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Avaliação da capacidade regenerativa em modelos de lesão da medula espinal

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Tese apresentada à Faculdade de Medicina da Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Patologia Experimental, realizada sob orientação científica da Doutora Mónica Mendes Sousa, Investigadora Principal do Grupo de Regeneração Nervosa do Instituto de Biologia Molecular e Celular-IBMC, Porto; e co-orientação do Professor Doutor António Manuel Silvério Cabrita, Professor e Coordenador do Mestrado em Patologia Experimental.

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ABSTRACT

Spinal cord injuries are amongst the most serious and debilitating health conditions with devastating impact on a patient´s life style, affecting not only its physical but also its psychological status and social life. The correct functioning of the nervous system depends on the formation of a complex pattern of connections during development. If these connections are subsequently lost through injury or degeneration, axons in the adult central nervous system fail to regenerate, which is a major clinical challenge. One of the main therapeutic strategies for spinal cord injury is to promote axonal regeneration. Thus, the use of experimental animal models of spinal cord injury is increasing. To allow the identification of molecular mechanisms responsible for axonal regeneration, our group has been using the conditioning lesion model, aiming at understanding the cellular and molecular mechanisms underlying the improvement of the regenerative capacity of central nervous system axons after a primary injury in the peripheral nervous system. This model is based on the fact that in dorsal root ganglion neurons, when an injury to the peripheral branch occurs before an injury to the central branch, the central branch gains regenerative capacity being able to grow through the glial scar. In this work we performed the validation of this model in rodents (mice and rats) and investigated the role of GSK-3β in promoting axonal regeneration. Our initial strategy was to assess the functional recovery of rats after spinal cord injury or conditioning lesion and, by retrograde labeling, evaluate, *in vivo*, the regeneration of central branch dorsal root ganglion axons. We established successfully the procedures of conditioning lesion and spinal cord injury in our animal facility, and developed a detailed post-surgical care of the paraplegic animals. Moreover, we confirmed the conditioning lesion effect in the ascending dorsal column tract axons by showing that they are capable of regenerating following conditioning lesion.

Glycogen Synthase Kinase 3β (GSK-3β) has been described as playing a central role in regulating axon genesis and elongation. The phosphorylation of GSK-3β Tyr216 results in the activation of the kinase, while the phosphorylation of Ser9 leads to its inactivation. Given the critical functions that have been attributed to GSK-3β in the context of axonal growth, the modulation of its activity may be an important strategy for developing successful therapies for central nervous system injuries. To elucidate

the role that inactivation of GSK-3β might play in the promotion of axonal regeneration, we tested several injury models in homozygous GSK-3 Ser21Ala/Ser9Ala knockin (GSK-3 KI) mice. In these mice, inactivation of GSK-3 through the phosphorylation of these residues is disabled. Through retrograde labeling of regenerating axons, we compared axonal regeneration in wild type and GSK-3 KI mice after spinal cord injury and conditioning lesion. Qualitative analyses led us to conclude that the conditioning lesion effect occurs independently of GSK-3β inactivation through Ser9 phosphorylation. However, further studies will be necessary to elucidate the role of this kinase in central nervous system regeneration.

In summary, currently, all the tools are available in our laboratory to evaluate functionally and at the molecular level the identity of the molecules involved in axonal regeneration and therapeutic approaches for spinal cord injury in mouse and rat models.

RESUMO

As lesões na medula espinal encontram-se entre os mais sérios e debilitantes problemas de saúde, com um impacto devastador no estilo de vida de uma pessoa, afectando não só o seu estado físico e psicológico, mas também a sua vida social. O correcto funcionamento do sistema nervoso depende da formação de complexas ligações nervosas durante o desenvolvimento embrionário. Se estas ligações forem perdidas após lesão ou degeneração, os axónios do sistema nervoso central adulto não conseguem regenerar, o que se tem revelado um grande desafio. Uma das principais estratégias em lesões medulares é promover a regeneração axonal. Assim, o uso de modelos animais experimentais, de lesões na medula espinal, tem vindo a aumentar. Para permitir a identificação de mecanismos moleculares responsáveis pela regeneração axonal, e visando comprender os mecanismos celulares e moleculares subjacentes ao aumento da capacidade regenerativa em axónios do sistema nervoso central, após uma lesão primária no sistema nervoso periférico, o nosso grupo tem vindo a utilizar o modelo de lesão condicionante. Este modelo baseia-se no facto de que, em neurónios do gânglio da raiz dorsal, quando uma lesão no ramo periférico ocorre antes de uma lesão do ramo central, a capacidade regenerativa do ramo central aumenta, tornando-se capaz de crescer através da cicatriz glial. Neste trabalho efectuámos a validação deste modelo em roedores (ratos e murganhos) e investigámos o papel da GSK-3β na promoção da regeneração axonal. A nossa estratégia inicial foi avaliar a recuperação funcional de ratos após lesão da medula espinal ou lesão condicionante e, através de marcação retrógrada, avaliar, *in vivo*, a regeneração de axónios do ramo central do gânglio da raiz dorsal. Conseguimos estabelecer, com sucesso, os procedimentos para a lesão condicionante e para a lesão medular no nosso biotério, e desenvolvemos cuidados pós-cirúrgicos detalhados para com os animais paraplégicos. Além disso, confirmámos o efeito da lesão condicionante no tracto ascendente de axónios dorsais, mostrando que são capazes de regenerar após lesão condicionante.

A *Glycogen Synthase Kinase 3β* (GSK-3β) tem sido descrita como tendo um papel central na regulação da elongação e da genese axonal. A fosforilação da Tyr216 GSK-3β resulta na activação da cinase, enquanto a fosforilação da Ser9 leva à sua inactivação.

Dadas as funções que têm sido atribuídas à GSK-3β, no contexto do crescimento axonal, a modulação da sua actividade pode ser uma estratégia importante para o desenvolvimento de terapias para lesões do sistema nervoso central. Para elucidar o papel que a inactivação de GSK-3β pode desempenhar na promoção da regeneração axonal, testámos vários modelos de lesão em murganhos homozigóticos GSK-3 Ser21Ala/Ser9Ala knockin (GSK-3 KI). Nestes animais, a inactivação da GSK-3 através da fosforilação destes resíduos está desactivada. Através da marcação retrógrada de axónios em regeneração, comparámos a regeneração axonal em murganhos *wild type* e GSK-3 KI após a lesão medular e lesão condicionante. Análises qualitativas levaram-nos a concluir que o efeito da lesão condicionante ocorre independentemente da inactivação da GSK-3β pela fosforilação da Ser9. No entanto, mais estudos serão necessários para elucidar o papel desta cinase na regeneração do sistema nervoso central.

Resumindo, actualmente, o nosso laboratório dispõe de todas as ferramentas necessárias, para avaliar funcionalmente e ao nível molecular quais as moléculas envolvidas na regeneração axonal, e abordagens terapêuticas para lesões da medula espinal em ratos e murganhos.

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PART 1

INTRODUCTION

"Only the man who is familiar with the art and science of the past is competent to aid in its progress in the future".

(Theodor Billroth)

1.1 History

The first descriptions of spinal cord injuries had origin in the ancient Egypt (3000 - 2500 years BC). During this era, a great amount of human resources were dedicated to the construction of the pyramids which led to a high incidence of trauma, enabling the description of a multiplicity of injuries within this population [1]. In 1862, Edwin Smith purchased documents (later known as the Edwin Smith Papyrus) from this period reporting different traumatic injuries in the human body [1]. Six cases of spine and spinal cord injuries were presented with the description of the patient´s history, diagnosis and prognosis. One case ("*Treatment instructions concerning a crushed vertebra of the back of his neck*") reports a complete spinal cord injury, caused by a fracture in the cervical region, presenting paralysis of both arms and legs, loss of sensation below the level of injury, and loss of urinary bladder control, concluding that it was "an ailment not to be treated" [1, 2].

Centuries later (460 - 377 BC), Hippocrates analyzed the correlation between vertebral and spinal cord injuries. He observed that if the spinal cord injury occurred in one side only, a subsequent paralysis would be located on the same side of the damage. He also described clinical conditions of chronic paralysis, such as constipation, bladder problems, pressure sores, and venous stasis of the lower limbs, as a result of a traumatic spinal cord injury. In order to reduce spinal deformities, Hippocrates created traction devices (Figure 1), where spinal manipulations were carried out. These extension techniques with the application of traction are still widely used today in the treatment of spinal disorders [3].

During the Greco-Roman era (1st century BC), Aulus Cornelius Celsus documented the rapid death as a consequence of cervical spinal cord injury. The Greek Galen (129–199 AD), described the anatomy of the brain, spinal column and spinal cord, as well as the effects of experimental incisions in the spinal cord. He noted that a longitudinal incision did not result in any symptoms but a transverse incision at the level of the cervical vertebras would result in paralysis and loss of sensory functions below the level of injury [1, 3]. He also observed that injuries at the first and second cervical vertebrae were fatal, while breathing would stop with injuries at the third or fourth vertebrae [4].

After several centuries of stagnation, in 1543, Flemish and Andreas Vesalius published the "*De Humani Corporis Fabrica*", showing for the first time drawings of the human nervous system correctly illustrated [1].

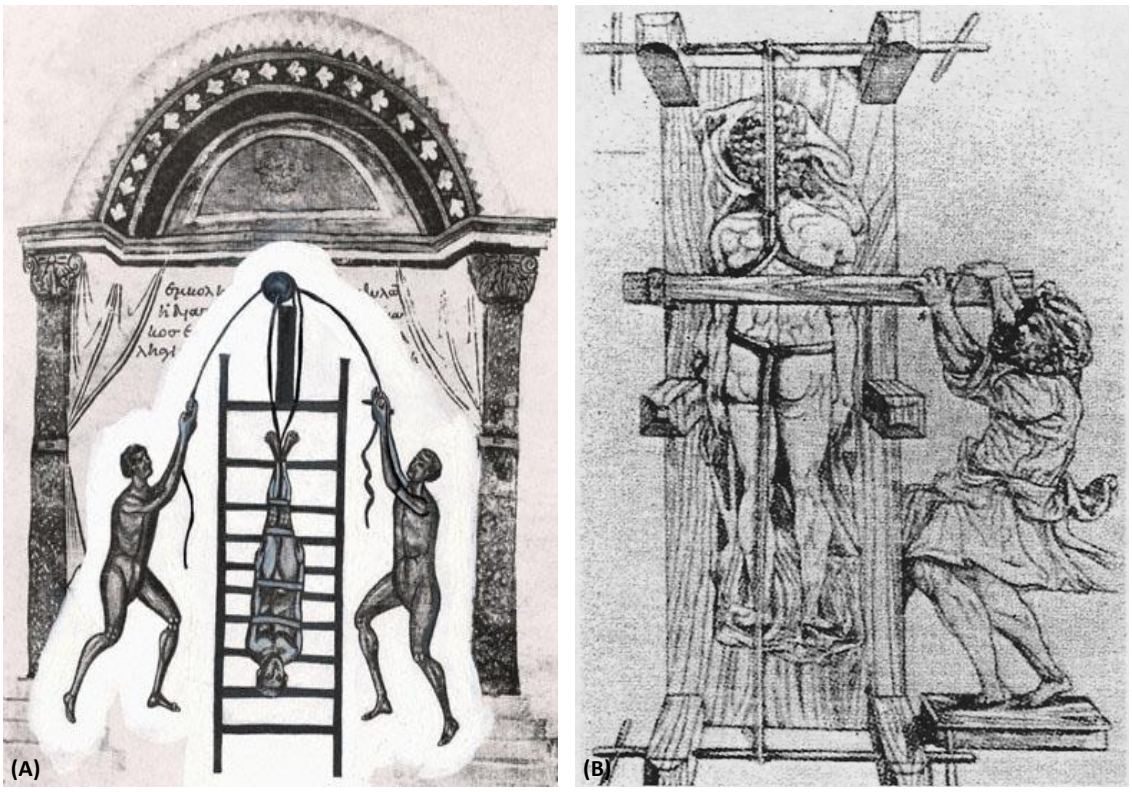


Figure 1 - Hippocratic Ladder (A) and Board (B) devices. To reduce spinal curvatures, the patient was shacked while tied on a ladder (A); the weight of the trunk and limbs would act as the pulling force, which would straighten the spine. In the Hippocratic Board (B) the patient was placed in a prone position and stretched from the shoulder area upward, and from the hips downwards; a wooden board would be placed crosswise over the injured area and its compression would lead to deformity reduction. From: [1, 5].

Charles Bell, in 1824, introduced the concepts of spastic and flaccid paralysis and described the concept of spinal shock [1]. Later, in 1858, Charles Edward Brown-Séquard described spinal cord functions through animal experiments and clinical examples and showed that the opening of the spinal canal and the exposure of the spinal cord to the air was on itself not dangerous. He also claimed that death following spinal fractures was a consequence of spinal cord damage and not due to the primary injury; additionally he correlated the urinary infections with spinal cord disorders [6]. During the 1st World War, spinal cord injuries associated mortality was as high as 80% within the first two weeks after injury. Meanwhile, after perceiving the importance of comprehensive care for spinal cord injured victims, the education for the care of spinal

cord injury, little addressed so far, became part of the programs in medical schools leading to the creation of rehabilitation programs and to the formation of specialized personnel in the care of patients with spinal cord injuries. Thus, from the middle of the 20th century, an important era of rehabilitation arised with the creation of spinal injury associations, which made great contributions to the diagnosis and comprehensive management of spinal cord injuries through annual meetings, teaching sessions, workshops, and research grants, and with the opening of rehabilitation centres. These centres opened the way for better care of spinal cord injured patients, not only increasing the patient's survival but also leading them to readapt to the society. Since then the implementation of treatment models and the development of medicine prolonged the survival of the patients in about 2000% [1, 3, 4].

The most recent advances in experimental spinal cord injuries investigation are due to the advances of neurobiology [3]. Ramon y Cajal (1900-1920) showed that, in mammals, although severed central nerve fibers commenced to regenerate, the attempts were abortive and the process was not functionally benefic to the animals. During the following decades, experiments on amphibians and reptiles showed that regeneration was possible after injury [4]. In the 70´s, Kao transplanted autogenous sciatic nerve into the injured spinal cord and succeeded in demonstrating axonal growth within the grafted tissue [7]. Research into genetic engineering and stem cell studies also began to give its first steps [4].

At present, the care of spinal cord injured patients requires knowledge within a variety of medical fields and also an interest in development and research. Neuroprotection and regeneration are currently the main focus of the latter. Unfortunately, the goal of healing the injured spinal cord is still out of reach. However, it is now possible to offer several therapeutic measures to improve the health and quality of life of spinal cord injured victims, with the aspiration of providing them a long and fulfilling life [1].

1.2 Spinal Cord Injury Epidemiology

According with the Council of Europe, in 2001, approximately 90 million people worldwide were suffering from some type of spinal cord injury and approximately 300,000 were paraplegic, only in Europe, as a result of the injury [8]. Unfortunately,

insufficient data exist to determine a global prevalence for spinal cord injuries. Nevertheless, the range of reported traumatic spinal cord injuries prevalence is between 236 and 1009 per million. In Asian countries, the overall prevalence is likely underestimated and the only existing data refers to India and Vietnam, with a prevalence of traumatic spinal cord injuries of 236 to 464 cases per million. In Western Europe, only Finland and Iceland have reported prevalence data, with 280 and 316 per million, respectively. In Canada, the prevalence of spinal cord injuries is approximately 1173 per million whereas in the USA it is estimated at approximately 853 per million people [9]. Also in Canada, in 2010, it was estimated that nearly 265,000 people were living with spinal cord injuries [10]. The number of injuries occurring around the world is alarming. In 2001 the Council of Europe predicted that, each year, 85,000 people would survive to a traumatic spinal cord injury, being destined to spend the rest of their lives in a wheelchair [8]. Just in the United States, approximately 40 cases per million population, not including those who die before reaching the hospital, is the estimated annual spinal cord injuries incidence [10], corresponding to 12,000 new cases each year [11]. Among the most frequent causes of spinal cord injuries (Figure 2A) are trauma (mostly related with motor vehicle accidents), falls, construction accidents, violence, sports incidents [10], and some illnesses like polio, multiple sclerosis, cancer, and arthritis [11].

