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Modulating Actin Dynamics during axon formation, growth and regeneration: The role of Adducin

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"Imagine uma nova história para a sua vida e acredite nela."

Paulo Coelho

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Index

Figures List I
Abbreviations III
Abstract V
Resumo VI
1. Introduction 1
1.1 Brief overview of the central nervous system and peripheral nervous system 1
1.2 Neuronal Polarization and Axon Formation 2
1.3 Axon Regeneration 5
1.3.1 The extrinsic factors 7
1.3.2 The intrinsic factors 8
1.3.3 Spinal cord injury as a model to study axonal regeneration 9
1.4 The importance of actin dynamics 10
1.4.1 Actin binding proteins 11
1.4.2 Adducin 12
1.5 Preliminary data 14
2. Objectives 19
3. Methodology 23
3.1 Animals 23
3.2 Cell culture 23
3.2.1 Culture of hippocampal neurons 23
3.2.2 Culture of DRG neurons 23
3.3 Immunocytochemistry 24
3.3.1 Immunocytochemistry for detection of the axon initial segment (AIS) 24
3.3.2 DRG cultures for immunocytochemistry and neurite tracing 24
3.4 Analysis of neuromuscular junctions 25
3.4.1 Analysis of neuromuscular junctions in pre- and postsynaptic structures 25
3.5 Lesions 26
3.5.1 Spinal cord injury 26
3.5.2 Evaluation of the glial scar 26
3.5.3 Analysis of regeneration of dorsal column fibers 27
3.5.4 Analysis of regeneration of serotonergic axons 28
4. Results and discussion 31
4.1 Analysis of neurite outgrowth of α-adducin KO DRG neurons 31

4.2 Analysis of axon regeneration following spinal cord injury 32
4.2.1 Regeneration of sensory axons (dorsal column tract) 32
4.2.2 Analysis of regeneration of serotonergic axons 33
4.2.3 Analysis of the glial scar 34
4.3 Adducin in neuronal polarization and synapse formation 35
4.3.1 Axon Initial Segment 35
4.3.2 Neuromuscular junction (NMJ) formation 36
4.3.3 Evaluation of the pre- and postsynaptic structure of the NMJ 38
5. Conclusions 41
Bibliography 45

Figures list

Figure 1 – The organization of the central nervous system and its subdivisions 1

Figure 2 – Representation of the neuronal structure.. 2

Figure 3 – Neuronal polarization *in vitro* and *in vivo*... 3

Figure 4 – Growth cone structure. 4

Figure 5 – Representative scheme of Wallerian Degeneration (WD) after PNS injury........ 6

Figure 6 – Structure of actin. 10

Figure 7 - A model for the cortical cytoskeleton in axons. 11

Figure 8 – Adducin structure and regulation.. 12

Figure 9 – Adducin activity towards F-actin is regulated by PKC phosphorylation. 13

Figure 10 – The conditioning lesion. 15

Figure 11– Quantification of neurite outgrowth of DRG neurons from WT and α-adducin KO animals.. 31

Figure 12 – Regeneration of dorsal column axons in α-adducin KO mice............................ 32

Figure 13 – Evaluation of regeneration of serotonergic axons. 33

Figure 14 – Evaluation of the glial scar structure. 34

Figure 15 – Evaluation of axon initial segment dynamics.. 35

Figure 16 – Evolution of postsynaptic structures in adducin α-adducin KO.......................... 37

Figure 17 – Morphological changes in the pre- and postsynaptic NMJ structure between α adducin KO and WT littermate mice at P30.. 38

Abbreviations

ABPs - actin binding proteins.

ADD - adducin.

AP-1 - activator protein 1.

ATF3 - activating transcription factor 3.

BDNF - brain-derived neurotrophic factor.

BMS - Basso mouse scale.

BSA - albumin serum fraction V.

BTX - bungarotoxin.

c-Jun - Jun proto-oncogene.

CL - conditioning lesion.

CNS - central nervous system.

CSPGs - chondroitin sulfate proteoglycans.

CTB - cholera toxin b.

DAB - 3,3'-diaminobenzidine.

DIV - days *in vitro.*

DRG - dorsal root ganglia.

ECM - extracellular matrix.

F-actin - filamentous actin.

FBS - fetal bovine serum.

FGF-2 - fibroblast growth factor-2.

G-actin - globular actin.

GDNF - glial-derived neurotrophic factor.

GSK3β - glycogen synthase kinase 3 beta.

HSPGs - heparan sulfate proteoglycans.

HTS - *hu-li tai shao* (adducin orthologue).

iTRAQ - isobaric Tag for Relative and Absolute Quantification.

JAK-STAT - Janus kinase and transducer and activator of transcription.

KLFs - Krüppel-like factors.

KO - knockout.

KSPGs - keparan sulfate proteoglycans.

MARCKS - myristoylated alanine-rich C-kinase substrate domain.

MTs - microtubules.

NB - neurobasal medium.

NFIL3 - nuclear factor IL-3.

NGF - nerve growth factor.

OCT - optimum cutting temperature.

PB - phosphate buffer.

PBS - phosphate buffered saline.

PFA - paraformaldehyde.

PIP_3 - phosphatidylinositol (3,4,5)-triphosphate.

PKA - AMP-dependent protein kinase.

PKC - protein kinase C.

PNS - peripheral nervous system.

PTEN - phosphatase and tensin homologue.

RAGs - regeneration associated genes.

ROCK - Rho-associated kinase.

STAT3 - signal transducer and activator of transcription 3.

TE - tris EDTA.

TSCI - tuberous sclerosis complex-1.

WD - Wallerian degeneration.

Abstract

Although actin is well recognized as a key player in axonal growth, how different actin-binding proteins control its dynamics is still not fully understood. In the adult CNS axonal regeneration may occur under specific conditions, namely when the intrinsic neuronal growth capacity is increased by a priming lesion. Using the conditioning lesion model, we determined by proteomics that the levels of some actin-binding proteins were differently regulated in regenerating axons. From these proteins, two different forms of phospho-adducin (alpha and gamma) were identified as being increased in its C-terminus serine residue. Adducins are actin capping proteins, that once phosphorylated are released from F-actin, promoting actin dynamics. *In vivo*, α-adducin knockout (KO) mice have impaired neuronal migration and decreased axonal density in the optic nerve. It is also known that the adducin KO mice develop hydrocephaly in 50% of the animals, further suggesting an important role of adducin in nervous system homeostasis. In this work we evaluated other aspects of neuron biology that could be impaired by the absence of adducin. Namely, we assessed the role of adducin in axonal polarization, growth, synapse formation and regeneration. Our results revealed that adducin is required for the proper nervous system development and regeneration. Although in α-adducin KO neurons the axon initial segment was correctly formed, *in vitro* relocation after chronic depolarization was impaired, suggesting that the absence of adducin inhibits axonal plasticity. Besides, neuromuscular junction maturation was delayed in the α-adducin KO mice with neuromuscular junctions displaying a decreased volume and complexity. Adducin revealed also to be an important molecule in the regeneration context. Using α-adducin KO mice to mimic the decreased adducin activity after conditioning injury, we found that the absence adducin enhances the capability of axons to extend into the inhibitory glial Schwann cellar. This suggests that regulation of adducin activity could be a target to improve axonal regeneration.

Resumo

Apesar de o citoesqueleto de actina ser um importante fator no crescimento axonal, a forma como as diferentes proteínas de ligação à actina controlam a sua dinâmica não é ainda completamente conhecida. No sistema nervoso central adulto a regeneração axonal pode ocorrer em condições específicas, nomeadamente aumentando o potencial regenerativo intrínseco de alguns neurónios através de uma lesão prévia. Utilizando o modelo de lesão condicionada foi determinado que certas proteínas de ligação à actina estão diferentemente reguladas em neurónios durante a regeneração. Dessas proteínas, duas diferentes formas (alfa e gama) da aducina fosforilada no seu resíduo serina C-terminal foram identificadas como estando aumentadas. As aducinas são proteínas que se ligam à extremidade positiva dos filamentos de actina, impedindo a sua polimerização e despolimerização. Quando fosforilada a aducina perde a capacidade de ligação à actina filamentosa, promovendo um aumento da dinâmica do citoesqueleto de actina. Murganhos deficientes para a produção da aducina têm um défice na migração neuronal e um decréscimo na densidade axonal no nervo ótico. Estes murganhos desenvolvem em 50% dos casos hidrocefalia, sugerindo o papel preponderante que a aducina tem na homeostasia do sistema nervoso. Neste projeto procurámos dissecar o papel da aducina na polarização crescimento axonal, na formação sináptica e na regeneração. Os nossos resultados demonstram que a aducina é necessária para o correto desenvolvimento e regeneração do sistema nervoso. Em neurónios deficientes para a α-aducina o segmento inicial axonal é corretamente formado embora *in vitro* a sua capacidade de recolocação após despolarização crónica seja deficiente, sugerindo que a ausência de aducina desregula a plasticidade axonal. Os processos de maturação neuromuscular apresentam um atraso nos animais deficientes para a produção de α-aducina, visto que junções neuromusculares apresentam um decréscimo no volume e na complexidade da estrutura durante a sua maturação. A α-aducina revelou-se também importante no contexto de regeneração axonal. O uso de animais deficientes para a α-aducina para mimetizar a diminuição da atividade da proteína após lesão condicionada revelou que estes animais possuem uma maior capacidade de estenderem axónios dentro da cicatriz glial. Estes dados sugerem, que a regulação da atividade da aducina pode ser um alvo a ter em consideração para melhorar a regeneração axonal.

1. Introduction

1. Introduction

1.1 Brief overview of the central nervous system and peripheral nervous system

The nervous system is about 3% of the total body weight. It is the smallest system in our body but at the same time, it is the most complex. The nervous system has three main functions: the first one is related to the sensory task (input), this type of information is provided by the cranial and spinal nerves; the second one is related with the integrative function as it is responsible for offering the correct response to certain stimuli. Last but not least, the motor function (output), according to the information that the nervous system receives (Tortora, 2012).

The nervous system is organized in two main systems: the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS is composed by the brain and spinal cord (figure 1). Its principal functions are related with the acquisition of knowledge, memory and emotions. This system is also responsible for the majority of the signals that control the muscles and for gland secretion (Siegel, 2011, Tortora, 2012).

The PNS is composed by nerves, ganglia, enteric plexuses and sensory receptors that sense alterations in the internal or external environment (figure 1). This system is subdivided in somatic nervous system, autonomic nervous system and enteric nervous system (Siegel, 2011, Tortora, 2012).

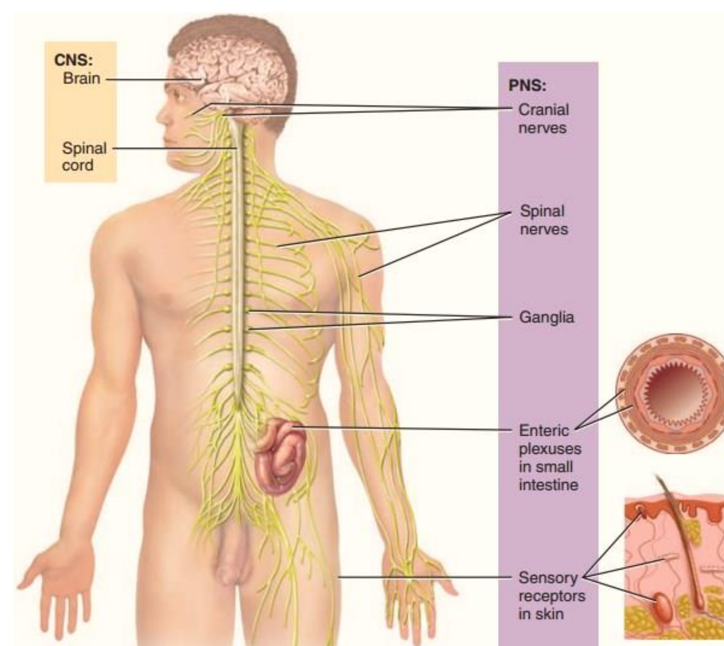


Figure 1 – The organization of the central nervous system and its subdivisions (Tortora, 2012).

