons: effects of NGF or prior peripheral axotomy. *Journal of Neuroscience Research* 39:634–645 Available at: [http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list\\_uids=7534832](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=7534832).
- Ikegami K, Heier RL, Taruishi M, Takagi H, Mukai M, Shimma S, Taira S, Hatanaka K, Morone N, Yao I, Campbell PK, Yuasa S, Janke C, MacGregor GR, Setou M (2007) Loss of alpha-tubulin polyglutamylation in ROSA22 mice is associated with abnormal targeting of KIF1A and modulated synaptic function. *Proceedings of the National Academy of Sciences of the United States of America* 104:3213–3218 Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1802010&tool=pmcentrez&rendertype=abstract>.
- Ikegami K, Mukai M, Tsuchida J, Heier RL, Macgregor GR, Setou M (2006) TTL7 is a mammalian beta-tubulin polyglutamylase required for growth of MAP2-positive neurites. *The Journal of Biological Chemistry* 281:30707–30716 Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3049811&tool=pmcentrez&rendertype=abstract>.
- Jacob JM, McQuarrie IG (1996) Assembly of microfilaments and microtubules from axonally transported actin and tubulin after axotomy. *Journal of Neuroscience Research* 43:412–419.
- Jana NR, Tanaka M, Wang GH, Nukina N (2000) Polyglutamine length-dependent interaction of Hsp40 and Hsp70 family chaperones with truncated N-terminal huntingtin: their role in suppression of aggregation and cellular toxicity. *Human Molecular Genetics* 9:2009–2018 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/10942430>.
- Janke C, Bulinski JC (2011) Post-translational regulation of the microtubule cytoskeleton: mechanisms and functions. *Nature Reviews Molecular Cell Biology* 12:773–786 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22086369>.



Janke C, Kneussel M (2010) Tubulin post-translational modifications: encoding functions on the neuronal microtubule cytoskeleton. *Trends in Neurosciences* 33:362–372 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/20541813>.

Kalbermatten DF, Erba P, Mahay D, Wiberg M, Pierer G, Terenghi G (2008) Schwann cell strip for peripheral nerve repair. *The Journal of hand surgery European volume* 33:587–594 Available at: [http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list\\_uids=18977829](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18977829).

Kimura-Kuroda J, Teng X, Komuta Y, Yoshioka N, Sango K, Kawamura K, Raisman G, Kawano H (2010) An in vitro model of the inhibition of axon growth in the lesion scar formed after central nervous system injury. *Molecular And Cellular Neurosciences* 43:177–187 Available at: <http://discovery.ucl.ac.uk/76649/>.

Lakämper S, Meyhöfer E (2006) Back on track - on the role of the microtubule for kinesin motility and cellular function. *Journal of muscle research and cell motility* 27:161–171 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/16453157>.

LaMonte BH, Wallace KE, Holloway BA, Shelly SS, Ascaño J, Tokito M, Van Winkle T, Howland DS, Holzbaur ELF (2002) Disruption of dynein/dynactin inhibits axonal transport in motor neurons causing late-onset progressive degeneration. *Neuron* 34:715–727 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/12062019>.

LeDizet M, Piperno G (1987) Identification of an acetylation site of Chlamydomonas alpha-tubulin. *Proceedings of the National Academy of Sciences of the United States of America* 84:5720–5724 Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=298934&tool=pmcentrez&rendertype=abstract>.

Leung T, Manser E, Tan L, Lim L (1995) A novel serine/threonine kinase binding the Ras-related RhoA GTPase which translocates the kinase to peripheral membranes. *The Journal of Biological Chemistry* 270:29051–29054 Available at: <http://www.jbc.org/cgi/doi/10.1074/jbc.270.49.29051>.

Li JY, Pfister KK, Brady ST, Dahlström A (2000) Cytoplasmic dynein conversion at a crush injury in rat peripheral axons. *Journal of Neuroscience Research* 61:151–161 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/10878588>.

Lindwall C, Kanje M (2005) Retrograde axonal transport of JNK signaling molecules influence injury induced nuclear changes in p-c-Jun and ATF3 in adult rat sensory neurons. *Molecular And Cellular Neurosciences* 29:269–282 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/15911351>.

Lingor P, Teusch N, Schwarz K, Mueller R, Mack H, Bähr M, Mueller BK (2007) Inhibition of Rho kinase (ROCK) increases neurite outgrowth on chondroitin sulphate proteoglycan in vitro and axonal regeneration in the adult optic nerve in vivo. *Journal of Neurochemistry* 103:181–189 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/17608642>.

