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Unraveling intrinsic mechanisms of Nerve Regeneration

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I have not failed.
I've just found 10000 ways that won't work.

Thomas Edison

Agradecimentos

Quando chegamos a esta meta final, é impossível não recordar as pessoas que de alguma forma marcaram o meu percurso pela FCT e também pelo grupo *Nerve Regeneration*.

Quero começar por agradeçer à Dra. Mónica Sousa, não só por me ter dado a oportunidade de fazer parte do grupo *Nerve Regeneration*, mas também pelo apoio, formação e conselhos oferecidos durante o ano que estive no Porto.

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Abstract

Unlike injury to the peripheral nervous system (PNS), where injured neurons can trigger a regenerative program that leads to axonal elongation and in some cases proper reinnervation, after injury to the central nervous system (CNS) neurons fail to produce the same response. The regenerative program includes the activation of several injury signals that will lead to the expression of genes associated with axonal regeneration. As a consequence, the spawned somatic response will ensure the supply of molecular components required for axonal elongation.

The capacity of some neurons to trigger a regenerative response has led to investigate the mechanisms underlying neuronal regeneration. Thus, non-regenerative models (like injury to the CNS) and regenerative models (such as injury to the PNS) were used to understand the differences underlying those two responses to injury. To do so, the regenerative properties of dorsal root ganglion (DRG) neurons were addressed. This particular type of neurons possesses two branches, a central axon, that has a limited capacity to regenerate; and a peripheral axon, where regeneration can occur over long distances.

In the first paradigm used to understand the neuronal regeneration mechanisms, we evaluated the activation of injury signals in a non-regenerative model. Injury signals include the positive injury signals, which are described as being enhancers of axonal regeneration by activating several transcription factors. The currently known positive injury signals are ERK, JNK and STAT3. To evaluate whether the lack of regeneration following injury to the central branch of DRG neurons was due to inactivation of these signals, activation of the transcription factors pELK-1, p-c-jun (downstream targets of ERK and JNK, respectively) and pSTAT3 were examined. Results have shown no impairment in the activation of these signals. As a consequence, we further proceed with evaluation of other candidates that could participate in axonal regeneration failure. By comparing the protein profiles that were triggered following either injury to the central branch of DRG neurons or injury to their peripheral branch, we were able to identify high levels of GSK3-β, ROCKII and HSP-40 after injury to the central branch of DRG neurons. While *in vitro* knockdown of HSP-40 in DRG neurons showed to be toxic for the cells, evaluation of pCRMP2 (a GSK3-β downstream target) and pMLC (a ROCKII downstream target), which are known to impair axonal regeneration, revealed high levels of both proteins following injury to the central branch when comparing with injury to their peripheral one. Altogether, these results suggest that activation of positive injury signals is not sufficient to elicit axonal regeneration; HSP-40 is likely to participate in the cell survival program; whereas GSK3-β and ROCKII activity may condition the regenerative capacity following injury to the nervous system.

In the second paradigm chosen, the *Conditioning lesion* was used as a regenerative model to evaluate some aspects of axonal transport and to identify the protein profile elicited by the nuclear changes under an axonal regeneration program. A particular characteristic of DRG neurons is that an injury to their peripheral branch - the conditioning lesion - prior to an injury to their central branch, besides increasing axonal transport, elicits regeneration of both branches. Since the signaling molecule

cAMP has been described as the central mediator of the *Conditioning lesion* regenerative effect, we asked whether by increasing cAMP levels through administration of rolipram, we could mimic the increased axonal transport observed following a conditioning lesion. Our results have shown that increasing cAMP levels did not elicit increased axonal transport. On the other hand, evaluation of the protein profile prompted by the nuclear response of DRG neurons following conditioning injury has allowed the identification of several proteins that where upregulated and being anterogradely transported to the injury site when comparing with uninjured DRG neurons. Amongst them, GNB-2 was identified as a potential enhancer of axonal regeneration.

Overall, this thesis elucidates some of the intrinsic mechanisms that determine whether following injury axonal regeneration occurs or fails.

Resumo

Ao contrário do que o que acontece após lesão no sistema nervoso periférico (SNP), onde os neurónios lesionados são capazes the activar um programa regenerativo capaz de induzir crescimento axonal e em certos casos correcta reinervação; após lesão no sistema nervoso central (SNC), os neurónios são incapazes de reproduzir a mesma resposta. O programa regenerativo inclui a activação de diversos sinais indicadores de lesão que induzem a expressão de genes associados à regeneração axonal. Como consequência, a resposta somática gerada vai providenciar os componentes necessários ao crescimento axonal.

A capacidade de alguns neurónios activarem um programa regenerativo, levou-nos a investigar os mecanismos associados à regeneração neuronal. Desta forma, modelos não-regenerativos (como lesão no SNC) e regenerativos (como lesão no SNP) foram usados para compreender as differenças inerentes a estas duas respostas. Neste sentido, fizemos uso das propriedades regenerativas dos neurónios dos ganglios da raiz dorsal. Este tipo peculiar de neurónios possui dois ramos, um central que possui uma capacidade limitada de regenerar, e um periférico, cuja regeneração axonal pode ocorrer por longas distâncias.

No primeiro paradigma utilizado para compreender os mecanismos de regeneração nervosa, foi proposto avaliar a activação de sinais indicadores de lesão num modelo não-regenerativo. Sinais indicadores de lesão incluem os sinais de lesão positivos, que estão descritos como sendo potenciadores da regeneração axonal através da sua capacidade de activar diversos factores de transcrição. Actualmente, os sinais de lesão positivos conhecidos são as proteinas ERK, JNK and STAT3. Com o objectivo de avaliar as causas responsáveis pela limitada capacidade regenerativa do ramo central dos neuronios dos ganglios da raiz dorsal, a activação dos factores de transcrição pELK-1, p-c-jun (respectivos alvos da actividade de ERK e JNK) e pSTAT3 foi verificada. Os resultados revelaram nenhuma limitação na sua activação. Como consequência, procedeu-se à avaliação de outros candidatos que poderiam de alguma forma contribuir para a ausência de regeneração axonal. Através da comparação dos perfis proteicos activados após lesão no ramo central ou no ramo periférico dos neurónios dos ganglios da raiz dorsal, foi possivel identificar elevados níveis das seguintes proteinas, GSK3-β, ROCKII and HSP-40, após lesão nos ramos centrais deste tipo de neurónios. Enquanto que diminuição *in vitro* dos níveis de HSP-40 em neurónios de ganglios da raiz dorsal mostrou ser tóxica para as células, a avaliação das fosforilações de CRMP2 e MLC (alvos da activadade da GSK3-β e ROCKII, respectivamente) revelou níveis altos destes substratos após lesão no ramo central dos neurónios dos ganglios da raiz dorsal em comparação com lesão nos seus ramos periféricos. Em conclusão, estes resultados mostraram que a activação de sinais de lesão positivos não é suficiente para induzir regeneração axonal; que a proteína HSP-40 poderá estar envolvida no programa de sobrevivência da célula; e que a actividade das proteínas GSK3-β e ROCKII condiciona a capacidade regenerativa dos neurónios após lesão do sistema nervoso.

No segundo paradigma utilizado, a lesão condicionada foi usada como modelo regenerativo para avaliar alguns aspectos do transporte axonal e para identificar o perfil proteico instigado pelas

modificações nucleares num programa de regeneração axonal. Uma característica dos neurónios dos ganglios da raiz dorsal é que a realização de uma lesão no seu ramo periférico - a lesão condicionante – antes de se proceder a uma lesão no seu ramo central, para além de induzir um maior transporte axonal, permite a regeneração de ambos os ramos. Uma vez que a molécula sinalizadora cAMP tem sido descrita como o mediador central do efeito regenerativo da lesão condicionante, colocou-se a questão se, aumentando os níveis de cAMP através da administração de rolipram, se poderia reproduzir um aumento no transporte axonal observado após lesão condicionante. Os resultados mostraram que um aumento nos níveis de cAMP, não induz um aumento no transporte axonal. Por outro lado, avaliação do perfil proteico activado pela resposta nuclear à lesão condicionante permitiu a identificação de várias proteínas que, para além de se encontrarem expressas em elevados níveis em comparação com neurónios não lesionados, estavam a ser transportadas do soma para o local de lesão. De entre essas proteínas, a molécula GNB-2 foi identificada como potencial promotor da regeneração axonal.

Em suma, esta Tese elucida alguns dos mecanismos intrinsicos aos neurónios que determinam se após lesão dos sistema nervoso regeneração ocorre ou não.

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Abbreviations

AAD	Axonal acute degeneration
AC	Adenyl cyclase
AMP	Adenosino-5'-monophosphate
ANX	Annexin
ARG	Arginase
ATF-3	Activating transcription factor 3
ATP	Adenosine-5'-triphosphate
BNB	Blood-nerve-barrier
BSA	Albumin bovine serum
BSCB	Blood-spinal cord-barrier
CAD	Cath.-a-differentiated cells
cAMP	Cyclic adenosine monophosphate
Cdc42	Cell division control protein 42 homolog
CL	Conditioning lesion
CNS	Central nervous system
CREB	cAMP response element-binding protein
CRMP	Collapsin response mediator protein
CSPG	Chondroitin sulphate proteoglycans
DAB	3,3´-diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
db-Camp	Dibutyryl cyclic adenosine monophosphate
DMEM	Dulbecco’s Modified Eagle Medium

DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DREZ	Dorsal root entry zone
DRG	Dorsal root ganglion
DRI	Dorsal root injury
ECM	Extracellular matrix
Elk-1	E twenty six like transcription factor 1
ERK	Extracellular signal regulated kinase
FBS	Fetal bovine serum
GAP-43	Growth associated protein 43
GCPR	G protein-coupled receptors
GDC	Granular disintegration of axonal cytoskeleton
GNB-2	Guanine nucleotide binding protein beta 2 subunit
GSK3-β	Glycogen synthase kinase 3 beta
H3	Histone 3
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HEK293T	Human Embryonic Kidney 293T cells
HPRT	Hypoxanthine-guanine phosphorobosyltransferase
HSP	Heat shock protein
JNK	c-Jun N-terminal kinase
MAPKK/MEK	Mitogen-activated protein kinase kinase
MLC	Myosin light chain
mRNA	RNA messenger
NGF	Nerve growth factor

NPY	Neuropeptide Y
P/S	Penicillin/Streptomycin
PBS	Phosphate buffered saline
PDE	Phosphosdiesterase
PFA	Paraformaldehyde
PFAC	Histone acetyltransferase p300/CBP-associated factor
PLL	Poly-L-Lysine
PNS	Peripheral nervous system
Rac	Ras-related C3 botulinum toxin
RAG	Regeneration-associated genes
RhoA	Ras homolog gene family, member A
RhoGDI	Rho GDP dissociation inhibitor
ROCK	Rho-associated kinase
RT	Room temperature
RT-qPCR	Real time-quantitative polymerase chain reaction
SCa	Slow component a
SCb	Slow component b
SCI	Spinal cord injury
SCs	Schwann cells
Sirt2	Sirtuin 2
SMAD	Mothers against decapentaplegic homolog
SNI	Sciatic nerve injury
STAT3	Signal transducer and activator of transcription 3
Vip	Vasointestine peptide

Prologue

In the first year of college, when asked what a biomedical engineer does, professor Pedro Vieira answered by saying something like "The biomedical engineer is the bridge between those who create the equipment and those who use it. He/she is the one that translates for engine language the requests in the biomedicine field, thus allowing the development of new technologies that will provide new therapies, new findings". Few years later, in the *Cell and Tissue Engineering* course, I had the opportunity to get in contact with the nerve regeneration field and all the possibilities that it offered. From pharmaceutical therapies, electric stimulation, scaffolds production to stem cell transplantation, there is an all-world of study trying to unravel mechanisms capable of improving function of the nervous system following injury. Being myself such passionate about the complexity of the nervous system, I knew that I would like to embark through this road. But how could a biomedical engineer focus its efforts to develop materials capable of improving nerve regeneration without getting in contact with the field? After almost 8 months of looking and several e-mails (and I must confess, of meditation too), I received a message from Dr. Mónica Sousa inviting me for get together and allowing me to perform this thesis work.

Saying this, before I present the structure of this thesis, there is one thing that should be taken in account: some injured neurons are capable of triggering intrinsic mechanisms that lead to axonal regeneration (such as neurons from the peripheral nervous system), while others fail to do it (like central nervous system neurons) (Liu et al 2011). Many have been the efforts to understand the mechanisms underlying these different capacities and, although much has been achieved, reports have failed to completely explain those mechanisms and much as yet to be unraveled.

In this sense, the goal of this thesis was to dissect some of the intrinsic factors that determine whether injured neurons switch to a non-regeneration or a regeneration-competent state. As a consequence, this work is divided in three chapters and an epilogue. While, in the first chapter, *Chapter 0*, some of the concepts that I acquired during the last year will be discussed, in the following two chapters, I will present the two approaches used to study the biological mechanisms of axonal regeneration. It is important to phrase that, although the two approaches have the same goal (to understand how axonal regeneration can be improved), they are focused in different features of axonal regeneration: whereas the first one, which will be addressed in *Chapter I*, took advantage of a non-regenerative model to understand axonal regeneration failure; the second, which will be discussed in *Chapter II*, used a regenerative model to understand intrinsic factors that elicit successful regeneration. Not less important, since my thesis work is part of a continuum investigation that started at the Nerve Regeneration lab, in the beginning of these two chapters I will also present some of the preliminary results that have allowed me to complete this thesis. Finally, in the epilogue, I will draw some general conclusions that resulted from the experience that I had during this last year.

Chapter 0

Introduction

The main goal of this chapter is to introduce some theoretical concepts that will put in context the work produced in this thesis. As a consequence, a brief description of the function and organization of the nervous system, as well as the constituents of the nervous tissue will be introduced. Then, some of the findings made in the nerve regeneration field will be dissected, giving special emphasis to the intrinsic mechanisms that modulate axonal regeneration, since it is the focus of this thesis.

1. Organization and function of the Nervous System

In mammals, including humans and rodents, the nervous system can be classified into two structures, namely the central nervous system (CNS) which comprises the brain and the spinal cord; and the peripheral nervous system (PNS) that encounters cranial and spinal nerves. Both peripheral and central nervous systems work in a dependent manner, sensing and executing somatic and autonomous tasks (Tortora & Derrickson 2011).

The CNS is known for being the main source of stimuli processing, like memories and thoughts. It is also responsible for the majority of the signals that encode muscle contraction and gland secretion; and also for the analysis of some of the sensory inputs. The PNS, on the other hand, covers all nervous tissue that doesn't belong to the CNS, including nerve fibers that have thousands of motor and sensory axons; ganglions which are clusters that contain cell bodies from sensory neurons; and sensory receptors, specialized neuronal cells that convert external stimuli into electrical messages. Because it is not in the interest of this thesis, the anatomy and physiology of the brain and cranial nerves will not be addressed, and only the spinal cord and spinal nerves will be covered (Tortora & Derrickson 2011).

Mammals' spinal cord can be classified in four main regions: cervical (c), thoracic (T), lumbar (L) and sacral (S). Depending on the species, each domain is divided in different segments. For instance, while in humans the cervical region is divided in 8 segments, thoracic in 12, lumbar in 5 and sacral also in 5 (Fig.1), in rodents, cervical, thoracic, lumbar and sacral have, respectively, 5, 13, 6 and 4 segments. Each segment connects with one pair of nerves, through the dorsal (sensory) and ventral (motor) roots. Also associated to each segment, there are two ganglions - the dorsal root ganglions (DRGs) - that precede the dorsal roots (Tortora & Derrickson 2011, Watson et al 2008).

Sensory information travels along the spinal nerve into the dorsal root and enters the spinal cord through the dorsal root entry zone (DREZ), as it is seen in figure 1. Depending on the signal's nature, it can be processed in the spinal cord or be transmitted to the brain along ascending tracks. Of note, each dorsal root contributes with about one third of its sensory neurons to the formation of these ascending paths, the dorsal column fibers. The other two thirds establish a synapse after entering the spinal cord to give origin to the spinothalamic tract. If the signal is processed in the brain, the message is then conducted along descending tracks to the corresponding ventral root; if analyzed in the spinal cord, the signal is immediately redirected into ventral root and finally to its target, in a process called reflex arc (Tortora & Derrickson 2011).

This well-established organization is crucial for a good and complete performance of body functions. For instances, severe aggressions to the spinal cord or nerves can interrupt the communication between the brain and the body, which results in most cases in loss of sensation and movement below the injury site. This means that an injury at lumbar level can result for example in paraplegia and bladder dysfunction, while an injury at sciatic nerve is more likely to end in member paralysis or chronic pain (Bradbury & McMahon 2006, Taylor et al 2010).

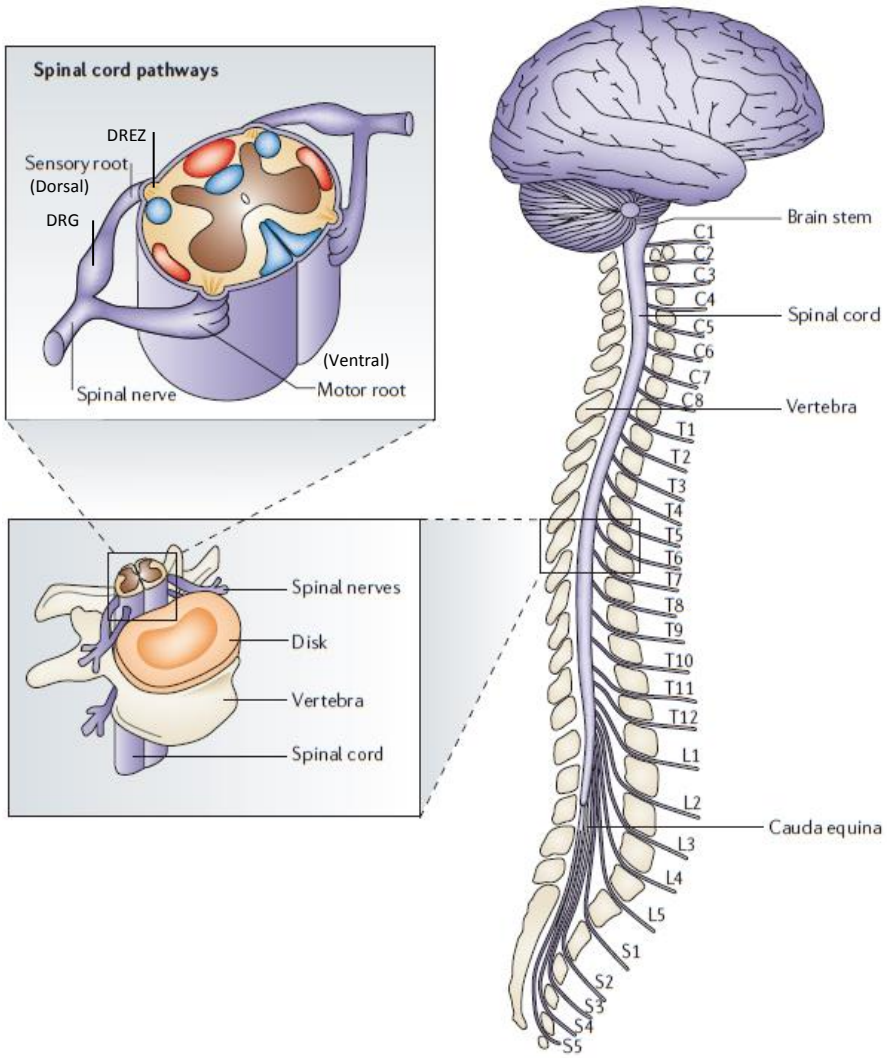


Figure 1 **| Human Spinal cord**

Communication between the body and the brain is made through the spinal cord which is segmented into four main groups: cervical (C), thoracic (T), lumbar (L) and sacral (S). Each nerve connects with a specific fragment of one of these 4 groups, through the ganglion and dorsal (sensory) root; and through the ventral (motor) root. Some of the sensory components of nerves are aligned in ascending tracks (colored in red), while motor neurons that come from the brain are lined up in descending tracks (colored in blue). The spinal cord is wrapped by vertebras that protect the nervous tissue from external aggressions. Adapted from (Bradbury & McMahon 2006)

2. The nervous tissue

The nervous tissue is a heterogeneous combination of cells that work together in order to keep the nervous system functional. Depending on their role and location in the nervous system these cells can be classified as neurons or neuroglia (also called glial cells). While neurons are responsible for conducting and processing external and internal stimuli, neuroglia has a more supportive and protective role, since it serves as scaffold, helps in electrochemical transmission and also provides nutrients to neurons (Tortora & Derrickson 2011). Knowing the histology of the nervous system is a crucial step to understand physiological changes, since each cell may contribute, either in a positive or negative manner, to nervous system survival. Therefore, in this section neurons' structure and function will be discussed, as well as some aspects of neuroglia.

2.1 Neurons

The simplest description of a neuron is that it is a polarized cell that possesses three well-established regions, namely a cell body (which contains the nucleus, membrane organelles, vesicles, endoplasmic reticulum and the Golgi apparatus), an axon that ends at the synaptic terminals, and branched structures called dendrites (Fig.2). While dendrites are responsible for receiving the action potentials, axons conduct them to the synaptic terminals, where stimulation of the adjacent cell is established through the release of neurotransmitters into the synaptic gap (Tortora & Derrickson 2011).

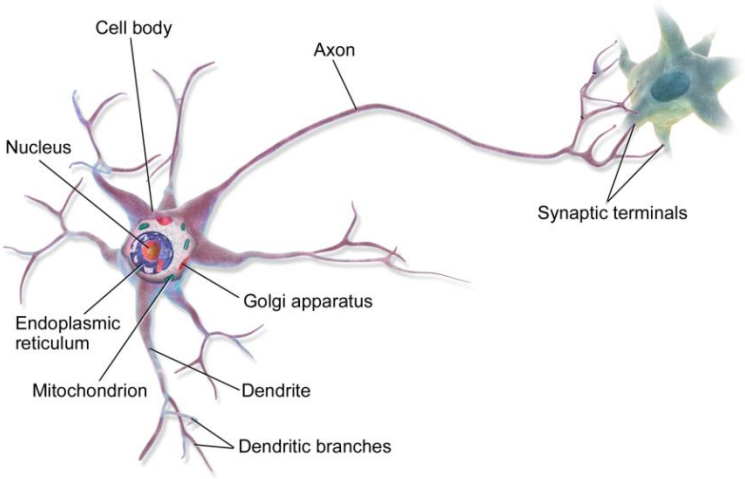


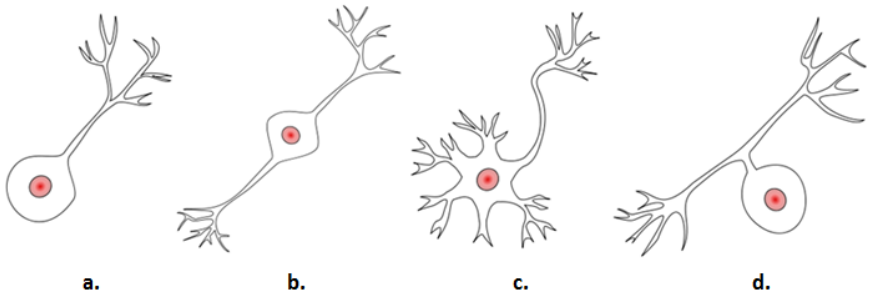
Figure 2 **| Neuron**

In the simplest representation of a neuron, neurons possess three regions, namely a cell body, that contains the nucleus, vesicles and membrane organelles, endoplasmic reticulum and Golgi apparatus; dendrites, which receive and transmit the action potentials; and an axon, that conducts the action potentials to their synaptic terminals. In synaptic terminals neurotransmitters are release to the synaptic gap, thus stimulating the adjacent cell. Adapted from (Communications 2013)

However, in the majority of the cases, neurons assume more complex shapes. Depending on their polarization they can be classified into four categories: unipolar, bipolar, pseudo-unipolar and multipolar (Fig.3) (Bear & Rintoul 2013).