Neurons are fundamental for the functions of the nervous system such as sensing, thinking, memorizing, monitoring muscle activity and regulating glandular secretions. This type of cell has electrical excitability that is the capacity to respond to a stimulus and translate it into an action potential. The neuron is constituted by a cell body, an axon and dendrites, as represented in figure 2 (Siegel, 2011, Tortora, 2012).

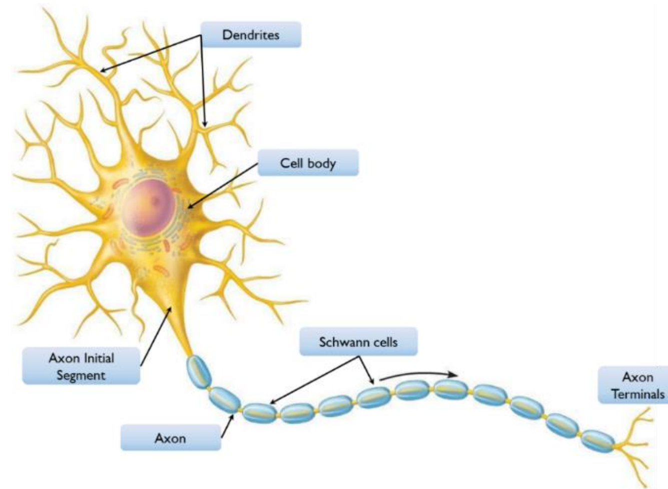


Figure 2 – Representation of the neuronal structure. Adapted from Siegel (2011) and Tortora (2012).

1.2 Neuronal Polarization and Axon Formation

For the development, differentiation and maintenance of tissues and organs cell polarization is required. Neurons are one of the most polarized cell types. This cell has distinct types of compartments, such as the cell body and the processes that arise from it, the axon and the dendrites. Axons are thin and usually long processes, heavily constituted of cytoskeleton proteins such actin, microtubules and neurofilaments. In the axon terminal, there is a synapse, where synaptic vesicles transmit signals to other neurons. Dendrites are short, thick and have neurotransmitter receptors, which get chemical signals from other neurons (Funahashi et al., 2014, Neukirchen and Bradke, 2011b, Neukirchen and Bradke, 2011a, Stiess and Bradke, 2011).

Cytoskeletal dynamics determines cell polarity. This dynamics enables the motility and forward motion of the growth cone, which defines the direction and elongation of the axon (Bradke and Dotti, 1999, Lowery and Van Vactor, 2009, Stiess and Bradke, 2011).

Neuronal polarization is clearly visualized in *in vitro* hippocampal neuron cultures and is composed by three stages. Shortly after plating, the stage 1, the dissociated neurons start forming several thin filopodia, in sites of increased actin dynamics. This increase in actin dynamics is fundamental for microtubules to be able to protrude and start exerting mechanical force against the cell membrane. The transition to the stage 2 occurs when the neurons start to originate multiple immature neurites (minor processes), often very dynamic but short lasting. Stage 3, occurs when one of the growing processes grows to at least twice the size of the others. When this happens, that particular process will become the axon, continuing to grow, whereas the other processes will remain shorter and will give rise to the dendrites. Interestingly, when the longest process is severed, one of the smaller processes takes its place as the axon, reinforcing the idea of a very robust control of axon polarization (figure 3 (a)) (Bradke and Dotti, 1999, Dent and Gertler, 2003, Funahashi et al., 2014, Neukirchen and Bradke, 2011b).

In vivo, neuronal polarization can be divided into two ways: the inheritance of polarity and the establishment of polarity (figure 3 (b) and (c), respectively) (Funahashi et al., 2014).

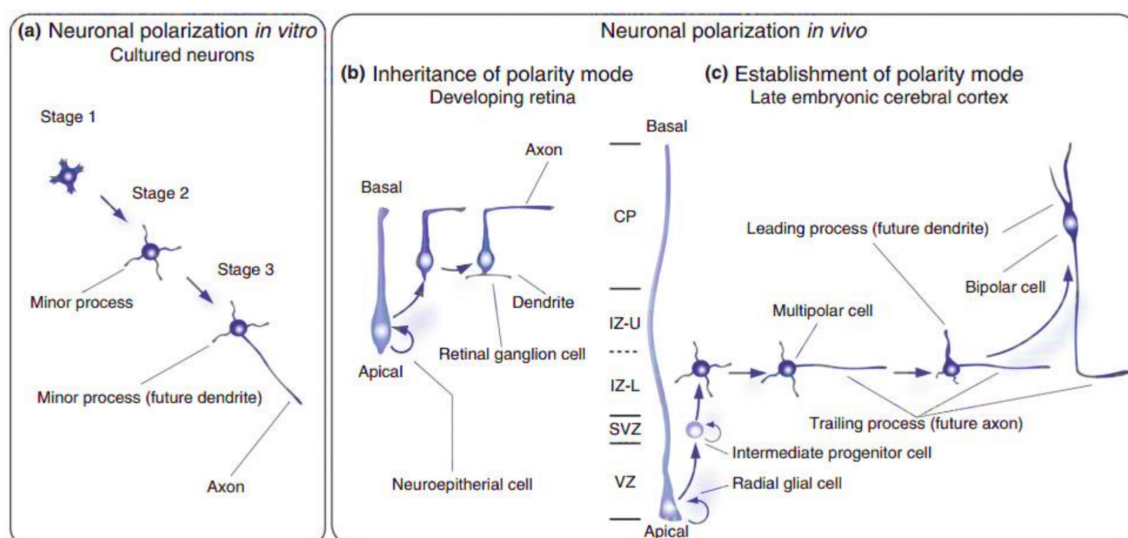


Figure 3 – Neuronal polarization *in vitro* and *in vivo*. (a) The stage 1 neurons create thin filopodia. The stage 2 neurons form multiple minor processes. The stage 3 neurons have only one axon and several minor processes. Therefore, the initial event during neuronal polarization in cultured neurons is axon specification. (b) In retinal ganglion cells, the apical process eventually advances into a dendrite, and the basal process turns into an axon. (c) Most pyramidal neurons are generated from the radial glial cells in the ventricular zone, through intermediate progenitor cells in the subventricular zone. The recently created neurons then extend multiple neurites in the lower part of the intermediate zone. The multipolar cells first elongate the trailing process tangentially and then generate the leading process. Finally, the multipolar cells convert into bipolar cells and migrate near the cortical plate via the upper part of the intermediate zone. The establishment of the trailing process (future axon) is the first phase of neuronal polarization (Funahashi et al., 2014).

Fundamental for these processes to occur is the capacity of the neuron to form a growth cone that will lead growth, until the axon reaches its target. Growth cone formation involves many different aspects, including cytoskeletal restructuring, calcium signalling, axonal transport, local translation of messenger RNAs, and inclusion of new membrane and cell surface molecules (Bradke et al., 2012).

The growth cone is characterized by the elevated density of actin filaments in the peripheral domain, reason why actin is one of the most important proteins in growth cone related processes (Gallo, 2013). The actin cytoskeleton is essential for the cell shape, structure, division, motility, contraction, adhesion, phagocytosis, protein sorting and signal transduction (Revenu et al., 2004, Uribe and Jay, 2009). The main support structures in the growth cone are the lamellipodia that is constituted by meshwork of filaments and the filopodia, which is formed by an aligned bundle of actin filaments (figure 4) (Bradke and Dotti, 1999, Stiess and Bradke, 2011, Bradke et al., 2012, Gallo, 2013).

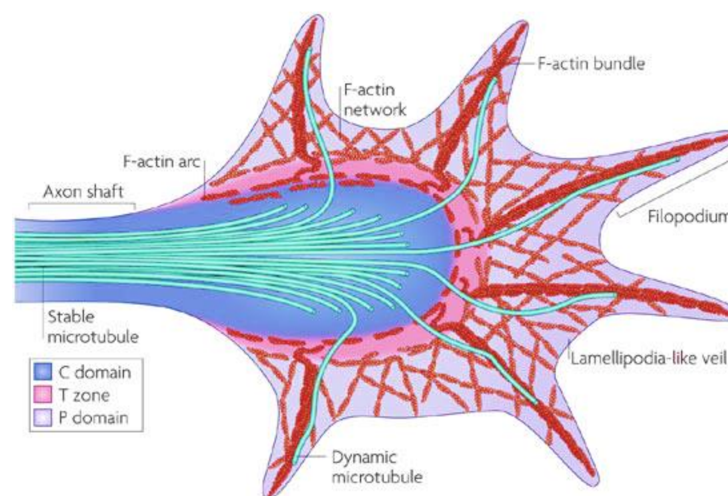


Figure 4 – Growth cone structure. The leading edge comprises dynamic, finger-like filopodia that explore the road ahead, separated by pieces of membrane between the filopodia named lamellipodia-like veils (in the figure). The cytoskeletal components in the growth cone underlie its form, and the growth cone can be divided into three domains based on cytoskeletal distribution. The peripheral (P) domain contains elongated, bundled actin filaments (F-actin bundles), which form the filopodia, as well as mesh-like branched F-actin networks, which give structure to lamellipodia-like veils. Additionally, individual dynamic 'pioneer' microtubules (MTs) explore this region, usually along F-actin bundles. The central (C) domain encloses stable, bundled MTs that enter the growth cone from the axon shaft, in addition to numerous organelles, vesicles and central actin bundles. Finally, the transition (T) zone sits at the interface between the peripheral and central domains, where actomyosin contractile structures (termed actin arcs) lie perpendicular to F-actin bundles and form a hemicircumferential ring. The dynamics of these cytoskeletal components determine growth cone shape, movement on its journey during development and axonal regeneration (Lowery and Van Vactor, 2009).

The regulation of actin dynamics and the directed growth of the growth cone are very important for neuronal processes, such as axonal regeneration (Stiess and Bradke, 2011, Bradke et al., 2012, Gordon-Weeks and Fournier, 2014).

1.3 Axon Regeneration

Regeneration recapitulates in part nervous system development, where the axons need to grow and target a specific tissue. Functional nerve regeneration requires cell survival, axon growth, synapse formation and re-myelination. For axonal regeneration to occur there are two main aspects that are critical for success: the intrinsic growth capacity and an extrinsic permissive environment (Horner and Gage, 2000, Kiryu-Seo and Kiyama, 2011).

The PNS and CNS are critically different in these two aspects: while in the PNS there is efficacy in the removal of regeneration inhibitors and the intrinsic capability of neurons to grow after an injury is high, in the CNS the non-removal of myelin inhibitors, the formation of the chemical/physical barrier that is the glial scar and also the lack of intrinsic capacity to express RAGs preclude successful axon regeneration (Diaz Quiroz and Echeverri, 2013, Mar et al., 2014, Shim and Ming, 2010). CNS neurons respond differently to an injury when compared to PNS neurons (Mar et al., 2014). Whereas in the CNS regeneration is usually abortive, PNS neurons not only regenerate but can also, in some cases, restore the original neuronal functions (Neumann and Woolf, 1999, Silver and Miller, 2004).