Lodish et al. (2008) *Molecular Cell Biology* (Ahr K, ed), 6th ed. Sara Tenney.

Lowery LA, Van Vactor D (2009) The trip of the tip: understanding the growth cone machinery. *Nature Reviews Molecular Cell Biology* 10:332–343 Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2714171&tool=pmcentrez&rendertype=abstract>.

Manzerra P, Brown IR (1996) The neuronal stress response: nuclear translocation of heat shock proteins as an indicator of hyperthermic stress. *Experimental Cell Research* 229:35–47 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/8940247>.

Martin-Villalba A, Winter C, Brecht S, Buschmann T, Zimmermann M, Herdegen T (1998) Rapid and long-lasting suppression of the ATF-2 transcription factor is a common response to neuronal injury. *Brain research Molecular brain research* 62:158–166 Available at: [http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list\\_uids=9813301](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=9813301).

Maruta H, Greer K, Rosenbaum JL (1986) The acetylation of alpha-tubulin and its relationship to the assembly and disassembly of microtubules. *The Journal of Cell Biology* 103:571–579 Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2113826&tool=pmcentrez&rendertype=abstract>.

- McDonald JW, Sadowsky C (2002) Spinal-cord injury. *The Lancet* 359:417–425 Available at: <http://www.sciencedirect.com/science/article/pii/S0140673602076031>.
- Miki H, Setou M, Hirokawa N (2003) Kinesin Superfamily Proteins (KIFs) in the Mouse Transcriptome. *Genome Research* 13:1455–1465 Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=403687&tool=pmcentrez&rendertype=abstract>.
- Morfini G, Szebenyi G, Elluru R, Ratner N, Brady ST (2002) Glycogen synthase kinase 3 phosphorylates kinesin light chains and negatively regulates kinesin-based motility. *The European Molecular Biology Organization Journal* 21:281–293 Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=125832&tool=pmcentrez&rendertype=abstract>.
- Mullins FH, Hargreaves AJ, Li JY, Dahlström A, McLean WG (1994) Tyrosination state of alpha-tubulin in regenerating peripheral nerve. *Journal of Neurochemistry* 10:227–234.
- Nagumo H, Ikenoya M, Sakurada K, Furuya K, Ikuhara T, Hiraoka H, Sasaki Y (2001) Rho-associated kinase phosphorylates MARCKS in human neuronal cells. *Biochemical and Biophysical Research Communications* 280:605–609 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/11162562>.
- Neumann S, Woolf CJ (1999) Regeneration of dorsal column fibers into and beyond the lesion site following adult spinal cord injury. *Neuron* 23:83–91 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/10402195>.
- Nixon RA, Yuan A (2011) *Cytoskeleton of the Nervous System* (Lajtha A, ed), 3rd ed. Springer.
- Orlinger MM, Lasek RJ (1984) A conditioning dorsal regeneration lesion ganglion of the peripheral cells accelerates their peripheral axons. *Journal of Neuroscience* 4:1736–1744 Available at: <http://www.jneurosci.org/content/4/7/1736.long>.
- Oudega M, Varon S, Hagg T (1994) Regeneration of adult rat sensory axons into intraspinal nerve grafts: promoting effects of conditioning lesion and graft predegeneration. *Experimental Neurology* 129:194–206 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/7957734>.

Oyinbo CA (2011) Secondary injury mechanisms in traumatic spinal cord injury: a nugget of this multiply cascade. *Acta neurobiologiae experimentalis* 71:281–299 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/21731081>.

Paschal BM, Shpetner HS, Vallee RB (1987) MAP 1C is a microtubule-activated ATPase which translocates microtubules in vitro and has dynein-like properties. *The Journal of Cell Biology* 105:1273–1282 Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2114794&tool=pmcentrez&rendertype=abstract>.

Perlson E, Hanz S, Ben-Yaakov K, Segal-Ruder Y, Seger R, Fainzilber M (2005) Vimentin-dependent spatial translocation of an activated MAP kinase in injured nerve. *Neuron* 45:715–726 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/15748847> [Accessed June 17, 2012].

Pfister KK, Wagner MC, Stenoien DL, Brady ST, Bloom GS (1989) Monoclonal antibodies to kinesin heavy and light chains stain vesicle-like structures, but not microtubules, in cultured cells. *The Journal of Cell Biology* 108:1453–1463 Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2115510&tool=pmcentrez&rendertype=abstract>.

Polleux F, Snider W (2010) Initiating and growing an axon. *Cold Spring Harbor perspectives in biology* 2:a001925 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/20452947>.