Figure 3| Types of neurons

Neurons assume different sizes and shapes. Depending on their polarization they can be classified in **a.** unipolar; **b.** bipolar; **c.** multipolar and **d.** pseudounipolar. Adapted from (Bear & Rintoul 2013)



The particular structure of each neuron allows proper connection between them and thus efficient electrochemical transmission. For instance, in the reflex arc process, sensory input induces the stimulation of the dorsal root ganglion neurons, which are pseudounipolar neurons. These cells have two axons responsible for afferent transmission to interneurons, present in the spinal cord, which in turn will stimulate motor neurons. Both interneurons and motor neurons are multipolar neurons, which mean that they receive the stimuli in dendrites and transmit it along their axons (Fig.4) (Reece et al 2011, Tortora & Derrickson 2011).

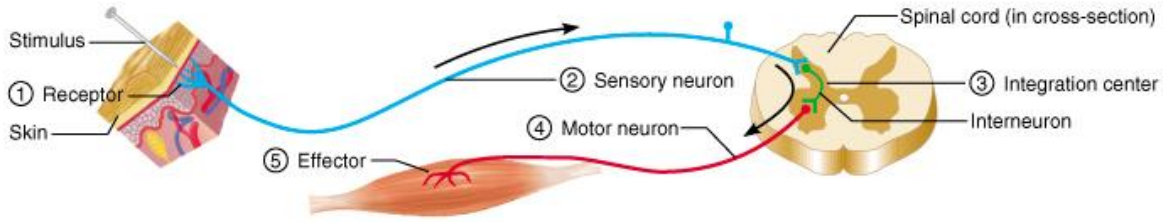


Figure 4**| Types of neurons that participate in the reflex arc**

In the arc reflex, the dorsal root ganglion (DRG) neurons (blue) are responsible for the afferent transmission, interneurons (green) integrate the information and motorneurons (red) allow efferent transmission. This capability is due to the unique structure of the neurons that participate in this process. While DRG neurons are pseudounipolar neurons, which means that they have two axons; interneurons and motorneurons are multipolar neurons which mean that they receive the stimuli through dendrites and transmit it through their axon (Reece et al 2011).

Efficient communication between neurons and, consequently competent performance of the nervous system, requires neuronal survival that depends on proper function of internal mechanisms.

2.1.1 Intrinsic mechanisms

Besides the unique ability of transmitting electrochemical messages, a lot happens within these cells, namely in soma which is the center of the genomic and metabolic changes that are essential for neuron survival and maintenance. However, cell metabolism is not restricted to the cell body, and both axons and dendrites require constantly support (Stahl 2008). Here, some neuron-specific features that allow cell survival and maintenance are addressed.

2.1.1.1 Protein synthesis

The major macromolecular components of neurons, as well as of any other cell, are proteins. Besides ensuring the unique structure of neurons, they are also crucial for neuronal function, since they participate in innumerous intracellular signaling mechanisms that allow proper response to extracellular stimulus, cell survival, and information storage and processing (Fallon & Taylor 2013). For years it was though that protein synthesis was exclusive of neuronal soma, although with the emergence of new evidences, it is now known that protein synthesis machinery is present in dendrites and axons during development, and also in mature peripheral axons (Twiss & Fainzilber 2009). Indeed, due to the extension of dendrites and axons, which in some cases stand several centimeters from the cell body, the presence of mRNA and translational machinery in different dendritic and axonal compartments allows a quick response to local requests by regulating local protein composition (Steward & Schuman 2003).

2.1.1.2 Axonal transport

An important feature of neuronal cells is their capacity to transport components along extended distances, which is fundamental for neuronal function and survival (Hirokawa et al 2010). This particular characteristic is due to a specialized microtubule and actin cytoskeleton that allows polarized transport by molecular motors (Kapitein & Hoogenraad 2011). Within dendrites and axons, longitudinal filaments of microtubules serve as road to molecular motors, such as dynein and kinesin, which are respectively responsible for retrograde and anterograde transport of molecules and organelles. While retrograde transport refers to the movement of cargoes that travel to the cell body, anterograde transport refers to the movement from the cell body (Fig. 5).

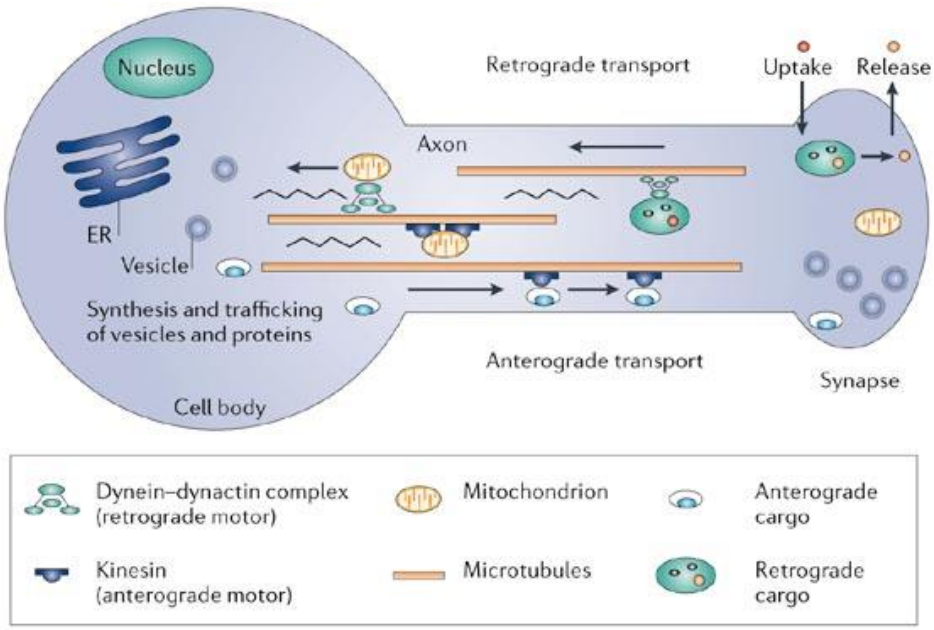


Figure 5 **| Axonal transport**

Axonal transport allows the transport of molecules and organelles through long distances. It can be classified in retrograde and anterograde transport. While retrograde transport is responsible for the movement of cargoes to the cell body. Anterograde transport corresponds to the movement from the cell body. (Pasinelli & Brown 2006)

In addition, in synaptic regions and in the tip of growing axons, the cytoskeleton is mainly composed by actin filaments and the transport of cargoes is coordinated by myosins (Kapitein & Hoogenraad 2011).

2.2 Glial cells

Glial cells are cells responsible for supporting neurons to perform their activities. Besides serving as physical support, they are responsible for modulating the environment that surrounds neurons (Bear & Rintoul 2013).

One of the main differences between the CNS and PNS is the neuroglia composition. While in PNS neuroglia is composed by Schwann and satellite cells, in CNS the majority of its components are astrocytes, oligodendrocytes, microglia and ependymal cells (Tortora & Derrickson 2011)(Fig. 6). Both Schwann cells, in PNS, and oligodendrocytes, in CNS, are responsible for axonal myelination, which not only offers physical support but also allows the rapid transmission of action potentials. Schwann cells also offer trophic support so that growing neurons reach their target and are also implicated in neuronal survival. Also in PNS, satellite cells regulate changes between neuronal soma and interstitial fluid. In CNS,

while astrocytes conduct nutrients and other substances from blood to neurons, microglia is responsible for the elimination of cell debris and pathogens through phagocytosis (Jessen 2004).

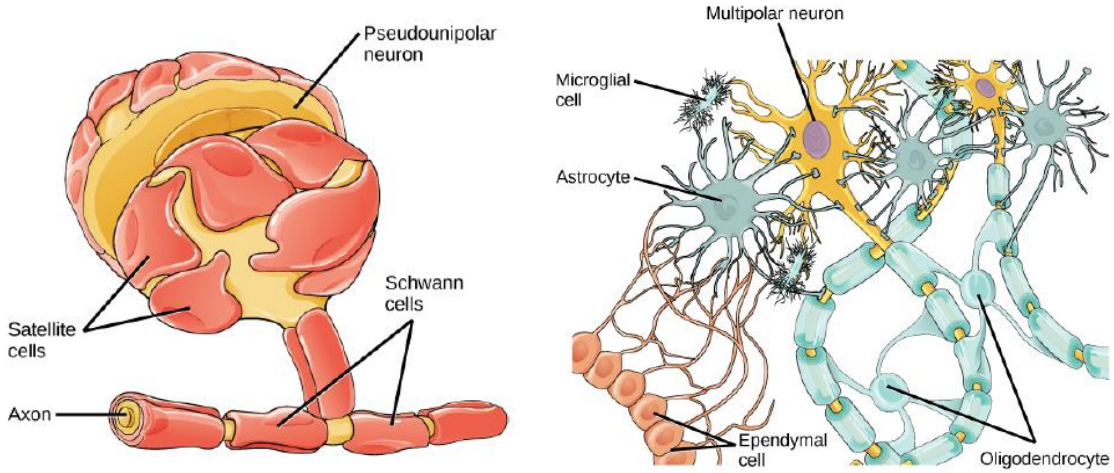


Figure 6 **| Glial cells found in the nervous system**

Glia cells provide support to neurons and regulate the external environment. **a.** Neuroglia from the peripheral nervous system includes Schwann cells and satellite cells; **b.** In central nervous system, astrocytes, oligodendrocytes, microglia and ependymal cells are the main glial cells (Bear & Rintoul 2013).

3. Injury to the nervous system

Axonal regeneration is possible

Following nervous system injury, neurons undergo a sequence of morphologic and metabolic changes that in most cases results in neuronal death and loss of nerve function (Bradbury & McMahon 2006). However, some neurons can trigger a regenerative program and axonal regeneration occurs (Liu et al 2011). Cruikshank was the first to suggest that damaged nerves could regenerate and regain their function, but it was Ramon y Cajal's work in nervous system degeneration and regeneration that supported those previous results (Ochs 1977). Since then, much has been done in order to understand the mechanisms underlying nerve regeneration, especially regarding the PNS which, unlike the CNS is able to trigger a regenerative program that leads to axonal elongation and in some cases reestablishment of neuronal connections (Huebner & Strittmatter 2009).

It is not new that the PNS and CNS have different responses when injured. In fact, in 1928, Ramon y Cajal highlighted these differences in a publication where several studies in neurons either from CNS and PNS were performed (Ramon y Cajal & May 1928). In one of those studies, it was shown that sensory central branches of DRGs once injured, either within the spinal cord or between the cord and the ganglion, behaved much likely to the peripheral nerves but regeneration was usually frustrated. Moreover, by showing that injured sensory roots stop growing when in contact with CNS tissue, he proposed that regeneration failure in CNS could be due to mechanical obstacles or lack of trophic factors. Five decades later, David and Aguayo demonstrated that injured CNS axons could regenerate into transplanted peripheral grafts, supporting the idea that interactions between axons and cellular environment modulate the different regenerative capacities of peripheral and central nervous system (David & Aguayo 1981).

The emergence of evidences that intrinsic signaling is also important for axonal regeneration, led Ambron and colleagues to raise the question whether a lesion could trigger intrinsic mechanisms that contribute to the neuronal response to injury. Indeed, by performing several studies in *Aplysia* they showed that the ability of injured neurons to regenerate their axons was also dependent on intracellular signaling (Ambron & Walters 1996). In addition, *in vitro* assays using dorsal root ganglia neurons from animals with either peripheral or central nervous system injury, revealed different growing patterns. While DRG neurons with previous injury on their central branch displayed short and branched neurites (projections that arise from the cell body), DRG neurons injured on their peripheral branches were able to extend long neurites, thus emphasizing that injury to the PNS and CNS elicit different responses and consequently different regeneration capacities (Smith & Skene 1997).

Altogether, these findings show that behind successful axonal regeneration relies a balance between extrinsic and intrinsic factors. Thus, in the following sections some of the most used injury models to study nervous system regeneration, namely those used in this thesis, will be presented and then the current knowledge about the extrinsic and intrinsic factors that determinates successful nerve

regeneration will be discussed, giving special emphasis to the intrinsic mechanisms that modulate axonal regeneration.

3.1 Injury Models to study Axonal Regeneration

Different are the paradigms used to study axonal regeneration. While some take advantage of the regenerative potential of the peripheral nervous system to study factors that prompt axonal regeneration, others use non-regenerative models, as is in the case of a spinal cord injury, to explore factors that contribute to axonal regeneration failure. Thus it is possible to classify these paradigms in two types, the non-regenerative and the regenerative models. Combinations between these two types of models have allowed some advances on the field and better understanding on the molecular mechanisms that contribute to the success or failure of axonal regeneration.

Non-regenerative models include injury to the spinal cord (SCI) and dorsal root injury (DRI) (Fig. 7, b. and c., respectively). As for the regenerative models, the most used are the sciatic nerve injury (SNI) and the conditioning lesion “paradigm” (Fig. 7, d. and e., respectively) (Bolsover et al 2008, Neumann & Woolf 1999). One characteristic shared by these models is that they can both use the regenerative capacity of DRG neurons.

DRG neurons are sensory pseudounipolar neurons with a peripheral branch located in the spinal nerve, and a central branch that arises from the DRG to the dorsal root, thus entering the spinal cord (Fig. 7, a.). Their cell bodies are clustered in the dorsal root ganglion (Tortora & Derrickson 2011). One important feature of these neurons is that when their peripheral branch is injured they are able to trigger a regenerative program that leads to axonal elongation and in some cases reinnervation of the correct targets. On the other hand, injury to their central branch, either by a SCI or a DRI, fails to trigger the same regenerative program and consequently axonal regeneration fails. However, despite the inefficiency of the SCI or even DRI to prompt axonal regeneration, the intrinsic regenerative capacity of these neurons in these two types of injury can be elicited by doing a conditioning lesion. Indeed, damaging the peripheral branch of DRGs – the conditioning lesion – prior to a lesion in their central one is sufficient to trigger intrinsic mechanisms that allow both branches to overcome an inhibitory environment and extend long neurites (Neumann & Woolf 1999).

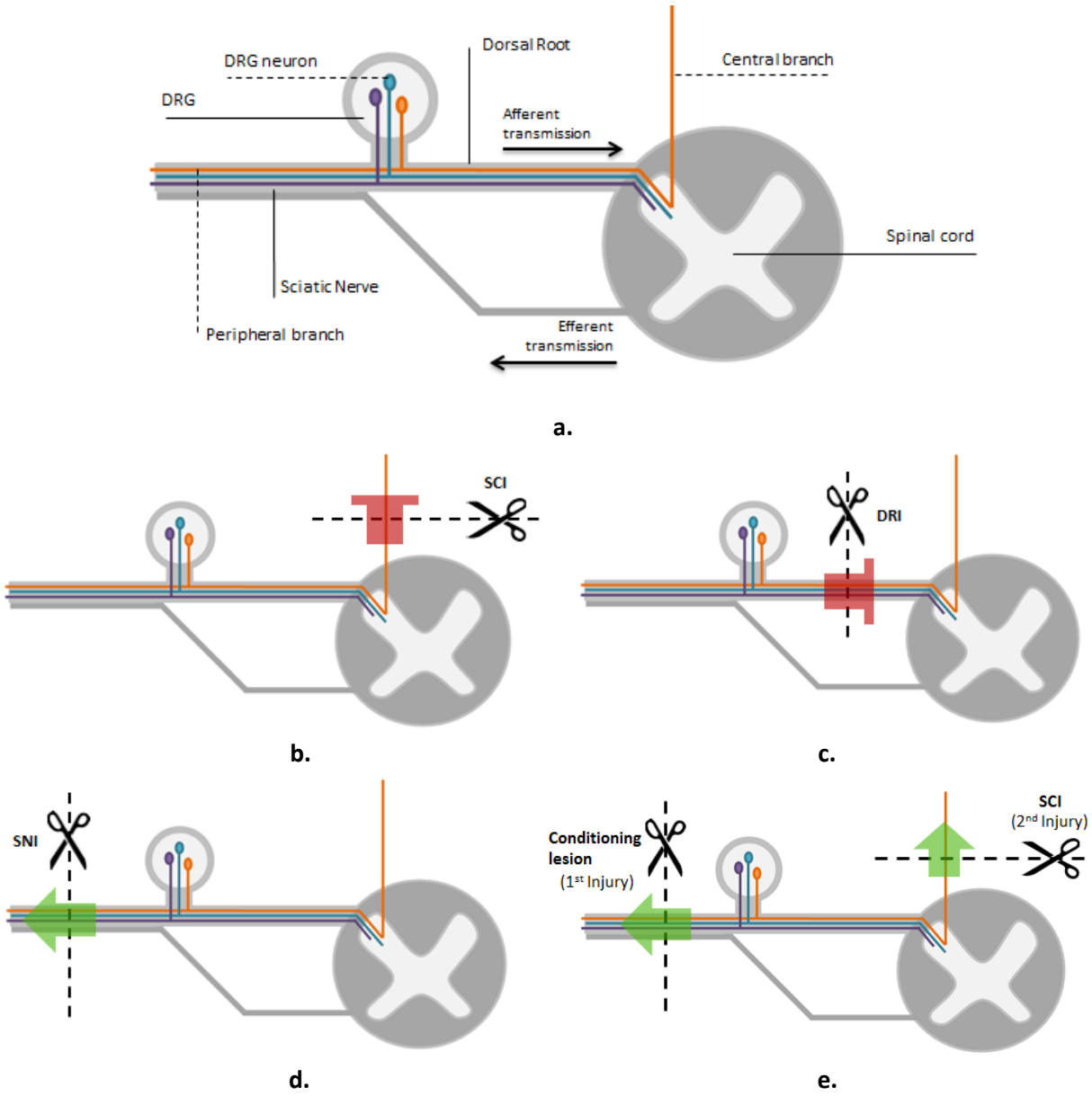


Figure 7 **| Injury models to study axonal regeneration**

DRG neurons are sensory pseudounipolar neurons widely used to study axonal regeneration. The middle panel illustrates some non-regenerative models, while the lower panel shows some regenerative models. **a.** Representative scheme of the spinal cross section and the sciatic nerve, including spatial localization of DRG neurons; **b.** Regeneration of DRG neurons central branch fails following spinal cord injury (SCI) or **c.** dorsal root injury (DRI); **d.** Sciatic nerve injury (SNI) triggers a regenerative program leading to the extension of long axons and eventually correct reiinervation; **e.** Regeneration of the central branch of DRG neurons can be elicited by performing a conditioning lesion prior to the SCI. Green arrows point to axonal outgrowth, while red arrows represent axonal regeneration failure.

3.2 Extrinsic Mechanisms that modulate Axonal Regeneration

Successful axonal regeneration is a process that depends on innumerous factors. In PNS, a crucial step to effective axonal regeneration relies on the rapid degeneration of the distal portion of the injured neuron and, competent clearance of myelin and cell debris. In the CNS these two events also occur, however, degeneration of the cut axon is a slow process and myelin and cell debris phagocytosis is less effective. Together, these molecular and cellular events constitute the Wallerian degeneration process, which is strongly modulated by the neuroglia and the immune system responses (Vargas & Barres 2007).

The first evidence of the Wallerian degeneration process emerged in 1850 when Augustus Waller described a process where the distal portions of axotomized neurons undergo disintegration (Waller 1950). Further studies have shown that there are at least three distinctive phases during axonal degeneration (Ambron & Walters 1996, Wang et al 2012). It starts with an acute axonal degeneration (also known as AAD) of both proximal and distal stumps of the injured neuron (Kerschensteiner et al 2005) which is followed by a latent period, where the distal portion starts to swell and irregular beads appear (George et al 1995) and finally, granular disintegration of axonal cytoskeleton (GDC) distal to the injury site occurs (Kerschensteiner et al 2005). This axonal degeneration takes about 24-48h to occur in rodents (Lubinska 1977) and several days in humans (Chaudhry et al 1992), however, the type of injury, the distance between the lesion and the cell body, as well as the axon's caliber also influence the time-course of these events (Beirowski et al 2005, Lubinska 1977). Wallerian degeneration is accomplished when the debris from axonal degeneration and myelin breakdown are cleared. Indeed, the different rates of Wallerian degeneration between the PNS and CNS are due to incompetent debris clearance, instead of a delay in the degeneration of the CNS neurons (George & Griffin 1994).

3.2.1 Wallerian degeneration in PNS

Successful regeneration within the PNS is dependent on the activity of Schwann and immune cells (Gaudet et al., 2011)(Fig.8).

Following injury, Schwann cells (SCs) along the distal segment, rapid dedifferentiate leading to demyelination of the cut axon, and myelin debris accumulation (Jessen and Mirsky, 2008). Injury also triggers downregulation of myelin proteins by SCs, which are known to have an inhibitory role in axonal outgrowth (He & Koprivica 2004, Trapp et al 1988). In addition, within the basal lamina, SCs start the clearance process by phagocytizing extracellular debris and their own myelin, and begin to proliferate creating a permissive substrate to axonal elongation (Stoll et al 1989). Besides, the physical support, they also secrete factors that elicit axonal outgrowth.

In the meantime, as soon as 8h post-injury resident neutrophils accumulate at the injury site. Besides its local phagocytic role, they also secrete factors that together with the onset of GDC recruit other immune cells. Indeed, within 48 h post-injury breakdown of the distal nerve's blood-nerve-barrier (BNB) occurs, allowing the influx of macrophages that once activated, start phagocytizing extracellular debris (Bouldin et al 1991, Bruck 1997).

In summary, SCs and immune cells create a proper environment that allows axons to regenerate over long distances (Gaudet et al 2011).

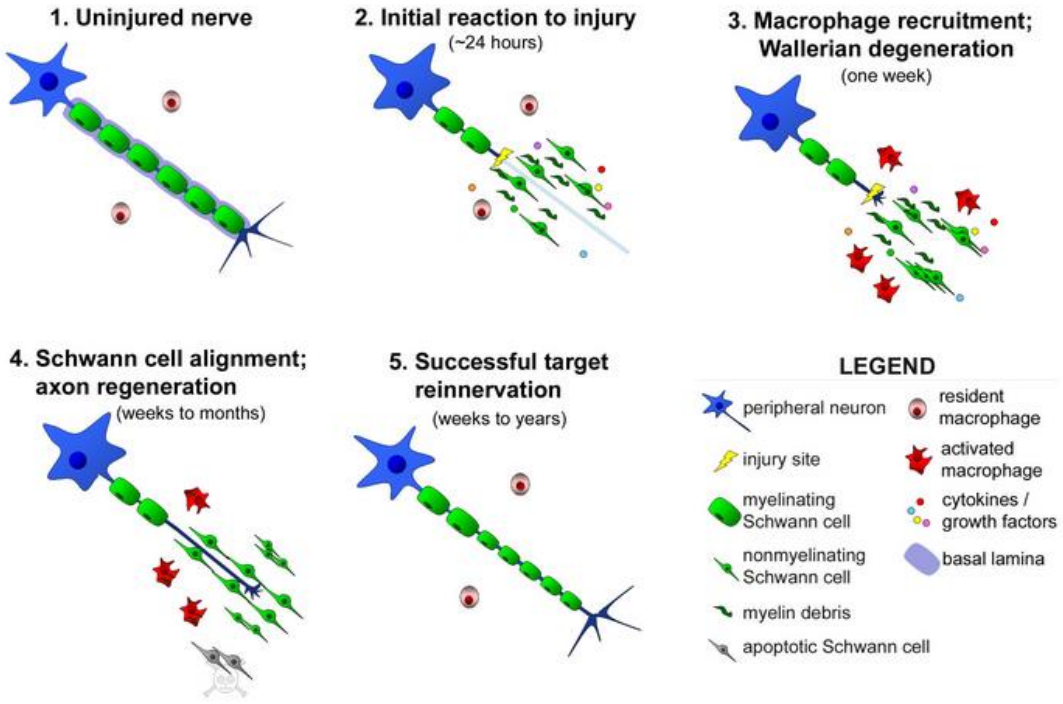


Figure 8 **| Wallerian degeneration in PNS and axon regeneration after peripheral nerve injury**

Successful axonal regeneration in the peripheral nervous system is dependent on the rapid axonal degeneration distal to the injury site and competent clearance of the extracellular debris, including myelin remains which are known to be inhibitory to axonal outgrowth. In parallel to the degeneration of the cut axons, Schwann cells release their myelin, dedifferentiate, phagocytize debris and begin to proliferate inside the intact basal lamina creating a permissive substrate for axonal elongation. In addition, the recruitment of immune cells, such as macrophages, speeds the myelin clearance process, thus allowing axonal elongation to take place. (Gaudet et al 2011)

3.2.2 Wallerian degeneration in CNS

In central nervous system, oligondendrocytes, unlike SCs, do not dedifferentiate or support debris clearance following injury, neither offer trophic support for axonal elongation. Instead, they undergo apoptosis or remain in a quiescent state. In addition, astrocytes become reactive and invade the

injury site, thus allowing the formation, together with oligodendrocytes, of a physical and chemical barrier to axon outgrowth - the glial scar (Fig. 9). Indeed, inhibitory cues either from oligondendrocytes myelin or astrocytes (that once reactive, start producing inhibitory molecules such as chondroitin sulfate proteoglycans - CSPG) are still present years after degeneration of the CNS (Buss et al 2004, Buss et al 2005).