Following an injury, the nervous system attempts to regenerate. The first process in order to regeneration to occur is Wallerian degeneration (WD), which in the PNS starts 24-72 hours after nerve injury (Camara-Lemarroy et al., 2012, Conforti et al., 2014, Lingor et al., 2012). WD starts in the distal part of the injured nerve with the Schwann cells becoming dedifferentiated and together with macrophages removing the axonal and myelin debris present in the lesion site. The debrie removal by both Schwann cells and macrophages clears the environment that will support the sprouting of the axon, allowing the protrusion of growth cones at the axons tips, elongating it. During the process, Schwann cells release a diversity of neurotrophic elements to sustain the axonal regrowth and guidance. (Camara-Lemarroy et al., 2012, Prinz and Priller, 2014, Salegio et al., 2011, Shim and Ming, 2010, Sta et al., 2014).

In the CNS, the oligodendrocyte capacity to clear the debris absent. This decreased capacity of clearing the debris and the occurrence of astroglyosis that will lead to the glial scar formation altogether with the lower intrinsic ability for adult CNS neurons to regrow are the main causes for the failure of regeneration in the CNS. On the other hand, the activation of microglia is very important in CNS regeneration (Lingor et al., 2012, Martini et al., 2013). A scheme representing the WD and the regeneration process that follows is presented in figure 5 (Shim and Ming, 2010).

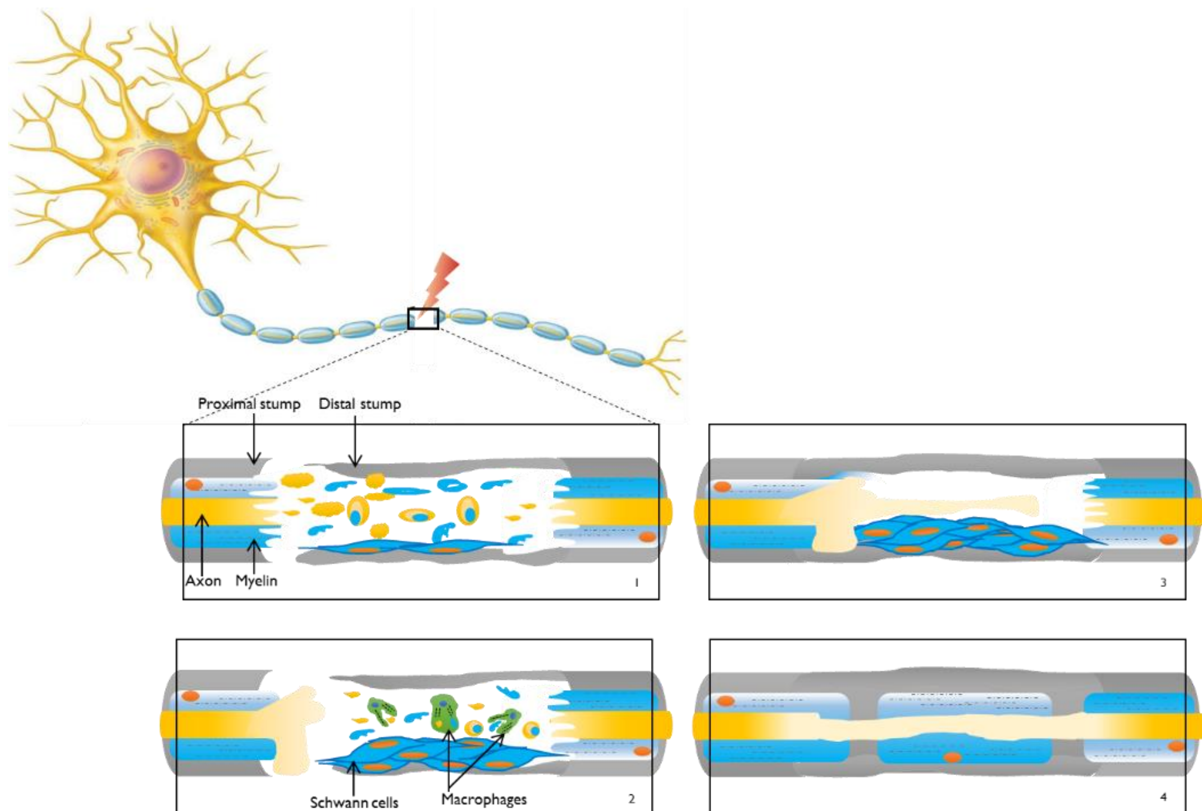


Figure 5 – Representative scheme of Wallerian Degeneration (WD) after PNS injury. After a nerve injury there is the disruption of the axons and the release of myelin debris from the distal stump (1). Dedifferentiated Schwann cells and macrophages then engulf degenerated axons and myelin debris (2). When the removal of debris is completed, axons can then extend processes through the cleared nerve (3). If regrowth is successful, the axon will extend and possibly re-enervate the original tissue, being also re-myelinated by the Schwann cells (4). Adaptation of Shim and Ming, (2010).

1.3.1 The extrinsic factors

When CNS injury occurs, the axons do not have the capacity to regenerate successfully while in the PNS there is a regenerative response that may provide a functional recovery (Dickendesher, 2013).

Most of the CNS particularities that are involved in the impairment of the regeneration capacity are well described: several inhibitory factors, as glial-based inhibitory molecules that are found at the lesion site of the injured CNS, the loss of neurotrophic factors, the incapacity of the oligodendrocytes to dedifferentiate, the macrophage-decreased migration to the lesion site and the aggregation of reactive astrocytes that will lead to the glial scar formation (Brosius Lutz and Barres, 2014, Dill et al., 2008, Hoffman-Kim et al., 2010, Hoffman, 2010, Reina et al., 2013, Silver and Miller, 2004). The mechanical barrier known as glial scar is enriched in molecules such as semaphorins and proteoglycans, namely the chondroitin sulfate proteoglycans (CSPGs), responsible for the decrease in axonal extension and regenerative failure (figure 6) (Barros et al., 2011, Smith et al., 2012, Mironova and Giger, 2013, Raposo and Schwartz, 2014). Besides the incapacity to degrade the myelin debris, oligodendrocytes also do not segregate basal lamina, leading to a decrease in myelination rate during regeneration (Barros et al., 2011, Diaz Quiroz and Echeverri, 2013, Brosius Lutz and Barres, 2014). For these reasons, the axonal regeneration in the CNS is less effective and the recovery is much limited (Smith et al., 2012, Dickendesher, 2013).

Extracellular calcium (Ca^{2+}) is also crucial for the regeneration outcome, entering into the damaged axon and raising axoplasmic calcium levels leading to the degradation of the axonal cytoskeleton, as is well described in *Aplysia* (Ferguson and Son, 2011). In the lesion site, the concentration of calcium is high and its transient increase is fundamental for retrograde signalling, membrane resealing, cytoskeleton rearrangement and formation of a new growth cone (Ferguson and Son, 2011, Rishal and Fainzilber, 2014). The lesion causes an increase in the intracellular concentration of Ca^{2+} that leads to a depolarization response to the cell body through the activation of voltage-dependent sodium channels (Ferguson and Son, 2011, Rishal and Fainzilber, 2014). In *Caenorhabditis elegans* and *Drosophila melanogaster* the calcium transients are also correlated to the regenerative growth, which depends of the dual leucine zipper kinase, a family member of mitogen-activated protein kinase (MAPK) (Abe and Cavalli, 2008, Ferguson and Son, 2011, Rishal and Fainzilber, 2014).

In the PNS an injury leads to numerous dynamic regenerative responses that can lead to a functional recovery (Thuret et al., 2006). In the PNS the main extrinsic factor responsible for the initiation of the regenerative pathway is the Schwann cell that produces various neurotrophic factors to support axonal regeneration (Hoffman-Kim et al., 2010, Bosse, 2012). Schwann cells also secrete a basal lamina in the mature PNS that secretes abundant extracellular matrix components (ECM) and plays an important role in PNS regeneration. This lamina is composed of growth promoting laminin (alpha- and gamma-), type IV collagen, keparan sulfate proteoglycans (KSPGs), and heparan sulfate proteoglycans (HSPGs), which are crucial to myelination (Yiu and He, 2006, Bosse, 2012, Yang and Yang, 2012, Diaz Quiroz and Echeverri, 2013, Mironova and Giger, 2013, Brosius Lutz and Barres, 2014).

Some molecules can stimulate the intrinsic capacity of axons to regenerate such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factors (GDNF) and fibroblast growth factor-2 (FGF-2) (Zhou and Snider, 2006, Abe and Cavalli, 2008, Hannila and Filbin, 2008, Bradke et al., 2012, Smith et al., 2012).

1.3.2 The intrinsic factors

In intrinsic factor that is important for axonal regeneration is the capacity of the injured neurons to express the RAGs necessary to establish a new and competent growth cone (Ferguson and Son, 2011).

When an injury occurs in the PNS, it activates the intrinsic machinery required for axonal growth. Most of these RAGs are transcription factors and growth associated proteins, namely proteins that regulate the axonal cytoskeleton (Yang and Yang, 2012). Some transcription factors that are increased after an injury are Krüppel-like factors (KLFs), Janus kinase and transducer and activator of transcription (JAK-STAT), signal transducer and activator of transcription3 (STAT3), nuclear factor IL-3 (NFIL3), activator protein 1 (AP-1), activating transcription factor 3 (ATF3) and Jun proto-oncogene (c-Jun) (Zhou and Snider, 2006, Abe and Cavalli, 2008, Ferguson and Son, 2011, Kiryu-Seo and Kiyama, 2011, Smith et al., 2012, Yang and Yang, 2012).

The intrinsic ability of neurons to regenerate is also related to the expression of growth suppressor molecules such as phosphatase and tensin homologue (PTEN), glycogen synthase kinase 3 beta (GSK3β) and tuberous sclerosis complex-1 (TSCI) (He, 2010,

Ferguson and Son, 2011, Harvey et al., 2012, Yang and Yang, 2012, Mironova and Giger, 2013, Brosius Lutz and Barres, 2014).

1.3.3 Spinal cord injury as a model to study axonal regeneration

Many CNS regeneration studies are performed using the acute spinal cord injury (SCI) model, which leads to the disruption (partial or total) of the axonal networks between the brain and spinal cord. In most of the cases this leads to the loss of motor and sensory capacity below the lesion site (Dickendesher, 2013, Guo et al., 2010, He, 2010, Yang and Yang, 2012).

New treatment approaches are required to promote axonal regeneration following a SCI. Most of the SCI are caused by fracture or compression in the column, which results in contusion or transection of neuronal tissue. Accordingly, the brutality of the injury may cause partial or total para- or tetraplegia (Dickendesher, 2013, Freeman, 2014, He, 2010, Thuret et al., 2006).

This injury can be divided in primary and secondary damage. The primary damage is caused by the impact that leads the destruction of neuronal network and the following cellular and molecular cascades that can lead to degeneration of neurons and cell death. The secondary damage is caused by the glutamate excitotoxicity and hypoxia occurs in the following minutes of SCI (Dickendesher, 2013).

1.4 The importance of actin dynamics

As referred to above, in neurons, one of the most polarized cell types, the cytoskeleton dynamics is fundamental for the establishment and maintenance of polarity and cargo transport, meaning that it is important for axonal initiation, growth, maintenance and regeneration (Gordon-Weeks and Fournier, 2014, Stiess and Bradke, 2011, Xu et al., 2013). The neuronal cytoskeleton is composed of actin filaments, intermediate filaments, and microtubules (Etienne-Manneville, 2013). The cytoskeletal assembly is a conserved process regulated by a common set of cytoskeletal regulating molecules through different cell types and species (Zhou and Snider, 2006). The actin cytoskeleton plays a crucial role in neuronal polarization, mediating axon formation and the maintenance of cellular structures such as lamellipodia, filipodia, stress fibers and focal adhesions (Etienne-Manneville, 2013). An important characteristic in all these processes is the dynamic transition between monomeric and filamentous actin and the asymmetry of the filament. Actin polymerization occurs preferentially at the fast growing end, the "barbed end", when it is in the ATP-globular-actin state, and depolymerization occurs in the opposite site of the filament, the "pointed end", which is usually in the ADP-bound state (figure 6) (Etienne-Manneville, 2013, Revenu et al., 2004).