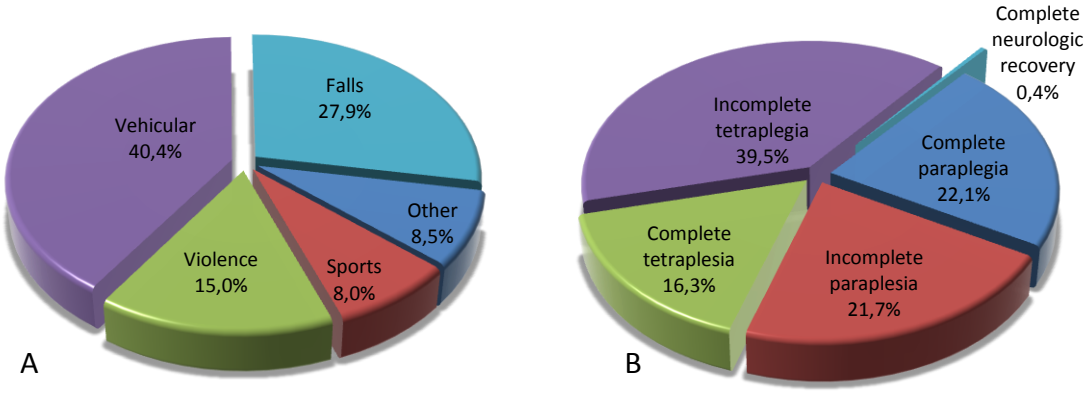


Figure 2 - Major causes (A) and consequences (B) of traumatic spinal cord injuries, in the United States, since 2005 [10].

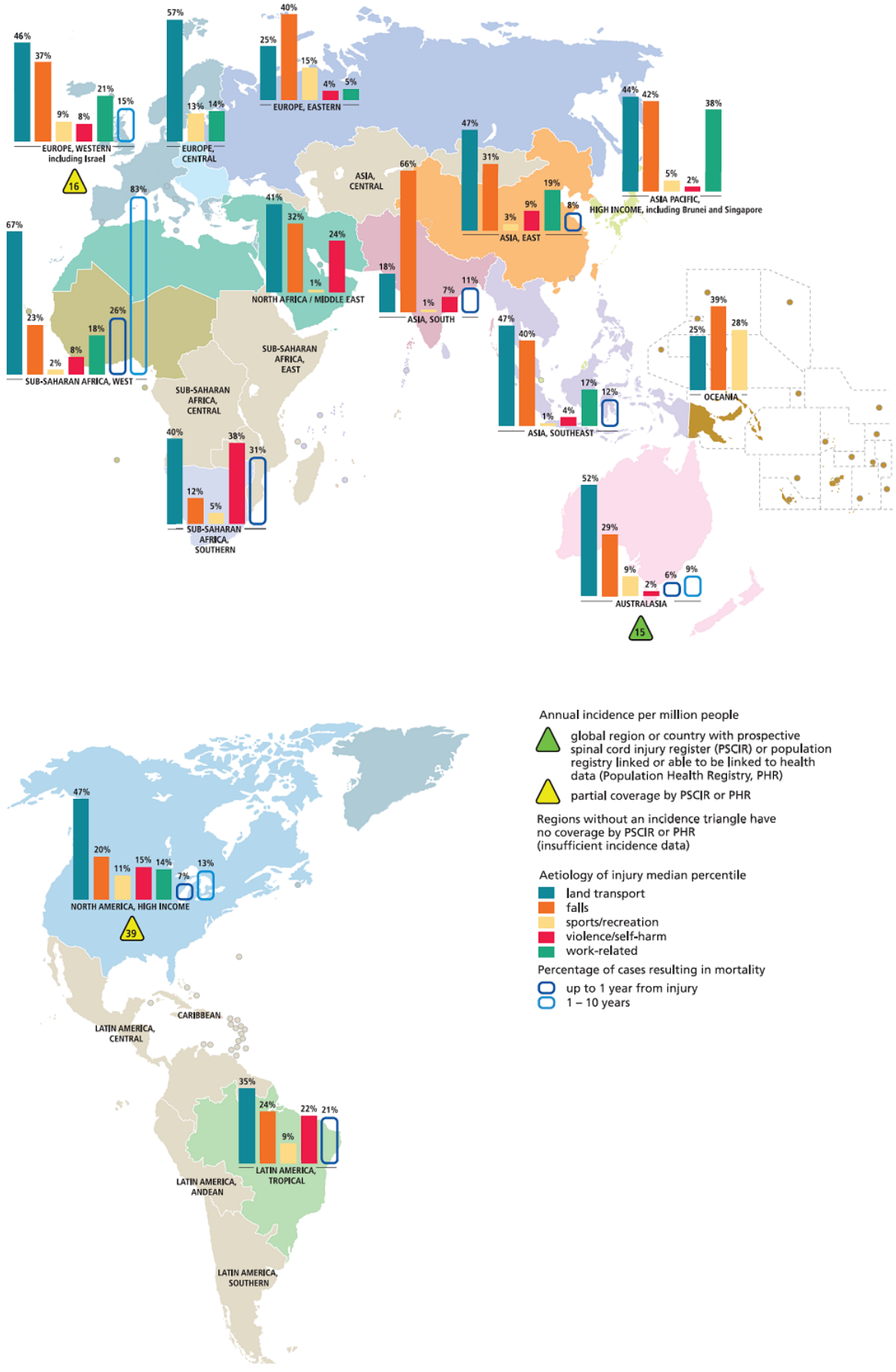


Figure 3 - Global mapping of spinal cord injury from traumatic causes by the World Health Organization regions, from 1959 to 2008. Adapted from [9].

Since 2005, the average age at injury is 40.7 years and the majority (80.7%) of spinal cord injuries reported in the United States database occurred among males. At hospital discharge, incomplete tetraplegia was the most frequent neurologic consequence of the injury, followed by complete and incomplete paraplegia and complete tetraplegia. Less than 1% of the patients have completely recovered their neurologic functions when leaving the hospital [10].

Although life expectancy for patients with spinal cord injuries remains below the life expectancy of those with no injury, it has been significantly improving over the last 40 years in developed countries when compared with under developed ones [9]. Moreover, the mortality rates are significantly higher through the first year after injury than in the subsequent years, reflecting the gravity of the injury. Generally, tetraplegics have lower survival rates than paraplegics [10]. Developing countries have the highest 1-year mortality rates and in some of them the occurrence of a spinal cord injury is likely to be fatal in the first year following injury [9]. Until recently, the leading cause of death among people with a spinal cord injury was renal failure, meanwhile the advances in urologic management have reduced this number of cases. Pneumonia, pulmonary embolism and septicemia are currently the main causes of the reduced life expectancy for this population [10].

Table 1 - Economic impact of spinal cord injuries, in the United States, in 2010.

Severity of injury	**Average Annual Expenses**		**Estimated Lifetime Costs by Age at Injury**	
	First Year	Each Subsequent Year	25 years old	50 years old
High Tetraplegia (C1-C4)	$985,774	$171,183	$4,373,912	$2,403,828
Low Tetraplegia (C5-C8)	$712,308	$105,013	$3,195,853	$1,965,735
Paraplegia	$480,431	$63,643	$2,138,824	$1,403,646
Incomplete Motor Functional at Any Level	$321,720	$39,077	$1,461,255	$1,031,394

Data source: Spinal Cord Injury Facts and Figures at a Glance, National Spinal Cord Injury Statistical Center, Birmingham, Alabama, February 2011 [10].

Due to the nonexistent cure, much has been spent on rehabilitation and comfort of the patients. The economic impact on the community with the long term living expenses for the care and social welfare support with survivors of a spinal cord injury are directly

related and depend from the severity of the injury, reaching however the tens of billions of dollars each year (Table 1) [10].

1.3 The Spinal Cord

The nervous system, broadly subdivided into Peripheral Nervous System and Central Nervous System, is responsible for controlling all the biological processes in the body. The peripheral nervous system is a collection of spinal and cranial nerves whose branches spread to all parts of the body transmitting electrical impulses to and from the central nervous system composed by the brain and the spinal cord (Figure 5A). Regardless of its size and widespread distribution, it contains only two major categories of cells (Figure 4): the neurons, which are the information processors and signaling elements, and the glial cells, playing a variety of supporting roles [12].

Located within the vertebral canal of the spine, the spinal cord is the part of the central nervous system that controls the voluntary muscles of the body, as well as most of the viscera and blood vessels of the trunk, and receives their sensory information [13].

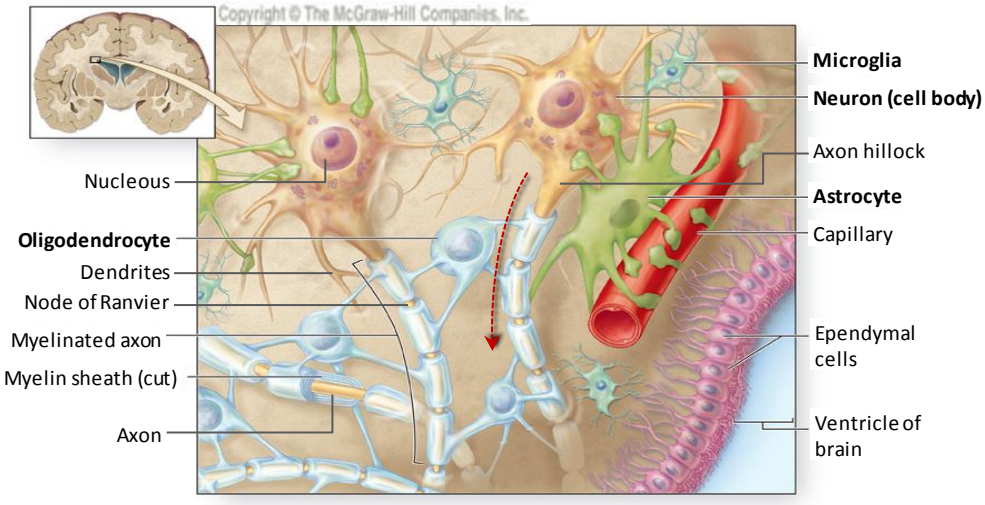


Figure 4 - Cells of the Central Nervous System and their relationships. In contrast to neurons, glial cells do not conduct electrical impulses, they surround neurons providing them support. Astrocytes are involved in neuronal metabolism, oligodendrocytes are involved in the production of myelin and microglia is part of the immune system. The arrow indicates the direction of impulse within the neuron. Adapted from [14].

The human spinal cord can be subdivided into 31 segments (12 thoracic, 5 lumbar, 5 sacral and 1 coccygeal) [13] (Figure 5B), which give rise to dorsal (posterior) and ventral (anterior) rootlets that unite into a pair of dorsal and ventral roots (Figure 6). The dorsal roots contain the spinal ganglions, located within the intervertebral foramina of the vertebrae and where the dorsal and ventral roots combine into the spinal nerves [5]. The spinal nerves are named and numbered according with the vertebrae from where they emerge from the vertebral canal. Cervical nerves 1-7 emerge above their respective vertebrae. Since there are only seven cervical vertebras, cervical nerve 8 arises between the 7th cervical (C7) and the 1st thoracic vertebras (T1). All the remaining nerves emerge below their respective vertebrae (Figure 5B) [13].

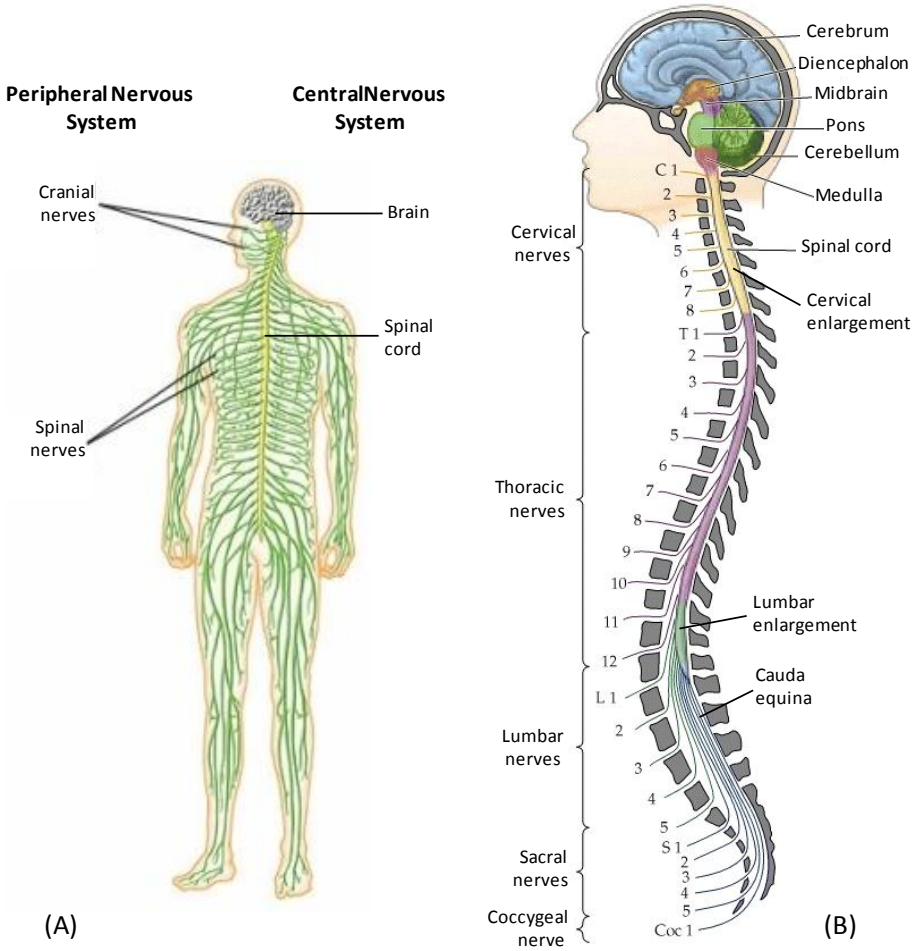


Figure 5 - Subdivisions and components of the central nervous system. (A) Division of the nervous system into peripheral nervous system and central nervous system. (B) Lateral view representing the major components of the central nervous system and indicating the emergence of the segmental nerves (The position of the brackets on the left of the image refers to the vertebra). Adapted from [15, 16].

Spinal nerves consist in sensory nerve roots, which enter the spinal cord through the dorsal root, and motor roots, which emerge from the cord via ventral roots, at each level. Therefore, dorsal roots contain mostly afferent (sensory) fibers whereas ventral roots contain efferent (motor) fibers [13]. The cell bodies from the afferent nerve fibers, belonging to the peripheral nervous system, are located within the dorsal root ganglion [5] where a single axon bifurcates (Figure 6) giving rise to one branch that connects with the periphery and another one that connects with the dorsal horn of the spinal cord [13].

Neuronal cell bodies, dendrites, axons and glial cells originate the grayish color of the spinal cord, the gray matter, surrounded by the white matter where the axons of the spinal cord are located [12].