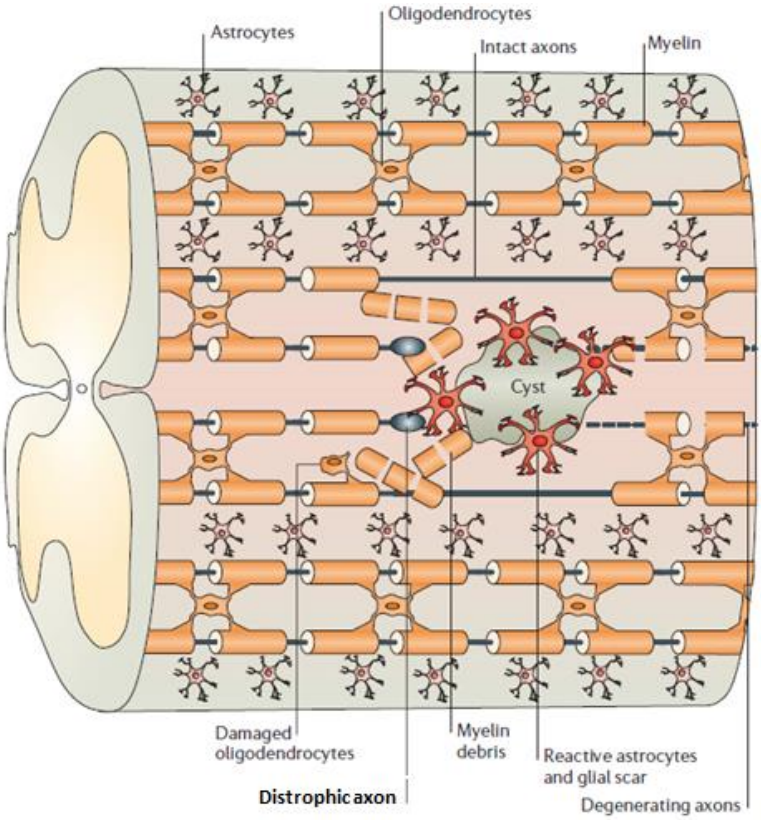


Figure 9 | Representation of CNS injury site and respective formation of the glial scar

Axon regeneration failure following injury to the spinal cord is a consequence of innumerous factors: astrocytes become reactive secreting inhibitory molecules and invading injury site; myelin debris from dead or quiscient oligodendrocytes contributes to the inhibitory environment; cyst formation may also occur, contributing to axonal regeneration imparment; and macrophage activity, in a different manner from what happen in PNS, is followed by the release of neurotoxic substances. Together, this results in the formation of the glial scar b. which constitutes a physical and chemical barrier to axonal outgrowth (Yiu & He 2006).

Also, unlike to what happens in PNS, whereas breakdown of the BNB occurs along the separated axon, breakdown of the blood-spinal cord-barrier (BSCB) is restricted to the injury site, impairing the clearance of the cut degenerated tracts. In addition, the activity of macrophages, although required to promote tissue repair, has also a neurotoxic effect that contributes for axon regeneration impairment, being this, a possible explanation why breakdown of the BSCB is restricted to the lesion site. Formation of fluid-filled cyst can also occur at the injury site. Together, these factors contribute for an inhibitory environment that leads to axonal regeneration failure following injury to the central nervous system (Gaudet et al 2011, Vargas & Barres 2007, Yiu & He 2006).

3.3 Intrinsic Mechanisms that modulate Axonal Regeneration

As evidenced before, external factors are crucial modulators of axonal regeneration. However, axonal outgrowth requires that injured neurons switch to a growth-competent state, otherwise regeneration fails. Indeed, regeneration-competent neurons are able to extend their axons even in the presence of an inhibitory environment (Neumann & Woolf 1999). Given this, it is appropriate to ask "Which are the intrinsic mechanisms that elicit axonal growth and the reinnervation of the correct targets?". Efforts to answer this question have allowed some advances on the field, although full recovery has never been achieved and some intrinsic mechanisms still need to be clarified.

So far, it is known that switching to a growth-competent state following injury requires in first place a local response, which involves the rearrangement of the cytoskeleton at the cut tip of the axon and the activation of several injury signals (Bradke et al 2012, Mar et al 2014a); followed by a somatic response where nuclear changes occur as a consequence of the signals received from the injury site (Kiryu-Seo & Kiyama 2011); and finally the transport of newly synthesized components to the cut tip (Mar et al 2014a). Altogether, these changes are capable of eliciting axonal elongation and guidance to the proper targets (Fig. 10).

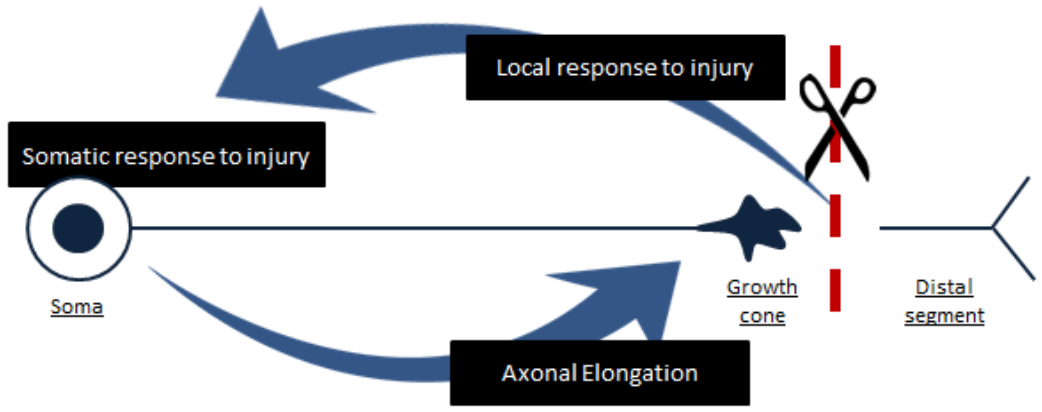


Figure 10 **|Activation of the regeneration program following injury**

Axonal injury triggers intrinsic changes which in some cases lead to the activation of a regeneration program capable of overcoming the inhibitory environment. Successful axonal regeneration is achieved when local changes elicit the rearrangement of the cytoskeleton at the cut tip and triggers a variety of intrinsic signals that will induce a somatic response. This somatic response to injury involves the synthesis of new components that, once transported to the cut tip, will allow axonal elongation and guidance to the correct targets.

3.3.1 Local response to injury

Following injury, axolemma is disrupted and the intracellular milieu is exposed to the extracellular content, leading to changes in homeostatic balance. Within 30 minutes, calcium influx activates calpain proteases that will induce a rapid disintegration of the cytoskeleton for a few hundreds of micrometers - AAD - and consequently membrane sealing (Eddleman et al 2000, Kerschensteiner et al 2005, Knoferle et al 2010).

After membrane sealing, some injured neurons are able to rearrange their cut end to form a competent growth cone in a process dependent on calcium influx and local protein synthesis (Kamber et al 2009, Verma et al 2005). The formation of this structure is crucial for axonal regeneration since it prepares the neuron for the next steps of axonal growth (Bradke et al 2012). In addition, activation of a variety of signaling mechanisms upon injury allows the cell body to receive precise information about the local changes caused by the lesion (Perlson et al 2004).

3.3.1.1 Growth cone assembling

The growth cone is a highly dynamic region found in the tip of growing axons and it is responsible for the integration of extracellular cues with intracellular signaling to guide axonal elongation (Bradke et al 2012, Kaplan et al 2014). Indeed, following injury, calcium influx allows the formation of a growth cone. Moreover, in a calcium free environment, axotomized neurons do not assemble a new growth cone and regeneration fails (Chierzi et al 2005).

Depending on their cytoskeleton distribution, it is possible to identify three different regions, namely the peripheral domain (P), the transition domain (T) and the central domain (C) (Fig. 11). The P domain is the outermost region of the growth cone and is mainly composed by actin filaments (F-actin) that are rearranged to form long bundles of F-actin (filopodia) separated by mesh-like networks of F-actin (lamellipodia). Due its long extension, the filopodia is the main responsible for exploring the surrounding environment. The C domain, which is the nearest region to the axon, is formed by dynamic microtubules that can protrude into P domain, and houses a variety of cytoplasmic vesicles and membranous organelles. The third and last region, the T domain, is the transition zone between P and C domains and is highly enriched in actin arcs (actomyosin contractile structures) that form a hemicircumferential curve (Lowery & Van Vactor 2009). Together, these domains determine the movement of the growth cone and, as a consequence, the elongation pathway of the growing axon.

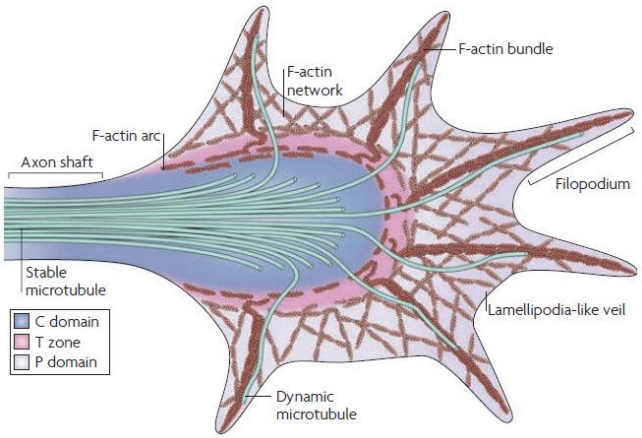


Figure 11 **| Growth cone structure and components**

The growth cone is a complex found in the tip of growing axons and is capable of changing its shape and direction in response to extracellular cues. In the periphery of the growth cone, it is possible to find two types of structures, the filopodia and lamellipodium, which mediates their movements. (Lowery & Van Vactor 2009).

Of equal importance are the extracellular cues, since they exhibit repulsive and attractive interactions over the growth cone, which then guide the axon through a pathway that in optimal conditions lead to the right target (Fig. 12). Indeed, some external cues, such as neurotrophins, are required to stimulate axonal growth and cell survival by triggering intracellular signals (Paves & Saarma 1997). When depleted of neurotrophic factors, growing neurons fail to innervate their target or die (Polleux & Snider 2010). Extracellular cues include diffusible chemotropic cues, such as guidance cues (netrins and semaphorins) and neurotropic factors (nerve growth factor (NGF)); adhesive substrate-bound cues, such as the extracellular matrix (ECM) protein laminin; and repellent substrate-bound cues that include the repulsive cue ephrin and the ECM inhibitors, CSPG (Lowery & Van Vactor 2009).

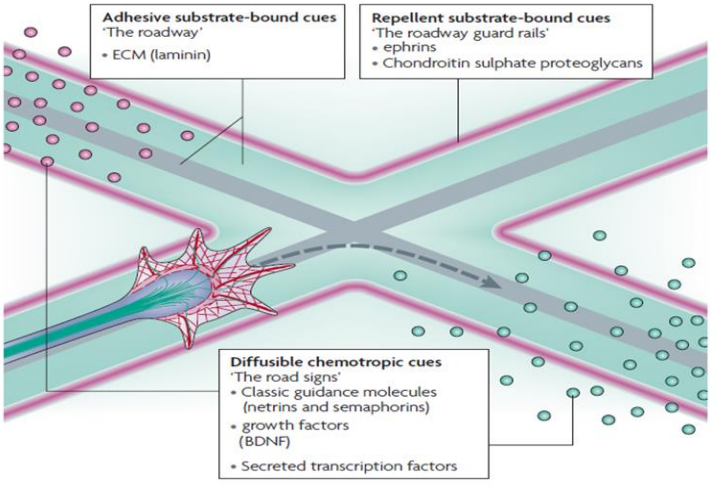


Figure 12 **| Extracellular cues determine the elongation pathway of the axon**

By exerting over the growth cone repulsive and attractive interactions, extracellular cues determine the elongation pathway of the growing axon. Extracellular cues include adhesive and repellent substrate-bound cues, and diffusible chemotrophic factors. Adapted from (Lowery & Van Vactor 2009)

As referred before, it is the interaction with external cues that allows the growth cone to change its shape and direction. But how the growth cone is capable of such changes is a question that has puzzled investigators for years. It is currently known that these interactions are mediated by cell-surface

receptors that trigger a variety of intracellular signaling pathways, thus leading to cytoskeleton rearrangement (Bradke et al 2012). One of the major players in regulation of growth cone dynamics is the Rho family of small GTP-binding proteins (RhoGTPases), being the Cdc42, Rac, and RhoA, the best studied ones (Huber et al 2003). By rearranging actin cytoskeleton they are capable of mediating growth cone guidance and axonal elongation. Indeed, activation of Cdc42 and Rac induce the formation of filopodia and lamellipodia, respectively, by promoting actin polymerization and consequently axon guidance and elongation. On the other hand, by activating ROCKII (Rho-associated protein kinase II) which phosphorylates myosin light chain (MLC), RhoA stimulates the constraining of the actin arcs and promotes the assembling of stress fibers (myosin filaments), thus leading to growth cone contraction and reduced motility (Fig. 13).

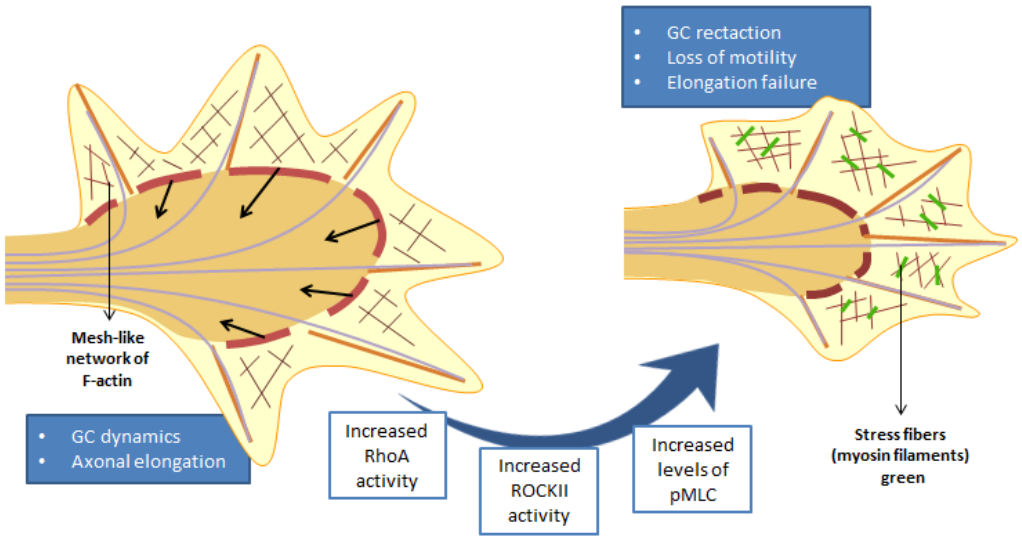


Figure 13 | RhoA/ROCKII pathway in growth cone

Increased activity of RhoA leads to the activation of ROCKII which then phosphorylates myosin light chain (MLC). This will lead to growth cone retraction, loss of motility and consequent elongation failure. Adapted from (Lowery & Van Vactor 2009, Luo 2000).

Typically, attractive cues activate Cdc42 and Rac, and inhibit RhoA; while repellent cues have the opposite effect (Guan & Rao 2003). In the presence of growth inhibitors the growing tip of the axon becomes "trapped" and the disregulation of intracellular signaling leads to growth cone retraction or even growth cone collapse in a process dependent on ROCKII activity (Mueller et al 2005). In addition, inhibition of Rho kinase activity has also been shown to enhance axonal regeneration either in injured central or peripheral nervous systems (Fournier et al 2003, Hiraga et al 2006), supporting the inhibitory role of RhoA/ROCKII and its effectors in axonal regeneration.

3.3.1.2 Injury signaling mechanisms

Regeneration of injured axons is a process that requires the synthesis of new components in soma, including cytoskeleton proteins, such as tubulin and actin, transmembrane proteins, like membrane receptors and voltage gated channels, membrane lipids, signaling molecules and metabolic enzymes (Goldberg 2003). Since in most cases the cell body stands several centimeters from the lesion site, retrograde signaling is required to "inform" the cell body that an injury has taken place (Ambron & Walters 1996, Rishal & Fainzilber 2010). Below some of the injury signals that elicit a proper somatic response (Fig. 14) are summarized.

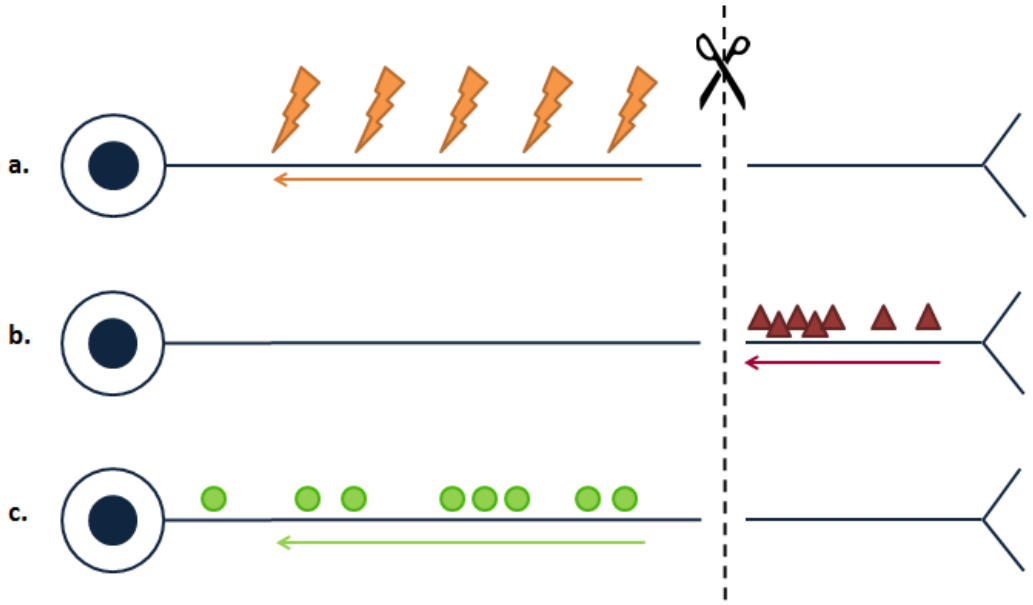


Figure 14 | **Injury signaling mechanisms**

Injury triggers a variety of signals that will inform the soma that an injury occurred. **a.** The first signal that arrives the cell body is the electrophysiological response caused by membrane depolarization. This is followed by motor-dependent signals either the loss of suppressors of axonal growth (Negative injury signals) **b.**, or the activation of injury signals at lesion site (Positive injury signals) that will be retrogradely transported to the cell body **c.** Adapted from (Rishal & Fainzilber 2010)

3.3.1.2.1 Depolarization

Membrane depolarization due to calcium and sodium entry through the axonal cut ends is one of the first changes that occur upon injury. These local alterations in ionic concentrations lead to activation of voltage-gated Ca^{2+} channels and reversal of the Na/Ca exchanger, thus increasing free-intracellular Ca^{2+} concentration and causing the propagation of a calcium wave from the lesion site to the cell body (Mandolesi et al 2004). In the soma, calcium elicits the activation of a variety of signals associated with axonal regeneration. In fact, increased intracellular calcium stimulates Ca^{2+}-dependent

enzymes, such as adenyl cyclase (AC), which will then convert ATP to cyclic adenosine monophosphate, cAMP (Fig. 15). cAMP is a signaling molecule that is increased following peripheral nerve injury, and it has been linked to a robust axonal regeneration (Lau et al 2013). Experiments that include the injection of the membrane-permeable analog of cAMP, dibutyryl-cAMP, in lumbar dorsal ganglia (Neumann et al 2002); or administration of rolipram, a phosphodiesterase inhibitor that impairs the conversion of cAMP into AMP (Nikulina et al 2004), have supported the importance of this signal to a successful axonal regeneration.

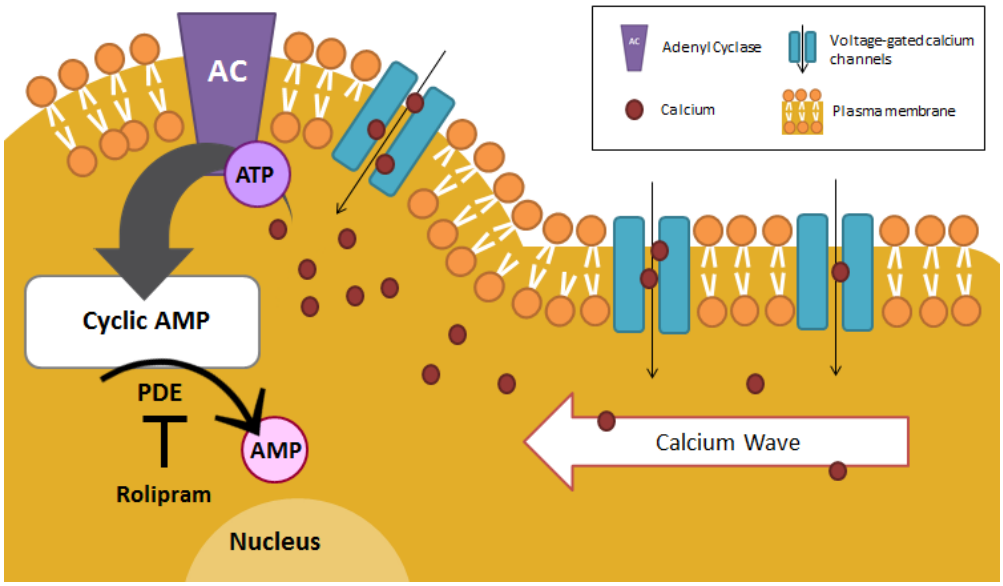


Figure 15 **| Activation of cAMP in regeneration-competent neurons**

Following lesion there is backpropagation of a calcium wave from the injury site to the soma leading to a general increase in free intracellular calcium concentration. Calcium will then activate adenyl cyclase (AC) responsible for converting adenosine triphosphate (ATP) into cAMP (cyclic-AMP). Increased levels of cAMP in the soma are associated with a robust regenerative response. Phosphodiesterases (PDE) are responsible for diminuishing cAMP levels by converting cAMP into AMP. This last conversion can be abolished by administrating Rolipram, a phosphodiesterase inhibitor, promoting axonal regeneration. Adapted from (Hannila & Filbin 2008, Hofer 2012, Nikulina et al 2004)

Recently, it has also been shown that the back-propagation of a calcium wave results in nuclear export of HDAC5 that leads to the activation of a proregenerative gene-expression program and successful axonal regeneration (Cho et al 2013).

Additionally to the calcium wave, it has been reported that in some neurons local depolarization may reach a threshold that triggers a burst of action potentials, thus contributing to the increased calcium levels found in the cell body upon injury and axonal regeneration (Mandolesi et al 2004, Ziv & Spira 1995). Therefore, it would be expected that electric stimulation would improve axonal regeneration. However, it is not clear whether this type of stimulation is capable of eliciting a regenerative response, since reports about this field are not consistent. For example, while electrical

stimulation caused axonal growth in sensory neurons (Udina et al 2008), transected rubrospinal tract was not able to regenerate when stimulated (Harvey et al 2005). A good explanation for these differences may rely on the paradigm used to stimulate the injured neurons, i.e, the pulse duration, numbered of pulses applied, the chosen system, among other aspects. In fact, Harvey and colleagues (2005) refer that the system used by them was not able to replicate other studies where electric stimulation had elicited axonal regeneration. Moreover, in the study performed in Fouad's lab, although electric stimulation was capable of promoting axonal outgrowth, regeneration was not sustained over long distances (Udina et al 2008).