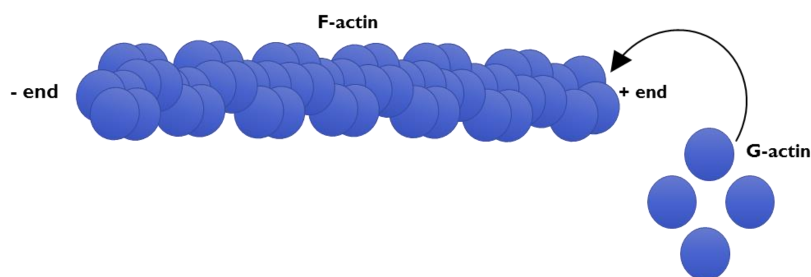


Figure 6 – Structure of actin. The monomers of G-actin are added to the barbed end (+ tip) of filamentous actin (F-actin), while monomers are depolymerized at the pointed end (- tip).

Actin dynamics at the growth cone and the leading edge is crucial for movement, neurogenesis and neurite elongation (Bradke and Dotti, 1999, Stiess and Bradke, 2011). Besides, β-actin has been described to be important to the development and maintenance of the specialized synapse between motor neurons and skeletal muscle, the neuromuscular junction (NMJ) (Cheever et al., 2011).

1.4.1 Actin binding proteins

Actin is ubiquitous to all cells and it has a tightly regulated dynamics. These dynamics are regulated by the intrinsic proprieties of actin, its interaction with the environment and by the presence of a class of proteins, the actin binding proteins (ABPs). ABPs closely interfere with the polymerization/depolymerization rates, severing, capping, nucleation and crosslinking of filamentous actin (Dent and Gertler, 2003, Lee and Dominguez, 2010). Several ABPs have been described in neurons, with critical impairments in neuronal functions when absent, such as ADF/Cofilin (Bradke and Dotti, 1999, Bradke et al., 2012, Kueh et al., 2008), Arp2/3 (Talman et al., 2014), Capping Protein (Fan et al., 2011), Spectrin (Machnicka et al., 2012, Uribe and Jay, 2009) and others. These proteins are required for proper polarization, neurite initiation and extension, synaptogenesis and synapse function, according with ABPs function they can be categorized in proteins that bind and/or sequester actin monomers, nucleate actin filaments, cap the barbed end or anticapping proteins, separate F-actin, and anchor F-actin to membrane association or specific regions of the membrane (Dent and Gertler, 2003).

Recently, a new conformation of the actin cytoskeleton in the axon was suggested, in which actin rings are part of the submembranous cytoskeleton (Xu et al., 2013) (figure 7). In this report, the actin rings were suggested to be spatially distributed along the axon by interaction with the ABPs, spectrin and adducin.

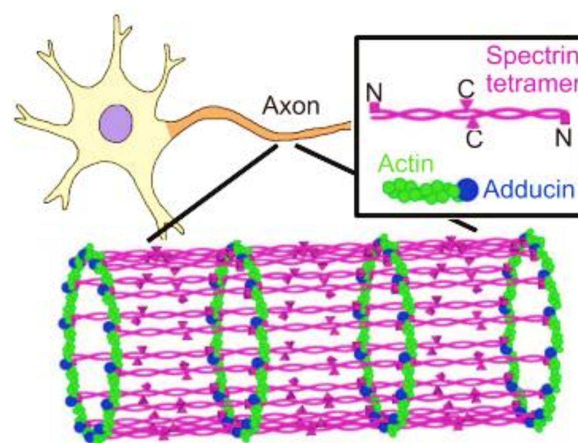


Figure 7 - A model for the cortical cytoskeleton in axons. Short actin filaments (green), capped by adducin (blue) at one end, form ring-like structures wrapping around the circumference of the axon. Spectrin tetramers (magenta) connect the adjacent actin/adducin rings along the axon, creating a quasi-1D lattice structure with a periodicity of ~180 to 190 nm. The letters “C” and “N” denote the C terminus (magenta triangles) and N terminus (magenta squares). Ankyrin and sodium channels, not shown in the model, also form semiperiodic patterns in coordination with the periodic cytoskeletal structure (Xu et al., 2013).

1.4.2 Adducin

Adducins (ADD) are a family of proteins that are part of the spectrin-based membrane cytoskeleton. This family is composed by three genes: ADD1, ADD2 and ADD3, that encode, α, β and γ adducin, respectively. These isoforms form dimers and tetramers α and β, or, α and γ. The α- and γ-adducin are ubiquitous in most tissues and the β-adducin is present in erythrocytes and brain (Bednarek and Caroni, 2011, Chan et al., 2014, Chen et al., 2007, Fukata et al., 1999, Matsuoka et al., 2000, Pielage et al., 2011, Robledo et al., 2008, Rotzer et al., 2014, Stevens and Littleton, 2011, Vukojevic et al., 2012, Wang et al., 2002). Adducins are important to the cellular morphology, motility and the regulation of ion membrane transport (Wang et al., 2002).

Adducins are composed by a head, a neck and a tail domain (figure 8). Between the neck and the tail are the phosphorylation sites recognized by AMP-dependent protein kinase (PKA) and Rho-kinase regulation. The tail contains the myristoylated alanine-rich C-kinase substrate (MARCKS) domain that assists in the link to protein kinase C (PKC), PKA and the binding of calcium-calmodulin (Ca^{2+}-calmodulin) (Babic and Zinsmaier, 2011, Bednarek and Caroni, 2011, Chan et al., 2014, Larsson, 2006, Matsuoka et al., 2000, Miyauchi et al., 2013, Rotzer et al., 2014, Stevens and Littleton, 2011, Vukojevic et al., 2012).

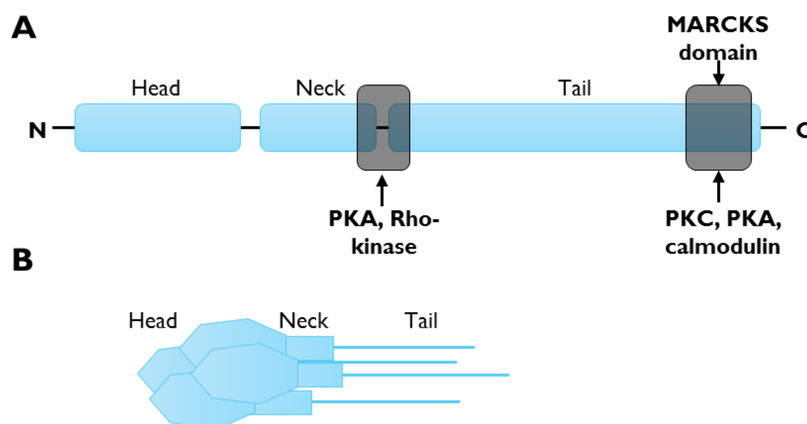


Figure 8 – Adducin structure and regulation. In A is represented the adducin monomer and it's regulation zones. In B, is the adducin tetramer; this is the regular conformation of this protein. Adapted from Miyauchi et al., (2013), Robledo et al., (2008) Stevens and Littleton, (2011), Vukojevic et al., (2012).

In vertebrates, the activity of adducin is regulated by PKC and PKA, which block the interactions with the actin cytoskeleton. On the other hand, Rho-associated kinase (ROCK) improves these interactions (Fukata et al., 1999, Matsuoka et al., 2000, Vukojevic et al., 2012).

Adducin has two main functions in the actin cytoskeleton: the capping activity towards the barbed end of F-actin, inhibiting the addition of G-actin monomers and the recruitment of spectrin to F-actin (Stevens and Littleton, 2011). This activity is critically regulated by PKC: when active PKC phosphorylates a C-terminal serine residue from adducin, it decreases its affinity to F-actin, releasing it from the barbed ends (figure 9).

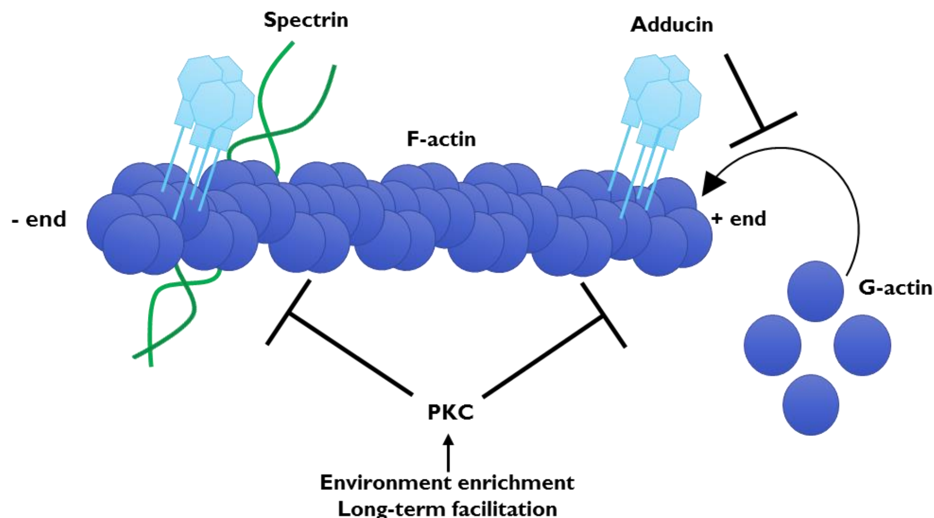


Figure 9 – Adducin activity towards F-actin is regulated by PKC phosphorylation. Upon phosphorylation, adducin is released from the + tip of the filaments, allowing increased actin dynamics to occur. Adapted from Stevens and Littleton (2011).

The adducin KO mice have been a useful resource to study the role of adducin and its different forms. The alpha KO mice is a complete KO for adducin, since no beta/gamma dimers are known to occur. These mice develop haemolytic anaemia and in 50% of the knockout animals the development of hydrocephaly leads to premature death (Robledo et al., 2008, Robledo et al., 2012).

Recently, adducin has been implicated in neuron biology in the adducin knockout fly (the adducin orthologue, hu-li tui shao, HTS) (Pielage et al., 2011) and in mice (Bednarek and Caroni, 2011). Synaptic plasticity is closely related to actin cytoskeleton remodelling, which is required for synapse formation and retraction. In these studies, adducin and its phosphorylation status (mediated by PKC) revealed to be crucial to the correct assembly of synapses. Work with knockdown of the adducin form of *C. elegans* also revealed problems in memory, that were associated with SNPs that also contribute for memory deficits in humans.

In summary, although actin dynamics is well recognized as a key player in axonal growth, how different ABPs control their process is still not fully understood.

1.5 Preliminary data

Dorsal root ganglia (DRG) neurons are a class of sensory neurons, which have the particular characteristic of having a peripheral and a central branch, the latest of which enters in the spinal cord. From these DRG neurons, some establish connections with neurons in the spinal cord and some extend their central branch through the brain, as dorsal column fibers (Silver, 2009). As DRG neurons have a peripheral and a central branch, they have been widely used for studies of regeneration, given the possibility of evaluating differences in neuronal behaviour depending on the CNS and PNS environment. DRG neurons react differently to a peripheral and central injury (Silver, 2009). When a peripheral axon is injured, Wallerian degeneration is followed by regeneration, contrarily to what happens following an injury to the central axon where no regeneration occurs. Interestingly, when injury to the central axon is preceded by a peripheral injury, the central axon gains regenerative capacity and grows beyond the lesion site (Neumann and Woolf, 1999). The priming peripheral injury, known as conditioning lesion (CL) (figure 10), also enhances neurite growth *in vitro*, even in an inhibitory environment such as in the presence of myelin (Silver, 2009). The enhanced neurite outgrowth following a peripheral injury is probably related to the increased expression of RAGs at the time when the second lesion is performed. The nature of these post-lesion signals is not known, although some of these have already been described (Benowitz and Routtenberg, 1997, Caroni, 1997a, Caroni, 1997b, Caroni, 2001, Silver, 2009, Skene, 1989).