Purves D, Augustine GJ, Fitzpatrick D, Hall WC, LaMantia A-S, McNamara JO, Williams SM (2004) *Neuroscience (Associates S, ed), 3rd ed.* Sunderland, Massachusetts U.S.A.

Raivich G, Hellweg R, Kreutzberg GW (1991) NGF receptor-mediated reduction in axonal NGF uptake and retrograde transport following sciatic nerve injury and during regeneration. *Neuron* 7:151–164 Available at: <http://discovery.ucl.ac.uk/830722/>.

Ramon y Cajal S (1928) *Cajal's Degeneration and Regeneration of the Nervous System* (DeFelipe J, Jones EG, eds). London: Oxford University Press.

Reed N a, Cai D, Blasius TL, Jih GT, Meyhofer E, Gaertig J, Verhey KJ (2006) Microtubule acetylation promotes kinesin-1 binding and transport. *Current biology: CB* 16:2166–2172 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/17084703> [Accessed February 28, 2013].

Richardson PM, Issa VM (1984) Peripheral injury enhances central regeneration of primary sensory neurones. *Nature* 309:791–793 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/6204205>.

Richardson PM, Verge VM (1986) The induction of a regenerative propensity in sensory neurons following peripheral axonal injury. *Journal of Neurocytology* 15:585–594 Available at: [http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list\\_uids=3772404](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=3772404).

Rishal I, Fainzilber M (2010) Retrograde signaling in axonal regeneration. *Experimental neurology* 223:5–10 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/19699198> [Accessed March 17, 2012].

Rossi F, Gianola S, Corvetti L (2007) Regulation of intrinsic neuronal properties for axon growth and regeneration. *Progress in neurobiology* 81:1–28 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/17234322> [Accessed April 1, 2012].

Sapunar D, Kostic S, Banozic A, Puljak L (2012) Dorsal root ganglion - a potential new therapeutic target for neuropathic pain. *Journal of pain research* 5:31–38 Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3287412&tool=pmcentrez&rendertype=abstract>.

Satkauskas S, Bagnard D (2007) Local protein synthesis in axonal growth cones: what is next? *Cell adhesion migration* 1:179–184 Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2634104&tool=pmcentrez&rendertype=abstract>.

Schmandke A, Schmandke A, Strittmatter SM (2007) ROCK and Rho: biochemistry and neuronal functions of Rho-associated protein kinases. *The Neuroscientist a review journal bringing neurobiology neurology and psychiatry* 13:454–469 Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2849133&tool=pmcentrez&rendertype=abstract>.

Schmied R, Ambron RT (1997) A nuclear localization signal targets proteins to the retrograde transport system, thereby evading uptake into organelles in aplasia axons. *Journal of Neurobiology* 33:151–160 Available at:

[http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list\\_uids=9240371](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=9240371).

Schofield JN, Day IN, Thompson RJ, Edwards YH (1995) PGP9.5, a ubiquitin C-terminal hydrolase; pattern of mRNA and protein expression during neural development in the mouse. *Brain research Developmental brain research* 85:229–238 Available at: <http://www.ncbi.nlm.nih.gov/htbin-post/Entrez/query?db=m&form=6&dopt=r&uid=7600671>.

Schreyer DJ, Skene JH (1993) Injury-associated induction of GAP-43 expression displays axon branch specificity in rat dorsal root ganglion neurons. *Journal of Neurobiology* 24:959–970 Available at: <http://dx.doi.org/10.1002/neu.480240709>.

Setler RA, Gan Y, Zhang W, Liou AK, Gao Y, Cao G, Chen J (2010) Heat shock proteins: Cellular and molecular mechanisms in the central nervous system. *Progress in neurobiology* 92:184–211.

Siegel GJ (2006) *Basic Neurochemistry: Molecular, Cellular and Medical aspects* (R. Wayne Albers, Scott T. Brady DLP, ed)., 7th ed. Elsevier Academic Press.

Simões-Pires C, Zwick V, Nurisso A, Schenker E, Carrupt P-A, Cuendet M (2013) HDAC6 as a target for neurodegenerative diseases: what makes it different from the other HDACs? *Molecular neurodegeneration* 8:7 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/23356410>.

Smith DS, Skene JH (1997) A transcription-dependent switch controls competence of adult neurons for distinct modes of axon growth. *Journal of Neuroscience* 17:646–658 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/8987787>.

Song HJ, Poo MM (1999) Signal transduction underlying growth cone guidance by diffusible factors. *Current Opinion in Neurobiology* 9:355–363 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/10395576>.