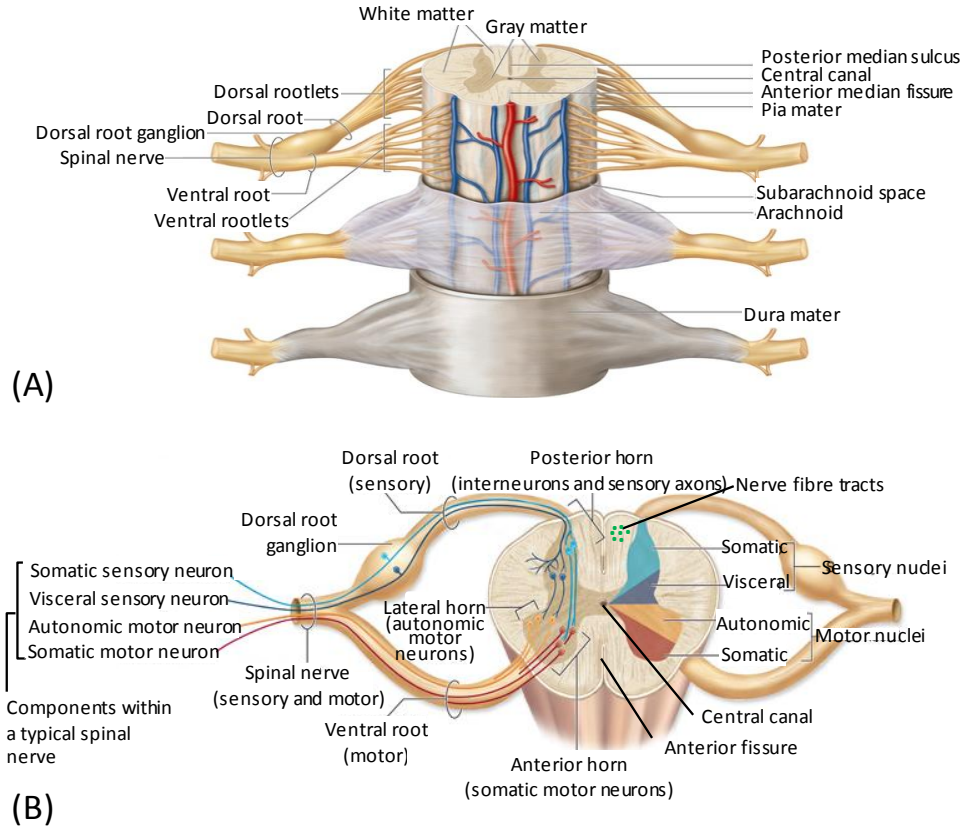


Figure 6 - Diagram of several spinal cord segments with associated nerve fibers. (A) Ventral view. (B) Representation of the spinal cord trough a transversal cut. Adapted from [14].

1.4 Spinal Cord Injury: from degeneration to regeneration

Although the spinal cord is well protected within the vertebral column, spinal cord injuries are normally caused by trauma or, but not so often, by health disorders. The traumatic event can be either endogenous, as a result for example of an intervertebral disc herniation, or exogenous, frequently caused by fractured or dislocated vertebras. The initial mechanical trauma to the neural tissue can result from contusion, compression, penetration or maceration of the spinal cord [17], and can injure both the central nervous system and the peripheral nervous system [18]. Spinal cord injury causes the disruption of nerve fibers and local neuronal circuits, interrupting the convey of information between the brain and the structures below the injury site [19], often resulting in the loss of sensory and motor function [20]. Yet, the consequences of injury are not just a break in communication between healthy neurons, but a cascade of events that can lead to neuronal degeneration and cell death [21]. Regardless of the cause of injury, the resultant pathology arises from primary and secondary injury mechanisms. The physical injury to the spinal cord represents the primary injury and can lead to severed axons, direct mechanical damage to cells, and ruptured blood vessels. Secondary injury is responsible for the expansion of the primary injury and is the result of alterations in local ionic concentrations, loss of regulation of local and systemic blood pressure, reduced spinal cord blood flow, crash of the blood-brain barrier, production of free radicals, imbalance of activated metalloproteinases, and release of cytotoxic neurotransmitters [22]. The results of both primary and secondary injury mechanisms are the conduction block of neuronal impulses as the result of local ionic changes and demyelination, ischemia, necrosis, and apoptosis of spinal cord tissue [22].

Whereas following peripheral nervous system injury, sensory and motor axons can and often do regenerate, the regenerative capacity of the injured adult mammalian central nervous system is very limited, usually resulting in permanent neurological deficits [23]. While embryonic central nervous system axons can regenerate quite readily, they lose this capacity with age [24]. Understanding why injured axons cannot regenerate after injury in the adult mammalian central nervous system has been a major challenge within the scientific community over the last decades [25]. Upon injury, adult central

nervous system axons undergo a spontaneous, but brief and eventually abortive attempt on repair reffered to as regenerative sprouting. This indicates that injured central nervous system neurons have not necessarily lost their intrinsic property to grow, but that they fail to initiate and/or maintain a specific growth programme required for axonal elongation [24]. Moreover, previous studies showed that adult central nervous system neurons can regrow if they have access to the permissive environment of a conditioned sciatic nerve. Further studies have also revealed that the failure of central nervous system neurons to regenerate is not an intrinsic deficit of the neuron, but rather a characteristic feature of the environment that either does not support or prevents regeneration [21]. Important regulators of axonal growth (GAP-43, cytoskeletal proteins, transcription factors, neuropeptides, integrins, growth factors and neurotrophin receptors) that are expressed during nervous system development are re-expressed upon nerve injury. However, in contrast to the peripheral nervous system, this event is often merely temporary or completely absent in the central nervous system. In addition to a reactivation of growth associated genes expressed during development, peripheral axotomy leads to the up-regulation of regeneration-associated genes (RAGs). These proteins promote axon growth when overexpressed in neurons. These observations suggest that expression of growth-associated molecules as well as RAGs may be required to trigger successful nerve regeneration [24].

1.4.1 Wallerian Degeneration

Following central nervous system lesion, the entire distal segment of the nerve degenerates, with desintegration of the distal axonal segment, degradation of myelin sheaths and axon cytoskeleton, and the subsequent apoptotic death of oligodendrocytes around the lesion site [24]. An additional reason for the different regenerative abilities of the peripheral nervous system and central nervous system may be the singular sequence of degenerative events after injury, known as Wallerian degeneration. Wallerian degeneration begins with axonal degeneration, after which, in the peripheral nervous system, the blood-tissue barrier permeability increases, the myelin sheath breaks down, and an influx of macrophages occurs to remove the cellular and myelin debris distal to the site of axonal injury (Figure 7A). Schwann cells

that formerly ensheathed the axons proliferate, align to form longitudinal arrays, and increase their production of neurotrophic factors that can promote axon regeneration. Proximal to the injury site, neuronal cell bodies react to injury by inducing expression of growth-related genes, including those for major components of axonal growth cones. The Schwann cell basal lamina and the extracellular matrix also provide a favorable substratum for the extension of regenerating axons [16]. In mammals, Wallerian degeneration in the peripheral nervous system is fast taking about 7–14 days. In the mammalian central nervous system, Wallerian degeneration is however dramatically slower, taking months to years. These differences are not only due to a delay in axonal degeneration in the central nervous system but rather to a failure to clear central nervous system myelin debris (Figure7B). The myelin debris have several inhibitors of axonal regeneration [26] namely Nogo, myelin-associated glycoprotein (MAG), oligodendrocyte-myelin glycoprotein (OMgp), tenascin-R, proteoglycans and chondroitin sulfate proteoglycans (CSPGs) [24]. Astrocytes at the site of injury also interfere with regeneration and neurons typically fail to activate the growth-associated genes [16].

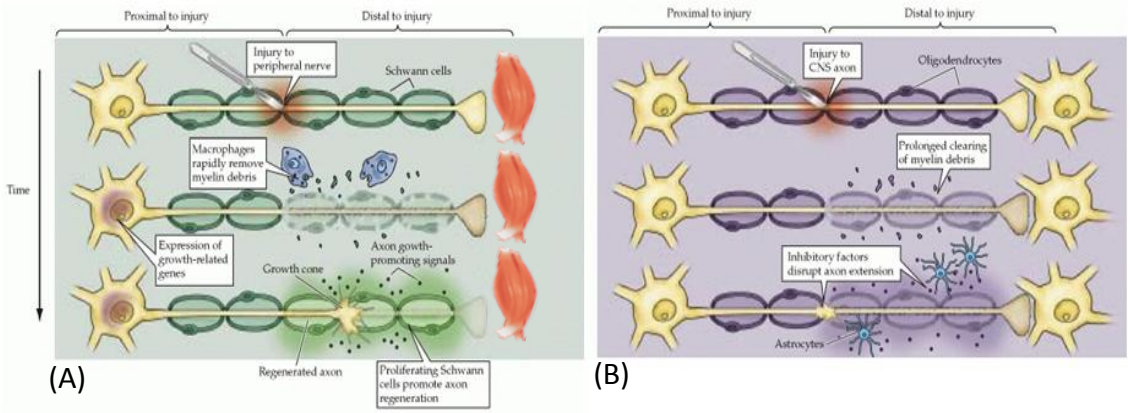


Figure 7 - Different responses to injury in peripheral (A) and central (B) nervous systems. Adapted from [16].

Following axonal injury, the primary role of the immune system is to remove toxic debris from the injury site. While the magrophages and Schwann cells in the distal stumps of the injured peripheral nerves are effective in removing the inhibitory proteins associated with myelin, the microglia and oligodendrocytes in the central nervous system are not [27]. Therefore, in the peripheral nervous system, rapid

Wallerian degeneration results in an extracellular environment that promotes axonal regeneration, whereas in the mammalian central nervous system, the slow Wallerian degeneration results in prolonged presence of myelin-associated inhibitors that likely contribute to the failure of central nervous system axons to regenerate [26].

Moreover, it is important to consider inflammation within the central nervous system as a potential source of cytokines and of other signaling molecules that can lead to the upregulation of inhibitory pathways after injury. Microglial cells from the central nervous system and activated macrophages from the periphery both respond to trauma in the brain and spinal cord, and this inflammatory response may contribute to secondary tissue damage after the primary insult [28].

1.4.2 The Glial Scar

Aside from the inhibitory properties of myelin, microglial cells proliferate along the degenerating axons, and hypertrophic astrocytes develop tightly packed filament-rich processes underling the formation of a glial scar in the location of the affected nerve fibres [29]. Central nervous system injury results in a rapid glial response around the injury site. The glial reaction to injury recruits microglia/macrophages, oligodendrocyte precursors, meningeal cells, astrocytes, stem cells and fibroblasts [24, 30], leading to the formation of a fibrotic glial scar (Figure 8) [24, 31].

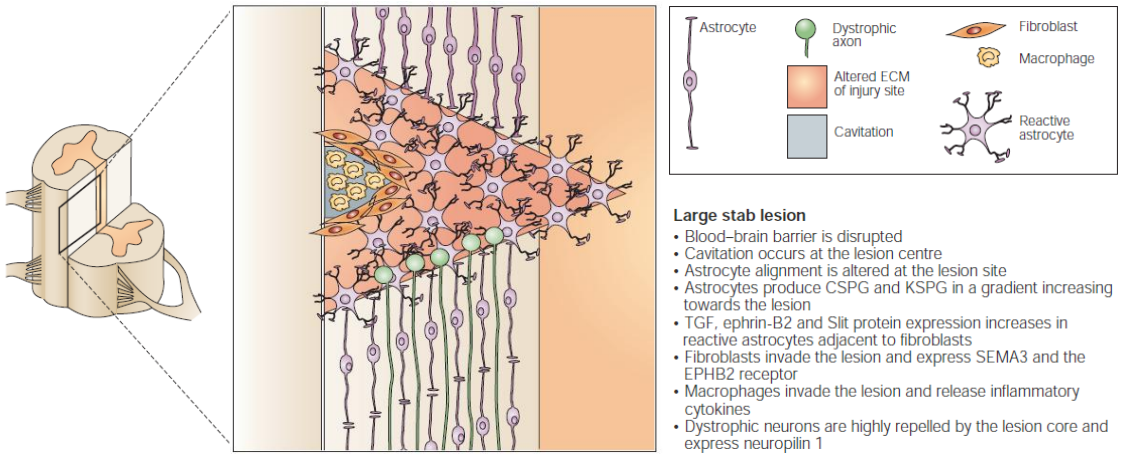


Figure 8 - Schematic representation of a stereotypical central nervous system lesion. Stab lesion that penetrates the meninges and allows fibroblast invasion in addition to macrophages. Axons are highly repulsed by the increasing gradient of chondroitin sulfate proteoglycans (CSPGs) and keratan sulfate proteoglycans (KSPGs). Several other inhibitory molecules are also made in this type of injury and are especially prevalent in the core of the lesion. ECM, extracellular matrix; TGF, transforming growth factor. Adapted from [32].

Although it contributes to seal the injury site from the spared tissue, possibly preventing the spread of the secondary injury, the glial scar is far more than just a physical barrier [28]. It is the source of factors such as tenascic acid, semaphorins, ephrins, and various proteoglycans, that make the biochemical milieu surrounding the injury site inhospitable for regenerating axons [22]. The glial scar thus forms both a mechanical and a molecular barrier to axonal growth [24]. Therefore axons cannot regenerate beyond it, and take on a dystrophic appearance of stalled growth. Recent studies have indicated that axons with dystrophic endings do not lose their ability to regenerate, and that they can in fact return to active growth states [32]. The significance of the persistence of such unusual "growth cones" within the lesion implies that some type of cytoskeletal and/or membrane plasticity must be occurring to maintain axonal viability and stability, even though the axons remain without forming synapses [32].

1.4.3 Axonal Regeneration

After injury to the adult mammalian central nervous system, the resulting failure of axonal regeneration can be attributed to myelin-associated inhibitors, the glial scar, and the neuronal intrinsic growth status [33]. Physical injury to the spinal cord results in the mechanical disruption and degeneration of ascending and descending axons. One of the main therapeutic strategies for spinal cord injuries is to promote axonal regeneration [22]. Although axonal regeneration is extremely limited in the adult mammalian central nervous system, partial lesions of the spinal cord can be followed by spontaneous, and often substantial, functional improvements [34]. Research on nerve regeneration has been focused on identifying the inhibitory factors present in the environment. However much less is known about the mechanisms that activate the intrinsic growth capacity of neurons following injury [35]. A substantial amount of research suggests that increasing the growth potential of damaged sensory neurons enables them to overcome inhibition in the injured peripheral nervous system and central nervous system [36].

1.5 The Consequences of Spinal Cord Injury

Acute traumatic spinal cord injury is an unexpected, catastrophic event. Its consequences often persist for the life of the patient and influence in several ways not only the patient, but also its family members and society at large [37].

Human spinal cord injury often results from an insult to the spinal column and by contusion injury. There is the subsequent displacement of the spinal cord due to the pressure from the broken bone, disk fragments and hematoma and swelling inside the closed vertebral canal [13]. This injury has a serious effect on the spinal cord segment with the breakdown of connections and networks and the development of a spinal cord scar. The functional consequences of such an injury are associated with autonomic paralysis including dysfunctional internal organs, limb muscle atrophy, sensory impairment, and chronic pain [38]. Phantom sensations are also frequent [39]. These symptoms are often lifelong persistent because of the inability of the lesioned fibres to regenerate [40]. The consequences of a spinal cord injury may vary depending on the type, level, and severity of injury. Complete injuries lead to the loss of function below the level of injury, resulting in the absence of motor and sensory function. In incomplete injuries, some sensation and/or movement below the level of injury is retained.

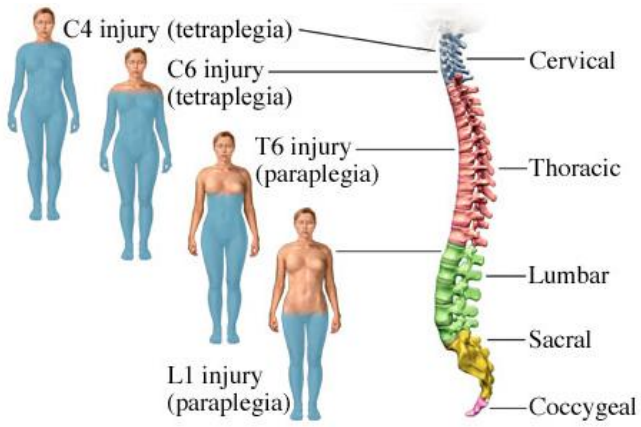


Figure 9 - Levels of injury and extent of paralysis. Injury to specific levels of the spine can cause spinal cord injuries. Varying degrees of nerve damage often result in paralysis bellow the lesion site. From: [44].