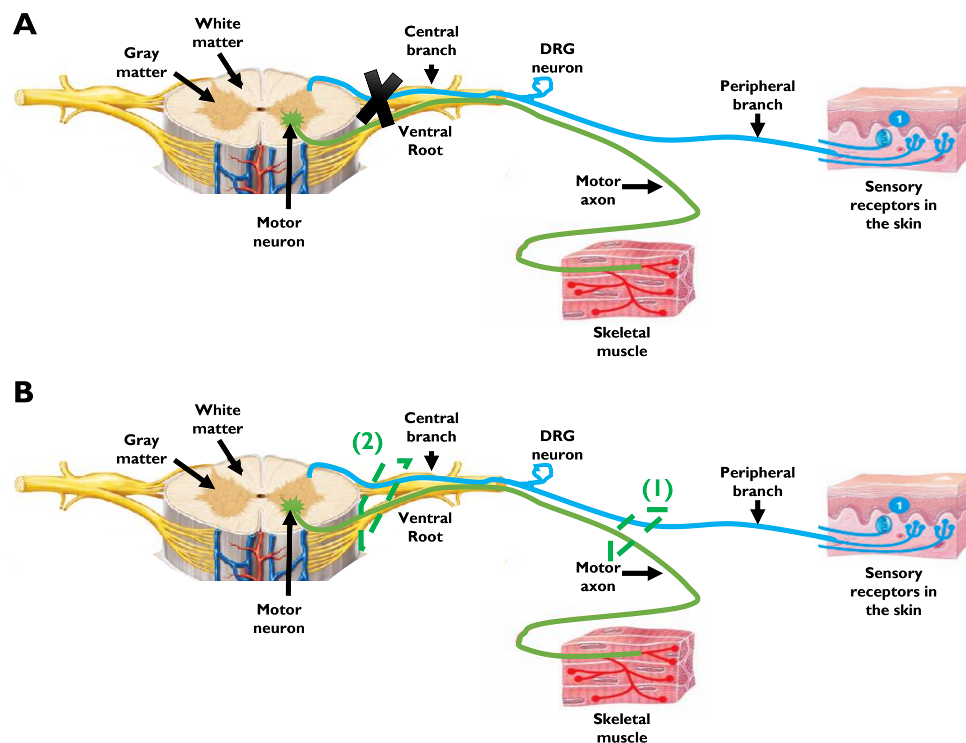


Figure 10 – The conditioning lesion. Primary sensory neurons inside DRG are particularly useful to study axonal regeneration. DRG neurons are unique in having two axonal branches; a long sensory CNS branch ascends the dorsal column in the spinal cord and a second branch projects through a peripheral nerve. Sensory axons in the adult spinal cord do not regenerate after injury (A), while peripheral injury result in a robust regenerative response. Regeneration of the central branch can be greatly enhanced by a prior injury to the peripheral branch, referred to as a "conditioning injury" (B). The conditioning injury suggests that distinct signalling mechanisms control responses to central vs. peripheral injury in DRG neurons and may contribute to their different abilities to axonal regrowth. Adapted from Abe and Cavalli (2008) and Tortora (2012).

In order to further understand the mechanism by which the CL enhances the growth capacity of the DRGs, a high through output proteomic analysis conducted in our group to identify the elements differently expressed/regulated in conditioned neurons, revealed the differential regulation of several cytoskeleton-related proteins. This analysis was performed in DRG from rats with spinal cord injury or a spinal cord injury preceded by a sciatic nerve transection (conditioning lesion). For proteomics two independent approaches were use, iTRAQ (isobaric Tag for Relative and Absolute Quantification) (Ross et al., 2004) and Kinexus phospho-site antibody array (www.kinexus.ca). From this comparison we found that several ABPs were differently regulated in the CL, namely spectrin and adducin, both alpha and gamma forms. The alpha and gamma adducin were found to have increased phosphorylation in the C-terminus serine residue (2,5 and 4,3 fold, respectively). This increase was then validated by ICC of conditioned DRGs and western blot analysis of spinal cord lesion sites of conditioned and non-conditioned animals.

2. Objectives

2. Objectives

In this work we aimed at:

- Understanding how adducin regulation can influence axonal regeneration after lesion in the central nervous system.
- Characterize how the absence adducin of influences axon biology namely:
    - Neuronal plasticity, more specifically formation of the axon initial segment.
    - Synapse formation, using the neuromuscular junctions as a study model.

3. Methodology

3. Methodology

3.1 Animals

Mice were handled according to European Union and National rules, maintained under a 12h light/dark cycle and fed with regular rodent's chow and tap water ad libitum. α-adducin KO mice were in a black6-129 background (Robledo et al., 2008) and were maintained by breeding heterozygous animals. Animals were euthanized with excessive dose of anesthetic (ketamine+medetomidine) or high concentration of isoflurane.

3.2 Cell culture

3.2.1 Culture of hippocampal neurons

For hippocampal neuron cultures E17.5 mouse embryos were used. Embryos were removed by C-section and placed in Hank's Balanced Salt Solution (HBSS). After the isolation, HBSS was removed and washed 2-3 times with fresh and sterile HBSS. The tissue was digested in 0,06% trypsin solution diluted in HBSS for 15 minutes at 37°C, agitated each 5 minutes. Trypsin was then removed and HBSS with 10% FBS was added to stop the reaction. After 2-3 washes with HBSS, the hippocampi were incubated in Neurobasal medium (NB) and dissociated using a 1mL micropipette followed by a 0,2mL micropipette and passed through a 70µm strainer. Cells were counted in a Neubauer chamber and then plated in NB supplemented with 2% B27 + 2mM L-glutamine + 1% penicilin/streptomycin. For axon initial segment assessment cells were plated at a density of 50,000 cells/well in a 24-well plate, in coverslips coated with 20µM Poly-l-lysine. At the 10th day *in vitro* (DIV) cells were incubated in complete medium either with 10mM NaCl or 10mM KCl solution for 2 days and were fixed in 2% paraformaldehyde (PFA) for 20 minutes.

3.2.2 Culture of DRG neurons

DRG cultures were performed as described. Briefly, DRGs were collected (with removal of the roots) to DMEM:F12 medium supplemented with 10% fetal calf serum. DRGs were treated with 0,125% collagenase (Sigma) for 90 minutes at 37°C. DRGs were then triturated to a single cell suspension using a sequence of lower diameter Pasteur pipettes. The cell

suspension was centrifuged in a 15% albumin serum fraction V (BSA) gradient for 10 minutes at 1000xg. The pellet obtained was then ressuspended in a medium containing DMEM:F12, B27, penicillin/streptomicin, L-glutamine, 50ng/µL of NGF, and the total number of cells was counted in a Newbauer chamber. Cells were plated in coverslips, previously treated with Poly-L-Lysine hydrobromide (PLL) (20µM) and laminin (10µg/ml). Cells were growth for 12 hours and then fixed in 2% PFA for 20 minutes.

3.3 Immunocytochemistry

3.3.1 Immunocytochemistry for detection of the axon initial segment (AIS)

Hippocampal neurons were washed in phosphate buffered saline (PBS) and incubated for 5 minutes in 0,2% Triton-X100 (in PBS) followed for 5 minutes incubation with 0,1% sodium borohydrade (in tris-EDTA (TE)). Cells were then blocked for 1 hour at 37ºC in 5% donkey serum (in PBS). Cells were incubated with primary antibodies against beta-III tubulin (Promega G7121, 1:600) and ankyrin G (Calbiochem NB20, 1:500) diluted in 5% donkey serum (in PBS) overnight at 4ºC. Cells were then washed in PBS and incubated with secondary antibody anti-mouse Alexa Fluor 488 (Invitrogen A21202, 1:500) and anti-rabbit Alexa Fluor 568 (Invitrogen A10042, 1:500) in 5% donkey serum (in PBS) for 1 hour at room temperature. Cells were then washed in PBS and mounted in fluoroshield with DAPI (Sigma-Aldrich F6057-20ML) for nuclear staining. For the AIS study we used a mixed sex population with 4 WT and 2 KO animals. For quantification, we utilized FIJI software, *is Just ImageJ* plugin.

3.3.2 DRG cultures for immunocytochemistry and neurite tracing

After fixation cells were washed in PBS and incubated for 5 minutes in 0,2% Triton-X100 (in PBS) followed for 5 minutes incubation with 0,1% sodium borohydrade (in TE). Cells were then blocked for 1 hour at 37ºC in 5% donkey serum (in PBS). Cells were incubated with primary antibody against beta-III tubulin (Epitomics 1967-1, 1:1000) diluted in 5% donkey serum (in PBS) overnight at 4ºC. Cells were then washed in PBS and incubated with secondary antibody anti-mouse Alexa Fluor 488 (Invitrogen A21202, 1:1000) in 5% donkey serum (in PBS) for 1 hour at room temperature. Cells were then washed in PBS and mounted in fluoroshield with DAPI (Sigma-Aldrich F6057-20ML) for nuclear staining. For

tracing we used a mixed sex population with 3 WT and 3 KO animals. For software quantification, we utilized the FIJI software, *is Just ImageJ* plugin (Meijering et al., 2004).

3.4 Analysis of neuromuscular junctions

Following the collection of the diaphragm, the muscle was washed with PBS for 5 minutes. The tissue was incubated overnight at 4°C with 0,1M glycine (in PBS), for 5 minutes. The tissue was washed again with PBS, and incubate with 0,2% Triton X-100 for 10 minutes. After a second wash, incubation with 0,1% Na Borohydrade (in TE) for 5 minutes was performed. After washing in PBS, the diaphragms were incubated for 1 hour in bungarotoxin (BTX) (Invitrogen T1157, 1:5000) in blocking buffer (1mg/ml BSA in 0,2% Triton X-100 in PBS) and washed again with PBS. The muscles were stored at 4°C in eppendorfs with 0,1% azyde-PBS. In this analysis we used a mixed sex population, at postnatal day 15 (P15), 3 WT and 4 KO, at P30, 5 WT and 5 KO and at P100, 6 WT and 5 KO animals. For quantification, we utilized the AxioVision LE and Huygens Professional software.

3.4.1 Analysis of neuromuscular junctions in pre- and postsynaptic structures

The *gastrocnemius*, *tibialis* and *femuralis* muscles were collected and washed with PBS. The muscles were incubated with 0,5% Triton X-100 (in PBS), for 30 minutes, washed with PBS for 5 minutes, incubated with 0,2M NH_4Cl for half hour and washed again with PBS. The muscles were blocked with blocking buffer (1mg/ml BSA in 0,1% Triton X-100 in PBS) for 1 hour at 37°C and incubated with the primary antibodies, rabbit anti-beta III tubulin (Epitomics 1967-1, 1:250) and anti-synaptophysin (Abcam ab68851, 1:250) in blocking buffer for 72 hours, at 4°C. After washing with PBS, the tissues were incubated in the secondary antibodies 1 hour at RT. The secondary antibodies were anti-rabbit Alexa Fluor 488 (Invitrogen A21206, 1:250), anti-mouse Alexa Fluor 488 (Invitrogen A21202, 1:250) and BTX (Invitrogen T1157, 1:5000) in blocking buffer. After washing 3 times with PBS, the muscle fibers were teased with the support of needles to separate fiber bundles, which were mounted with fluoroshield with DAPI (Sigma-Aldrich F6057-20ML). The muscles were stored at 4°C in eppendorfs with 0,1% azyde-PBS. For this analysis we used a mixed

sex population, with 5 WT and 6 KO animals. For quantification, we used the Huygens Professional and FIJI software.