Together these results suggest that electric stimulation may be required in the early stages of axonal regeneration, but it is not sufficient to trigger a capable regenerative response.

3.3.1.2.2 Negative Injury signals

Although little is known about this type of signals, they are referred in literature as being suppressors of axonal growth so that synaptic formation could take place. In the same line of thoughts, it is hypothesized that, as a consequence of injury, neuronal disconnection from its targets leads to down-regulation of these signals, thus allowing axonal regeneration. As for my knowledge the only signal that completes these requisites is SMAD2 since is down-regulated following peripheral nerve injury (Abe & Cavalli 2008).

3.3.1.2.3 Positive injury signals

In the 90s, studies in *Aplysia* suggested that axotomy was able to produce a variety of axoplasmic molecules responsible for the regenerative response (Ambron et al 1995). Today, it is known that the activation of these molecules – the positive injury signals - is dependent on local phosphorylation of proteins as well as local translation, and that retrograde transport machinery ensures that these signals are properly carried to the nucleus (Mar et al 2014a, Rishal & Fainzilber 2014). Moreover, the improvement of new techniques and several studies had allowed so far the recognition of three positive injury signals. Those are the signal transducer and activator of transcription factor 3 (STAT3) (Ben-Yaakov et al 2012, Sheu et al 2000), the extracellular signal regulated kinase (ERK) (Perlson et al 2005, Sheu et al 2000) and the c-Jun N-terminal kinases (JNK) (Cavalli et al 2005). In the following paragraphs the mechanisms that trigger the activation of these signals and their retrograde transport will be discussed, as well as their importance in axonal regeneration.

STAT3 is a transcription factor associated with JAK-STAT pathway and belongs to the STAT family. Extracellular signals, such as cytokines and growth factors, lead to the activation of this pathway and subsequent phosphorylation of STAT proteins. In PNS, after injury, specifically STAT3 is locally translated, subsequent phosphorylated and retrogradely transported to the nucleus (Ben-Yaakov et al 2012, Sheu et

al 2000). Moreover, it had been suggested that activated STAT3 links to the dynein motor to be retrogradely transported through the axoplasmic importin-α5 and the post-injury locally translated importin-β (Ben-Yaakov et al 2012) (Fig. 16). Its activation and transport to the nucleus have been described as being important to enhance the regenerative potential and cell survival of dorsal root ganglion neurons by inducing the transcription of genes (Ben-Yaakov et al 2012, Miao et al 2006, Qiu et al 2005). Recently, it has also been shown that STAT3 activity is required in the initial steps of axonal growth, since its depletion delayed axonal elongation but did not abolish it (Bareyre et al 2011).

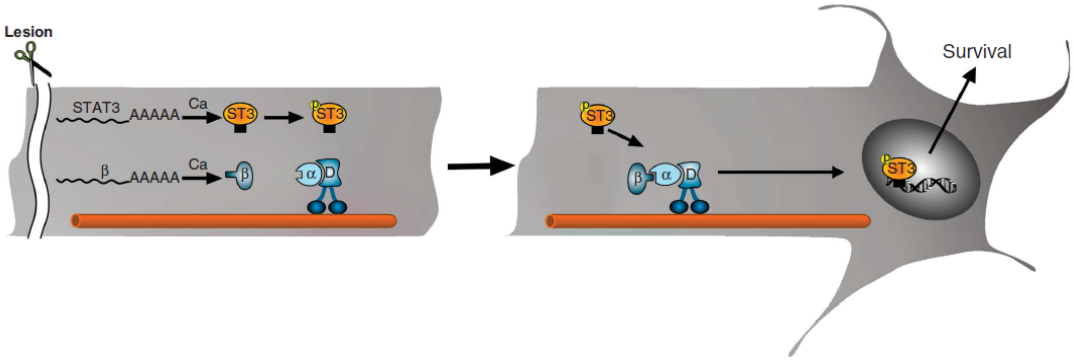


Figure 16 | Retrograde transport of pSTAT3

Following injury, there is local translation of the transcription factor STAT3 followed by its phosphorylation. Local translation of importin-β, also triggered by injury, increases their affinity to the dynein motor complex, leading to the retrograde transport of p STAT3 to the nucleus, thus contributing for the initiation of the regenerative program. (Rishal & Fainzilber 2010)

The other two positive injury signals, ERK and JNK, are protein kinases and belong to the mitogen-activated protein kinases (MAPKs) family. They are key regulators of cell function by coordinating the activity of several proteins through the addition of phosphate groups to substrate proteins (Pearson et al 2001).

Following injury, ERK is immediately phosphorylated and the local translation of vimentin and importin-β increases pERK affinity to the retrograde transport machinery (Fig. 17). Moreover, binding of pERK to vimentin not only allows its retrograde transport by dynein, but also protects phosphorylated ERK from phosphatase inhibitors. In sciatic nerve injury, it takes approximately 20h to pERK be transported to the cell body, where it will activate the transcription factor Elk-1 (E twenty-six like transcription factor 1). Also, blocking retrograde transport of pERK following injury will impair in part the somatic response to injury (Perlson et al 2005, Perlson et al 2006).

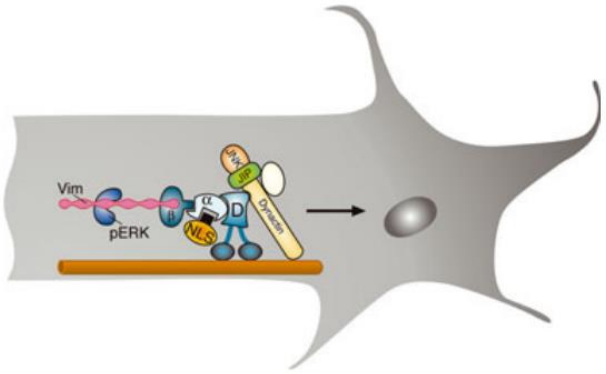


Figure 17 **| Retrograde transport of pERK**

Injury triggers the phosphorylation of the positive injury signal ERK. Through the local translation of importin-β and vimentin, pERK binds to the dynein complex and is retrogradely transported to the neuronal soma, where it activates transcription factors, such as ELK-1, associated with the regenerative program. (Hanz & Fainzilber 2004)

In a similar way to what happens with ERK, within an hour after injury, JNK is phosphorylated and coupled with sunday driver to the dynein molecular motor, to be then retrogradely transported in axonal vesicles (Cavalli et al 2005). In the cell body, activated JNK will phosphorylate the transcription factor c-jun (Lindwall & Kanje 2005), thus allowing the expression of genes associated with axonal regeneration. In addition, while absence of c-jun in the nervous system leads to drastic effects on axonal regeneration (Raivich et al 2004), overexpression of both c-jun and signal transducer and activator of transcription 6 (STAT6) increases total neurite length in cortical neurons (Lerch et al 2014).

3.3.2 Somatic response to injury

Somatic changes are also required to prompt axonal regeneration. In regeneration-competent neurons, the activation of several transcription factors coordinates the expression of several genes that elicit axonal regeneration – the regeneration associated genes (RAG) (Fig. 18). Indeed, one of the main differences between the peripheral and central responses to injury relies in the capacity of eliciting RAG expression. While PNS injury is followed by the activation of several RAG, CNS injury *per se* fails to activate those genes (Ylera et al 2009).

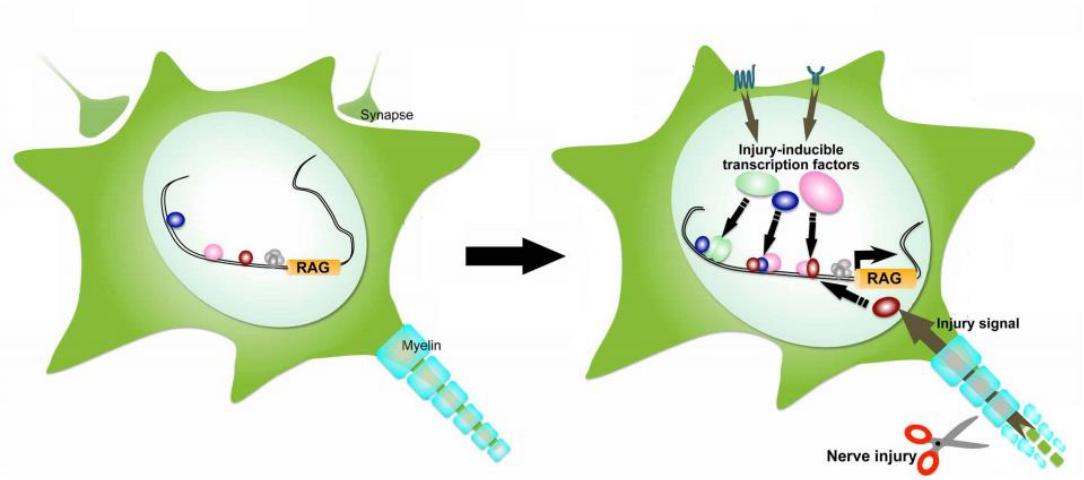


Figure 18 **| Nuclear changes following nerve injury**

Following injury, activation of the transcriptions factors, as well as upregulation of the regeneration-asssociated genes (RAG) elicit the intrinsic capacity of neurons to prompt axonal regeneration. (Kiryu-Seo & Kiyama 2011)

Large scale screening approaches have allowed the identification of several transcription factors and genes that contribute for axonal regeneration. Some of the current known transcription factors that participate in the regeneration program include cAMP response element-binding protein (CREB) (Hannila & Filbin 2008), signal transducer and transcription factor 3 (STAT3) (Ben-Yaakov et al 2012), ETS-like transcription factor 1(Elk-1) (Perlson et al 2005), c-Jun (Raivich et al 2004), activating transcription factor 3 (ATF3) (Seijffers et al 2007), sex determinant region Y-box 11 (sox11) (Jankowski et al 2006) and Mothers against decapentaplegic homolog 1 (Smad1) (Zou et al 2009). Also, hundreds of RAGs have been identified, namely neuropeptide-Y (NPY), vasoactive intestinal peptide (Vip), arginase (ARG), growth associated protein (GAP-43), activating transcription factor 3 (ATF3) and cytoskeleton genes, such as tubulin and actin (Hoffman 2010, Xiao et al 2002, Ylera et al 2009).

The mechanisms by which transcription factors mediate gene expression are quite complex since one single transcription factor is capable of activating/repressing several genes. Moreover, a proper somatic response to injury depends on a balanced activation of the transcriptions factors, which is achieved when a favorable cross-talk between several intracellular signaling pathways occurs (van Kesteren et al 2011).

Recent studies have also raised the importance of epigenetic modifications in axonal regeneration. Gene expression requires the formation of transiently opened chromatin regions which is achieved through histone modifications that are regulated by chromatin-modifying enzymes, such as histone aceltyltransferases (HATs) and histone deacetylases (HDACs)(Kiryu-Seo & Kiyama 2011), (Fig. 19). Indeed, chromatin-modifying enzymes can be targeted to a specific DNA sequence by their binding with transcription factors (Borrelli et al 2008).

Also, the nuclear export of HDAC5 was shown to be essential for axon regeneration following peripheral nerve injury (Cho et al 2013). In another recent paper, it was shown that peripheral nerve injury induces histone 3 acetylation by the HAT p300/CBP-associated factor (PCAF), and that the same does not occur following central nervous system injury (Puttagunta et al 2014). In addition, Puttagunta and colleagues revealed that the activation of the PCAF-regeneration program following conditioning was dependent on activated ERK, and that PCAF injection in CNS damaged mice was capable of increasing nerve fiber regeneration. Together these evidences highlight the importance of chromatin-modifying enzymes, and suggest that they may offer some therapeutic opportunities.

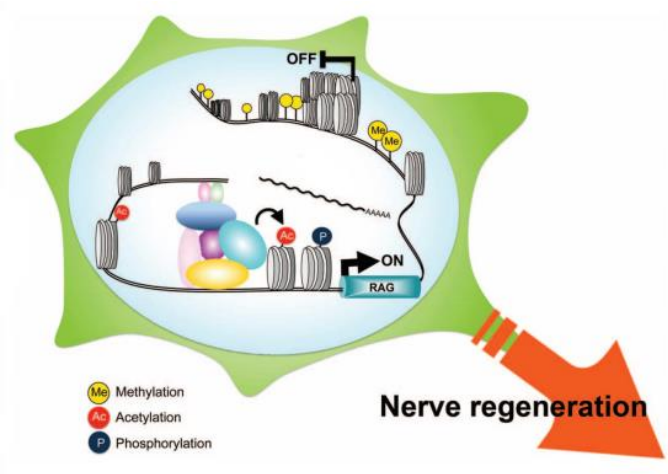


Figure 19 **| Epigenetic changes following nerve injury**

In addition to the activation of injury-induced transcription factors and upregulation of RAG, evidences are supporting the importance of epigenetic modications in axonal regeneration, which include chromatin remodeling by activated histones, thus allowing successful transcription of RAG. Adapted from (Kiryu-Seo & Kiyama 2011)

3.3.3 Axonal elongation

Growing axons require a continuum support of newly synthesized components, such as cytoskeleton, membrane and cytoplasmic molecules. Although recent reports suggest that local protein synthesis may help on this process (Gumy et al 2010), the majority of these components are transported from the cell body to growing tips (Mar et al 2014a). Thus, anterograde transport is a fundamental tool in axon outgrowth. Anterograde transport is divided into three components, the slow component a (SCa), which is responsible for the transport of tubulin, microtubule-associated proteins and neurofilaments; the slow component b (SCb), that transports actin and cytoplasmic proteins; and finally the fast component that transports components of membrane organelles, such as vesicles and mitochondrion (Lasek et al 1984). Interestingly, axon regeneration rate is similar to the speed of SCb (Wujek & Lasek 1983).

Chapter 1

Intrinsic changes following dorsal root injury

Why does axonal regeneration fail?

In this chapter, through the use of non-regeneration models, evaluation of some of the intrinsic mechanisms that impair injured neurons to switch to a growth-competent state was proposed. Thus, I will start to present the preliminary results obtained at the Nerve Regeneration lab that had allowed me to do define the research goals of this chapter. The materials and methods used to achieve those research goals will be also included here. Finally, the results obtained will be discussed.

Preliminary results

Unlike the central nervous system (CNS), whereas an injury *per se* cannot trigger an efficient regenerative program, neurons from peripheral nervous system (PNS) can respond to injury, regrow their axons and in some cases reinnervate their targets. Due to its particular structure and capacity to regenerate, dorsal root ganglion (DRG) neurons have been widely used to study nerve regeneration. In fact, while an injury at DRGs' peripheral branch is followed by axonal regeneration, the same response is not observed when the central branch is injured. *In vitro* assays have revealed that neurons which had suffered a sciatic nerve injury (SNI) extended long processes, whereas neurons with a spinal cord injury (SCI) or dorsal root injury (DRI), instead of long axons, displayed shorter and highly branched neurites (Smith & Skene 1997). Some authors have linked these differences to the environment that surrounds both branches, being the presence of inhibitory molecules the main reason why neurons stop growing when in contact with CNS tissue (Zhang et al 2001). However, several studies revealed that different injuries can trigger different intrinsic responses. To support this, evaluation of gene expression has shown that following peripheral injury regeneration associated genes (RAG) are expressed, whereas damaging the CNS fails to induce the same response (Mason et al 2002, Schreyer & Skene 1993, Ylera et al 2009). Again, evidences suggest that different signaling pathways are triggered following either DRI or SNI.

In order to assess those differences, DRI and SNI models were used to explore the local activation of injury signals and their importance in axonal regeneration. To do so, L4 and L5 DRG neurons from adult Wistar rats with either SNI or DRI were plated on permissive and inhibitory substrates; L4,5 DRGs from naïve animals were used as control. The longest neurite and the percentage of cells with neurites were then measured, confirming that only a SNI could trigger a regenerative program that *per se* could overcome an inhibitory environment (Fig. 1.1).

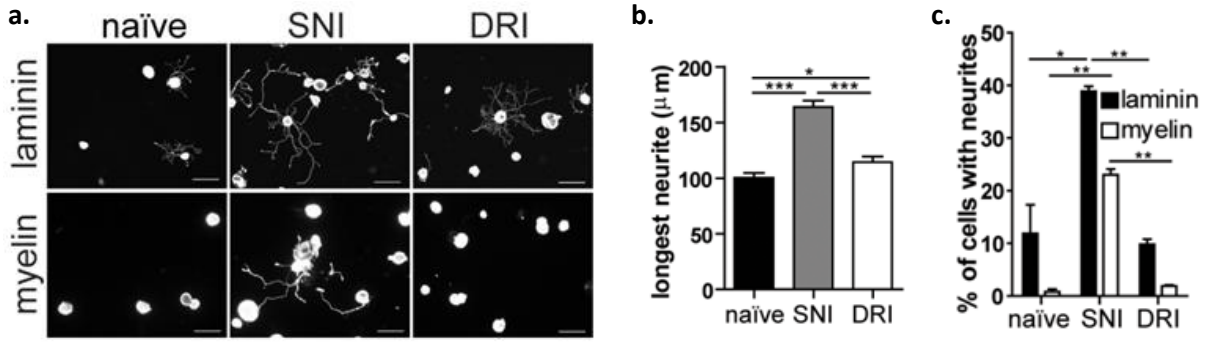


Figure 1.1 **| DRI does not prompt an efficient axonal regeneration**

a. Representative images of β-III tubulin immunohisctochemestry of L4,5 DRG neurons from naïve Wistar rats or rats with SNI or DRI (n=4/group), plated on either a permissive (laminin) or inhibitory (myelin) substrate; scale bar 100 μm. **b**. Longest neurite measurements of a; **c.** Percentage of cells with neurites from a. Results represent the mean ± SEM. *p<0.05, **p<0.01 and ***p<0.001. (Nerve Regeneration group, unpublished)

Gene expression of some of the best characterized RAG, namely neuropeptide-Y (NYP), vasoactive intestinal peptide (Vip), growth associated protein (GAP-43), arginase (ARG1) and activating transcription factor 3 (ATF-3), was also evaluated under the conditions described above. As expected, RAG expression was significantly lower in DRG neurons from animals with DRI (Fig. 1.2), meaning that a DRI fails to activate intrinsic mechanisms that allow axonal regeneration.

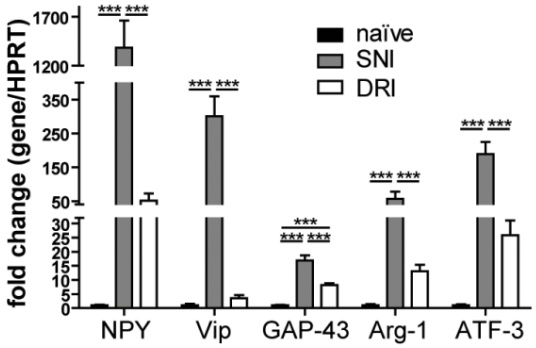


Figure 1.2 | **DRI fails to efficiently upregulate RAG expression**

RT-qPCR performed in L4, 5 DRGS from Wistar rats with SNI or DRI; naïve animals were used as control. Results represent the mean ± SEM. *p<0.05, **p<0.01 and ***p<0.001. (Nerve Regeneration group, unpublished)

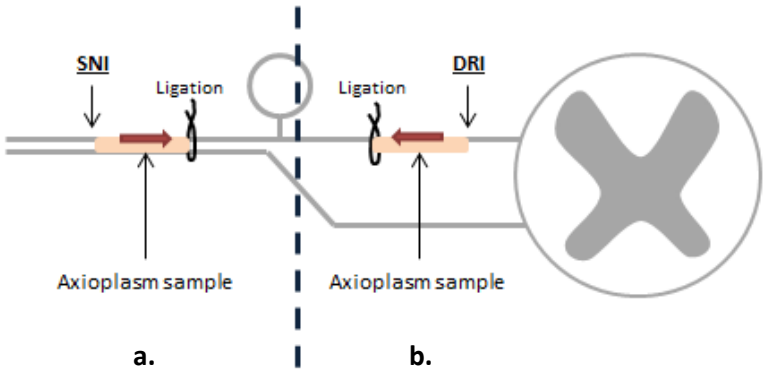
Together these results suggest that different signaling mechanisms are mounted following either SNI or DRI. In order to understand the reasons underlying these differences, two strategies were used: evaluate the local activation of the current known positive injury signals following DRI; and identify eventual differences in protein profiles that are locally activated and retrogradely transported following either DRI or SNI. Thus, in the following two parts these two strategies will be discussed.

Local activation of positive injury signals following DRI

To understand if the inability of DRG neurons to mount a regenerative program following DRI was due to an inefficient activation of the known positive injury signals, axoplasm from dorsal roots (central branch) or peripheral nerves was collected using the crush-ligation paradigm (Fig. 1.3).

Figure 1.3 | **Injury-ligation paradigm**

Representative scheme of the injury-ligation model used to collect axoplasm samples from **a.** sciatic nerve following (SNI) or **b.** dorsal root after (DRI). Orange boxes represent axoplasm samples, while red arrows indicate the movement orientation of the dynein-signal complex. Adapted (Mar et al 2014b)



Dynein immunoprecipitation (Perlson et al 2005) followed by immunoblot analysis against pERK were then performed. The levels of pSTAT3 and pJNK were also evaluated in the injured axoplasm. Surprisingly, it was observed that after DRI, ERK, JNK and STAT3 were also activated in a similar way to what happens after a SNI (Fig. 1.4).

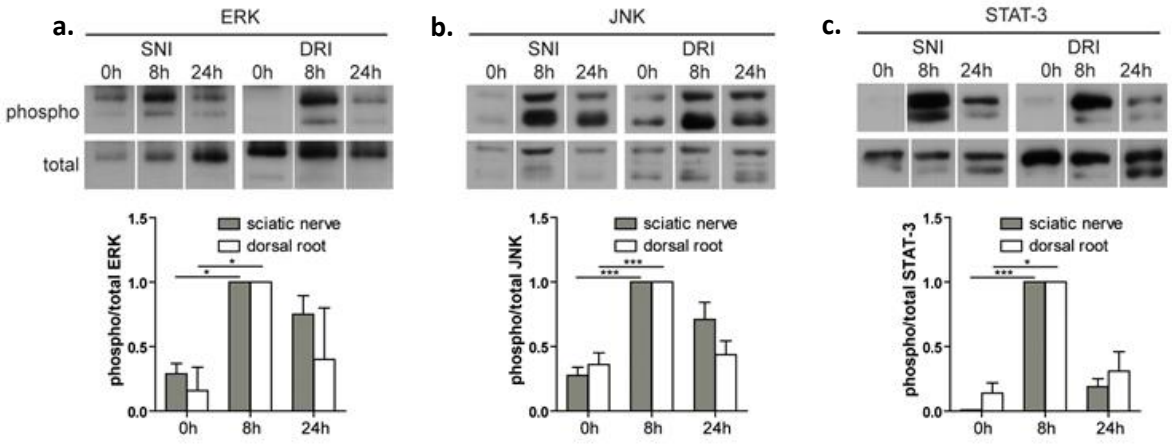


Figure 1.4 **| DRI induces local activation of positive injury signals**

Representative Western blots of axoplasm collected at different time points following SNI or DRI and respective quantification of **a.** pERK and total ERK; **b.** pJNK and total JNK; and **c.** pSTAT-3 and total STAT-3. Results represent the mean ± SEM. *p<0.05, **p<0.01 and ***p<0.001. (Nerve Regeneration group, unpublished)

Although these results confirm the local activation of positive injury signals and suggest that they are being efficiently transported, their activity in the cell body needed to be confirmed. In order to evaluate that, L4 and L5 DRGs from animals with SNI or DRI were collected 20-17h following injury, respectively; naïve animals were used as control. Western blot analysis of pERK, pJNK and pSTAT3 revealed that both signals are present in the cell body (Fig. 1.5) and immunohistochemistry confirmed that pSTAT3 is being translocated to the nucleus in both types of injuries (Fig. 1.6).