A spinal cord injury at the neck level may result in tetraplegia and impair the ability to breathe. Injuries to the lower spine may cause weakness and loss of sensation in the legs and lower parts of the body, and paraplegia (Figure 9). Complications such as loss of bladder and bowel control, increased risk for urinary tract infections, sexual dysfunction, skin breakdown and pressure sores, spasticity, inability or reduced ability to regulate heart rate, blood pressure and body temperature, autonomic dysreflexia, muscle atrophy, blood clots, osteoporosis and cardiovascular disease can result from injury and from the consequent reduced physical activity [41]. Also, the inability to maintain body weight within prescribed ranges occurs in a significant proportion of the human spinal cord injured population. In general, clinical reports indicate that spinal cord injured patients are at risk for a lifelong inability to maintain a neutral energy balance. Nutritional deficits leading to an underweight body mass in spinal cord injury present a number of risk factors to the individual in both the acute and chronic phases of injury. Low body weight increases the risk of developing infection and prolongs the recovery process from major traumatic injury. Furthermore, insufficient subcutaneous fat mass increases the risk of developing pressure ulcers. The presentation of recurrent pressure ulcers, in turn, is a comorbidity that triggers proinflammatory cytokine release, which can exacerbate the cachexic state of the patient [42].

The symptoms of spinal cord damage usually appear immediately after injury. However, if an infection or tumor is gradually increasing pressure on the spinal cord, they can develop slowly [41].

1.5.1 Bladder Dysfunctions

Bladder dysfunction is considered one of the top concerns among paraplegics and tetraplegics, usually of higher importance than the loss of locomotion [43]. The functions of the lower urinary tract to store and periodically release urine are regulated by a complex neural control system located in the brain and spinal cord, which functions like a switching circuit to maintain a reciprocal relation between the bladder, urethra and urethral sphincter [44]. This dependence on central nervous system control distinguishes the lower urinary tract from many other visceral structures that maintain a certain level of function even after elimination of extrinsic

neural input [45].

Voluntary micturition is regulated by a mechanism in the spinal and supraspinal neural pathways [44]. The central nervous system communicates with the lower urinary tract via three sets of peripheral nerves: sacral parasympathetic nerves that provide an excitatory input to the bladder and an inhibitory input to the urethra; lumbar sympathetic nerves that excite the urethra; and sacral somatic nerves that excite the urethral sphincter. Sympathetic and somatic pathways to the urethra and the sphincter are activated by spinal reflex mechanisms during urine storage. However, during voiding, parasympathetic excitatory input to the bladder, as well as inhibition of sympathetic and somatic control of the urethral outlet, are mediated by supraspinal mechanisms [46]. Spinal cord injury rostral to the lumbosacral level disrupts this voluntary and supraspinal control of voiding, leading to a marked reorganization of the micturition reflex pathways that coordinate bladder and sphincter function [44].

Previous studies have shown that chronic spinal cord injured rats exhibited impaired voiding, distended, hypertrophied bladders, and hypertrophied afferent neurons [47].

Spinal cord injury initially induces an areflexic bladder and urinary retention followed by the emergence of automatic micturition mediated by spinal reflex pathways. The urinary bladder becomes hypereflexic along with tonic activity of urethral sphincter. These lower urinary tract dysfunctions then produce various urological problems such as urinary incontinence, recurrent urinary tract infections, that can result in sepsis, and vesicoureteral reflux with or without upper urinary tract deterioration [44]. An additional consequence of spinal cord injury is a high intravesical bladder pressure, which can lead to damage and hypertrophy of the smooth muscle mass, accompanied by an enormous enlargement of the urinary bladder [48].

1.6 Animal Models of Spinal Cord Injury

There is an increased usage of experimental animal models of spinal cord injury. The aims of these models are to better understand the acute and chronic morphological, cellular and molecular consequences of spinal cord injuries and, with this information, develop and test new therapies to enhance anatomical repair and functional recovery. From a therapeutic point of view, even partial restoration of damaged spinal tracts

following spinal cord injury could result in improvements in respiratory function, upper/lower limb function, and bowel/bladder/sexual function [13]. The development of a rational approach for the treatment of traumatic spinal cord injury requires a standardized and reproducible animal model in which a quantifiable trauma can be correlated with functional recovery and morphology of the lesion [49]. Of the many traumatic spinal cord injury models, the most frequently used methods of injury include complete transection or hemissection (dorsal transection). Although these types of laceration injuries are not typically seen clinically, they can effectively disconnect both ascending and descending axonal pathways at designated levels of the spinal cord. This approach allows the study of mechanisms that govern the inhibition or successful regeneration of axons across or around injury as well as of the resulting functional deficits and potential recovery. Importantly, the severity and level of the spinal cord injury entirely dictate the applicability of quantitative assessments for impaired motor, sensory, and/or autonomic functions [50].

Mice and rats have been the models of choice for evaluating the effects of various procedures aimed at reducing damage to the spinal cord or at promoting axonal regeneration [49]. As experimental models they appear to be a satisfactory species for spinal cord studies, since they are able to tolerate the rigors of experimentation and have the further advantage of economy and availability [37].

Mice and rats are in many ways well suited to the assessment of nerve regeneration. However, they present special challenges because of their small size, their inherent capacity for regeneration, and the potential strain effects. For regeneration of interrupted fibers, histological and physiological measures have the advantages of allowing the direct correlation with restoration of function [51].

1.6.1 Mouse / Rat Spinal Cord

There are many differences between the nervous system of humans and that of the animals commonly used in spinal cord injury studies. Issues such as size, gait, neuroanatomical, neurophysiological and behavioral differences, as well as disparities in immunological and inflammatory responses following spinal cord injury, point to potential limitations of animal models when assessing the efficacy and safety of

possible spinal cord injury treatments in humans. The importance of size differences between animal spinal cord injury models and humans is reflected in the distances over which axons are required to regenerate after injury. This may affect comparisons between regeneration studies in rodents versus primates, especially with injuries to the cervical and thoracic spinal cord [13].

In contrast to humans, where the spinal cord has 31 segments, the rat spinal cord is made up of 34 segments: 8 cervical (C1 to C8), 13 thoracic (T1 to T13), 6 lumbar (L1 to L6), 4 sacral (S1 to S4), and 3 coccygeal (Co1 to Co3) [13].

1.7 The Conditioning Lesion Model

Primary sensory neurons with cell bodies in the dorsal root ganglion, that have a central axon that ascends in the dorsal column, provide an useful model system to study the mechanisms that regulate regeneration [35]. Dorsal root ganglion neurons have two axonal branches stemming from an unipolar axon, a peripheral branch that regenerates when injured and a central one that enters the central nervous system and does not regenerate [52]. Remarkably, injury to the peripheral branch before injury to the central branch promotes regeneration of central axons (Figure 10).

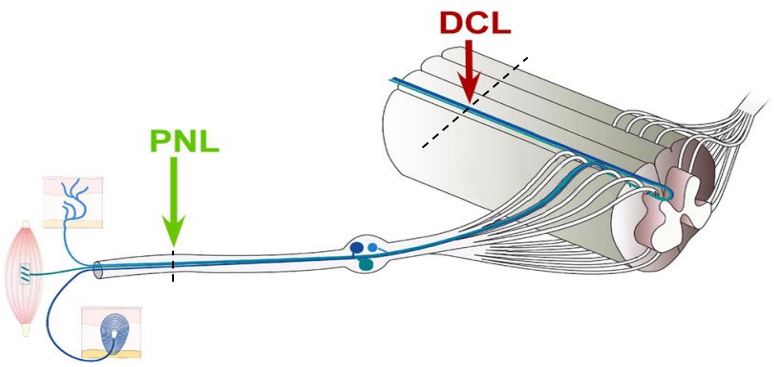


Figure 10 - Conditioning injury paradigm. Dorsal root ganglion neurons have two axonal branches; a long sensory central nervous system branch that ascends the dorsal column in the spinal cord and a second branch that projects trough a peripheral nerve. In the conditioning lesion, a peripheral nerve lesion (PNL) prior to the dorsal column lesion (DCL) leads to regeneration in the central nervous system branch. Adapted from [53].

This phenomenon known as Conditioning Lesion paradigm suggests that retrograde injury signals travel from the peripheral injury site back to the cell body to increase the

intrinsic growth capacity of the neuron [35]. Under these circumstances, not only does the peripheral axon regrow in the peripheral nervous system, but their central axon in the dorsal column of the central nervous system is also capable of regenerating (Figure 11) [53]. This conditioning effect has been documented in both sensory and motor neurons in the mammalian peripheral nervous system [35]. Although central regeneration is relatively meager (on the order, at top, of a few millimeters) this result has stimulated a renewed excitement on understanding the conditioning effect [36].

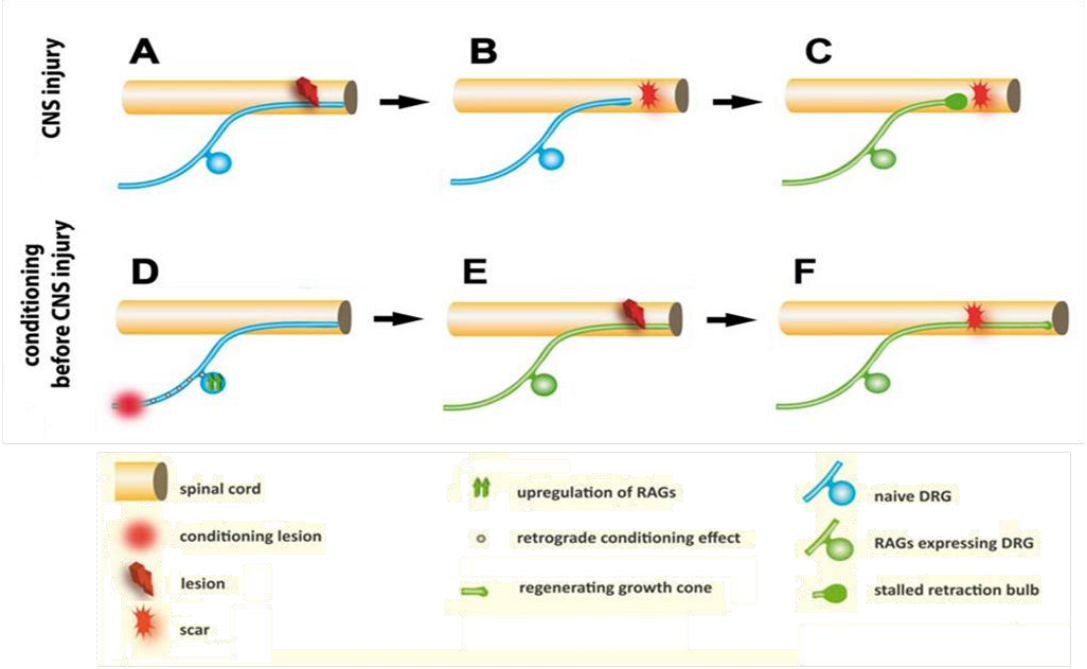


Figure 11 - Conditioning lesion effect. Sensory axons in the adult spinal cord do not regenerate after injury (A-C), while prior peripheral injury results in a robust regenerative response (D-E). Pre-conditioned neurons express RAGs and are growth competent prior to central injury (D). They start to regenerate immediately upon a central lesioning (E) and cross the injury site (F). Several experimental manipulations result in axonal regeneration in the central nervous system when applied before or at the time of injury, but not when initiated after a delay, which would be clinically more relevant. Adapted from [54].

Furthermore, other studies showed that dorsal root ganglion neurons could regenerate their central axons into peripheral nerve explants (inserted into the dorsal column of the spinal cord) only if the peripheral axon of the dorsal root ganglion had been previously cut. These observations suggested that the acquired regenerative competence of dorsal root ganglion neurons depended on some form of cell body activation in response to the peripheral injury. It is now known that axotomy of the peripheral axon, in contrast to axotomy of the central one, triggers altered expression of several RAGs in the dorsal root ganglion cell bodies [55].

Axonal regeneration is enhanced if second injury occurs 1-2 weeks after the intrinsic growth state has been increased by the prior conditioning lesion. Improved regeneration is characterized by accelerated outgrowth, a reduction in the lag time between the time of injury and the onset of elongation, and an increase in the number of regenerating sprouts. The intrinsic growth state increases substantially after the injury of peripheral branches, increases less effectively after dorsal root lesion, and does not increase at all after spinal cord injury [56].

Nevertheless, the cell body of injured neurons must receive accurate and timely information on the site and extent of axonal damage in order to orchestrate an appropriate response leading to successful regeneration. The conditioning lesion suggests that distinct signaling mechanisms regulate the responses to a central versus a peripheral injury that may contribute to the different abilities of axonal regrowth [35]. Several studies provided evidence for the existence of multiple injury signals functioning in a temporal sequence: injury-induced discharge of axonal potentials, interruption of the normal supply of retrogradely transported target-derived factors (also called negative injury signals) and retrograde injury signals traveling from the injury site back to the cell body (also called positive injury signals) (Figure 12)[35].

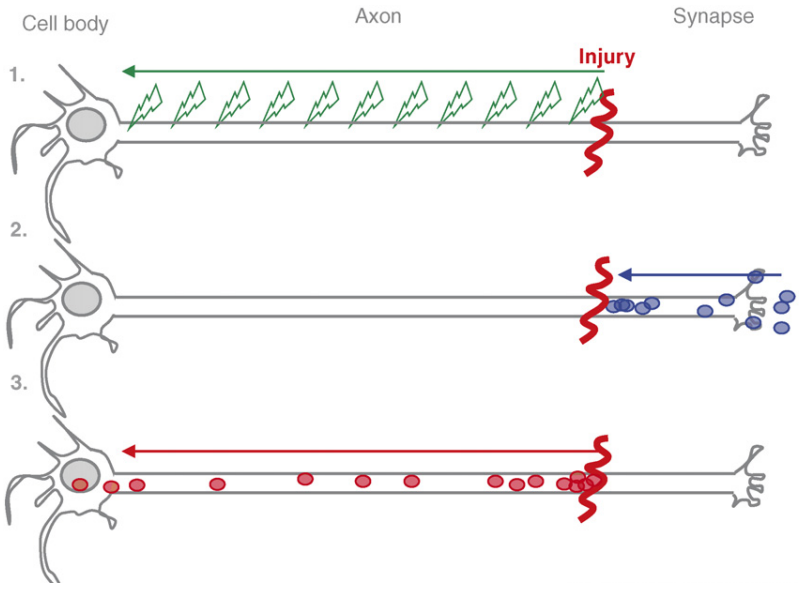


Figure 12 - Signaling mechanisms. Distinct signaling mechanisms may act in complementary and synergistic manners: (1) injury-induced discharge of axonal potentials, (2) interruption of the normal supply of retrogradely transported trophic factors or negative regulators of neuronal growth derived from the target, and (3) retrograde transport of activated proteins emanating at the injury site, termed positive injury signals. From: [35].

The retrograde transport of injury signals is one of the essential cellular mechanisms leading to regeneration. Coordination between several injury-signaling pathways is necessary to regulate the appropriate genes to promote neuronal survival and increase the intrinsic growth state of injured neurons [35]. Furthermore, neurons projecting into the peripheral nervous system respond to injury of their peripheral axon with characteristic changes in gene expression of transcription factors, cytoskeletal and cytoskeletal regulatory proteins, cell adhesion and axonal guidance molecules, trophic factors and cytokines (including their receptors), neuropeptides and neurotransmitter-synthesizing enzymes, ion channels, and membrane transporters [55].