3.5 Lesions

3.5.1 Spinal cord injury

α-Adducin KO mice (n=19) and WT littermates (n=16) (10-12 weeks old), were anesthetized with ketamine/medetomidine. The fur was shaved and the spinal cord was exposed at the thoracic level. Laminectomy was performed at the T6-T8 level with a microscissor. The injury performed was either a dorsal hemisection or a complete transection, using an ophthalmologic scalpel. The animals received analgesia (Butorphanol) twice a day, for 72 hours and fluid therapy once a day (5ml of Duphalyte), for 72 hours. Animals also required manual voiding of the bladder twice a day for the rest of the experimental time. Wet food was placed in the cage floor and water with antibiotic (0,016% Baytril) was supplied in long nipple bottles.

3.5.2 Evaluation of the glial scar

α-Adducin KO mice (n=3) and WT littermates (n=3) (10-12 weeks old) with a complete spinal cord transection, were anesthetized with ketamine/medetomidine and perfused with saline and thereafter in 4% PFA and post-fixed overnight in 4% PFA. Then, spinal cords were incubated overnight in 30% sucrose in PBS and incubated in optimum cutting temperature (OCT) and 10µm sections were obtained in the cryostat and mounted in Superfrost Plus treated slides. The spinal cord slices were hydrated in decreasing alcohol concentrations, 100%, 100%, 96%, 90%, 80% and 70%, for 5 minutes each. The endogenous peroxidase was quenched with 3% H_2O_2 in methanol, 30 minutes at room temperature. The slices were washed 2 times with PBS for 5 minutes and activity was blocked with blocking buffer (10% of fetal bovine serum (FBS) in 0,3% Triton X-100 (in PBS)) for 1 hour at room temperature. The slides were incubated with primary antibodies in blocking buffer, overnight at 4ºC. Primary antibodies were rabbit anti-GFAP (DAKO Z0334, 1:500) and mouse anti-CSPG (Sigma C8035, 1:200). The slices were washed 3 times with PBS for 5 minutes, with agitation, and then incubated with secondary antibodies in blocking buffer for 30 minutes at room temperature. The secondary antibodies were anti-mouse biotinylated

IgG (Vector BA-2000, 1:200) and anti-rabbit biotinylated IgG (Vector BA-1100, 1:200). After washing 3 times with PBS, 5 minutes each, the slices were incubated with ABC reagent (Streptavidin-HRP), 30 minutes at room temperature and developed with 3, 3'-diaminobenzidine (DAB) (Vector, DakoCytomation K3464). When the brown color appeared, reaction was stopped by putting the slides in H_2O for 5 minutes. After washing with water, the slices were dehydrated in alcohols and mounted with mounting medium for microscopy DPX (VWR 1.01979.0500). For analysis of immunostaining, we used Photoshop software.

3.5.3 Analysis of regeneration of dorsal column fibers

For this analysis of regeneration of dorsal column fibers, sciatic nerves were injected with cholera toxin b (CTB) 2µl 1% CTB 3 weeks and half after spinal cord injury. Three days later (4 weeks after the original spinal cord injury) animals were sacrificed and a transcardial perfusion was performed with 4% PFA to fix the animal tissues. Post-fixation (immersion) in 4% PFA was done for 72 hours. The slices were placed at -20°C in a 30% sucrose solution until sectioning and washed in 0,1M phosphate buffer (PB) and embedded with OCT. In the cryostat 50 µm spinal cord sections were done. Slices were washed for 10 minutes with 0,1M PB, 40 minutes with 50% ethanol, 2 times for 10 minutes with 0,1M PB, 1 hour with 0,6% H_2O_2 (in 0,1M PB), 2 times for 10 minutes with 0,1M PB. The slices were incubated for 1 hour in blocking solution (5% donkey serum + 0,3% Triton X-100 in 0,1M PB) at room temperature and overnight at 4°C with goat anti-CTB (List Biological Laboratories Inc 703, 1:30000) in blocking solution and put 2 hours in anti-goat biotinylated IgG (Vector BA-5000, 1:200) in blocking buffer. After washing 3 times for 10 minutes with 0,1% Triton X-100 TBS (in 0,1% triton), slices were incubated with extravidin peroxidase (in Triton X-100 TBS) (Sigma E2886, 1:1000) 1 hour at room temperature. To develop DAB (5mg / 10mL TB) with1µL of H_2O_2 (/1 mL DAB) was used. Slices were mounted mounted in chrome-potassium sulphate gelatin solution and let to air-dry overnight. The slices were then incubated for 5 minutes with toluidine blue, washed 1 minute with 96% ethanol, 1 minute with absolute ethanol, 2 times for 5 minutes with xylol and coverslipped with DPX (VWR 1.01979.0500). For this analysis we used a mixed sex population, with 10 WT and 8 KO animals. For image analysis, we used Photoshop software.

3.5.4 Analysis of regeneration of serotonergic axons

Animals were transcardial perfused with 4% PFA. Post fixation (immersion) in 4% PFA was performed for 2 hours. Spinal cords were dissected and incubated in 30% sucrose in PBS, overnight at 4°C, washed with 0,1M PB and embedded in OCT. Cryostat sections of the spinal cords were done. Sections were washed for 10 minutes with 0,1M PB, 40 minutes with 50% ethanol, 2 times for 10 minutes with 0,1M PB and treated for 1 hour with 0,6% H_2O_2 (in 0,1M PB). Slices were incubated for 1 hour in blocking solution (10% FBS + 0,3% Triton X-100 in 0,1M PB) at room temperature and with rabbit anti-5-HT (Immunostar 20080, 1:20000) in blocking buffer, overnight at 4ºC. After washing 3 times for 10 minutes with 0,1M PB, slices were incubated for 2 hours with anti-rabbit biotinylated IgG (Vector BA 1100, 1:200) in blocking buffer. After washing 3 times for 10 minutes with Triton X-100 TBS (0,1% triton), slices were incubated with Extravidin Peroxidase (in Triton X-100 TBS) (Sigma E2886, 1:1000), for 1 hour at room temperature. The development was performed with DAB. Slices were mounted in chrome-potassium sulphate gelatin solution and let to air-dry overnight. For this analysis we used a mixed sex population, with 6 WT and 5 KO animals. For image analysis, we used the Photoshop software.

4. Results and Discussion

4. Results and discussion

4.1 Analysis of neurite outgrowth of α-adducin KO DRG neurons

To evaluate if DRG neurons from α-adducin KO mice have abnormal axon formation and growth, we analysed their neurite outgrowth *in vitro*. In our measurements, we did not see any significant differences in neurite outgrowth of α-adducin KO and WT neurons, in the three parameters analysed, which were, number of segments, total neurite length and length of the longest neurite (figure 11).

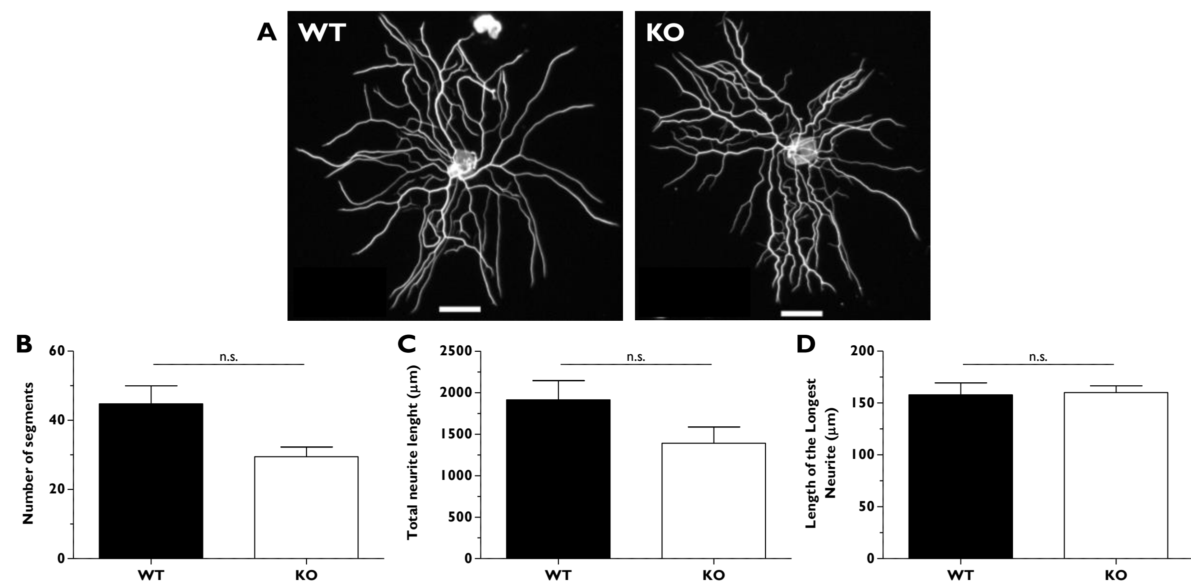


Figure 11– Quantification of neurite outgrowth of DRG neurons from WT and α-adducin KO animals. A, representative images of a DRG neuron of a WT animal and a DRG neuron of α-adducin KO mouse. Neurite outgrowth analysis. Number of segments (B), total neurite length (C), and length of longest neurite (D) in the two groups. Scale bar, 50 µm.

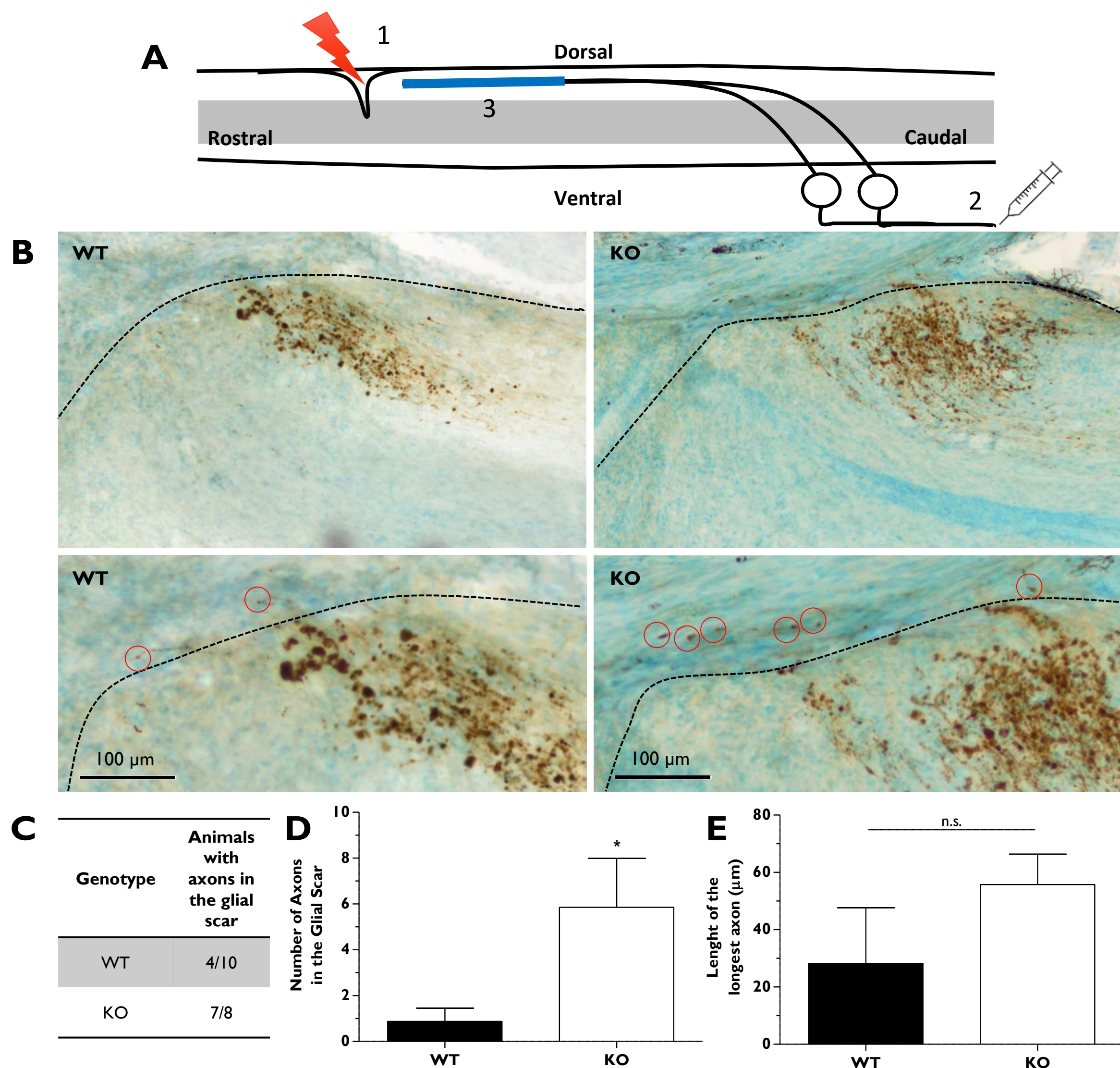
Our hypothesis was that in the absence of adducin, the axonal cytoskeleton would be more dynamic probably enabling an increased growth. However, this hypothesis was not validated.