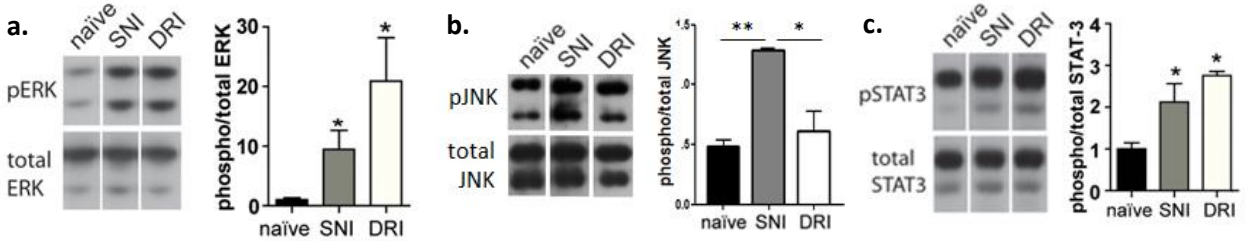


Figure 1.5 **|DRI elicits the retrograde transport of positive injury signals to DRGs**

Representative Western blots of L4, 5 DRGs from naïve Wistar rats or rats with SNI or DRI and respective quantifications against **a.** pERK and total ERK; **b.** pJNK and total JNK; and **c.** pSTAT-3 and total STAT-3. Results represent the mean ± SEM. *p<0.05, **p<0.01 and ***p<0.001. (Nerve Regeneration group, unpublished)

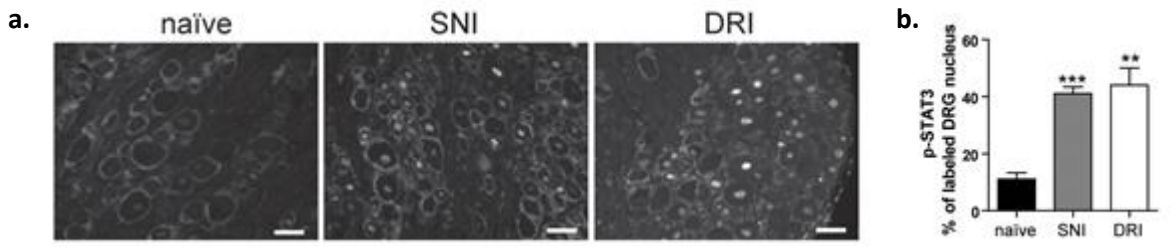


Figure 1.6 **| pSTAT3 is translocated to nucleus of DRG neurons following DRI**

a. Representative images of the immunohistochemistry performed against pSTAT-3 in L4,5 DRG of naïve rats or with either SNI or DRI; **b.** Percentage of the labeled nucleus in a. Results represent the mean ± SEM. *p<0.05, **p<0.01 and ***p<0.001. (Nerve Regeneration group, unpublished)

Since STAT3 is described as being crucial for the initial axonal elongation but not for the subsequent steps (Bareyre et al 2011), other players must be involved in triggering an efficient regenerative response, meaning that an eventual failure of its activation could delay axonal regeneration but not explain the lack of regeneration seen following DRI.

Hereupon, it is important to confirm if both p-c-jun and pELK-1 (pJNK and pERK respective downstream targets) are present in the nucleus following DRI, since their non-activation could explain, in part, the inability of a DRI to trigger an efficient regenerative response.

Local activation of a specific protein profile following DRI

Since all positive injury signals evaluated are also efficiently activated following a DRI, we aimed at identifying differences between the protein profiles that are locally activated following both dorsal root and SNI.

In order to assess that, the crush-ligation paradigm was again used and axoplasm was collected from injured dorsal roots or sciatic nerves. Dynein immunoprecipitation of the collected samples was then performed and the Kinex^{TM} Antibody Mircroarray (Kinexus) used to evaluate protein expression and phosphorylation. The antibody microarray revealed that, in comparison with SNI, 28 proteins were found in higher amounts following DRI, while 16 were present in lower levels (Supplementary information, Table S1) thus suggesting that there is differential retrograde transport following dorsal root and sciatic nerve injuries. From the Kinexus list, most of the candidates were subjected to validation by immunoblot of total axoplasm collected from rats with either SNI or DRI. Whereas only 14 had been detected by this technique, just 8 had shown differences similar to those revealed by the Kinexus analysis on samples of dynein immunoprecipitated axoplasm (Table 1.1). Of note, the fact that the antibody microarray is more sensitive than a standard immunoblot could explain why many candidates were not detected in the validation assay.

Table 1.1 **| Axoplasmic proteins differentially regulated as assessed by both Kinexus analysis and immunoblot validation after DRI when compared with SNI**

From the list of proteins that were differentially regulated in the Kinexus analysis after DRI or SNI, only 8 had shown similar differences when subjected to immunoblot validation. Of note, owning to the low sensitivity of the immunoblotting techniques in comparison with the Kinexus microarray, total axoplasm were used for immunoblot validation, while for the Kinexus analysis dynein immunprecipitated axoplasmic samples were selected.

Candidates	Kinex™ Antibody Mircroarray DRI/SNI (z-ratio)	Immunoblotting DRI/SNI (fold change)
HSP40	**1,40**	**1,4**
Plk3	1,36	2,2
RockII	**1,18**	**1,3**
MEK6	1,11	3,0
Csk	1,05	1,9
GSK3β	**1,04**	**3,2**
Src	-1,18	0,6
Caspase 4	-1,20	0,7

The 8 candidates were further validated by Western blot and immunohistochemestry of the L4,5 dorsal root ganglions from rats with either SNI or DRI. Naïve animals were used as control. For two of the eight candidates, heat shock protein-40 (HSP-40) and glycogen synthase kinase 3 beta (GSK3-β), immunoblot analysis clearly revealed higher levels of these proteins in the DRGs from animals with dorsal root injury (Fig. 1.7 a.). Immunohistochesmistry to DRGs in the same conditions also confirmed this tendency for HSP-40 and GSK3-β (Fig. 1.7 b.). In the case of Rho kinase II (ROCKII), although no differences were observed by immunoblotting of DRG samples from animals with either DRI or SNI (Fig. 1.7 a.), by immunohistochemistry a significant increase following DRI was found (Fig. 1.7 b.)

Both ROCK-II and GSK3-β have been described as being regulators of cytoskeleton organization (Maekawa et al 1999, Yoshimura et al 2005). Indeed, activation of the RhoA/ROCK pathway, that signals through its effectors, namely myosin light chain (MLC), is associated with axonal regeneration failure (Mueller et al 2005). Thus, the increased levels of ROCKII found following dorsal root injury may certainly contribute to the lack of regeneration seen following this type of injury. Either way, confirmation of its increased activity following DRI should be further accessed by evaluation of the phosphorylation levels of its downstream effector, MLC. Regarding GSK3-β, it is known that GSK3-β activity depends on phosphorylation of its residues, serine 9 (S9) or tyrosine 216 (Y216), and also that it is responsible for regulating the phosphorylation of collapsin response mediator protein-2 (CRMP2). In addition, phosphorylation of CRMP-2 impairs microtubule polymerization and thereby axon outgrowth (Yoshimura et al 2005). Thus, increased levels of p-CRMP-2 following DRI may also explain the regeneration impairment seen upon this type of injury.

Although ROCKII and GSK3-β have been widely studied in the nerve regeneration field, few is known about the impact of HSP-40 in axonal regeneration. It is known that HSP-40 are molecular chaperones involved in protein translation, folding, unfolding, translocation and degradation (Qiu et al 2006). Therefore, assessing their importance in axonal regeneration through neurite outgrowth assays may give a clue why their levels are increased following dorsal root injury.

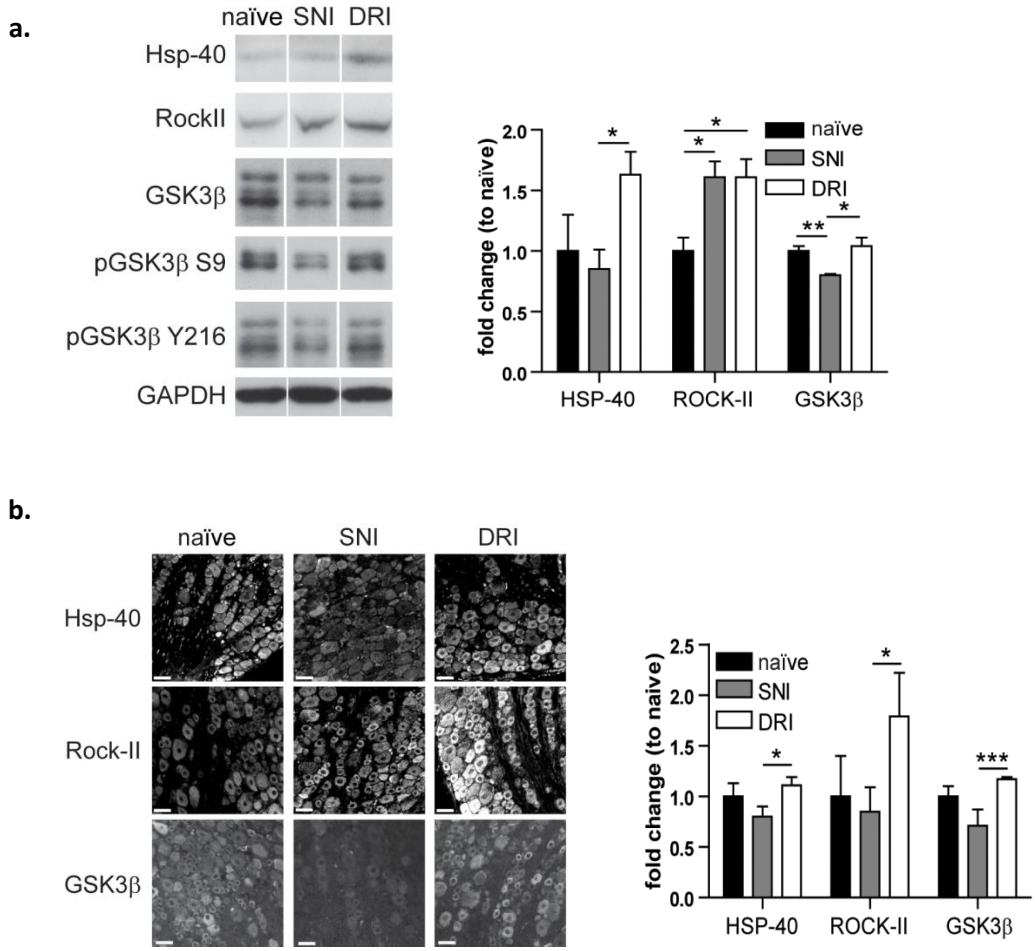


Figure 1.7 **| HSP-40, ROCKII and GSK3-β are increased in the DRG following DRI**

a. Representative Western blot for HSP-40, ROCKII, GSK3-β, GSK3-β (S9), GSK3-β (Y216) and GAPDH in L4,5 DRG from animals with SNI or DRI, and respective quantification; **b.** Representative immunohistochemistry against HSP-40, ROCKII and GSK3-β and respective quantification in DRG from animals in the same conditions. Naïve animals were used as a control. Results represent the mean ± SEM. *p<0.05, **p<0.01 and ***p<0.001. (Nerve Regeneration group, unpublished)

Research goals

Taken the previous data obtained at the Nerve Regeneration group, the research goals of this chapter are:

Local activation of positive injury signals following DRI

- Evaluate p-c-jun and pELK-1 presence in the nucleus of DRG neurons following DRI

Local activation of a specific protein profile following DRI

- Evaluate GSK3-β phosphorylation in its S9 and Y216 residues, as well as the activity of its downstream target, CRMP2;
- Confirm ROCK-II activity by assessing the phosphorylation levels of MLC;
- Characterize the impact of HSP-40 in axonal regeneration

Materials and methods

Animals

Animals used for the experiments were handled according European Union, National and veterinary rules. A 12 hours dark/light cycle was assured, as well as proper chow and ad libitum tap water. Eight weeks old Wistar rats, of either sex, were chosen for the following assays. Animals' sacrifice was performed in a proper chamber by administrating low doses of CO_2, followed by increased concentrations once asleep.

Surgical procedures

Before all procedures a ketamine/medetomidine (75mg/kg; 0,5mg/kg) anesthesia was applied. For DRI, dorsal roots of DRGs L4,5 were exposed by doing a laminectomy at lumbar vertebrae 2. Then, L4,5 dorsal roots were crushed twice for 15 seconds using Pean forceps. For SNI, sciatic nerves were exposed by doing incisures at mid-thigh and crushed twice for 15 seconds with Pean forceps. Surgeries were aseptically executed. To revert the anesthesia effect, atipamezole (1mg/kg) was used. Analgasia (butorphanol, 3mg/kg) was performed before surgery and then buprenorphine (0,04mg/kg) administrated twice a day for 48 h.

DRG immunohistochemistry

Animals with either SNI or DRI were sacrificed 17-20h after injury, and L4,5 DRGs were collected, formalin fixed and processed for paraffin blocks. As control, L4, 5 DRGs from naïve animals were used (n=4/group). After paraffin section clearance and hydration, antigen retrieval was performed and samples were blocked in 5% fetal bovine serum (FBS, Invitrogen) in tris-buffered saline (TBS) for 1 h at room temperature (RT). The following primary antibodies were then incubated overnight at 4^{o}C: anti-p-c-jun (1:200, Cell Signaling) and anti-pELK-1 (1:100, Cell Signaling). For p-c-jun, samples were next incubated in biotinilated secondary antibody diluted in saline-0,1% Tween-20 (TBST, 1:200, Vector Laboratories) for 1h at RT, and extravidin-HRP (1:1000, Sigma) was added and left for 1h at the same temperature. Sections were then incubated with 3,3'-diaminobenzidine (DAB, Vector Laboratories) until a brown precipitate was formed, and washed in water. Finally, sections were dehydrated, mounted in vectashield and image acquisition was performed at a 10 x magnification in Olympus BX50 microscope with CCD camera DP50. For pELK-1, after primary antybody incubation, sections were stained with secondary antibody diluted in 5%FBS in TBST (alexa488-anti-rabbit 1:1000, Cell Signaling) for 1h at RT and mounted in Vectashield with DAPI (Vector Laboratories). Image acquisition was performed at 20 x magnification in AxioImager Z1.

DRG protein extract

L4, 5 DRG of animals with either SNI or DRI were collected 20-17 h after injury, respectively; naïve animals were used as control (n=4/group). Homogenization was held in phosphate buffered saline (PBS, Gibco) containing protease inhibitors (GE Healthcare), 1mM ortovanadate (Sigma-Aldrich) and

0,3% Triton X-100 (Sigma-Aldrich). After sonication, the suspension was centrifuged for 10 minutes at 15.000 g, 4^{o}C, supernatant collected and protein concentration was determined.

Immunoblots

25 µg of protein from each sample were separated in 12% SDS-PAGE (Bio-Rad) and then transferred to nitrocellulose membrane (GE Healthcare). The membrane was blocked in 5% skim milk (Sigma Aldrich) and incubated with the respective primary antibodies (Table 1.2) diluted in 5% albumin bovine serum (BSA, Sigma Aldrich). The membrane was the washed and incubated with appropriate secondary antibodies also prepared in 5% skim milk. Luminata^{TM} Cresecendo Western HRP substrate (Millipore) was used for development.

Table 1.2 **| List of primary antibodies used for immunobloting**

Primary Antibody	Source	Company	Dilution
pGSK3-β (S9)	rabbit	Cell Signaling	1:1000
pGSK3-β (Y216)	rabbit	Santa Cruz Biotechnology	1:2000
Total GSK3-β	mouse	Cell Signaling	1:1000
pCRMP2 (Y509/Y514)	sheep	Kinasource	1:1000
Total CRMP2	sheep	Kinasource	1:1000
pMLC (S19)	rabbit	Cell Signaling	1:500
Total MLC	rabbit	Cell Signaling	1:500

Neurite outgrowth assay

All the following procedures were performed at an aseptically room or in the cabinet flow. Cells were maintained at 37^{o}C, 5%CO_2.

Lentiviral production

Eighty per cent confluent HEK293T cells were transfected using a 1:4 DNA/lipofectamine ratio: 6 µg of pPAX plasmid DNA (a kind gift from Dr. João Relvas), 3 µg of VSVG envelope plasmids DNA (a kind gift from Dr. João Relvas), 6 µg from the specific small harpin plasmid DNA (Sigma-Aldrich) and 60 µl of Lipofectamine 2000 (Invitrogen) in an Opti-MEM solution (Gibco). Reagents were gently mixed. To obtain high efficiency, the solution was briskly added to about 15% of the cells. Six hours later, the medium was replaced by DMEM (Sigma-Aldrich) enriched with 10%FBS and 1%Penicellin/Streptomycin (P/S, Invitrogen), and 48h upon transfection the supernatant was collected, filtered and aliquotted. Sh plasmid DNA is designed to show puromycin and ampicillin resistance.

Lentiviral titration

For lentiviral titration, two different viral dilutions were prepared (1:1000; 1:10000) and added to plated HEK293T cells (a kind gift from Dr. João Relvas) with 15-25% confluence. As control, non-transduced HEK293T cells were used. Twenty four hours upon transduction, medium was replaced by growth medium (DMEM +10%FBS + 1%P/S) and for the following days, growth medium supplemented with 1,5µg/mL of puromycin (Merck) was daily changed until no cells were found in the control wells. The number of puromycin resistant colonies was then counted.

Primary cultures of DRG neurons

DRG neurons were collected to DMEM:F12 (Sigma-Aldrich) enriched with 10% FBS and 1% P/S, and digested with 0,125% collagenase (Sigma-Aldrich) for 2h at 37^{o}C. Once digested, ganglions were dissociated with Pasteur pipettes and suspensions were carefully poured to 15% BSA in DMEM:F12. Gradients were then centrifuged at 1.000 rpm for 10 minutes and once finished, pellet was resuspended in complete medium (DMEM:F12 + 1% P/S + 1% L-Glutamine (Invitrogen) +2% B-27 (Invitrogen) + 50 ng/ml NGF (Millipore)) . Six thousand cells were plated in previously coated coverslips with Poly-L-lysine (Sigma-Aldrich) at 20 µg/ml and laminin (Sigma-Aldrich) at 5 µg/ml.

Transduction and Puromycin Selection

Twenty four hours after plating, transduction was performed using 10.000 infection units per well. Sixteen hours later, supernatant was removed and replaced by complete medium. For the following 3 days, puromycin selection was performed with a 5 µg/ml concentration.

Replating

Replating was performed as described elsewhere (Saijilafu et al 2013). On the third day of puromycin selection, neurons were treated with trypsin for 2 minutes at 37^{o}C, plus 1 minute at RT. The reaction was stopped by adding DMEM + 10%FBS + 1%P/S to the wells. Then, the suspension was centrifuged for 5 minutes at 800 rpm, the pellet was resuspended in complete medium enriched with 5 µg/ml of puromycin and 5.000 cells were replated in coated coverslips. Thirteen hours later, cells were fixed in 4% paraformaldehyde (PFA, Bio-Optica).

<u>β-III tubulin Immunocytochemistry</u>

After fixation in 4% PFA, cells were permeabilized in 0,2% Triton X-100 (Sigma-Aldrich) for 5 minutes, and blocked for 1 h at RT with blocking buffer (5%FBS + 0,4% tween-20 (Sigma-Aldrich) in PBS). The coverslips were then incubated with anti-β-III-tubulin antibody (1:2000, Promega) for 1h at RT, washed in PBS and again incubated with anti-mouse IgG-alexa488 (1:1000, Invitrogen) for 1h at RT. Coverslips were then washed and mounted in Fluroshield^{TM} with DAPI (Sigma). Image acquisition was performed at 10x magnification in a Leica DMI 6000B microscope. NeuronJ, a plugin from ImageJ, was used for the measurement of the longest neurite. Measurements were performed for at least 60 cells, from three different wells.

Results and discussion

Local activation of positive injury signals following DRI

In DRGs of animals with either SNI or DRI, immunohistochemistry performed against the transcription factors, pELK-1 and p-c-jun, have shown that following both SNI and DRI there is increased phosphorylation of both ELK-1 and c-jun. Both phosphorylated forms were significantly increased in the nucleus following either SNI or DRI: for pELK-1 a 1,2- and 1,3-fold were respectively obtained (Fig. 1.8), while for p-c-jun, a 11- and 7-fold change were respectively observed (Fig. 1.9).

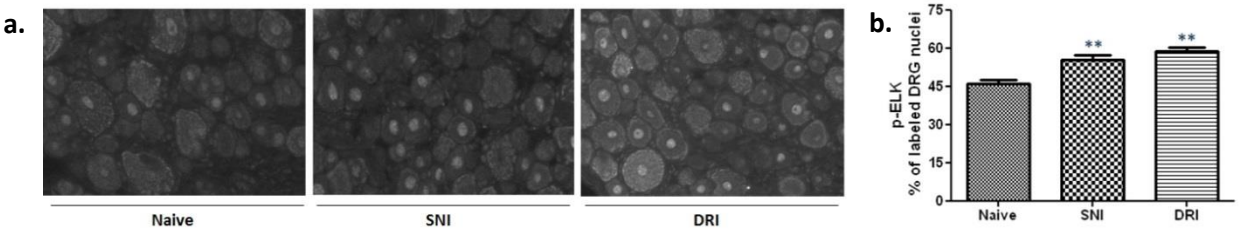


Figure 1.8 **| The transcription factor pELK-1 is translocated to the nucleus following DRI**

a. Representative immunohistochemistry against pELK-1 in L4,5 DRGs from animals with SNI or DRI; **b.** Respective quantification of a. Naïve animals were used as a control. Results represent the mean ± SEM. *p<0.05, **p<0.01 and ***p<0.001.

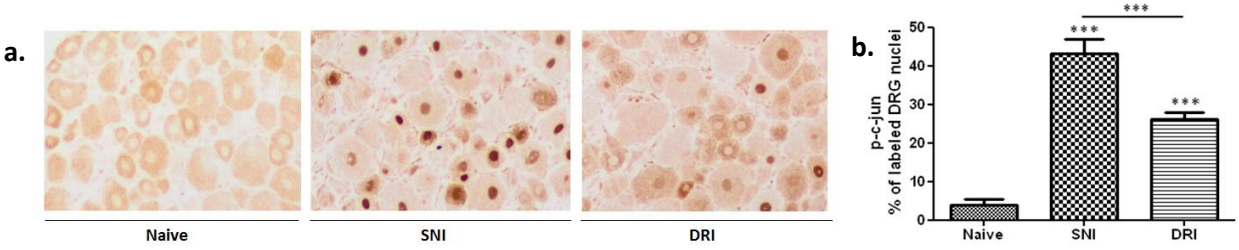


Figure 1.9 **| The transcription factor p-c-jun is translocated to the nucleus following DRI**

a. Representative immunohistochemistry against p-c-jun in L4,5 DRGs from animals with SNI or DRI; **b.** Respective quantification of a. Naïve animals were used as a control. Results represent the mean ± SEM. *p<0.05, **p<0.01 and ***p<0.001.

Surprisingly, it seems that both JNK/c-jun and ERK/ELK pathways are activated following either SNI or DRI. Although these results suggest that activation of c-jun and Elk is not essential to prompt an efficient regenerative program, many reports support the opposite. For instance, deletion of c-jun in motor neurons reduced axonal regeneration speed and functional recovery, since most of the cut axons were not able to reconnect their targets (Ruff et al 2012). Moreover, pERK retrograde transport blockage or treatment with MEK inhibitors had shown to impair Elk-1 phosphorylation and neuronal regeneration (Perlson et al 2005).

Taken these evidences together, it is likely that after DRI other factors suppress and/or compete with the activity of these positive injury signals and for that reason axonal regeneration fails. In fact, inhibition of intrinsic cellular pathways, such as Rho signaling pathway, has shown to improve axonal regeneration (Fournier et al 2003).