1.8 The Glycogen Synthase Kinase - 3β Pathway in Axonal Regeneration

Development and regeneration of the nervous system requires the precise formation of axons and dendrites [57], in which biochemical signaling pathways are known to have a critical role [58]. A growing body of evidence is accumulating to suggest that signaling pathways also underlie neurodegeneration and neurodegenerative diseases [58]. Kinases and phosphatases are pervasive regulators of cellular function and have been implicated in controlling axodendritic development and regeneration. Aside from regulating neuronal differentiation, including axon formation and elongation, phosphorylation controls most cellular processes, including the cell cycle, proliferation, metabolism and apoptosis [57]. In addition, signaling pathways like MAPK, growth factor signaling, PIP3, cytoskeletal, and calcium-dependent pathways have been shown to impinge on or control neuronal process development [57].

Glycogen Synthase Kinase 3 (GSK-3) is a serine/threonine kinase that was first isolated and purified as a key enzyme involved in the glycogen synthesis. Beyond its role in glycogen metabolism and insulin signaling [59], GSK-3 plays a role in the transduction of regulatory and proliferative signals arising from out of the cells [60], and is thought to play an important role in critical physiological processes, such as the cell cycle, neuronal function, oncogenesis, apoptosis, and embryonic development [61]. The pathways in which GSK-3 acts as a key regulator have been implicated in the development of a wide variety of human diseases, such as diabetes, cancer,

inflammation, Alzheimer´s disease, schizophrenia, attention deficit hyperactivity disorder, and bipolar disorder [60]. Thus, it is not surprising that GSK-3 inhibitors are being actively developed as drugs for the treatment of various disorders [59].

In vertebrates, GSK-3 is found as two isoforms: GSK-3α and GSK-3β [59]. They both have similar functions; however they are not functionally redundant since deletion of GSK-3β leads to embryonic lethality due to hepatocyte apoptosis. Nonetheless, in certain pathways both of the isoforms are implicated, as is the case, in axon formation in hippocampal neurons [59]. Unlike other kinases, GSK-3 is constitutively active in cells and its activity, controlled by phosphorylation, is responsible for cell signaling [62].

Upstream signals downregulate its activity by phosphorylation at specific residues. The most important phospho-residues are Ser21 for GSK-3α and Ser9 for GSK-3β, which inhibit its kinase activity, while phosphorylation on Tyr residues (Tyr216 for GSK-3β and Tyr279 for GSK-3α), is required for its activation [63]. Structural and biochemical studies indicate that phosphorylated Ser9 acts as a pseudosubstrate for GSK-3β, by binding to Arg96, a residue that normally interacts with the phosphorylated site of primed substrates [58]. Numerous kinases, including the Ser/Thr protein kinase C (PKC) and Akt-1 [60] can phosphorylate GSK-3β at Ser9 . The ability of Akt to phosphorylate GSK-3β at its inhibitory site is intriguing, given the prominent role of the Akt signaling pathway in neuroprotection [58]. GSK-3 is also inhibited in response to secreted glycoproteins, termed Wnts, that function in a pathway that is crucial for the specification of cell fates during embryonic development [64]. Phosphorylation of the residue Tyr216 results in the constitutive activity of GSK-3β [62]. Physiological levels of calcium have been shown to increase Tyr216 phosphorylation of GSK-3β. In addition, proapoptotic stimuli increase GSK-3β activity through increased Tyr216 phosphorylation [58]. In mammalian cells, this seems to be an intramolecular autophosphorylation event that plays an important role in stabilizing the enzyme [64], and it is believed to be an important target for signal transduction [62]. Several studies provide evidence that activation of GSK-3β contributes to neuronal apoptosis and that the phosphatidylinositol 3-kinase (PI3K)/Akt pathway may protect neurons by inhibiting GSK-3β [58]. GSK-3β sits at the convergence of several signaling pathways that are critical for neuronal viability and proper function. Several apoptotic stimuli

appear to be involved in pathways that activate GSK-3β. Conversely, neuroprotective stimuli lead to an inactivation of GSK-3β. Prominent in this latter category is the PIK3/Akt pathway. Thus, GSK-3β activity appears to be correlated inversely with neuronal viability [58].

Its role in phosphorylation of cytoskeletal proteins impacts on neuronal plasticity, as cytoskeletal constituents are involved in the development and maintenance of neurites, and changes in the rate of stabilization/destabilization of microtubules could influence major cellular compartments of neurons, such as dendrites, spines, axons, and synapses [63]. It has been shown that Akt-1 and GSK-3β have multiple roles in axonal and dendritic development and establishment and maintenance of neuronal polarity [60].

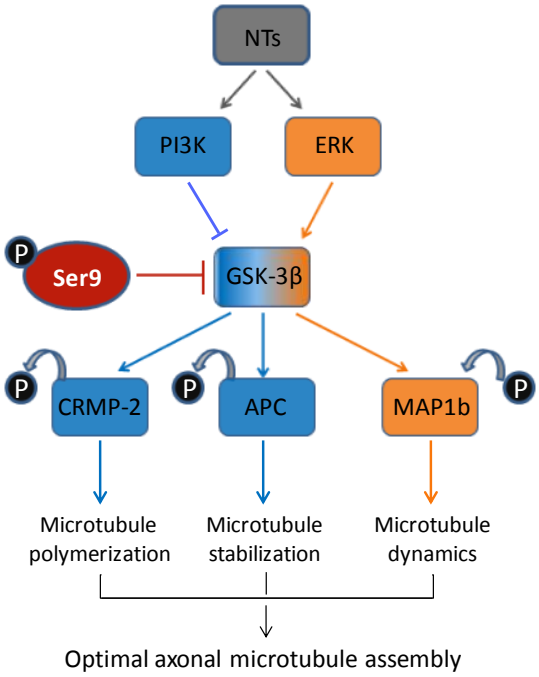


Figure 13 - Schematic representation of the essential aspects for axonal microtubule assembly in axons, regulated by GSK-3β: microtubule polymerization, stabilization and dynamics. To promote axonal assembly, neurotrophins inactivate GSK-3β by phosphorylation of Ser9 via the PI3K/Akt signaling pathway. Phosphorylation of GSK-3β allows dephosphorylation of CRMP-2 and APC, promoting microtubule polymerization and stabilization. Neurotrophins enhance the activity of GSK-3β toward MAP1b (independent of Ser9 phosphorylation) via the ERK pathway. Phosphorylation of MAP1b ensures maintenance of microtubule dynamics. Coordinated regulation of these pathways ensures optimal axonal microtubule assembly. Adapted from: [65]

GSK3 activity also depends on its cellular localization. Althoug GSK-3 is predominantly located in the cytosol, it is also present in nuclei and mitochondria, where it is highly activated when compared to the cytosolic pool. Nuclear GSK-3 regulates the expression of diverse genes via various transcription factors, such as Ap-1, β-catenin, c-myc, and p53. Subtle control of GSK3-mediated activation and inhibition is required to ensure proper balance among cell morphoregulation, proliferation, and growth. Prolonged inhibition of GSK-3 is associated with hypertrophic cell growth, while sustained activation is associated with neurodegeneration [63].

Microtubules are the principal cytoskeletal components of axons. Thus, control of microtubule polymerization and stability is a key regulatory step in axon growth during development and regeneration after nerve injury [65]. Given the critical function of GSK-3 in regulating axon genesis and elongation, modulation of GSK-3β activity may be an important strategy for developing successful therapies for central nervous system axonal injuries [66].

PART 2

INDUCTION OF THE INTRINSIC CAPACITY OF AXONAL REGENERATION THROUGH A CONDITIONING LESION

"The frog instantly dies when the spinal cord is pierced;
And previous to this it lived without head, without heart
or any bowels or intestines or skin; and here therefore
it would seem lies the foundation of movement and life".

(Leonardo da Vinci)

2.1 INTRODUCTION

Upon injury, regeneration of adult central nervous system axons is abortive. This lack of regeneration appears to be due to both a decreased intrinsic ability of central fibers to grow, and to the inhospitable environment [24]. In contrast to the central nervous system, after axonal injury, peripheral nerve fibres are able to regenerate [67]. A frequent injury occurring in the central nervous system is the spinal cord injury. This is a global concern affecting millions of people worldwide. Every year the number of patients who survive spinal cord injuries increase, leading thousands of people to initiate a daily routine of medical care.

Due to the inexistence of a cure, the identification of mechanisms that allow central nervous system regeneration is a major challenge in the neuroscience field.

To increase the knowledge in this area, the use of animal models of central nervous system injury is of major importance, allowing to test and identify mechanisms promoting axonal regeneration.

The conditioning lesion model has been widely used in our group. This model is described as being able to promote de intrinsic capacity of central nervous system axons to regrow upon injury. This model is based on the manipulation of sensory neurons in the dorsal root ganglion. These neurons possess two axonal branches: a peripheral axon that regenerates when injured and a centrally projecting axon that does not regenerate following injury. However, in this model, an injury to the peripheral branch promotes regeneration of the central axons [35].

In order to validate the conditioning lesion model in our laboratory and identify additional molecules capable of inducing regeneration, this model was evaluated and characterized.

2.2 OBJECTIVES

The main objective of this work was to evaluate and characterize the conditioning lesion model, in rats. This will allow the validation of this model in our laboratory and the future identification of new molecules/therapeutic strategies with the ability of increasing axonal regeneration. In order to achieve these goals we evaluated:

1. The functional recovery of rats post-surgery (either spinal cord injury or conditioning lesion), particularly the ability of the different groups to regain bladder control;
2. Axonal regeneration by retrograde labeling of ascending dorsal column tract axons through cholera toxin injection in the sciatic nerve.

2.3 MATERIAL AND METHODS

2.3.1 Animals and Animal Care

As animal models of injury, 8-10 weeks old Wistar rats were used. All animals were handled according to the European Communities Council Directive (86/609/EEC) and National rules and all procedures were approved by the Portuguese General Veterinarian Board. Animals were maintained at 22 ± 1°C under a 12 hr light/dark cycle and fed with regular rodent chow and tap water *ad libitum*. All surgical procedures were preceded by anesthesia with a Medetomidine and Ketamine mixture (0.5 mg/kg and 75 mg/kg body weight, respectively) via intra peritoneal injection. At the end of the study, or at humane endpoints, all animals were euthanized with a 20% Pentobarbital overdose. Humane endpoints were determined trough the visualization of persistent pain or distress. Prolonged abnormal posture, dyspnea, chronic weight loss (more than 20% after sciatic nerve injury or more than 25% after spinal cord injury), prolonged porphyrin staining, self-induced trauma, rupture of the urinary bladder and extended dehydration were also signs for humane endpoint. After death confirmation, skeletal remains were frozen and sent for incineration according to the dispatch 242/96 August 13.

2.3.2 Surgical Procedures and Post-Surgical Care

2.3.2.1 Sciatic Nerve Injury

Animals were anesthetized with a Medetomidine and Ketamine mixture (0.5 mg/kg and 75 mg/kg body weight, respectively) via intra peritoneal injection, and 3.0 mL of a 5% glucose saline solution was injected subcutaneously. In order to expose the sciatic nerve, the mid thigh was shaved and a 2.0 cm long incision was made. Local anesthesia (Articaine Hydrochloride / Epinephrine, 72 mg and 0.018 mg / 1.8 mL) was injected on the thigh (0.3 mL in case of unilateral lesion and 0.2 mL in each thigh in case of bilateral lesion). Transection with a microscissor or crush using Pean forceps, was performed in the sciatic nerve immediately distal from the sciatic notch. The animals were awaked with Atipamesol (5 mg/kg) and analgesia was performed through

subcutaneous injection of Butorphanol (1.0 mg/kg) twice a day for 48 hr following injury.

2.3.2.2 Spinal Cord Injury

Animals were anesthetized as previously described and kept warm with heating pads. In order to compensate for the diuresis caused by anesthesia, and for the blood loss, a fluid supplement of 3.0 mL of 5% glucose saline solution was subcutaneously injected. The skin was then shaved along the vertebral column and one incision was performed through the thoracic vertebras 6 to 11 (T6-11). Under a microscope, the vertebral column was exposed and with a bone rongeur, the thoracic vertebra 7 (T7) was removed followed by spinal cord hemisection with a microscissor. Post surgical treatment consisted in fluid therapy with daily subcutaneous injection of 5.0 mL of Duphalyte® and analgesia, every 12 hr, through subcutaneous injection of Butorphanol (1.0 mg/kg) for the next 72 hr after injury. Crede's manoeuvre (manual bladder voiding) was performed twice a day until the end of the experiment or until the animals recovered their bladder control. Wet food was placed in the cage floor and the drinking water, supplemented with 0.016% Enrofloxacin to prevent infections, was supplied in long nipple bottles.

2.3.2.3 Conditioning Lesion

Animals were subjected to sciatic nerve injury and one week later spinal cord injury was performed.

2.3.3 Assessment of Recovery After Surgery

The observation and evaluation of the animals was carried out by recording daily the body weight, by analyzing the presence of blood in the urine, and by assessment of recovery of bladder control and mobility. Score sheets were kept for all animals.

2.3.4 Assessment of Axonal Regeneration *in vivo*

Six weeks after spinal cord injury or conditioning lesion, animals were anesthetized and the left sciatic nerve was exposed as previously described. To label ascending regenerating axons, 2.0 µL of 1% Cholera Toxin β (CTβ, List Biologicals) was injected in the sciatic nerve distally of the sciatic notch. The skin was then sutured and analgesia was carried out as described for sciatic nerve injury procedures. Four days later, animals were anesthetized and transcardially perfused with 40.0 mL Phosphate Buffered Saline (PBS), followed by 80.0 mL of 4% Paraformaldehyde (PFA). The spinal cords were then collected, postfixed overnight at 4°C in 4% PFA and cryoprotected in a 30% sucrose solution in PBS for 48 hr, after which they were frozen and kept at -20°C until sectioning. In order to detect labeled regenerating neurons, each spinal cord was sliced through their sagittal plane. Spinal cords were embedded with OCT (Tissue-Tek® O.C.T. Compound) and serial 50 µm thick frozen sections were performed in a Cryostat (CM 3050S, Leica). Slices were serially collected into a 24 well plate and processed for anti-CTβ free floating immunohistochemistry. Briefly, endogenous peroxidase was inhibited with H_2O_2, and sections were then blocked in blocking buffer (5% Normal Rabbit Serum, 0.3% Triton-X100 in 0.1M Phosphate Buffer) for 1 hr at room temperature and incubated overnight at 4°C with anti-CTβ antibody (List Biologicals; 1:30,000 diluted in blocking buffer) followed by incubation in biotinylated rabit anti-goat IgG (Vector; 1:200 in blocking buffer). Antigen detection was performed with Extravidin Peroxidase (Sigma; 1:1000 in Triton-X100 Tris Buffered Saline) for 1 hr and development was done with diaminobenzidine (DAB) (Sigma). Sections were mounted with Chrome-Potassium Sulphate-gelatin solution, air dried overnight and stained with 1% Toluidine Blue.

2.4 RESULTS

2.4.1. Assessment of Recovery After Surgery

After surgery, animals were carefully daily monitorized. Parameters such as body weight, mortality rate, recovery of motor function of the hind limbs, and recovery of bladder control were recorded in scoring sheets.

From a total of 99 animals subjected to surgical procedures, 91 survived until the end of the study and only those were considered for data analysis. Animals included were grouped as described below (Table 2).

Table 2 - Distribution of the animals through the different surgical procedures.

Animals / Surgery	SCI	CL
Male	28	28
Female	25	10

2.4.1.1 The cause of death is independent from the surgery type

From the total 99 animals initially included in the study, 8 died within the first days after injury or even almost immediately after surgery (data not shown), resulting in a total mortality rate of 8%. Whereas in the spinal cord injury group 4% of the animals died, in the conditioning lesion group this rate was about 14%. These deaths were mostly due to bladder rupture at the time of manual voiding. Both in the spinal cord injury and in the conditioning lesion group, all the dead animals were male. In spinal cord injured animals, 7% of the males died before the end of the study (Figure 14A), while in conditioning lesion this number was 21% (Figure 14B). Although only males have died, there is no significant statistical value that correlates the animal's death with the type of surgery.