The substrate is very important for axonal growth. According to the substrate used, the culture can have more or less adherence that needs to be surpassed such that neurite growth occurs. Future experiments should address the study of different substrates to better understand whether the lack of adducin has consequence for axon growth.

4.2 Analysis of axon regeneration following spinal cord injury

4.2.1 Regeneration of sensory axons (dorsal column tract)

In α-adducin KO animals the number of sensory axons, capable of entrying the lesion site was increased (figure 12 C e D). However, axonal regeneration of sensory axons was similar in the two groups (figure 12 E).



Genotype	Animals with axons in the glial scar
WT	4/10
KO	7/8

Figure 12 – Regeneration of dorsal column axons in α-adducin KO mice. A, representation of the dorsal column hemisection model with tracing of dorsal column axons with cholera toxin b. Briefly, animals were subjected to a spinal cord injury hemisection (1) and let to recovery for 4 weeks. Four days prior to sacrifice, injection of CTB in the sciatic nerve was performed (2) immunohistochemistry against CTB was done (3). B, representative images of the spinal cord injury site in WT and KO animals. Red circles highlight the axons that displayed axons capable of passing through the glial scar border. C, number of animals genotype that can pass the glial scar border. D, number of axons present in the glial scar in of each genotype (p=0,03). E, average length of the longest axons within the glial scar in each genotype.

This increase appears to be related to the ability of sensory axons be less inhibited by glial scar components (myelin or CSPG, among others), which may lead to an increased growth.

This effect can be associated with our observations in the conditioning lesion paradigm, wherein, in the presence of inhibitory molecules, the regeneration potential is raised and the activity of adducin is decreased (as suggested by the increased phosphorylation levels). During conditioning lesion several players that modulate adducin activity are increased such as molecules that increase the levels of phospho-adducin namely PKC (Fukata et al., 1999, George et al., 2010, Stevens and Littleton, 2011).

4.2.2 Analysis of regeneration of serotonergic axons

To analyze the descending serotonergic tract we used immunolabeling after a complete lesion (Tuszynski and Steward, 2012). In this specific tract (figure 13 A), no significant differences between groups were observed (figure 13 B). This results lead us to raise the hypothesis that the absence of adducin can have different effects on axonal regeneration of distinct CNS tracts.

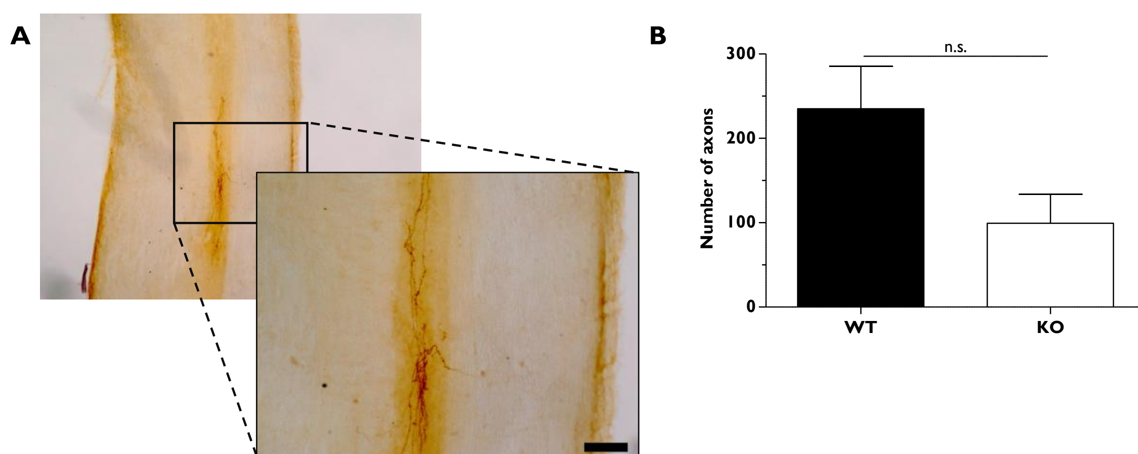


Figure 13 – Evaluation of regeneration of serotonergic axons. A, representation of 5-HT positive regenerating axons. B, analysis of the number of serotonergic axons regenerating in the spinal cord of WT and α-adducin KO mice. Scale bar, 100 μm.

4.2.3 Analysis of the glial scar

As the glial cells in α-adducin KO mice are also targeted, for a complete understanding on how the absence of adducin can modulate axonal regeneration, the following step was to evaluate the glial scar structure. For this we analysed the glial scar area using the GFAP staining (figure 14 A) and CSPG staining (figure 14 B). While there was a trend for a decrease in the area of the glial scar in the α-adducin KO mice as evaluated by GFAP and CSPG staining, the difference was not significant (figure 14 C).

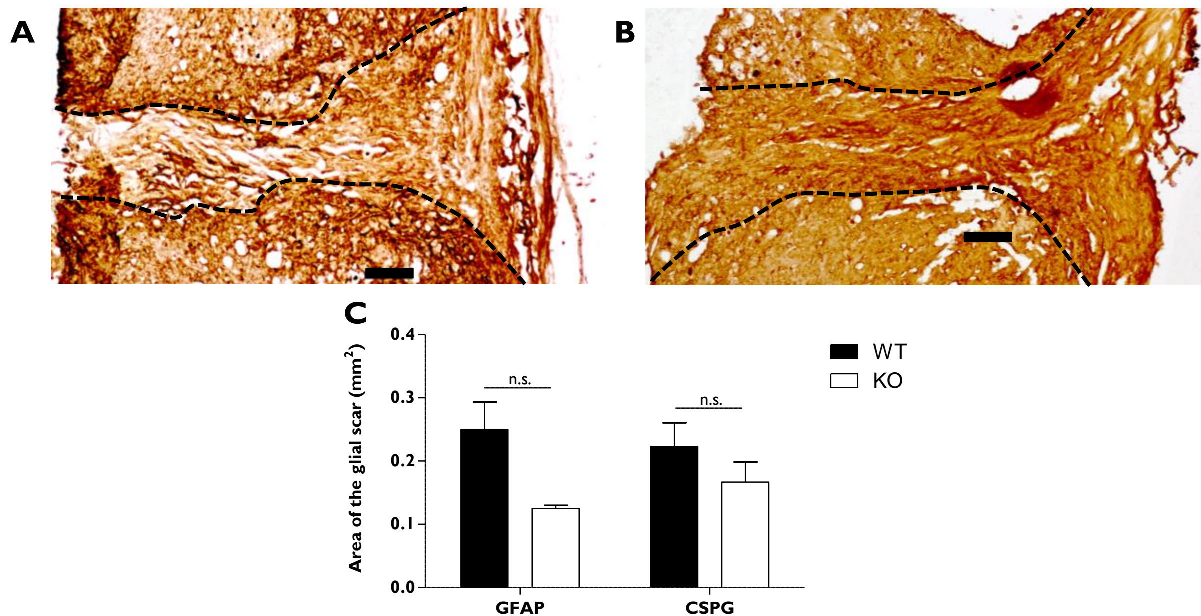


Figure 14 – Evaluation of the glial scar structure. A, representative glial scar image with GFAP staining in the borders of the lesion. B, representative glial scar with CSPG labelling inside the lesion site. C, quantification of the glial scar area using either the area excluded by GFAP staining or the area with CSPG staining in α-adducin KO and WT littermates. Scale bar, 100 μm.

Given the trend for a reduced glial scar area in α-adducin KO mice, one can raise the possibility that events such as the proliferation and/or migration of astrocytes might be affected in this paradigm. Future studies should investigate this possibility.

In summary, our results suggest that at least for axons of the sensory tract, the absence of adducin is related to a decreased inhibition by components of the glial scar. This data goes along our observation in the conditioning lesion model and should be reinforced by additional experiments evaluating the growth and WT and α-adducin KO DRG neurons in inhibitory substrates.

4.3 Adducin in neuronal polarization and synapse formation

4.3.1 Axon Initial Segment

The axon initial segment (AIS) is the structure responsible for the initiation of the action potential and the maintenance of neuronal polarity (Adachi et al., 2014, Xu and Shrager, 2005, Yoshimura and Rasband, 2014). The AIS is highly enriched in actin, ankyrin G (Jones et al., 2014, Watanabe et al., 2012, Xu and Shrager, 2005, Yoshimura and Rasband, 2014) and spectrin (Yoshimura and Rasband, 2014). The chronic depolarization of neurons leads to the distal relocation of the AIS (figure 15 A) (Adachi et al., 2014, Yoshimura and Rasband, 2014).

Given the importance of adducin in regulating the actin cytoskeleton, we analyzed the formation and relocation of the AIS in WT and α-adducin KO mice (figure 15).

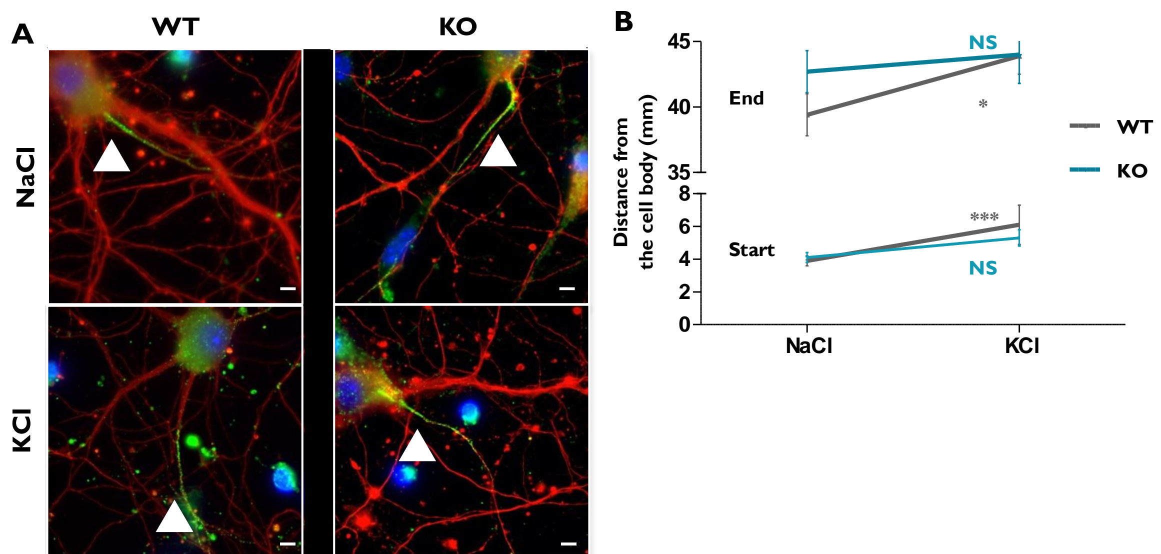


Figure 15 – Evaluation of axon initial segment dynamics. A, representative images of hippocampal cultures and AIS dynamics (white arrow) in neurons grown with NaCl or with a depolarizing solution (KCl). When the depolarization occurs, the AIS moves. This plasticity does not occur in the α-adducin KO animals contrarily to what happens in WT littermates. Phalloidin staining (red), ankyrin-G (green) and DAPI (blue). B, quantification of the distance from the cell body to the start and end of the AIS. Scale bar, 5 µm.