Another plausible hypothesis is that activation of positive injury signals *per se* is not sufficient to prompt a successful regeneration, i. e., it is likely that an injury to the periphery triggers factors besides the ones identified so far, that together will trigger a regenerative response. To support this, recent published data had shown that following peripheral injury, back-propagation of a calcium wave induces the export of HDAC5 from nucleus to the injury site, eliciting axonal regeneration (Cho et al 2013). In this same paper, it is also shown that HDAC5 elicits the expression of regeneration genes, such as jun. Moreover, following optic nerve crush, HDAC5 fails to accumulate at the tips of the injured retinal ganglion cells, which could explain why these axons fail to regenerate. Thus, it would be interesting to correlate whether the lack of regeneration seen following DRI could be explained by transport failure of HDAC5 from the nucleus to the injury site. Of equal importance is the recent finding of the activation of a regenerative program dependent on the acetylation of histone 3 by histone acetyltransferase p300/CBP-associated factor (PCAF) and on the retrograde transport of pERK (Puttagunta et al 2014). Additionally, it is shown that acetylation of histone 3 by PFAC does not occur following injury to the spinal cord, and the intrinsic ability of dorsal column fibers to regenerate after this type of injury could be elicited by injecting PCAF into the CNS damaged tissue.

In summary, further studies should be performed in other to clarify the importance of both p-c-jun and p-ELK, since their activation seems to be necessary but not sufficient to prompt axonal regeneration. In this sense, the inability of a DRI to trigger certain epigenetic modifications could be an explanation for the reason why neurons fail to regenerate their axons following this type of injury.

Local activation of a specific protein profile following DRI

To confirm the increased activity of ROCKII following DRI, Western blot analysis of L4,5 DRGs extracts from rats with either SNI or DRI against phospho and total myosin light chain (MLC), downstream target of ROCKII, was performed. Naïve animals were used as control. A 1,8- and 3,0- fold increase was observed when comparing with either uninjured animals or animals with SNI (Fig. 1.10), thus confirming the increased activity of ROCKII following DRI, and consequently its contribute for axonal regeneration failure. However, these results were not statistically significant, suggesting that other factors may contribute for lack of axonal regeneration seen upon this type of injury.

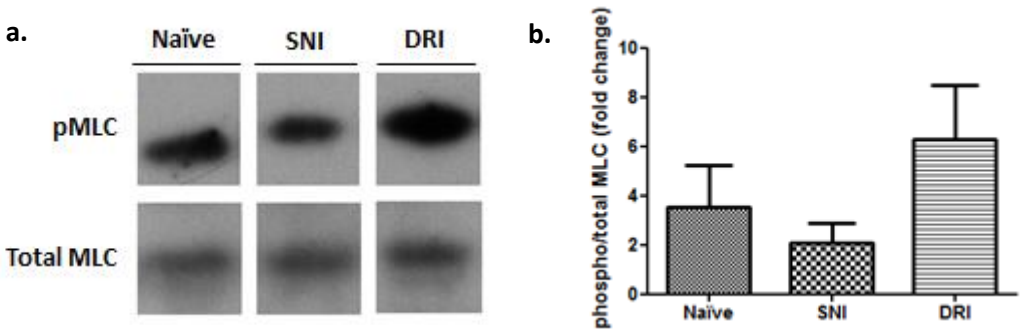


Figure 1.10 **|DRI increases the phosphorylation of MLC**

High levels of pMLC upon DRI confirm the increased activity of ROCKII, thus indicating ROCKII as a responsible for the lack of regeneration seen after DRI. **a.** Representative immunoblot of o phospho and total MLC in L4,5 DRGs extracts from animals with SNI or DRI; **b.** Respective quantification of **a**. Naïve animals were used as control. Results represent the mean ± SEM.

Regarding GSK3-β, it is known that its activity depends on the residue where it is phosphorylated, serine 9 (S9) or tyrosine 216 (Y216). In mature cells GSK3-β is found phosphorylated in its tyrosine 216 residue, which is commonly associated with its active form. Under this condition, GSK3-β phosphorylates proteins that are associated with the plus end of microtubule filaments, thus preventing microtubule polymerization and therefore axon outgrowth inhibition. When the cell is exposed to migratory cues, GSK3-β is phosphorylated in serine 9 becoming inactive, i.e., dephosphorylation of its effectors, such as collapsin response mediator protein 2 (CRMP2), occur and as a consequence axon elongation (Liz et al 2014, Yoshimura et al 2005, Zhou & Snider 2005).

In order to evaluate GSK3-β activity, immunoblot of L4,5 DRGs extracts from animals with SNI or DRI was performed against pGSK3-β (S9), pGSK3-β (Y216), and total GSK3-β. Results have shown that GSK3-β phosphorylation tends to be more pronounced following DRI (Fig.1.11). However, these trends were not statistical significant.

As no specific conclusion could be withdraw in relation to GSK3-β activity just by studying its phosphorylation pattern, phosphorylation of its downstream target, CRMP2, was evaluated. Immunoblot analysis revealed that following DRI, CRMP2 phosphorylation is significantly increased when comparing with SNI (2,1-fold change) but no significant changes are seen when comparing with naïve animals (Fig. 1.12). By phosphorylating CRMP2, active GSK3-β uncouples CRMP2 from the plus tip of microtubules filaments, thus preventing microtubule polymerization and axonal elongation (Liz et al 2014, Yoshimura et al 2005). Thus, the increased levels of pCRMP2 found following DRI may account for the inability of a DRI to induce axonal regeneration.

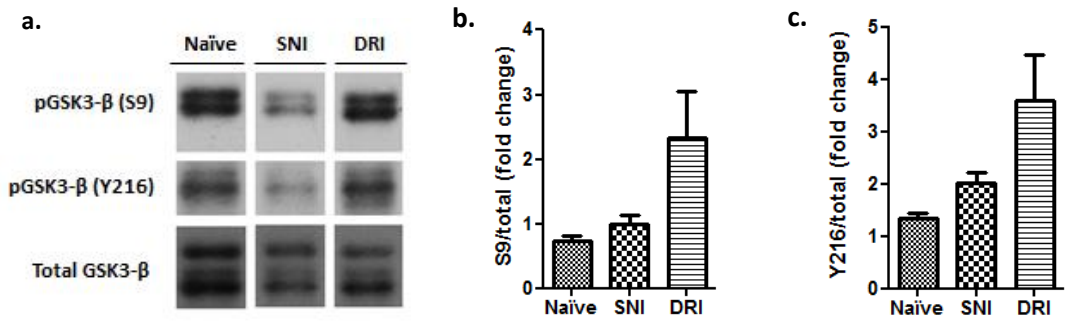


Figure 1.11 **| GSK3- β phosphorylation is increased following DRI**

a. Representative images of the immunoblots performed against pGSK3-β(S9), pGSK3-β(Y216) or total pGSK3-β in L4,5 DRGs extracts from animals with SNI or DRI; **b.** Western blot quantification of pGSK3-β(S9); **c.** Western blot quantification of pGSK3-β(Y216). Naïve animals were used as control. Results represent the mean ± SEM.

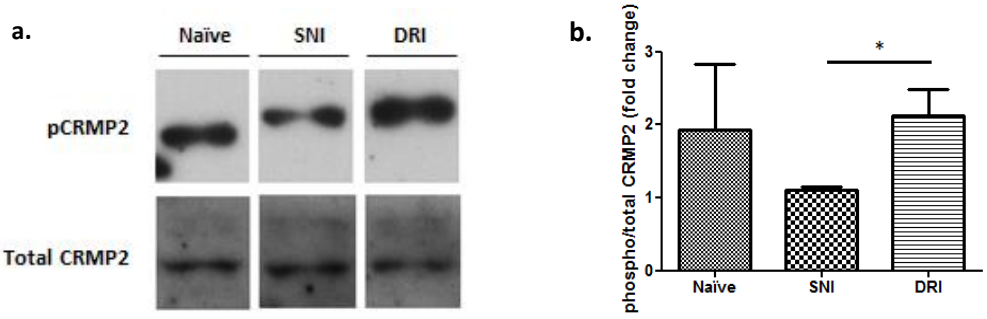


Figure 1.12 **| Phosphorylation of CRMP2 is increased in DRG following DRI**

a. Representative immunoblot of phospho and total CRMP2 in DRG from animals with SNI or DRI; **b.** Respective quantification of a. Naïve animals were used as control. Results represent the mean ± SEM. *p<0.05, **p<0.01 and ***p<0.001.

As for the evaluation of HSP-40 in axonal regeneration, neurite outgrowth *in vitro* following knock down of HSP-40 by ShRNA revealed no statistically significant differences in neurite length (Fig. 1.13). Besides, the total number of cells with neurites in this condition was consistently insufficient in all the experiments performed, even when replating (data not shown) suggesting that HSP-40 knock down is toxic for neurons.

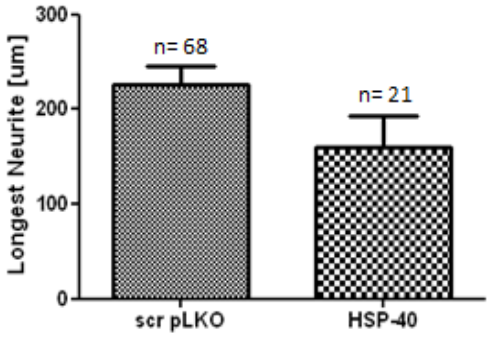


Figure 1.13 **| HSP-40 knock down is toxic for the cells**

The number of cells obtained (n) was consistently insufficient to assess whether HSP-40 could be responsible for the lack of regeneration seen following DRI. Results represent the mean ± SEM.

In a series of experiments performed in different neuronal populations, Tanaka and colleagues observed that HSP40 was upregulated as a response to ischemia in ischemia-resistant and tolerance-acquired neurons, suggesting that upregulation of this protein may be required for cell survival (Tanaka et al 2002). In addition, similar conclusions were withdrawn in hepatoma cells where HSP-40 knock down diminishes cell survival to drug exposure (Sharma et al 2009). In relation to our results with our results, it is likely that, following DRI, neurons are exposed to such drastic changes that the necessity of triggering a survival program, probably mediated by HSP-40 neuroprotective role, overlaps with their capacity to initiate axonal regeneration.

Chapter 2

Intrinsic changes following a conditioning injury

What makes axonal regeneration possible?

In this chapter, through the use of regeneration models, it was proposed to evaluate some of the intrinsic mechanisms that allow successful axonal regeneration to occur.
Thus, similar to Chapter I, preliminary results will be presented, followed by the research goals, materials and methods and finally, the discussion of the results obtained.

Part of the results of this chapter had been included in the following publication:
Mar FM et al. 2014. CNS axons globally increase axonal transport after peripheral conditioning. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 34: 5965-70

Preliminary results

Although regeneration in the central nervous (CNS) is limited, the intrinsic capacity of some axons to regenerate can be elicited, as is in the case in the *Conditioning Lesion* (CL) paradigm (Neumann & Woolf 1999). Doing an injury in the peripheral branch of dorsal root ganglions (DRGs) triggers intrinsic mechanisms that elicit axonal regeneration of the central branches of these neurons irrespective of the presence of an inhibitory environment.

Some of these mechanisms have been dissected along the years. However, none is capable by itself of eliciting a robust regeneration program. In fact, for years elevation of intracellular cAMP levels following peripheral nerve injury has been linked to an increased capacity of axonal regeneration and even described as the crucial mediator of the conditioning effect (Neumann et al 2002, Qiu et al 2002). However, recent reports have shown that elevating cAMP levels per se is not sufficient to explain the robust regeneration that is seen following a conditioning lesion (Blesch et al 2012). Studies performed in our lab, also support this idea, since both DRI and SNI were capable of increasing intracellular cAMP concentrations, despite of the poor regeneration seen after DRI (data not published). Together these results suggest that although intracellular elevation of cAMP is important to induce axonal regeneration, other mechanisms are required to build a robust regeneration.

As a matter of fact, following conditioning injury, different pathways are activated, including alterations in molecular profiles that are retrogradely transported (Rishal & Fainzilber 2010). These changes are crucial for neuronal survival and regeneration, since only a precise and timely retrograde signaling between injury site and cell body will allow an efficient regeneration of both branches (Smith & Skene 1997). Under these conditions, nuclear modifications occur, namely upregulation of transcription factors and newly synthesized molecules, such as cytoskeletal proteins, axon guidance molecules, neurotrophic factors and membrane receptors (Goldberg 2003). Since growing axons require high amounts of macromolecules and metabolites at their very tips, anterograde transport ensures the supply at long distances from the cell body, of newly synthesized proteins, vesicles and membranous organelles such as mitochondria (Mar et al 2014a). Moreover, it has been suggested that following conditioning injury there is increased axonal transport in DRG neurons (Hoffman 2010, Hoffman & Luduena 1996).

Axonal transport appears therefore as a crucial tool in regeneration since it informs the soma about changes that occur at long distances and is able to supply different locations within the cell. For this reason, the conditioning lesion paradigm was used to assess some aspects of axonal transport that will be discussed in the following two parts.

cAMP as the central mediator of the conditioning effect?

Since cAMP has been described for years as the principal mediator of the conditioning effect (Neumann et al 2002), and knowing that following a conditioning lesion (CL) there is a general increase in axonal transport of different proteins, like cytoskeleton and membrane components (Hoffman 2010, Hoffman & Luduena 1996), transport of lysosomes and synaptic vesicles was evaluated in order to understand whether this increase after CL could be expanded to the transport of moving vesicles.

To do so, L4,5 DRGs from naïve or conditioned mice were collected and used for *in vitro* transduction with either lysotracker or synaptophysin-GFP. Velocity measurements in both directions revealed a general increase, being the anterograde transport favored following a conditioning injury (Fig. 2.1).

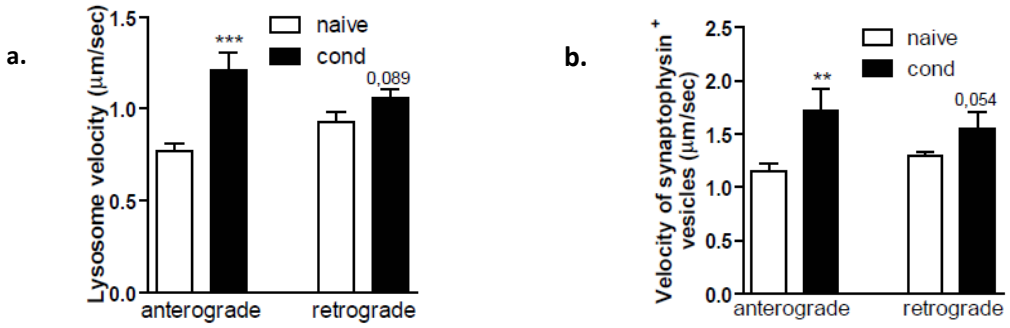


Figure 2.1 **| Conditioning lesion elicits the movement of lysosomes and vesicles**

Naïve and conditioned DRGs were used to measure the velocity of anterogradely and retrogradely **a.** moving lysosomes (at least 48 lysossomes/condition) and **b.** synaptophysin-positive moving vesicles (at least 44 synaptophysin-positive moving vesicles/condition). Results represent the mean ± SEM. *p<0.05, **p<0.01 and ***p<0.001. (Nerve Regeneration group, unpublished)

Thus, similar to what is described by Hoffman and colleagues, a conditioning injury also increases the movement of lysosomes and vesicles. Whether these alterations can be mimicked by increasing cAMP levels is not known. Thus, what it is proposed in this section is to see if treatment of DRG neurons with rolipram, a phosphodiesterase inhibitor that prevents cAMP to be converted in AMP, could mirror the effect of the conditioning lesion in the movement of lysosomes and vesicles.

Novel modulators of axonal regeneration ?

Due to the demands of growing axons, anterograde transport of proteins ensures that all necessary components are provided to the growth cone. For instance, cytoskeleton components have been widely described in neuroregeneration due to their importance for cell integrity (Galbraith & Gallant 2000). However, growth cone dynamics is not restricted to the assembly of cytoskeleton

proteins; regulation of molecular pathways that allow axon guidance, branching and finally synaptic formation are also crucial for growth cone function. So, to evaluate the somatic response to injury in a regeneration model it seems indispensible to understand the mechanisms underlying successful regeneration. In this sense, the protein profile that is instigated in the cell body and the anterograde transport to the growth cone following conditioning injury were subjected to evaluation. To assess that, a radiolabeling assay (Oblinger & Lasek 1988)was performed (Fig. 2.2).

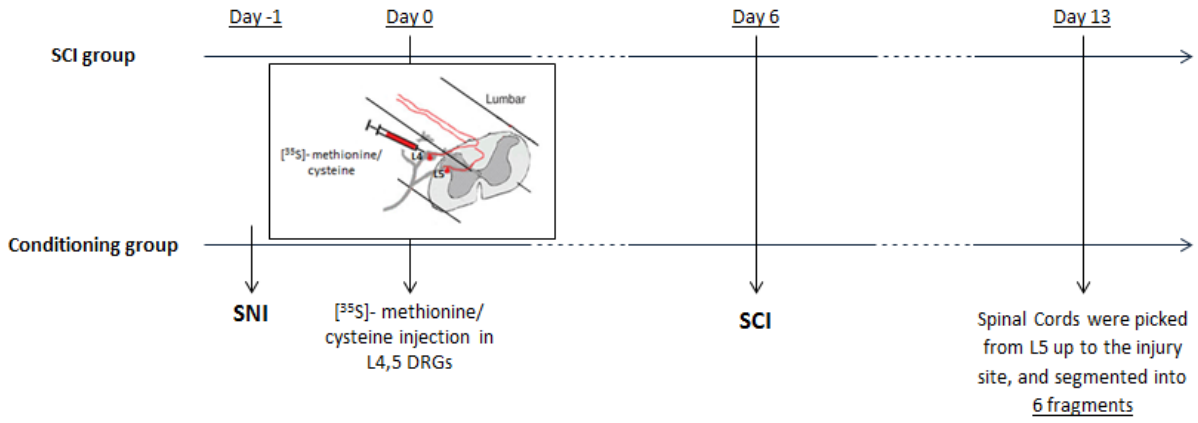


Figure 2.2 **| Representative scheme of the radiolabeling assay used to assess the protein profile transported from the soma to the spinal cord**

L4,5 DRGs from naïve rats or from rats with sciatic nerve injury performed on the previous day were injected with [^{35}S]-methionine/cysteine. Six days later, spinal cord injury was performed on the two groups of animals. After one week, spinal cords were collected from L5 up to the injury site and divided into 6 fragments. Each fragment was then homogenized and counts per minute were measured on 50 µg of protein. From L4,5 spinal cord segments, a 2D gel was performed and exposed to a phosphor screen for 5 weeks. Radioactive spots were identified by MALDI-TOF/TOF mass spectrometry.

Results from our lab, revealed that following CL there is a general increase in the amount of the radiolabeled proteins that are being synthesized and/or anterogradely transported (Fig. 2.3 a.). A 2D gel analysis of the L4, 5 spinal cord segments also confirmed this upturn (Fig. 2.3 b.) and, by taking advantage of MALDI-TOF/TOF mass spectrometry, radiolabeled proteins were identified. The most abundant proteins were cytoskeleton components, such as tubulin and actin (spots 2 and 5, respectively), and metabolic enzymes, like NADH-dehydrogenase (NDUS1, spot 1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, spots 16 and 17). Increased levels of heat shock protein 70 (HSP-70, spot 1), collapsin response mediator protein-5 (CRMP5, spot 4), guanine nucleotide binding protein beta-2 (GNB-2, spot 13), annexins 2 and 5 (ANX2, spot 16; ANX5, spot 18), sirtuin 2 (Sirt2, spot 16), 14-3-3 proteins (spot 19), Rho GDP-dissociation inhibitor 1 (RhoGDI, spot 20) and the neuron-specific ubiquitin carboxyl-terminal hydrolase 1 (PGP9.5, spot 20), were also found following CL (Fig. 2.3 b.). For further detail see Supplementary information, Table S2. Of note, 2D gel analysis was also performed in naïve animals or in animals with SNI, and radioactive content revealed profiles similar to those presented by the group with SCI or conditioning, respectively (data not shown).

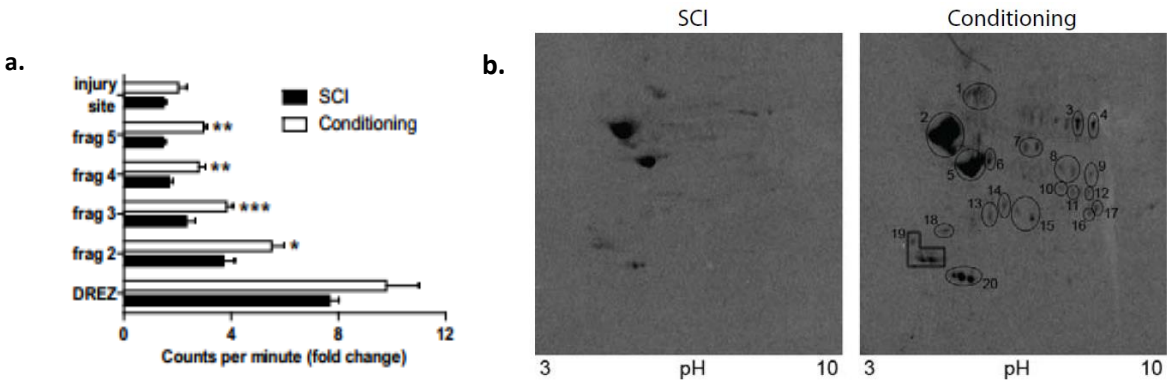


Figure 2.3 **| Conditioning increases the synthesis and transport of proteins to the central branch of DRGs**

a. Counts per minute in spinal cord fragments of rats with either SCI or SCI and previous conditioning (SNI). **b.** Representative 2D gels obtained from L4,5 spinal cord segments of animals in the same conditions. *p<0.05, **p<0.01 and ***p<0.001.(Mar et al 2014b)

To evaluate whether this increase in protein levels was due to gene upregulation, the proteins mentioned above were selected for gene expression analysis. For this effect, L4, 5 DRGs were collected 1 day or 1 week after SNI; naïve animals were used as control. Although significant changes were not observed for tubulin, actin and ANX5, the remaining proteins had shown substantial expression one day and in some cases 1 week after SNI (Fig. 2.4)

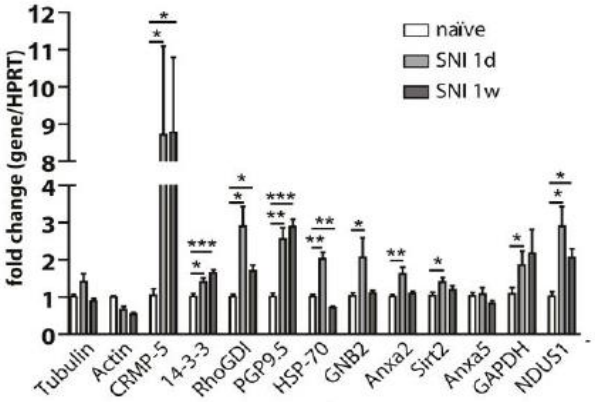


Figure 2.4 **|A conditioning lesion upregulates protein levels**

Quantitative PCR of DRGs colleted from animals with SNI either 1 day or 1 week following injury. Naïve animal were used as a control. Results represent the mean ± SEM. *p<0.05, **p<0.01 and ***p<0.001. (Mar et al 2014b)

To evaluate the impact of these proteins in axonal regeneration, neurite outgrowth assays were performed. So far, inhibition of RhoGDI, 14-3-3, PGP9.5 and CRMP-5 have revealed that only the decrease of the first two leads to neurite outgrowth impairment.

Other interesting candidates besides the ones mentioned above were identified, namely heat shock protein 70 (HSP-70), annexins 2 and 5, GNB-2 and Sirt 2. Since they were upregulated following conditioning injury, in this section we purposed to assess their importance in axonal regeneration through the use of neurite outgrowth assays.

Research goals

Taken the previous data obtained at the Nerve Regeneration group, as research goals of this chapter, we proposed:

cAMP as the principal mediator of the conditioning effect ?

- Assess whether increasing cAMP levels could mimic the conditioning lesion effect on moving vesicles and lysosomes

Novel modulators of axonal regeneration ?