Sun F, He Z (2010) Neuronal intrinsic barriers for axon regeneration in the adult CNS. *Current Opinion in Neurobiology* 20:510–518 Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2911501&tool=pmcentrez&rendertype=abstract>.

- Takahashi Y, Edamatsu M, Toyoshima YY (2004) Multiple ATP-hydrolyzing sites that potentially function in cytoplasmic dynein. *Proceedings of the National Academy of Sciences of the United States of America* 101:12865–12869 Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=516486&tool=pmcentrez&rendertype=abstract>.
- Tashiro T, Komiya Y (1992) Organization and slow axonal transport of cytoskeletal proteins under normal and regenerating conditions. *Molecular Neurobiology* 6:301–311 Available at: [http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list\\_uids=1282336](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=1282336).
- Tom VJ, Steinmetz MP, Miller JH, Doller CM, Silver J (2004) Studies on the Development and Behavior of the Dystrophic Growth Cone, the Hallmark of Regeneration Failure, in an In Vitro Model of the Glial Scar and after Spinal Cord Injury. *The Journal of Neuroscience* 24 :6531–6539 Available at: <http://www.jneurosci.org/content/24/29/6531.abstract>.
- Tyxo (2006) Human anatomy: The spinal nerves. *Encyclopedia Lubopitko* Available at: [http://encyclopedia.lubopitko-bg.com/The\\_Spinal\\_Nerves.html](http://encyclopedia.lubopitko-bg.com/The_Spinal_Nerves.html) [Accessed May 10, 2013].
- Udina E, Furey M, Busch S, Silver J, Gordon T, Fouad K (2008) Electrical stimulation of intact peripheral sensory axons in rats promotes outgrowth of their central projections. *Experimental Neurology* 210:238–247 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/18164293>.
- Vargas ME, Barres B a (2007) Why is Wallerian degeneration in the CNS so slow? *Annual review of neuroscience* 30:153–179 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/17506644> [Accessed March 25, 2012].
- Verma P, Chierzi S, Codd AM, Campbell DS, Meyer RL, Holt CE, Fawcett JW (2005) Axonal protein synthesis and degradation are necessary for efficient growth cone regeneration. *Journal of Neuroscience* 25:331–342 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/15647476>.
- Willis DE, Twiss JL (2006) The evolving roles of axonally synthesized proteins in regeneration. *Current opinion in neurobiology* 16:111–118 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/16418002> [Accessed March 10, 2012].

Woolf CJ (2001) Turbocharging neurons for growth: accelerating regeneration in the adult CNS. *Nature Neuroscience* 4:7–9 Available at: [http://www.nature.com/neuro/journal/v4/n1/full/nn0101\\_7.html](http://www.nature.com/neuro/journal/v4/n1/full/nn0101_7.html).

Wujek JR, Lasek RJ (1983) Correlation of axonal regeneration and slow component B in two branches of a single axon. *Journal of Neuroscience* 3:243–251 Available at: [http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list\\_uids=6185656](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=6185656).

Yamazaki M, Miyazaki H, Watanabe H, Sasaki T, Maehama T, Frohman MA, Kanaho Y (2002) Phosphatidylinositol 4-Phosphate 5-Kinase Is Essential for ROCK-mediated Neurite Remodeling. *The Journal of Biological Chemistry* 277:17226–17230 Available at: [http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list\\_uids=11877391](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11877391).

Yin Z-S, Zhang H, Bo W, Gao W (2010) Erythropoietin promotes functional recovery and enhances nerve regeneration after peripheral nerve injury in rats. *AJNR American journal of neuroradiology* 31:509–515 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/20037135>.

Yiu G, He Z (2006) Glial inhibition of CNS axon regeneration. *Nature reviews Neuroscience* 7:617–627 Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2693386&tool=pmcentrez&rendertype=abstract> [Accessed March 10, 2012].

Ylera B, Ertürk A, Hellal F, Nadrigny F, Hurtado A, Tahirovic S, Oudega M, Kirchhoff F, Bradke F (2009) Chronically CNS-injured adult sensory neurons gain regenerative competence upon a lesion of their peripheral axon. *Current biology: CB* 19:930–936 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/19409789> [Accessed April 20, 2012].

Zheng JQ, Kelly TK, Chang B, Ryazantsev S, Rajasekaran AK, Martin KC, Twiss JL (2001) A functional role for intra-axonal protein synthesis during axonal regeneration from adult sensory neurons. *Journal of Neuroscience* 21:9291–9303 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/11717363>.



Zurn AD, Bandtlow CE (2006) Regeneration failure in the CNs: cellular and molecular mechanisms. *Advances in experimental medicine and biology* 557:54–76 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/16955704>.