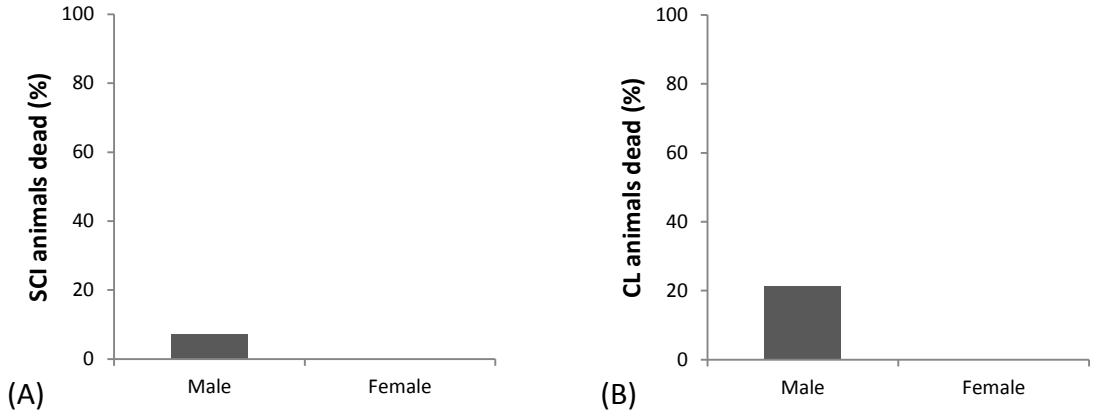


Figure 14 - Comparison between the percentage of males and females that died before the end of the study in spinal cord injury and conditioning lesion group. (A) Animals subjected to spinal cord injury (SCI). (B) Animals subjected to conditioning lesion (CL). Although only males have died, there is no statistical evidence that correlates their death with the injury type.

2.4.1.2 Recovery of motor function and bladder control are independent from the injury type

As a consequence of the spinal cord injury, all animals lost their hind limb motor function (data not shown). 68% of the animals from the spinal cord injury group and 47% of the animals from the conditioning lesion group recovered hind limb motor function until the end of the study (Figure 15).

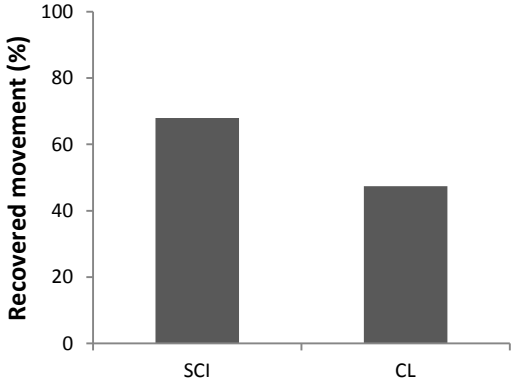


Figure 15 - Comparison between the percentage of animals that recovered the motor function after spinal cord injury (SCI) and conditioning lesion (CL).

A similar percentage of animals from the spinal cord injury group (40%) and from the conditioning lesion group (42%) recovered bladder control, (Figure 16A). However, it was among males that this recovery was more evident. 57% of the males recovered their bladder control after spinal cord injury whereas only 20% of the females regained this function (Figure 16B). In the conditioning lesion group the bladder control recovery was achieved only by males, representing a total of 57% of the animals (Figure 16C).

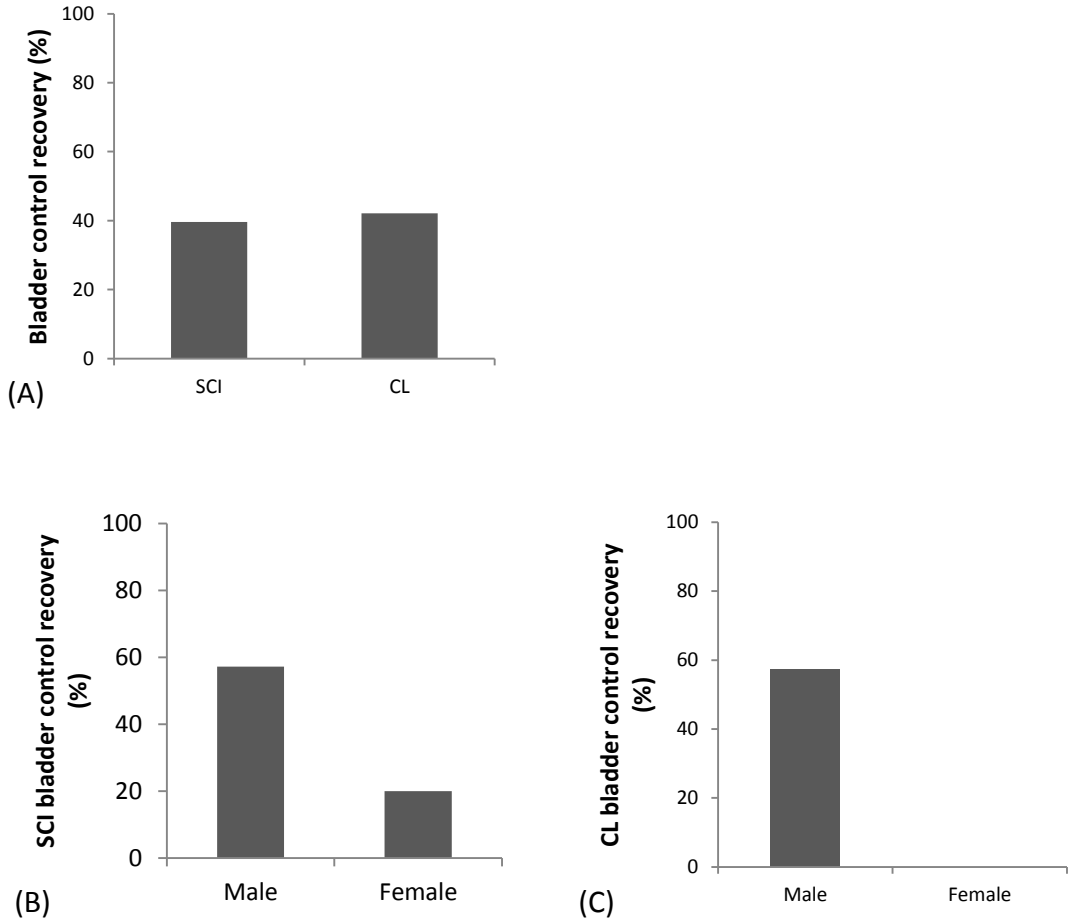


Figure 16 - Comparison between the percentage of animals that recovered bladder control. (A) Percentage of animals that recovered bladder control after spinal cord injury (SCI) and conditioning lesion (CL). (B) Animals subjected to spinal cord injury. (C) Animals subjected to conditioning lesion.

2.4.1.3 Animals did not present a significant weight loss both following spinal cord injury or conditioning lesion

An important value to consider after surgical procedures, indicative of recovery, is body weight. An animal losing weight is an animal with a diminished or even absent food intake, and is frequently an evidence that the animal´s welfare is compromised. Animals with chronic body weight loss are not maintained in the study and humane end points are applied.

Throughout the experiment none of the animals presented severe body weight loss. From a total of 91 animals included in the study, the majority didn´t lose more than 12% of body weight. There were even some animals that gained weight. Between injury type it was not observed a statistical difference in the body weight loss of the animals.

A significant difference in weight loss was however observed between males and females in the spinal cord injury group (Figure 17). Although this tendency was also observed in the conditioning lesion group, it had no statistical significance. With an average body weight of 285g, males lost about 7% of their weight, while in females, with 181g of average body weight, this value was of only 1%.

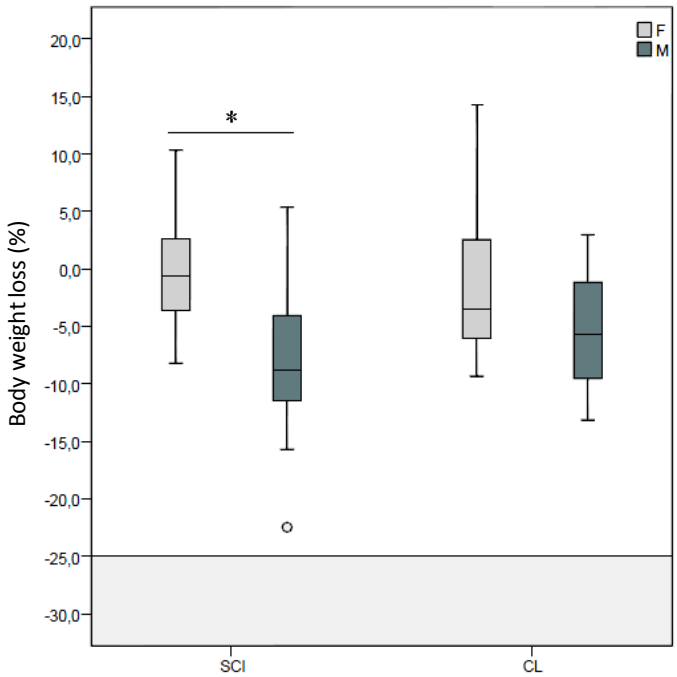


Figure 17 - Distribution of the animal's body weight loss. Results are presented as % of body weight loss in a total of 91 animals. Pearson´s Chi-Squared test: $p < 0.005$ (*).

2.4.2 Assessment of Axonal Regeneration

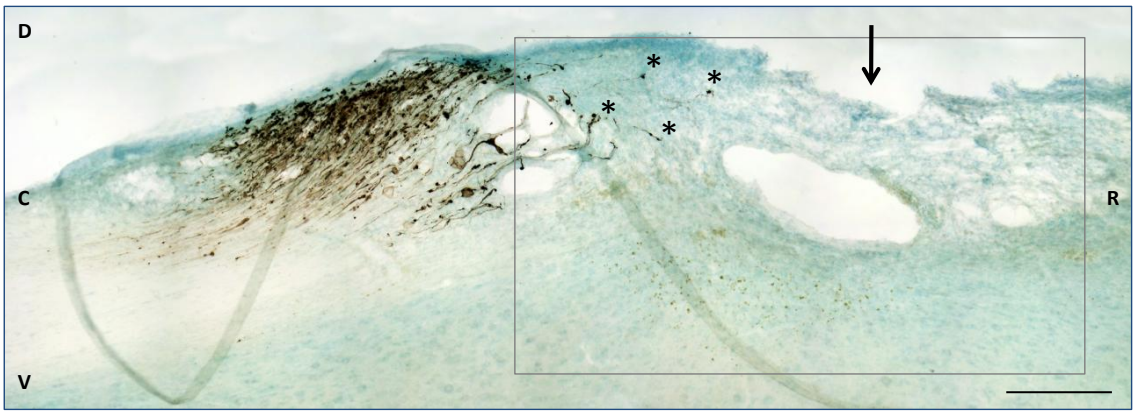
Several strategies are able to promote the intrinsic axonal regeneration capacity of the central nervous system after injury, as already described. In our lab, the conditioning lesion model has been widely used in order to identify molecules that might contribute to this increase.

2.4.2.1 Evaluation of axonal regeneration after conditioning lesion, in rats

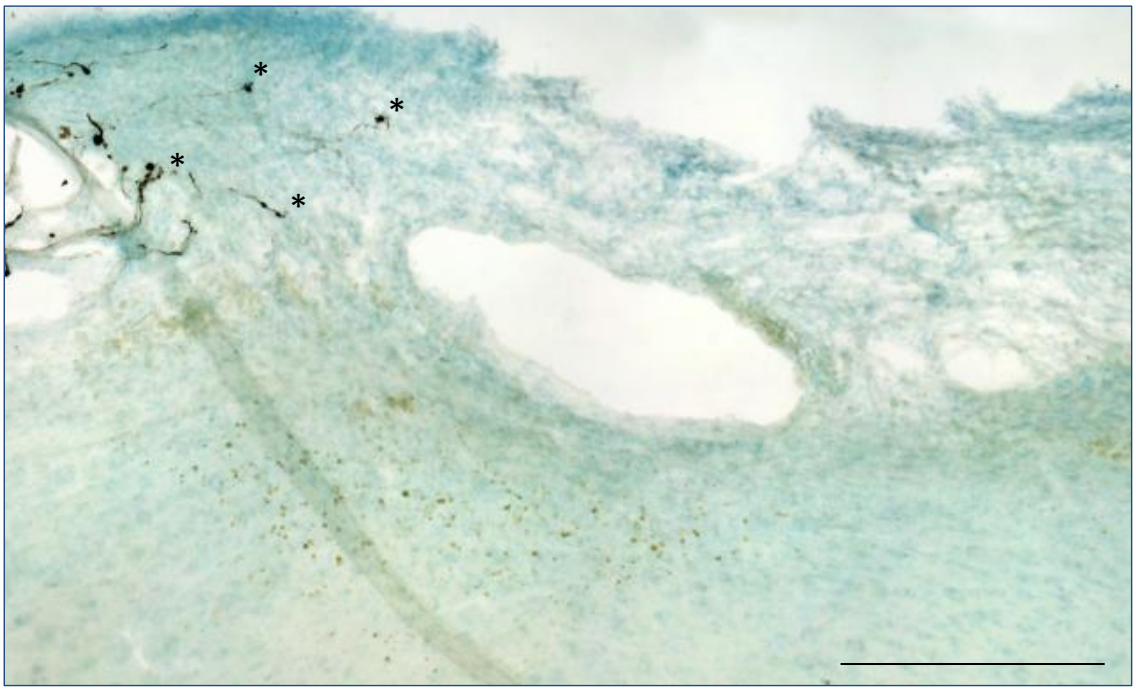
In order to assess axonal regeneration within the spinal cord, cholera toxin β was injected in the left sciatic nerve of animals with either spinal cord injury or conditioning lesion. Through immunohistochemistry, regenerating axons were assessed.

2.4.2.1.1 The conditioning lesion increases the intrinsic capacity of axonal regeneration in the central nervous system of rats

After transection, adult central nervous system axons are not able to regenerate and cross the glial scar formed following injury (Figure 18). However, if the spinal cord injury is preceded by one week by a lesion in the sciatic nerve, a reduced number of axons within the central nervous system gain regenerative capacity and are able to slightly cross the glial scar (Figure 19).