- Evaluate the importance for axonal regeneration of newly synthesized proteins that are anterogradely transported following conditioning lesion

Materials and methods

Animals

Animals used for the experiments were handled according European Union, National and veterinary rules. A 12 hours dark/light cycle was assured, as well as proper chow and ad libitum tap water. Surgeries were aseptically executed. Eight weeks old Wistar rats, of either sex, were choosen for the following assays.

Neurite outgrowth assays

Lentivirus production and titration, as well as primary cultures of DRG neurons, transduction and replating were performed as described in Chapter I - Materials and Methods.

Assessing the movement of lysossomes and synaptophysin positive vesicles

For the assessment of lysosomes movement, DRG neurons were incubated with 100nM lysotracker (Invitrogen) for 45 min at 37^{o}C. Medium was then replaced by complete medium without phenol red. Cells were then incubated for 1h at 37ºC and then imaged.

For the assessment of synaptophysin positive vesicles, one day following plating, DRG neurons were infected overnight with baculovirus expressing synaptophysin-GFP (Invitrogen). On the next day, medium was replaced by complete medium without phenol red. Cells were then incubated for 1h at 37^{o}C and then imaged.

Rolipram (0,5 µM in DMSO) was added to the cells immediately after DRGs collection, and to all culture mediums. All videos were acquired with confocal Leica SP5 at 0,5Hz for 2 min, 40x magnification. At least 26 vesicles per condition were analyzed.

Validation of knock down efficiency in Cath.-a-differentiated (CAD) cells

CAD cells (Sigma-Aldrich), a mouse cell line derived from a cathecholaminergic neuronal tumor, were used for knock down evaluation of the ShRNA. Transfection was performed using a DNA/Lipofectamine ratio of 1:4 and 2µg of DNA were added per well. As control, non-transfected cells were used. For the following days, growth medium with puromycin at 1,5 µg/ml was added until no cells were found in the control well. RNA extraction was performed using NZY Total Isolation kit (NZYTech), followed by cDNA synthesis with NZY First-Strand cDNA Synthesis kit (NZYTech). RT-qPCR was then performed to evaluate ShRNA efficiency.

Validation of knock down efficiency in DRG neurons

RNA extraction, followed by cDNA synthesis and RT-qPCR were performed in *in vitro* transduced and puromycin selected DRGs. The procedures used were the same described above.

RT-qPCR

RT-qPCR to evaluate protein expression either in transfected CAD cells or in transduced DRG neurons was performed by using the iQ Supermix (Bio-Rad) and the following designed primers (Beacon software).

Table 2.1 | **Primers used for knock down validation**

Target	Sense	Antisense
ANX2	5'-ATGTCTACTGTCCACGAA- 3'	5'-GCGGTTAGTCAGAATGTT- 3'
ANX5	5'-GCTGGGACAGATGAGAAAG- 3'	5'-AAGGAGGACCACCAACAT- 3'
GNB-2	5'-GACAGACATTCATAGGTCAC- 3'	5'-GATGTTGTCGTGGGAATAC- 3'
Sirt-2	5'-TATTGACACTCTGGAACG- 3'	5'-TTACCACATTCTGACACTT- 3'
HPRT	5'-ATGGACTGATTATGGACAGGACTG- 3'	5'-GCAGGTCAGCAAAGAACTTATAGC- 3'

Results and discussion

cAMP as the central mediator of the conditioning effect ?

Since a conditioning injury was able to increase the movement of lysosomes and synaptophysin-positive vesicles, we asked whether by increasing cAMP levels, axonal transport of these vesicles could be elicited in a similar way to what was observed following conditioning injury. To do so, cultured DRG neurons were treated with rolipram which is known to improve axonal regeneration by elevating cAMP levels (Nikulina et al 2004). Treatment with rolipram did not mimic the conditioning effect in axonal transport. Indeed, although lysosome velocity tended to be decreased in both directions, no significantly differences were observed (Fig. 2.5 a.). Also, analysis of the anterograde and the retrograde transport of synaptophysin-positive vesicles did not show significant differences (Fig. 2.5 b.).

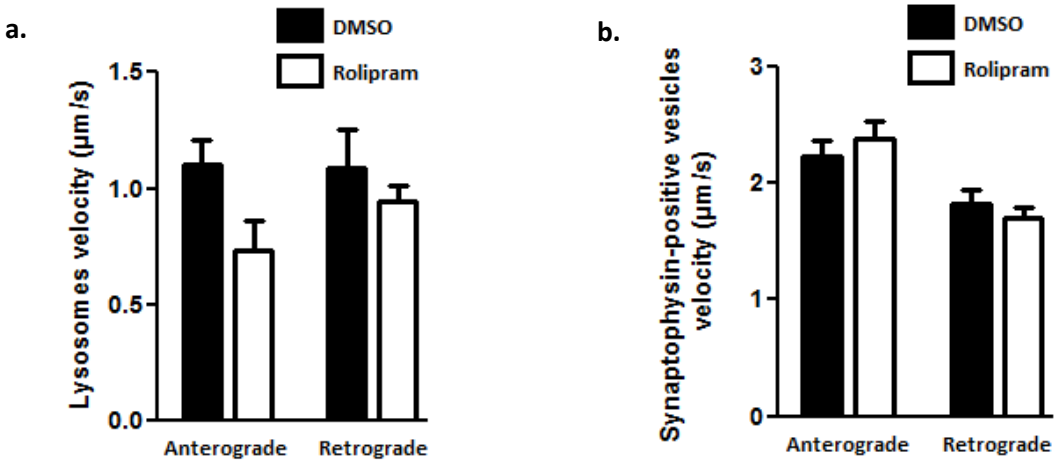


Figure 2.5 **| Treatment with rolipram in cultured DRG neurons did not elicit an increase in the velocity of transport of lysosomes and synaptophysin-positive vesicles**

Results obtained from videoimaging of moving organelles in either anterograde or retrograde direction. a. Lysosomes velocity (µm/s); b. Synaptophysin-positive vesicles velocity (µm/s). DMSO was used as a vehicle control. Results represent the mean ± SEM.

Different studies have indicated cAMP as being essential for the conditioning effect. The increased levels of this molecule seen following conditioning injury (Qiu et al 2002), as well as the improvement in CNS axonal regeneration seen with treatment with a cAMP homolog, dibutyryl cyclic adenosine monophosphate (db-cAMP) (Lau et al 2013, Monsul et al 2004), support this hypothesis. However, Han and colleagues showed that cAMP elevates tubulin expression without increasing the intrinsic capacity to regenerate of DRG neurons (Han et al 2004), suggesting that cAMP *per se* is not sufficient to trigger a regenerative program. This evidence is supported by our findings, which point to the fact that cAMP is probably required for several aspects of axonal regeneration but it is likely that

other factors mediate the regenerative program, suggesting that the notion of cAMP as central mediator of conditioning effect may be overestimated.

Novel modulators of axonal regeneration ?

From the list of candidates found upregulated and anterogradely transported to the spinal cord injury site, in the conditioning lesion paradigm, it remained to clarify the importance in axonal regeneration of the following proteins: HSP-70, ANX2, ANX5, GNB-2 and Sirt-2. Since activity of HSP-70 is dependent on stabilization by its partner heat shock protein 40 (HSP-40) (Qiu et al 2006), which knock down was previously shown to be toxic to the cells, we excluded HSP-70 from analysis.

For the remaining proteins, ShRNA transduced DRG neurons were used to measure the longest neurite and CAD cells to confirm ShRNA efficiency. Of note, Sirt-2 ShRNA efficiency was not confirmed in CAD cells, since primers where designed for rats and CAD cells come from a mouse cell line. Knockdown of Sirt-2 in cultured DRG neurons was further evaluated, but no decreased in mRNA levels was observed (data not shown), and for that reason measurement of the longest neurite was not addressed.

In CAD cells, ShRNA efficiency was confirmed for ANX2, ANX5 and GNB-2 by obtaining, respectively, 44%, 69% and 55% decrease in mRNA levels when comparing with control (Fig. 2.6).

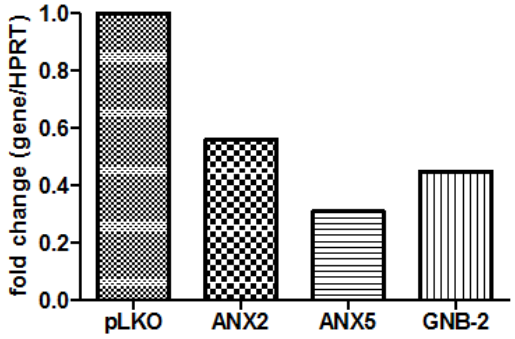


Figure 2.6 **| Validation of knock down efficiency in Cath.-a-differentiated (CAD) cells**

RT-qPCR of transfected CAD cells with annexin 2 (ANX2), annexin 5 (ANX5) or guanine nucleotide binding protein beta 2 subunit (GNB-2) Sh plasmids. All knock downs showed 44% to 69% reduced mRNA levels. An empty pLKO was used as control.

Measurement of the longest neurite from *in vitro* transduced DRG neurons revealed a 1,4- fold decrease for GNB-2, whereas ANX2 and ANX5 knockdown produced no significant effect (Fig.2.7). These results suggest that GNB-2 has an important role in axonal regeneration.

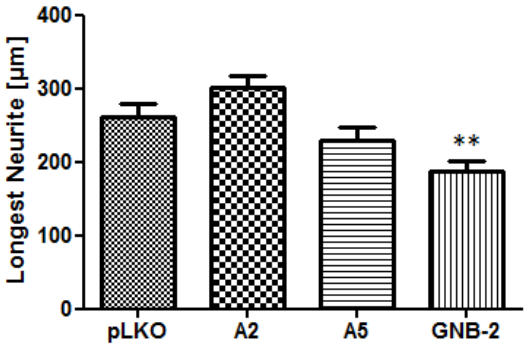


Figure 2.7 | ***In vitro*** **knockdown of GNB-2 exhibits a significant decrease in the length of the longest neurite**

Neurite outgrowth measurements of cultured DRG neurons transduced with annexin 2, annexin 5 or GNB-2 ShRNAs. An empty pLKO was used as control. Results represent the mean ± SEM. *p<0.05, **p<0.01 and ***p<0.001.

To confirm that GNB-2 could impair axonal regeneration, *in vitro* neurite outgrowth assays were again performed in DRG neurons. From the obtained transduced cells, while part was used to assess the longest neurite (Fig. 2.8 a. and b.), the remaining cells were used to confirm knockdown for this experience through the use of RT-qPCR (Fig, 2.8 c.) Results were very consistent showing a 1,3- fold decrease (p=0,01) whereas mRNA expression in DRG neurons was almost 90% reduced (Fig. 2.8).

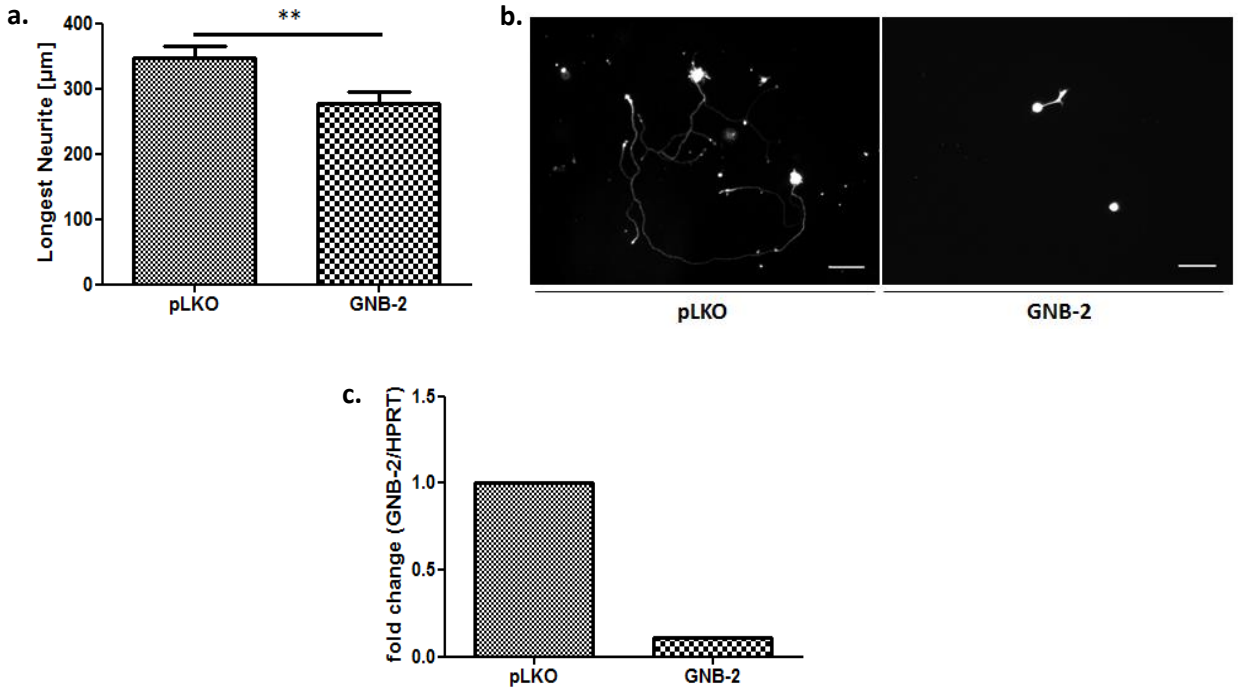


Figure 2.8 | **GNB-2 knock down impairs neurite outgrowth**

a. Neurite outgrowth of culture DRG neurons transduced with GNB-2 ShRNA. Results represent the mean ± SEM. *p<0.05, **p<0.01 and ***p<0.001; **b.** Representative βIII-tubulin immunocytochemistry of a.; **c.** RT-qPCR showed a reduction in mRNA levels of almost 90%.

These results suggest that GNB-2 is required for successful axonal regeneration. In this sense, it will be interesting to see whether overexpression of this protein will improve axonal regeneration. In the meantime, an overview of the function of this protein may give us a clue on how it regulates the axonal regeneration program.

GNB-2, also called G protein beta 2 subunit, belongs to the family of G proteins which are involved in innumerous intracellular signaling pathways. Besides the subunit beta (β), there are the subunits alpha (α) and gamma (γ). Within the cell, these three subunits combine to form a heterotrimeric complex that couples with G protein-coupled receptors (GCPRs). When an extracellular stimuli activates GCPRs the heterotrimeric complex is cleaved forming two molecules, the G protein alpha (G_{α}) and the heterodimic complex G protein beta-gamma ($G_{\beta\gamma}$). These two molecules will then be responsible for activating different signaling pathways that will eventually lead to a proper physiological response (Oldham & Hamm 2008). Specifically $G_{\beta\gamma}$-dimmer can interact with numerous effectors, such as K+ and Ca^{2+} channels, phospholipase C (PLC), adenyl cyclases (ACs), phosphoinositide 3-kinase (PI_3 kinase) and elements of the MAPK cascade (Cabrera-Vera et al 2003). Interestingly, it was identified a direct interaction between the $G_{\beta\gamma}$ subunit and the Raf-1 protein kinase that depends on the amino terminus of Raf-1 and the carboxyl terminus of $G_{\beta 2}$ subunit (Pumiglia et al 1995). In addition, in a series of paradigms using Raf1 and B-Raf[1] conditioned null mice expressing Cre recombinase, it was shown that this family is crucial for axon growth during development (Zhong et al 2007) and more recently, conditional activation of B-Raf has been reported to promote axon outgrowth of injured CNS neurons from adult mice (O'Donovan et al 2014). Of note, Zhong and colleagues also refer that compensation between the Raf homologs is likely to occur (Zhong et al 2007). Whether GNB-2 can participate in the Raf kinase-dependent regeneration program is not known.

Further studies should be performed, but altogether these evidences suggest that GNB-2 may have a role in eliciting/supporting axonal regeneration, perhaps through the modulation of Raf-1 activity or even through of other Raf homologs. In summary, GNB-2 appears as a potential enhancer of axonal regeneration.

[1]Mammalian Raf homolog. There are three currently known mammalian Raf homologs: A-Raf, B-Raf and C-Raf (Raf1).

Epilogue

Injury triggers a variety of changes that will determine the somatic response and consequently whether axonal regeneration occurs or not. One of the first determinants for successful axonal regeneration is the activation of injury signals, namely alteration of the membrane potential, suppression of negative injury signals and local activation of positive injury signals. Initiation of the regeneration program is strongly dependent on the achievement of these three steps.

The results from our lab surprisingly showed that non-regenerative models, such as a dorsal root injury, could also trigger positive injury signals. This led me to question whether regeneration models, such as a sciatic nerve injury, could stimulate the activation of other factors required for successful axonal regeneration. And indeed it has been recently shown that injury to the peripheral nerve system elicits certain epigenetic changes that are required for a successful regeneration program. What triggers those changes is not completely understood. In the study performed by Cho and colleagues, they showed that following sciatic nerve injury, backpropagation of a calcium wave elicits epigenetic modifications that include actelylation of histone 3 (H3) and nuclear export of HDAC5 to the injury site, thus promoting axonal regeneration. In contrast, when analyzing retinal ganglion cells following optic nerve crush they observed a decrease in the level of Ac-H3 in nuclei and also accumulation failure of HDAC5 in the injury site (Cho et al 2013). Puttagunta and colleagues also state the importance of the histone acetyltransferase, PFAC, in H3 acetylation following sciatic nerve injury, and refer that following spinal cord injury H3 acetylation does not occur (Puttagunta et al 2014). It seems that backpropagation of the calcium wave is required as an early signal to elicit epigenetic changes that, dependent on the late injury response (retrograde transport of injury signals), will trigger a competent regeneration program. As far as I know, evaluation of the calcium wave following injury *in vivo* was never addressed in the central branch of DRG neurons following central injury, or even in retinal ganglion cells following optic nerve injury, probably due to lack of high sensitive equipment capable of assessing those parameters. In addition, a particular characteristic of the central branch of DRG neurons and also of the axons from retinal ganglion cells is that electric transmission occurs from the cell body to the tip of the axons, which in a situation of injury will have the opposite direction of the calcium wave. Therefore, it would be interesting to draw a mathematical model that address whether these two ionic currents could conflict with each other, thus impairing the backpropagation of the calcium wave and consequently the epigenetic modifications required for the regeneration program.

Moreover, since it has been reported that electric stimulation in intact peripheral branches of DRG neurons promotes outgrowth of their central branches (Udina et al 2008) and that blocking voltage-gated sodium channels diminishes the amplitude of the calcium wave that propagates from the injury site to the cell body following (Mandolesi et al 2004), it would be interesting to check whether this first early response mediates epigenetic changes and the parameters (amplitude, frequency, duration, …) that may lead to it. Unluckily, in the study performed by Cho and colleagues (Cho et al 2013), similar to what had been described in Fouad's lab (Udina et al 2008), blockage of voltage-gated sodium channels

did not impair propagation of the calcium wave. However, they did not test whether that blockage could affect acetylation of H3.

The mechanisms by which neurons can regenerate their axons following injury are becoming cleared. However, there are several questions that remain unanswered. The early electrophysiological response may contribute to axonal regeneration, but in my modest opinion full recovery of neuronal function is a process that will require combined therapies, meaning that a long road still needs to be covered, either in the study of neurobiology or the development of new technologies.

References

Abe N, Cavalli V. 2008. Nerve injury signaling. *Current opinion in neurobiology* 18: 276-83

Ambron RT, Dulin MF, Zhang XP, Schmied R, Walters ET. 1995. Axoplasm enriched in a protein mobilized by nerve injury induces memory-like alterations in Aplysia neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 15: 3440-6

Ambron RT, Walters ET. 1996. Priming events and retrograde injury signals. A new perspective on the cellular and molecular biology of nerve regeneration. *Molecular neurobiology* 13: 61-79

Bareyre FM, Garzorz N, Lang C, Misgeld T, Buning H, Kerschensteiner M. 2011. In vivo imaging reveals a phase-specific role of STAT3 during central and peripheral nervous system axon regeneration. *Proceedings of the National Academy of Sciences of the United States of America* 108: 6282-7

Bear R, Rintoul D. 2013. Neurons and Glial Cells. ed. O College. http://cnx.org/

Beirowski B, Adalbert R, Wagner D, Grumme DS, Addicks K, et al. 2005. The progressive nature of Wallerian degeneration in wild-type and slow Wallerian degeneration (WldS) nerves. *BMC neuroscience* 6: 6

Ben-Yaakov K, Dagan SY, Segal-Ruder Y, Shalem O, Vuppalanchi D, et al. 2012. Axonal transcription factors signal retrogradely in lesioned peripheral nerve. *The EMBO journal* 31: 1350-63

Blesch A, Lu P, Tsukada S, Alto LT, Roet K, et al. 2012. Conditioning lesions before or after spinal cord injury recruit broad genetic mechanisms that sustain axonal regeneration: superiority to camp-mediated effects. *Experimental neurology* 235: 162-73

Bolsover S, Fabes J, Anderson PN. 2008. Axonal guidance molecules and the failure of axonal regeneration in the adult mammalian spinal cord. *Restorative neurology and neuroscience* 26: 117-30

Borrelli E, Nestler EJ, Allis CD, Sassone-Corsi P. 2008. Decoding the epigenetic language of neuronal plasticity. *Neuron* 60: 961-74

Bouldin TW, Earnhardt TS, Goines ND. 1991. Restoration of blood-nerve barrier in neuropathy is associated with axonal regeneration and remyelination. *Journal of neuropathology and experimental neurology* 50: 719-28

Bradbury EJ, McMahon SB. 2006. Spinal cord repair strategies: why do they work? *Nature reviews. Neuroscience* 7: 644-53

Bradke F, Fawcett JW, Spira ME. 2012. Assembly of a new growth cone after axotomy: the precursor to axon regeneration. *Nature reviews. Neuroscience* 13: 183-93

Bruck W. 1997. The role of macrophages in Wallerian degeneration. *Brain pathology* 7: 741-52

Buss A, Brook GA, Kakulas B, Martin D, Franzen R, et al. 2004. Gradual loss of myelin and formation of an astrocytic scar during Wallerian degeneration in the human spinal cord. *Brain : a journal of neurology* 127: 34-44

Buss A, Pech K, Merkler D, Kakulas BA, Martin D, et al. 2005. Sequential loss of myelin proteins during Wallerian degeneration in the human spinal cord. *Brain : a journal of neurology* 128: 356-64

Cabrera-Vera TM, Vanhauwe J, Thomas TO, Medkova M, Preininger A, et al. 2003. Insights into G protein structure, function, and regulation. *Endocrine reviews* 24: 765-81

Cavalli V, Kujala P, Klumperman J, Goldstein LS. 2005. Sunday Driver links axonal transport to damage signaling. *The Journal of cell biology* 168: 775-87

Chaudhry V, Glass JD, Griffin JW. 1992. Wallerian degeneration in peripheral nerve disease. *Neurologic clinics* 10: 613-27

Chierzi S, Ratto GM, Verma P, Fawcett JW. 2005. The ability of axons to regenerate their growth cones depends on axonal type and age, and is regulated by calcium, cAMP and ERK. *The European journal of neuroscience* 21: 2051-62

Cho Y, Sloutsky R, Naegle KM, Cavalli V. 2013. Injury-induced HDAC5 nuclear export is essential for axon regeneration. *Cell* 155: 894-908

Communications BM. 2013. Anatomy of a multipolar neuron. http://blausen.com/

David S, Aguayo AJ. 1981. Axonal elongation into peripheral nervous system "bridges" after central nervous system injury in adult rats. *Science* 214: 931-3

Eddleman CS, Bittner GD, Fishman HM. 2000. Barrier permeability at cut axonal ends progressively decreases until an ionic seal is formed. *Biophysical journal* 79: 1883-90

Fallon JR, Taylor AB. 2013. Protein Synthesis in Neurons In *eLS*

Fournier AE, Takizawa BT, Strittmatter SM. 2003. Rho kinase inhibition enhances axonal regeneration in the injured CNS. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 23: 1416-23

Galbraith JA, Gallant PE. 2000. Axonal transport of tubulin and actin. *Journal of neurocytology* 29: 889-911

Gaudet AD, Popovich PG, Ramer MS. 2011. Wallerian degeneration: gaining perspective on inflammatory events after peripheral nerve injury. *Journal of neuroinflammation* 8: 110