In α-adducin KO animals, although the AIS was formed normally, it did not move after chronic depolarization, while in the WT littermates the AIS was relocated directly. In sum, the α-adducin KO animals were incapable to relocate the AIS after chronic axonal depolarization with KCl (figure 15 B). The specific role of adducin in the plasticity of the AIS should be further investigated.

The diffusion barrier created with the AIS, is dependent on the dynamic filaments of actin. Through the AIS development, the levels of ankyrin G and actin are increased (Jones et al., 2014, Xu and Shrager, 2005, Yoshimura and Rasband, 2014), so the loss of adducin can lead to an impairment in the formation and relocation of the AIS.

Our results show that the absence of α-adducin is not important for the AIS formation but affects its relocation, in contrast to what happen with spectrin (Xu et al., 2013).

4.3.2 Neuromuscular junction (NMJ) formation

Regulation of adducin's activity has already been shown to be important for the stability of synaptic structures, learning and memory (Babic and Zinsmaier, 2011, Bednarek and Caroni, 2011, Stevens and Littleton, 2011). Adducin specially β-adducin, is important for the synaptogenesis processes in adulthood. Bednarek and Caroni, observed that the synapse disassembly is promoted by the phosphorylation with PKC in as mice environment enriched (Bednarek and Caroni, 2011). The β-adducin KO mice had deficits in behavior, motor coordination and learning (Porro et al., 2010), as the absence of β-adducin affected synaptic plasticity (Bednarek and Caroni, 2011).

Besides, in *Drosophila melanogaster*, it was observed that Hts, the adducin ortologue, is an important player in the mechanisms that control both the stability and growth of the NMJ. The Hts knockdown effect leads to increases the NMJ size, with increase filopodia and increase rate of elimination of the NMJs (Pielage et al., 2011). Moreover, the PKC regulated adducin protein, is important for the metabolism and contraction of muscle fibers. Of note, the phosphorylation by PKC can regulate the neurotransmitter release in synapses (Lanuza et al., 2014). Given these findings, it was interesting to analyse in the α-adducin KO mice, if the formation and maturation of the NMJ pattern was affected.

The innervation pattern and number in α-adducin KO and WT animals was identical at postnatal day 30 (P30) and 100 (P100) (data not shown). However, after a detailed analysis of the postsynaptic region using BTX staining in P15, P30 and P100 animals, differences were observed (figure 16). In the postsynaptic structure, we analysed four parameters: fragmentation, volume, area and length of each NMJ. Although at P15, no significant differences were observed, at P30 α-adducin KO mice had a decreased complexicity, as assessed by measurement of fragmentation and length. At P100 although the differences in

fragmentation were not seen, α-adducin KO mice had a trend for a decreased volume, area and length.

With this evaluation, we noted that the α-adducin KO NMJ appears to be more immature throughout development than that of WT littermates but that this effect is normalized in the adult. As such our findings for the role of adducin in NMJ do not recapitulate the findings in synaptic structures of the CNS (Babic and Zinsmaier, 2011, Bednarek and Caroni, 2011) neither in the *Drosophila* NMJ (Pielage et al., 2011).

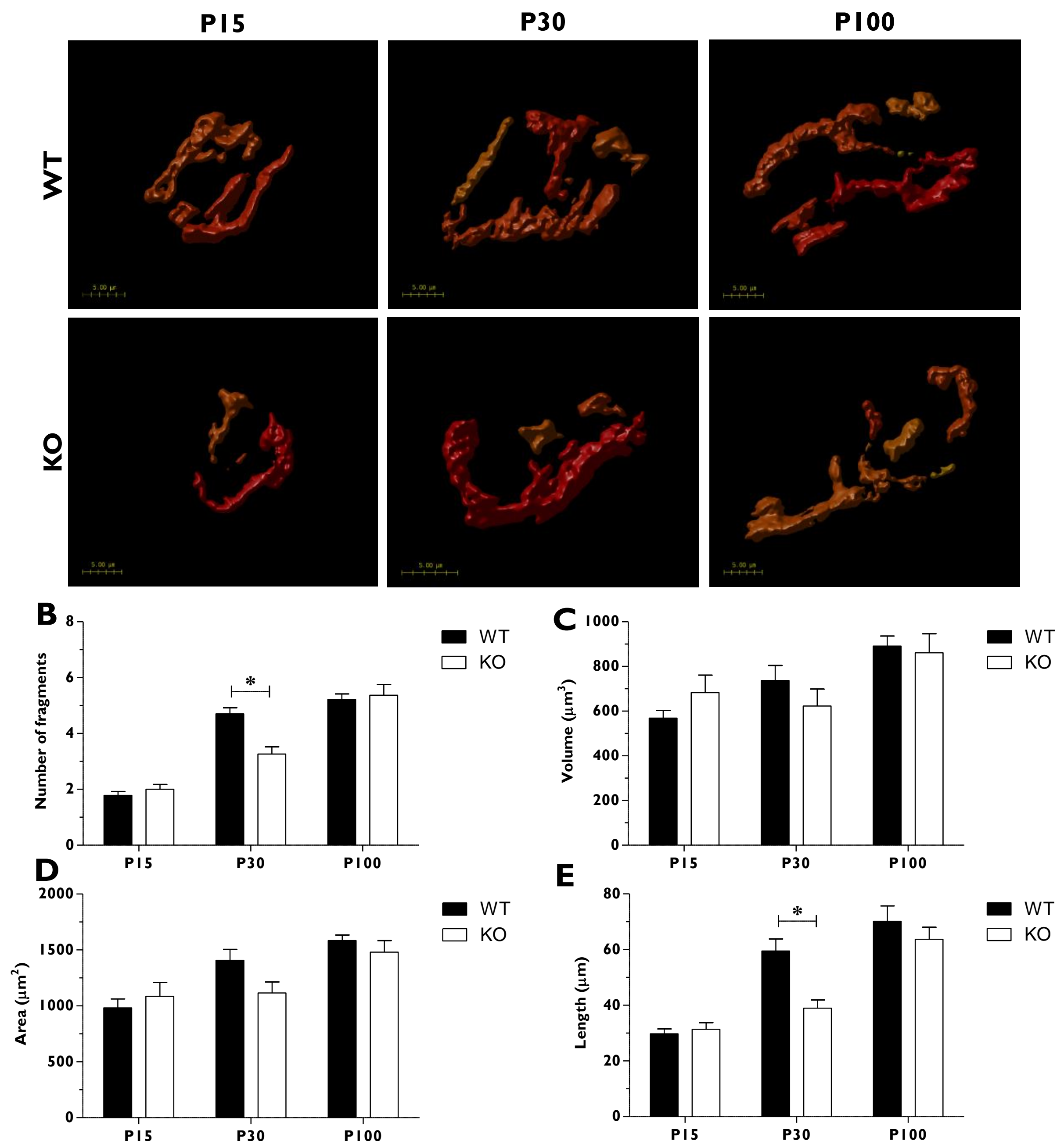


Figure 16 – Evolution of postsynaptic structures in adducin α-adducin KO. A, 3-D images of NMJs of each age and genotype after Huygens Professional analysis. B, results for the morphometric analysis of NMJs in the three age groups and genotypes.

4.3.3 Evaluation of the pre- and postsynaptic structure of the NMJ

Given that in *Drosophila melanogaster* Hts can affect post- and presynaptic structure formation, we evaluated whether similar events occured in α-adducin KO mice.

Since BTX staining only allows the evaluation of the pre-synaptic compartment, we performed an evaluation in teased fibers to better understand if co-localization occurred in the pre- and postsynaptic compartment of the NMJ. We observed that the α-adducin KO mice co-localized in the same way that WT littermates at P30. Another confirmed aspect that was in this analysis was the delay in the maturation of α-adducin KO NMJ that displayed a reduced complexicity as can be observed in figure 17.

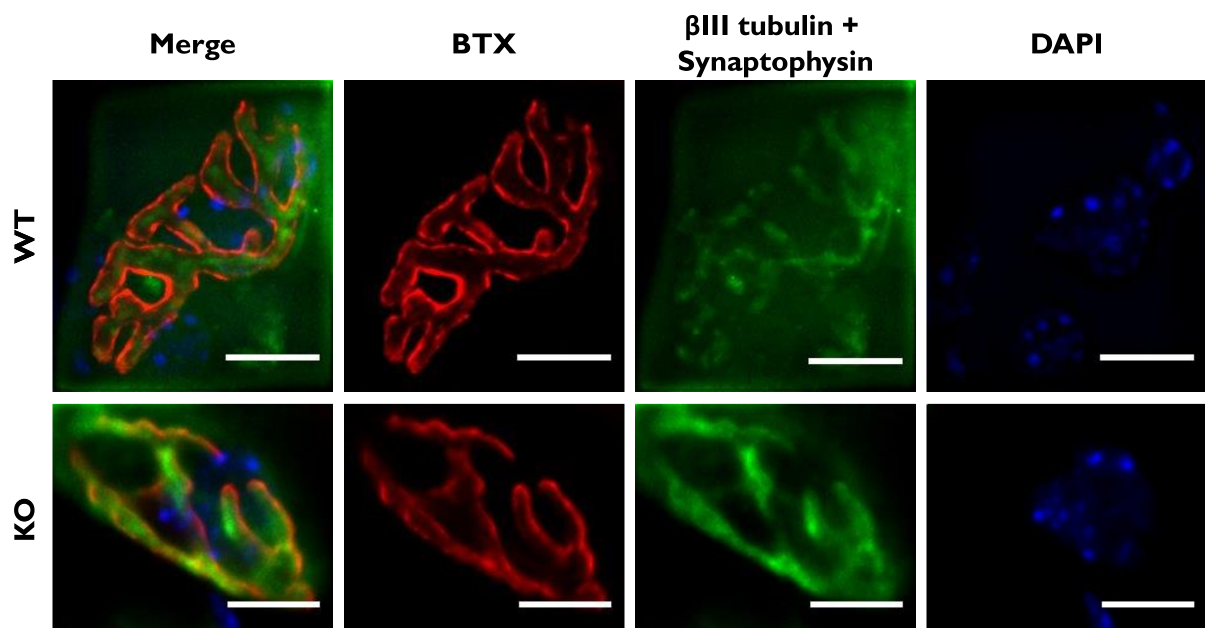


Figure 17 – Morphological changes in the pre- and postsynaptic NMJ structure between α adducin KO and WT littermate mice at P30. The bungoratoxin (BTX) staining labels the postsynaptic structure. The staining with βIII tubulin and Synaptophysin revealed the presynaptic structure. DAPI staining labels the perisynaptic nuclei. Scale bar, 10 μm.

5. Conclusions

5. Conclusions

In summary, our work demonstrated that:

The activity of α-adducin might be important for the sensory axonal sensory of the inhibitory environments. This effect does not seem to occur in other axonal populations.

Adducin is an important protein in axonal plasticity namely in the relocation of the AIS.

The effect of adducin in the actin cytoskeleton needed to form NMJ is probably limited to defining the initial complexicity of the synapse.

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