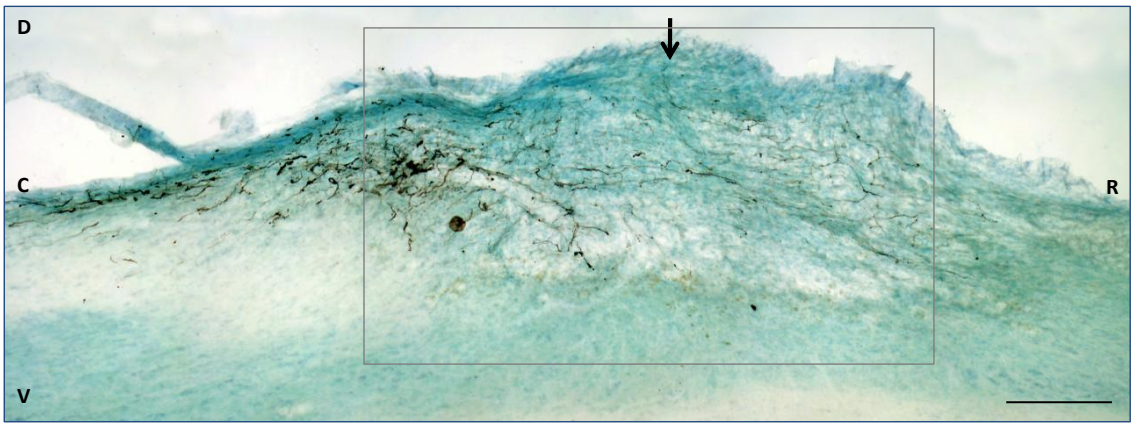


(A)

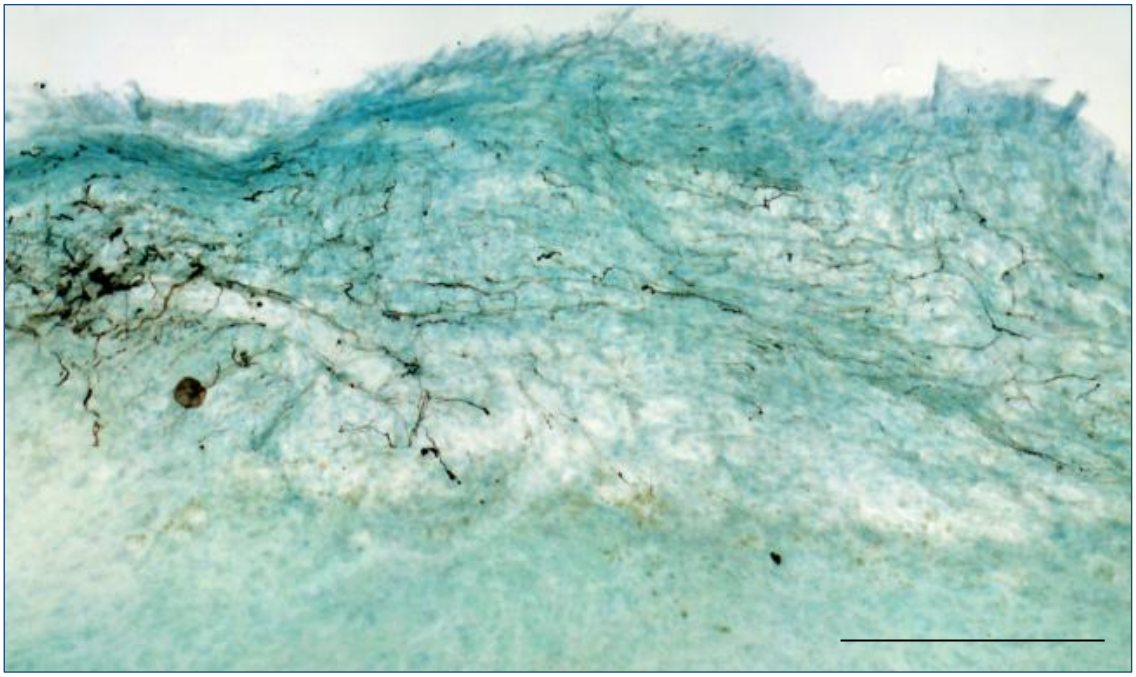


(B)

Figure 18 - Representative sagittal sections of the rat dorsal spinal cord after spinal cord injury. CTβ-immunolabeled axons are shown in dark brown. Lesioned axons (highlighted by*) fail to cross the lesion site. (A) Spinal cord section where the injury site is represented. D – dorsal, V – ventral, C – caudal, R – rostral. The arrow indicates the lesion site. (B) Zoom in on the lesion site. (Scale bar = 200 µm).



(A)



(B)

Figure 19 - Representative sagittal sections of the rat dorsal spinal cord after conditioning lesion. CTβ-immunolabeled axons through the lesion site are shown in dark brown. Regenerating axons succeeded in crossing the lesion site. (A) Spinal cord section where the injury site is represented. D – dorsal, V – ventral, C – caudal, R – rostral. The arrow indicates the lesion site. (B) Zoom in on the lesion site. (Scale bar = 200 μm).

2.5 DISCUSSION

Understanding the mechanisms responsible for the gain of regenerative capacity following conditioning lesion is one of the major goals of our group. Thus, the correct establishment, characterization and evaluation of this model is mandatory. To achieve this goal, Wistar rats were subjected to spinal cord injury or conditioning lesion. These animals were observed daily and their movement and bladder control recovery was assessed, as well as their body weight loss.

The first conclusion of our study was that we were able to successfully establish this procedure in our animal facility as in our experimental setup we had a 96% survival rate. Nevertheless, more animals died when subjected to conditioning lesion and only males were affected. These deaths were mostly related with bladder voiding and not to the surgery itself. Since the male urethra is longer and contains a prominent bend, it is not surprising that bladder voiding is more difficult, often resulting in bladder rupture and consequent dead. Surprisingly, more dead animals occurred in the conditioning lesion group (14%) when compared to the spinal cord injury group (4%). Nevertheless, the fact that these animals were subjected to more surgical procedures may justify this result, since their health status is more fragile after surgeries. The rate of animals that recovered motor function after spinal cord injury (68%) was higher than that of animals after conditioning lesion (47%). It should however be noted that as animals with conditioning lesion have a sciatic nerve injury their motor function cannot be directly compared to the spinal cord injury group where both sciatic nerves are intact. Another noteworthy result was the identical rate in the recovery of bladder control between the two groups (40% in spinal cord injured animals *vs* 42% in the conditioned lesion group). Although the bladder is not directly innervated by the axons affected by the conditioning lesion, if any type of regenerative plasticity would be enhanced by the conditioning lesion, one would expect that it would lead to a functional improvement in these animals.

A common consequence after spinal cord injury is weight loss [42]. Several reports indicate that a maximum acceptable body weight loss in rats subjected to spinal cord injury, is 25%. Above this value animals should be euthanized. With our procedures, however, none of the animals experienced this percentage of body weight loss.

Moreover, the body weight loss rate was equally distributed between animals subjected to either spinal cord injury or conditioning lesion. In fact, 24% of the animals subjected to these injuries gained weight. Thus, this is a good indication of the animal´s welfare after these surgical procedures.

Finally, through retrograde labeling of regenerating axons it was possible to confirm the differences between both injury models. After spinal cord injury, regenerating axons were not able to cross the glial scar formed at the lesion site. However, if the spinal cord injury is preceded by a lesion in the sciatic nerve, axons within the central nervous system gained regenerative capacity and were able to slightly cross the glial scar. Thus, we confirmed *in vivo*, the enhanced intrinsic capacity of axonal regeneration after a conditioning lesion. This effect was previously observed in our group, *in vitro*, using cultures of dorsal root ganglion neurons, where an increase in neurite length was seen in conditioned dorsal root ganglion neurons.

Finally, it is important to highlight that these surgeries are performed manually, thus a certain variation in, for example, the injury depth may occur.

PART 3

EVALUATION OF THE PARTICIPATION OF GSK-3β IN AXONAL REGENERATION

"The important thing in science
is not so much to obtain new facts as to
discover new ways of thinking about them".

(William Bragg)

3.1 INTRODUCTION

The reduced intrinsic growth capacity of adult central nervous system neurons and the poor environment for axon extension contribute greatly to the failure of central nervous system axonal regeneration [66]. However, if an injury to peripheral nervous system axons precedes the central nervous system injury, regeneration of central axons is increased [35]. To reveal the intrinsic mechanisms enabling central nervous system axonal regeneration after conditioning lesion, our group used two proteomic approaches that identified the GSK-3β pathway as being differentially regulated following conditioning lesion. GSK-3β has been described as a key regulatory kinase in the nervous system being regulated by phosphorylation. Under resting conditions, GSK-3β is constitutively active by phosphorylation of the Tyr216 residue, and phosphorylation of Ser9 leads to its inactivation. This inactivation has been correlated with the promotion of optimal axonal microtubule assembly [66]. In our model, Akt levels were increased following conditioning lesion (Akt leads to inactivation of GSK-3β by phosphorylation of Ser9), and the inactive form of GSK-3β, p-GSK3βSer9P, was also increased as expected due to the increase of Akt. Moreover, the phosphorylated form of CRMP-2 (a GSK-3β substrate) was decreased. This data suggests that the conditioning lesion leads to a reduction in GSK-3β activity that might be responsible for the increased regenerative capacity in this model.

However, the role of GSK-3β in axonal growth following injury remains controversial. Dill *et al*, reported that inhibition of GSK-3β overcomes growth suppression of central nervous system inhibitory substrates and promotes axonal regeneration and functional recovery *in vivo* after central nervous system injuries [66]. In contrast Alabed *et al* showed that GSK-3β is directly phosphorylated and inactivated by myelin-associated inhibitors contributing to the failure of regeneration in the central nervous system [68]. These findings raised doubt as to the possibility of GSK-3β inhibition being capable of promoting central nervous system regeneration following spinal cord injury. To elucidate the role that inactivation of GSK-3β plays, and the mechanism through which it is modulated during central nervous system axonal regeneration, we tested several injury models in homozygous GSK-3α/β Ser9Ala/Ser21Ala knockin mice [69]. In

these knockin mice, the phosphorylation sites Ser21 and Ser9 are changed to Ala [69], thus disabling the possibility of inactivating GSK-3 through phosphorylation.

3.2 OBJECTIVES

The main objective of this work was to assess the role of GSK-3β in axonal regeneration. In order to achieve this aim we evaluated:

1. The functional recovery of wild type and GSK-3β knockin mice post-surgery, particularly the ability of the different groups to regain bladder control;
2. Axonal regeneration by retrograde tracing of regenerating axons through cholera toxin injection in the sciatic nerve of GSK-3β knockin and wild type mice after spinal cord injury or conditioning lesion.

3.3 MATERIALS AND METHODS

3.3.1 Animals and Animal Care

All animals were handled according to the European Communities Council Directive (86/609/EEC) and National rules and all procedures were approved by the Portuguese General Veterinarian Board. Animals were maintained at 22 ± 1°C under a 12 hr light/dark cycle and fed with regular rodent chow and tap water *ad libitum*. All surgical procedures were preceded by anesthesia and at the end of the study all animals were euthanized and their skeletal remains were frozen and sent for incineration, according to the dispatch 242/96 August 13. Humane endpoints were determined trough the visualization of chronic pain or anomalous health status of the animal. Prolonged abnormal posture, respiratory distress, severe weight loss (more than 20%), continued porphyrin staining, self-induced trauma, rupture of the urinary bladder and persistent dehydration were also signs for humane endpoint.

As animal models of injury, 8-9 weeks old homozygous GSK-3α/β$^{21A/21A/9A/9A}$ knockin mice (referred to as GSK-3 KI from now on) were used and wild type heterozygous littermate animals were used as control group.

GSK-3 KI mice, kindly provided by Dario R. Alessi (MRC Protein Phosphorylation Unit, Dundee, Scotland) [69] were bred at our animal facilities with C57/Bl6 mice. Littermates were then crossed between them until the desired genotype was achieved. Animals were selected according to their genotype, which was confirmed at post natal day 10 (P10), from ear punch extracted DNA (performed at the CCgen service, IBMC) [69].

3.3.2 Surgical Procedures

3.3.2.1 Sciatic Nerve Injury

Animals were anesthetized with a Medetomidine and Ketamine mixture (1.0 mg/kg and 75.0 mg/kg body weight, respectively) via intra peritoneal injection, and 1.0 mL of a 5% glucose saline solution was injected subcutaneously. To expose the sciatic nerve, the left mid thigh was shaved and a 1.0 cm long incision was made. One drop of local

anesthesia (Articaine Hydrochloride / Epinephrine, 72 mg and 0.018 mg / 1.8 mL) was placed at the *fascia lata* over the sciatic nerve which was then sectioned immediately distally from the sciatic notch with a microscissor. The animals were awaked with Atipamesol (5 mg/kg) and analgesia was performed with subcutaneous injection of Butorphanol (1.0 mg/kg) twice a day for 48 hr following injury.

3.3.2.2 Spinal Cord Injury

Animals were anesthetized as mentioned above and kept warm with heating pads. To compensate for the diuresis caused by anesthesia, and for the blood loss, a fluid supplement of 1.0 mL of 5% glucose saline solution was subcutaneously injected. The skin was then shaved along the vertebral column and, under a microscope, one incision was made through the thoracic vertebras 6 to 11 (T6-11). The vertebral column was exposed and with a microscisor, the thoracic vertebra 7 (T7) was removed. A spinal cord hemisection was then performed with an ophthalmic scalpel. Post surgical treatment consisted in fluid therapy with daily subcutaneous injection of 0.5 mL of Duphalyte® and through subcutaneous injection of Butorphanol (1.0 mg/kg). Analgesia was applied every 12 hr for the next 72 hr after injury. Manual bladder voiding was performed twice a day until the end of the study or until animals recovered their bladder control. Wet food, supplemented with Anima Strath, was placed in the cage floor and the drinking water was supplied in long nipple bottles with 0.016% Enrofloxacin, to prevent infections.

3.3.2.3 Conditioning Lesion

Animals were subjected to sciatic nerve injury followed by spinal cord injury one week later.

3.3.2.4 Assessment of Axonal Regeneration in vivo

Six weeks after spinal cord injury or conditioning lesion, animals were anesthetized and the left sciatic nerve was exposed as previously described. To label ascending regenerating axons, 2.0 µL of 1% Cholera Toxin β (CTβ, List Biologicals) was injected in the left sciatic nerve distally of the sciatic notch. The skin was then sutured and

analgesia was carried out as described for sciatic nerve injury procedures. Four days later, animals were anesthetized and transcardially perfused with 20.0 mL PBS, followed by 50.0 mL of 4% PFA. The spinal cords were collected, postfixed overnight at 4°C in 4% PFA and cryoprotected in a 30% sucrose solution in PBS for 48 hr, after which they were frozen and kept at -20°C until sectioning. To detect labeled regenerating neurons, each spinal cord was sliced through their sagittal plane. Spinal cords were embedded with OCT (Tissue-Tek® O.C.T. Compound) and serial 50 µm thick frozen sections were performed in a Cryostat (CM 3050S, Leica). Slices were serially collected into a 24 well plate and processed for anti-CTβ free floating immunohistochemistry, as already described.

3.3.2 Assessment of Recovery After Surgery

The observation and evaluation of the animals was carried out by recording daily the body weight, by analyzing the presence of blood in the urine, and by assessment of recovery of bladder control and mobility. All data was recorded in scoring sheets.

3.4 RESULTS

3.4.1 Assessment of Recovery After Surgery

After surgery, a follow-up of all the animals was performed. Parameters such as body weight, mortality rate, recovery of functional movement of the posterior limbs, and recovery of bladder control, were taken into account and recorded daily in scoring sheets.

From a total of 31 animals subjected to surgical procedures, only 25 survived until the end of the study and were considered for data analysis. Animals included in the study were grouped as described below (Table 3).

Table 3 - Distribution of the animals through the different surgical procedures.

Surgery /Animals	**GSK3 KI**		**WT**	
	Male	Female	Male	Female
SCI	0	4	2	1
CL	3	5	1	9

3.4.1.1 Only males died and the majority of deaths occurred within GSK-3 KI animals subjected to conditioning lesion

As a consequence of the spinal cord injury, animals lose the motor function of their posterior limbs and lose bladder control. The lack of bladder voiding can lead to low urinary tract infections, and in extreme situations can result in bladder rupture. Daily manual bladder voiding was performed in all the animals, however, not all survived to this clinical condition. From the 31 animals subjected to spinal cord hemissection, 6 died within the first days after injury (data not shown). These deaths were related with bladder rupture either at the time of manual voiding or while the animals were freed in their cages. 25% of the GSK-3 KI animals died before the end of the study, while in wild type animals this percentage was 13% (Figure 20A). Among the dead GSK-3 KI animals, 75% of the deaths occurred after conditioning lesion (Figure 20B), while in the wild type animals 100% of deaths occurred after spinal cord injury (data not shown). All dead animals were males.