George EB, Glass JD, Griffin JW. 1995. Axotomy-induced axonal degeneration is mediated by calcium influx through ion-specific channels. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 15: 6445-52

George R, Griffin JW. 1994. Delayed macrophage responses and myelin clearance during Wallerian degeneration in the central nervous system: the dorsal radiculotomy model. *Experimental neurology* 129: 225-36

Goldberg JL. 2003. How does an axon grow? *Genes & development* 17: 941-58

Guan KL, Rao Y. 2003. Signalling mechanisms mediating neuronal responses to guidance cues. *Nature reviews. Neuroscience* 4: 941-56

Gumy LF, Tan CL, Fawcett JW. 2010. The role of local protein synthesis and degradation in axon regeneration. *Experimental neurology* 223: 28-37

Han PJ, Shukla S, Subramanian PS, Hoffman PN. 2004. Cyclic AMP elevates tubulin expression without increasing intrinsic axon growth capacity. *Experimental neurology* 189: 293-302

Hannila SS, Filbin MT. 2008. The role of cyclic AMP signaling in promoting axonal regeneration after spinal cord injury. *Experimental neurology* 209: 321-32

Hanz S, Fainzilber M. 2004. Integration of retrograde axonal and nuclear transport mechanisms in neurons: implications for therapeutics. *The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry* 10: 404-8

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. *The European journal of neuroscience* 22: 3025-35

He Z, Koprivica V. 2004. The Nogo signaling pathway for regeneration block. *Annual review of neuroscience* 27: 341-68

Hiraga A, Kuwabara S, Doya H, Kanai K, Fujitani M, et al. 2006. Rho-kinase inhibition enhances axonal regeneration after peripheral nerve injury. *Journal of the peripheral nervous system : JPNS* 11: 217-24

Hirokawa N, Niwa S, Tanaka Y. 2010. Molecular motors in neurons: transport mechanisms and roles in brain function, development, and disease. *Neuron* 68: 610-38

Hofer AM. 2012. Interactions between calcium and cAMP signaling. *Current medicinal chemistry* 19: 5768-73

Hoffman PN. 2010. A conditioning lesion induces changes in gene expression and axonal transport that enhance regeneration by increasing the intrinsic growth state of axons. *Experimental neurology* 223: 11-8

Hoffman PN, Luduena RF. 1996. The axonal transport of beta III-tubulin is altered in both branches of sensory axons after injury of the rat sciatic nerve. *Brain research* 708: 182-4

Huber AB, Kolodkin AL, Ginty DD, Cloutier JF. 2003. Signaling at the growth cone: ligand-receptor complexes and the control of axon growth and guidance. *Annual review of neuroscience* 26: 509-63

Huebner EA, Strittmatter SM. 2009. Axon regeneration in the peripheral and central nervous systems. *Results and problems in cell differentiation* 48: 339-51

Jankowski MP, Cornuet PK, McIlwrath S, Koerber HR, Albers KM. 2006. SRY-box containing gene 11 (Sox11) transcription factor is required for neuron survival and neurite growth. *Neuroscience* 143: 501-14

Jessen KR. 2004. Glial cells. *The international journal of biochemistry & cell biology* 36: 1861-7

Kamber D, Erez H, Spira ME. 2009. Local calcium-dependent mechanisms determine whether a cut axonal end assembles a retarded endbulb or competent growth cone. *Experimental neurology* 219: 112-25

Kapitein LC, Hoogenraad CC. 2011. Which way to go? Cytoskeletal organization and polarized transport in neurons. *Molecular and cellular neurosciences* 46: 9-20

Kaplan A, Kent CB, Charron F, Fournier AE. 2014. Switching responses: spatial and temporal regulators of axon guidance. *Molecular neurobiology* 49: 1077-86

Kerschensteiner M, Schwab ME, Lichtman JW, Misgeld T. 2005. In vivo imaging of axonal degeneration and regeneration in the injured spinal cord. *Nature medicine* 11: 572-7

Kiryu-Seo S, Kiyama H. 2011. The nuclear events guiding successful nerve regeneration. *Frontiers in molecular neuroscience* 4: 53

Knoferle J, Koch JC, Ostendorf T, Michel U, Planchamp V, et al. 2010. Mechanisms of acute axonal degeneration in the optic nerve in vivo. *Proceedings of the National Academy of Sciences of the United States of America* 107: 6064-9

Lasek RJ, Garner JA, Brady ST. 1984. Axonal transport of the cytoplasmic matrix. *The Journal of cell biology* 99: 212s-21s

Lau BY, Fogerson SM, Walsh RB, Morgan JR. 2013. Cyclic AMP promotes axon regeneration, lesion repair and neuronal survival in lampreys after spinal cord injury. *Experimental neurology* 250: 31-42

Lerch JK, Martinez-Ondaro YR, Bixby JL, Lemmon VP. 2014. cJun promotes CNS axon growth. *Molecular and cellular neurosciences* 59: 97-105

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: 269-82

Liu K, Tedeschi A, Park KK, He Z. 2011. Neuronal intrinsic mechanisms of axon regeneration. *Annual review of neuroscience* 34: 131-52

Liz MA, Mar FM, Santos TE, Pimentel HI, Marques AM, et al. 2014. Neuronal deletion of GSK3beta increases microtubule speed in the growth cone and enhances axon regeneration via CRMP-2 and independently of MAP1B and CLASP2. *BMC biology* 12: 47

Lowery LA, Van Vactor D. 2009. The trip of the tip: understanding the growth cone machinery. *Nature reviews. Molecular cell biology* 10: 332-43

Lubinska L. 1977. Early course of Wallerian degeneration in myelinated fibres of the rat phrenic nerve. *Brain research* 130: 47-63

Luo L. 2000. Rho GTPases in neuronal morphogenesis. *Nature reviews. Neuroscience* 1: 173-80

Maekawa M, Ishizaki T, Boku S, Watanabe N, Fujita A, et al. 1999. Signaling from Rho to the actin cytoskeleton through protein kinases ROCK and LIM-kinase. *Science* 285: 895-8

Mandolesi G, Madeddu F, Bozzi Y, Maffei L, Ratto GM. 2004. Acute physiological response of mammalian central neurons to axotomy: ionic regulation and electrical activity. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 18: 1934-6

Mar FM, Bonni A, Sousa MM. 2014a. Cell intrinsic control of axon regeneration. *EMBO reports* 15: 254-63

Mar FM, Simoes AR, Leite S, Morgado MM, Santos TE, et al. 2014b. CNS axons globally increase axonal transport after peripheral conditioning. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 34: 5965-70

Mason MR, Lieberman AR, Grenningloh G, Anderson PN. 2002. Transcriptional upregulation of SCG10 and CAP-23 is correlated with regeneration of the axons of peripheral and central neurons in vivo. *Molecular and cellular neurosciences* 20: 595-615

Miao T, Wu D, Zhang Y, Bo X, Subang MC, et al. 2006. Suppressor of cytokine signaling-3 suppresses the ability of activated signal transducer and activator of transcription-3 to stimulate neurite growth in rat primary sensory neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 26: 9512-9

Monsul NT, Geisendorfer AR, Han PJ, Banik R, Pease ME, et al. 2004. Intraocular injection of dibutyryl cyclic AMP promotes axon regeneration in rat optic nerve. *Experimental neurology* 186: 124-33

Mueller BK, Mack H, Teusch N. 2005. Rho kinase, a promising drug target for neurological disorders. *Nature reviews. Drug discovery* 4: 387-98

Neumann S, Bradke F, Tessier-Lavigne M, Basbaum AI. 2002. Regeneration of sensory axons within the injured spinal cord induced by intraganglionic cAMP elevation. *Neuron* 34: 885-93

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

Nikulina E, Tidwell JL, Dai HN, Bregman BS, Filbin MT. 2004. The phosphodiesterase inhibitor rolipram delivered after a spinal cord lesion promotes axonal regeneration and functional recovery. *Proceedings of the National Academy of Sciences of the United States of America* 101: 8786-90

O'Donovan KJ, Ma K, Guo H, Wang C, Sun F, et al. 2014. B-RAF kinase drives developmental axon growth and promotes axon regeneration in the injured mature CNS. *The Journal of experimental medicine* 211: 801-14

Oblinger MM, Lasek RJ. 1988. Axotomy-induced alterations in the synthesis and transport of neurofilaments and microtubules in dorsal root ganglion cells. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 8: 1747-58

Ochs S. 1977. The early history of nerve regeneration beginning with Cruikshank's observations in 1776. *Medical history* 21: 261-74

Oldham WM, Hamm HE. 2008. Heterotrimeric G protein activation by G-protein-coupled receptors. *Nature reviews. Molecular cell biology* 9: 60-71

Pasinelli P, Brown RH. 2006. Molecular biology of amyotrophic lateral sclerosis: insights from genetics. *Nature reviews. Neuroscience* 7: 710-23

Paves H, Saarma M. 1997. Neurotrophins as in vitro growth cone guidance molecules for embryonic sensory neurons. *Cell and tissue research* 290: 285-97

Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, et al. 2001. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocrine reviews* 22: 153-83

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: 715-26

Perlson E, Hanz S, Medzihradszky KF, Burlingame AL, Fainzilber M. 2004. From snails to sciatic nerve: Retrograde injury signaling from axon to soma in lesioned neurons. *Journal of neurobiology* 58: 287-94

Perlson E, Michaelevski I, Kowalsman N, Ben-Yaakov K, Shaked M, et al. 2006. Vimentin binding to phosphorylated Erk sterically hinders enzymatic dephosphorylation of the kinase. *Journal of molecular biology* 364: 938-44

Polleux F, Snider W. 2010. Initiating and growing an axon. *Cold Spring Harbor perspectives in biology* 2: a001925

Pumiglia KM, LeVine H, Haske T, Habib T, Jove R, Decker SJ. 1995. A direct interaction between G-protein beta gamma subunits and the Raf-1 protein kinase. *The Journal of biological chemistry* 270: 14251-4

Puttagunta R, Tedeschi A, Soria MG, Hervera A, Lindner R, et al. 2014. PCAF-dependent epigenetic changes promote axonal regeneration in the central nervous system. *Nature communications* 5: 3527

Qiu J, Cafferty WB, McMahon SB, Thompson SW. 2005. Conditioning injury-induced spinal axon regeneration requires signal transducer and activator of transcription 3 activation. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 25: 1645-53

Qiu J, Cai D, Dai H, McAtee M, Hoffman PN, et al. 2002. Spinal axon regeneration induced by elevation of cyclic AMP. *Neuron* 34: 895-903

Qiu XB, Shao YM, Miao S, Wang L. 2006. The diversity of the DnaJ/Hsp40 family, the crucial partners for Hsp70 chaperones. *Cellular and molecular life sciences : CMLS* 63: 2560-70

Raivich G, Bohatschek M, Da Costa C, Iwata O, Galiano M, et al. 2004. The AP-1 transcription factor c-Jun is required for efficient axonal regeneration. *Neuron* 43: 57-67

Ramon y Cajal S, May RM. 1928. *Degeneration and Regeneration of the Nervous System*. New York: Oxford University Press.

Reece JB, Taylor M, Simon E, Dickey J. 2011. *Campbell Biology: Concepts & Connections*. pp. 928. San Francisco: Benjamin Cummings.

Rishal I, Fainzilber M. 2010. Retrograde signaling in axonal regeneration. *Experimental neurology* 223: 5-10

Rishal I, Fainzilber M. 2014. Axon-soma communication in neuronal injury. *Nature reviews. Neuroscience* 15: 32-42

Ruff CA, Staak N, Patodia S, Kaswich M, Rocha-Ferreira E, et al. 2012. Neuronal c-Jun is required for successful axonal regeneration, but the effects of phosphorylation of its N-terminus are moderate. *Journal of neurochemistry* 121: 607-18

Saijilafu, Hur EM, Liu CM, Jiao Z, Xu WL, Zhou FQ. 2013. PI3K-GSK3 signalling regulates mammalian axon regeneration by inducing the expression of Smad1. *Nature communications* 4: 2690

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: 959-70

Seijffers R, Mills CD, Woolf CJ. 2007. ATF3 increases the intrinsic growth state of DRG neurons to enhance peripheral nerve regeneration. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 27: 7911-20

Sharma A, Upadhyay AK, Bhat MK. 2009. Inhibition of Hsp27 and Hsp40 potentiates 5-fluorouracil and carboplatin mediated cell killing in hepatoma cells. *Cancer biology & therapy* 8: 2106-13

Sheu JY, Kulhanek DJ, Eckenstein FP. 2000. Differential patterns of ERK and STAT3 phosphorylation after sciatic nerve transection in the rat. *Experimental neurology* 166: 392-402

Smith DS, Skene JH. 1997. A transcription-dependent switch controls competence of adult neurons for distinct modes of axon growth. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 17: 646-58

Stahl S. 2008. *Stahl's Essential Psychopharmacology Neuroscientific Basis and Practical Applications*. New York, NY: Cambridge Medicine. 1132 pp.

Steward O, Schuman EM. 2003. Compartmentalized synthesis and degradation of proteins in neurons. *Neuron* 40: 347-59

Stoll G, Griffin JW, Li CY, Trapp BD. 1989. Wallerian degeneration in the peripheral nervous system: participation of both Schwann cells and macrophages in myelin degradation. *Journal of neurocytology* 18: 671-83

Tanaka S, Kitagawa K, Ohtsuki T, Yagita Y, Takasawa K, et al. 2002. Synergistic induction of HSP40 and HSC70 in the mouse hippocampal neurons after cerebral ischemia and ischemic tolerance in gerbil hippocampus. *Journal of neuroscience research* 67: 37-47

Taylor KS, Anastakis DJ, Davis KD. 2010. Chronic pain and sensorimotor deficits following peripheral nerve injury. *Pain* 151: 582-91

Tortora G, Derrickson B. 2011. *Principles of Anatomy and Physiology*. Wiley, John & Sons, Incorporated. 1344 pp.

Trapp BD, Hauer P, Lemke G. 1988. Axonal regulation of myelin protein mRNA levels in actively myelinating Schwann cells. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 8: 3515-21

Twiss JL, Fainzilber M. 2009. Ribosomes in axons--scrounging from the neighbors? *Trends in cell biology* 19: 236-43

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: 238-47

van Kesteren RE, Mason MR, Macgillavry HD, Smit AB, Verhaagen J. 2011. A gene network perspective on axonal regeneration. *Frontiers in molecular neuroscience* 4: 46

Vargas ME, Barres BA. 2007. Why is Wallerian degeneration in the CNS so slow? *Annual review of neuroscience* 30: 153-79

Verma P, Chierzi S, Codd AM, Campbell DS, Meyer RL, et al. 2005. Axonal protein synthesis and degradation are necessary for efficient growth cone regeneration. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 25: 331-42

Waller A. 1950. Experiments on the section of the glossopharyngeal and hypoglossal nerves of the frog, and observations of the alterations produced thereby in the structure of their primitive fibres. *Philosophical Transactions of the Royal Society of London* 140: 423-29

Wang JT, Medress ZA, Barres BA. 2012. Axon degeneration: molecular mechanisms of a self-destruction pathway. *The Journal of cell biology* 196: 7-18

Watson C, Paxinos G, Kayalioglu G. 2008. *The Spinal Cord: A Christopher and Dana Reeve Foundation Text and Atlas*. 408 pp.

Wujek JR, Lasek RJ. 1983. Correlation of axonal regeneration and slow component B in two branches of a single axon. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 3: 243-51

Xiao HS, Huang QH, Zhang FX, Bao L, Lu YJ, et al. 2002. Identification of gene expression profile of dorsal root ganglion in the rat peripheral axotomy model of neuropathic pain. *Proceedings of the National Academy of Sciences of the United States of America* 99: 8360-5

Yiu G, He Z. 2006. Glial inhibition of CNS axon regeneration. *Nature reviews. Neuroscience* 7: 617-27

Ylera B, Erturk A, Hellal F, Nadrigny F, Hurtado A, et al. 2009. Chronically CNS-injured adult sensory neurons gain regenerative competence upon a lesion of their peripheral axon. *Current biology : CB* 19: 930-6

Yoshimura T, Kawano Y, Arimura N, Kawabata S, Kikuchi A, Kaibuchi K. 2005. GSK-3beta regulates phosphorylation of CRMP-2 and neuronal polarity. *Cell* 120: 137-49

Zhang Y, Tohyama K, Winterbottom JK, Haque NS, Schachner M, et al. 2001. Correlation between putative inhibitory molecules at the dorsal root entry zone and failure of dorsal root axonal regeneration. *Molecular and cellular neurosciences* 17: 444-59

Zhong J, Li X, McNamee C, Chen AP, Baccarini M, Snider WD. 2007. Raf kinase signaling functions in sensory neuron differentiation and axon growth in vivo. *Nature neuroscience* 10: 598-607

Zhou FQ, Snider WD. 2005. Cell biology. GSK-3beta and microtubule assembly in axons. *Science* 308: 211-4

Ziv NE, Spira ME. 1995. Axotomy induces a transient and localized elevation of the free intracellular calcium concentration to the millimolar range. *Journal of neurophysiology* 74: 2625-37

Zou H, Ho C, Wong K, Tessier-Lavigne M. 2009. Axotomy-induced Smad1 activation promotes axonal growth in adult sensory neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 29: 7116-23

Supplementary information

Table S1 **| Target proteins for which a significant variation was found by microarray analysis of dynein-immunoprecipitated axoplasm from DRI and SNI samples**

Grey- targets for which additional analysis was performed by Western blot of axoplasm
Red- targets for which Western blot results validated the microarray data

Full Target Protein Name	Phospho Site (Human)	Microarray Z-ratio (DRI/SNI)	Western blot fold change (DRI/SNI)
Acetylated Lysine	Pan-specific	1,28	not detected
B-cell lymphoma protein 2 alpha (Bcl2)	Pan-specific	2,14	not detected
Breast cancer type 1 susceptibility protein (BRCA1)	S1497	1,29	not detected
Caspase 4	Pan-specific	-1,2	0,7
Caspase 5	Pan-specific	-1,26	2,2
Caveolin 1	Y14	-1,32	not detected
Cell division cycle 2-like protein-serine kinase 5 (CDC2L5, CHED)	Pan-specific	-1,21	not detected
C-terminus of Src tyrosine kinase (Csk)	Pan-specific	1,05	1,9
Cyclin-dependent protein-serine kinase 5 (CDK5)	Pan-specific	-1,07	1,7
Cyclin-dependent protein-serine kinase 8 (CDK8)	Pan-specific	4,35	1,1
DnaJ homolog, subfamily B member 1(Hsp40)	Pan-specific	1,4	1,4
Epidermal growth factor receptor-tyrosine kinase (EGFR)	Y1110	1,79	not detected
Extracellular regulated protein-serine kinase 2 (Erk1 + Erk2)	Pan-specific	1,32	-
Glycogen synthase-serine kinase 3 beta (GSK3b)	Pan-specific	1,04	3,2
Heat shock 27 kDa protein beta 1 (Hsp27)	S78	2,12	0,9
Heat shock 60 kDa protein 1 (chaperonin, CPN60)	Pan-specific	1,37	not detected
Histone H2A variant X	S140	-1,22	not detected
Histone H2B	S15	-1,14	not detected
Integrin-linked protein-serine kinase 1 (ILK1)	Pan-specific	-1,21	not detected
Jun N-terminus protein-serine kinase (JNK1/2/3)	Pan-specific	-1,46	-
Kit/Steel factor receptor-tyrosine kinase (Kit)	Y936	1,7	not detected
LIM domain kinase 1 (LIMK1)	Pan-specific	1,86	not detected
LIM domain kinase 2 (LIMK1 + LIMK2)	Y504+T505	1,19	not detected
MAPK/ERK protein-serine kinase 1 (MKK1, MEK1, MAP2K1)	Pan-specific	1,09	-
MAPK/ERK protein-serine kinase 2 (MKK2, MEK2, MAP2K2)	T394	1,14	not detected
MAPK/ERK protein-serine kinase 6 (MKK6, MEK6, MAP2K6)	Pan-specific	1,11	3,0
Mitogen-activated protein-serine kinase p38 alpha (p38a MAPK)	Pan-specific	1,21	0,5
Mitogen-activated protein-serine kinase p38 gamma (MAPK12, p38g MAPK, Erk6)	Pan-specific	1,03	not detected
NF-kappa-B p65 nuclear transcription factor (NFkappaB p65)	S276	-1,03	not detected
nucleophosmin, numatrin, nucleolar protein NO38 (B23)	T234/T237	1,68	not detected
Polo-like protein kinase 3 (Plk3)	Pan-specific	1,36	2,2
Protein-serine kinase B beta (PKBb; Akt2)	Pan-specific	2,77	0,9
Protein-serine kinase C beta 1/2 (PKCb1/2)	T500	1,2	not detected
Protein-serine kinase C beta 2 (PKCb2)	Pan-specific	1,2	0,5
Protein-serine kinase C eta (PKCh)	T655	-5,22	not detected
Protein-serine kinase C lambda/iota (PKCl/i)	T564	1,1	1,0
Protein-serine kinase C theta (PKCq)	Pan-specific	1,32	not detected
Protein-serine phosphatase 2A - alpha and beta isoforms (PP2A/Aa/b)	Pan-specific	-1,12	1,9
Retinoblastoma-associated protein 1	S807	-2,12	not detected

Retinoblastoma-associated protein 1	T356	-1,51	not detected
RhoA protein-serine kinase alpha (ROCK2)	Pan-specific	1,18	1,3
Signal transducer and activator of transcription 3 (STAT3)	S727	1,25	-
Sphingosine kinase 2 (SPHK2)	Pan-specific	-1,12	not detected
Src proto-oncogene-encoded protein-tyrosine kinase (Src)	Pan-specific	-1,18	0,6

Table S2 **| Protein identification by MALDI-TOF/TOF of 2D gel spots of spinal cord extracts**

Spot	Protein identified	Swissprot ID
2	Tubulin	TBB3_RAT
5	Actin	ACTB_RAT
4	Dihydropyrimidinase-related protein 5 (CRMP-5)	DPYL5_RAT
19	14-3-3 protein	
	gamma isoform	1433G_RAT
	theta isoform	1433T_RAT
	zeta/delta isoform	1433Z_RAT
	eta isoform	1433F_RAT
	beta/alpha isoform	1433B_RAT
	epsilon isoform	1433E_RAT
20	Rho GDP-dissociation inhibitor 1 (RhoGDI)	GDIR1_RAT
20	Ubiquitin carboxyl-terminal hydrolase (PGP 9.5)	UCHL1_RAT
1	Heat shock protein 70 (HSP70)	HSP7C_RAT
13	Guanine nucleotide binding protein beta-2 (G protein subunit 2)	GBB2_RAT
16	Annexin A2	ANXA2_RAT
16	NAD-dependent deacetylase sirtuin-2 (Sirtuin2)	SIRT2_RAT
18	Annexin A5	ANXA5_RAT
3	Pyruvate kinase isozymes M1/M2	KPYM_RAT
4	Pyruvate kinase isozymes M1/M2	KPYM_RAT
7	Alpha-enolase	ENOA_RAT
9	Phosphoglycerate kinase 1 (PGK1)	PGK1_RAT
10	Fructose-bisphosphate aldolase C	ALDOC_RAT
16	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	G3P_RAT
17	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	G3P_RAT
14	Malate dehydrogenase, cytoplasmic	MDHC_RAT
15	Malate dehydrogenase, cytoplasmic	MDHC_RAT
4	Catalase	CATA_RAT
1	NADH dehydrogenase	NDUS1_RAT
14	L-lactate dehydrogenase B chain	LDHB_RAT
6	Creatine kinase B-type	KCRB_RAT
11	Acetyl-CoA acetyltransferase (ACAT)	THIC_RAT
12	Acetyl-CoA acetyltransferase (ACAT)	THIC_RAT
8	Glutamine synthetase	GLNA_RAT
11	Cytosolic acyl coenzyme A thioester hydrolase	BACH_RAT
11	Peptidyl-prolyl cis-trans isomerase D (prolyl isomerase)	PPID_RAT
12	Cytosolic acyl coenzyme A thioester hydrolase	BACH_RAT
12	Peptidyl-prolyl cis-trans isomerase D (prolyl isomerase)	PPID_RAT