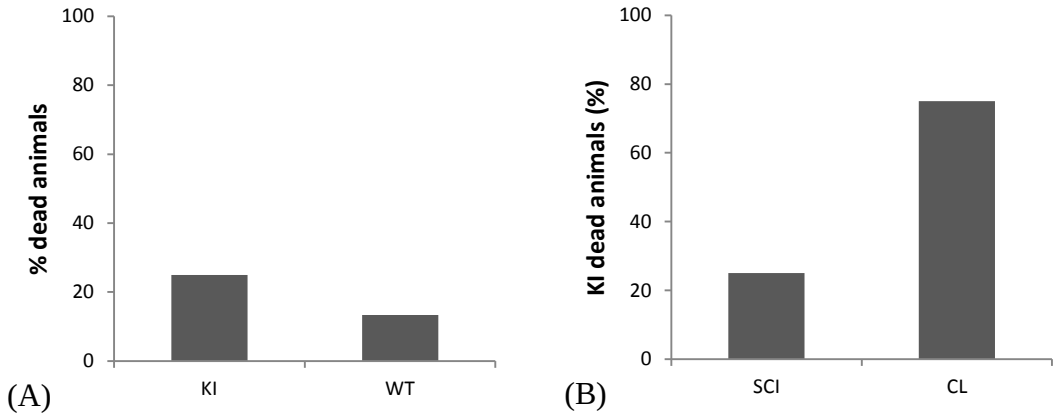


Figure 20 - Percentage of dead animals. (A) Comparison between the percentage of GSK-3 KI (KI) and wild type (WT) animals that died before the end of the study. (B) Percentage of GSK-3 KI animals that died after spinal cord injury (SCI) or conditioning lesion (CL).

3.4.1.2 Constitutively active GSK-3β did not influence the motor function recovery

Upon surgery, all the animals lost their hindlimb motor function (data not shown) which they recovered until the end of the study, irrespective of the fact that they were subjected to spinal cord injury or conditioning lesion and irrespective of their genotype (GSK-3 KI or wild type).

3.4.1.3 Bladder control recovery was independent from the genotype

Both GSK-3 KI and wild type animals had aproximately the same rate of recovery of bladder control (8.3% and 7.7% respectively, Figure 21A). However, within each genotype the only animals where bladder control was recovered were those subjected to conditioning lesion (data not shown). Among these, only females achieved this functional output (Figure 21B).

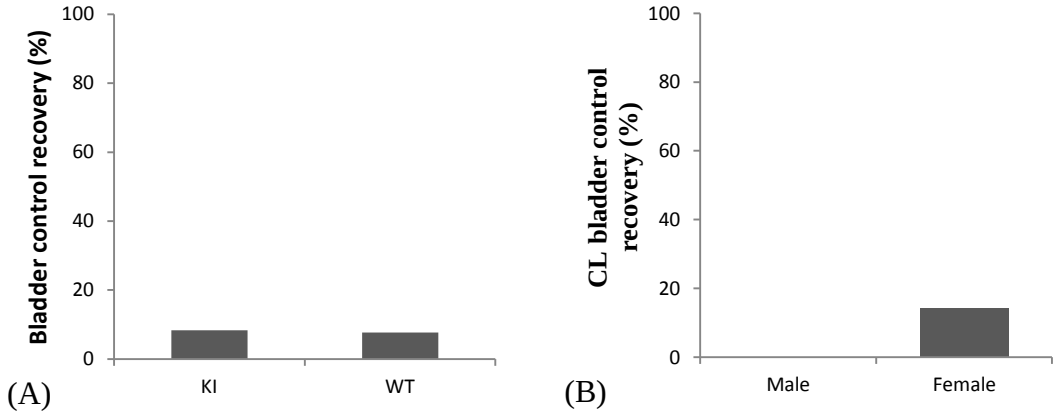


Figure 21 - Recovery of bladder control. (A) Bladder control recovery among GSK-3 KI (KI) and wild type (WT) animals. (B) Comparison between the recovery of bladder control among males and females.

3.4.1.4 Animals did not have significant weight loss

Body weight loss is an important value to determine whether animals are consuming food or not after the surgical procedure. This is one of the measures that can be indicative of the animal´s welfare. Severe body weight loss is often indicative of humane endpoint. If excessive body weight loss is observed, animals should be euthanized and removed from the study.

From the beginning of the experiment, none of the animals presented chronic body weight loss. From a total of 25 animals included in the study, none lost more than 20% of their body weight; 12% of the animals lost between 11-20%; 32% had a body weight loss between 6-10%; and 12% of the animals did not lose more than 5% of body weight. A small group of animals (4%) did not change their body weight from the beginning of the study (Figure 22A), while 20% gained weight. Whether animals were subjected to spinal cord injury or conditioning lesion, the amount of body weight loss was not significantly different (Figure 22B). Also no differences were found between GSK-3 KI and wild type animals (Figure 22C). However, males lost more body weight than females. With 20g of average body weight, females lost an average of 2.4%, while males, with 26g average body weight, lost an average of 7% of their body weight (Figure 22D).

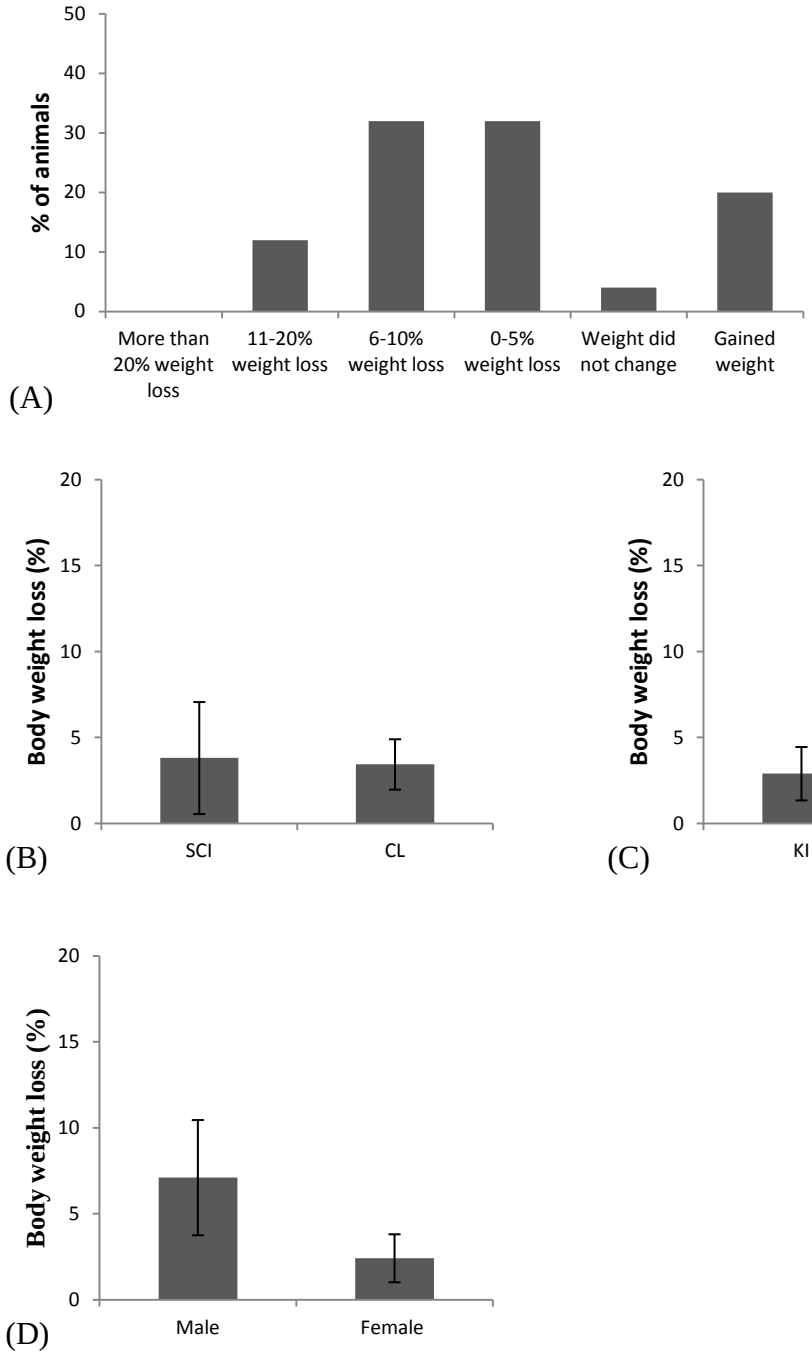


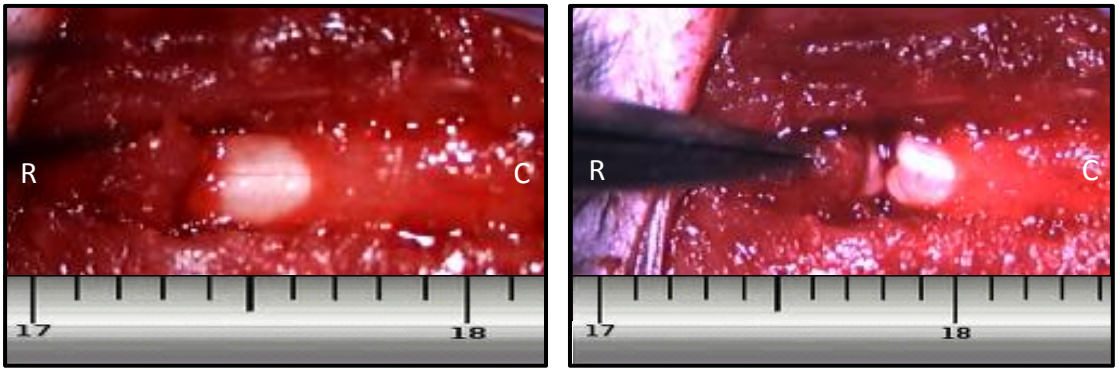
Figure 22 - Body weight loss. (A) Global body weight loss amongst all animals. Results are presented as % of animals in a total of 25 mice. (B) Average percentage of body weight loss between animals subjected to spinal cord injury (SCI) or conditioning lesion (CL). (C) Average percentage of body weight loss among GSK-3 KI (KI) and wild type (WT) animals. (D) Comparison between the percentage of body weight loss among males and females.

3.4.2 Assessment of Axonal Regeneration

Several strategies are able to promote the intrinsic axonal regeneration capacity of the central nervous system after injury. In our group, the conditioning lesion model has been widely used to identify molecules that might contribute to this increase.

3.4.2.1 Evaluation of Axonal Regeneration After Conditioning Lesion

In order to assess axonal regeneration within the spinal cord, cholera toxin β (CTβ) was injected in the left sciatic nerve of animals subjected to either spinal cord injury or conditioning lesion. The regenerating axons were visualized by immunohistochemistry against this toxin.



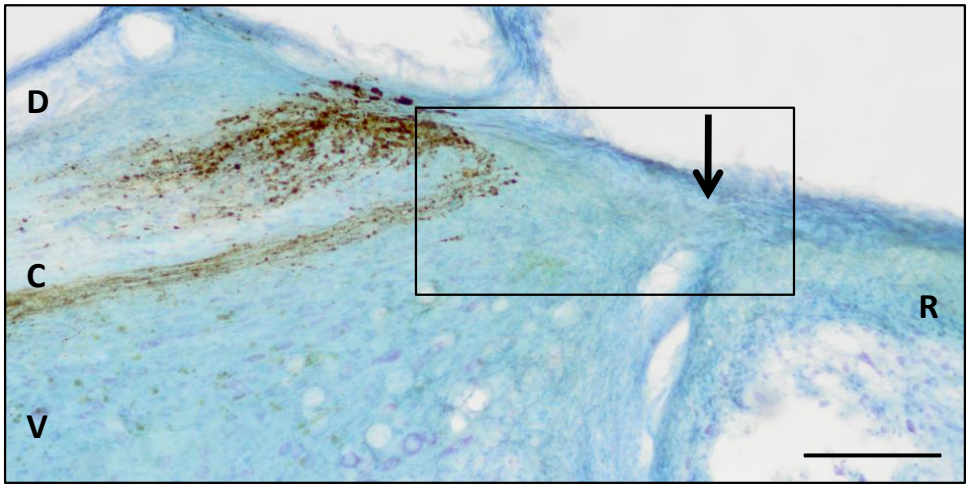
(A) (B)

Figure 23 - Mouse spinal cord injury surgery. (A) Exposed spinal cord, before injury. (B) Spinal cord immediately after hemissection. R – rostral; C – caudal.

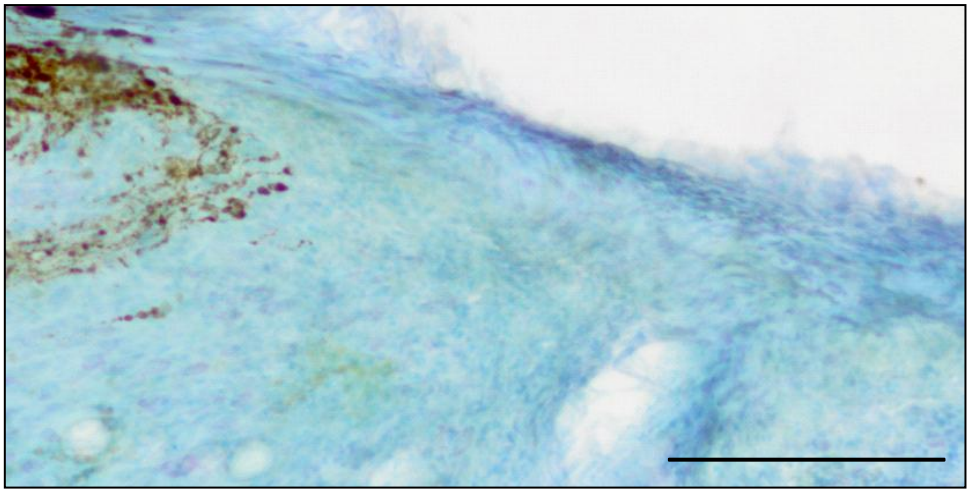
3.4.2.1.1 The conditioning lesion increases the intrinsic capacity of axonal regeneration in the central nervous system

After processing the tissues and performing anti-CTβ free floating immunohistochemistry, spinal cord sections were observed under a light microscope.

In wild type animals, after spinal cord injury, axons in the spinal cord are not able to cross the lesion site (Figure 24). However, if the spinal cord injury is preceded in one week by a lesion in the sciatic nerve, a reduced number of axons within the central nervous system gain regenerative capacity and are able to slightly cross the glial scar (Figure 25).

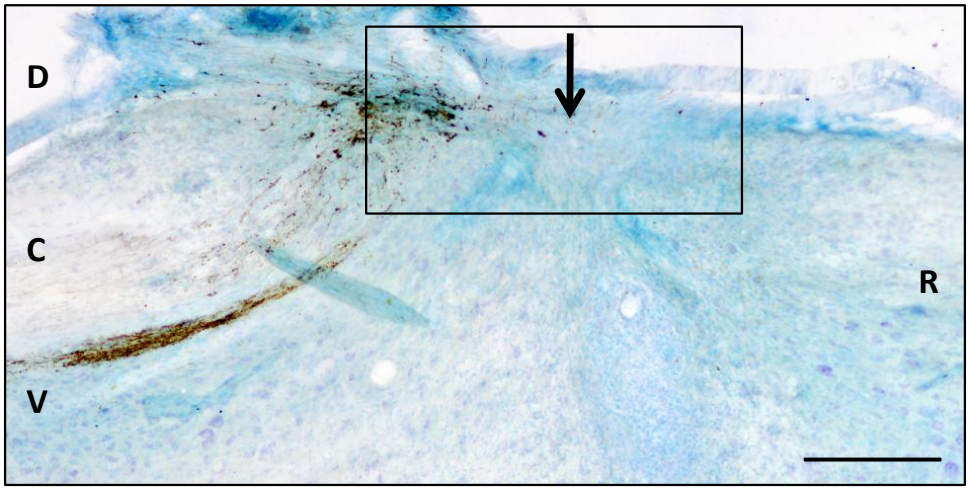


(A)

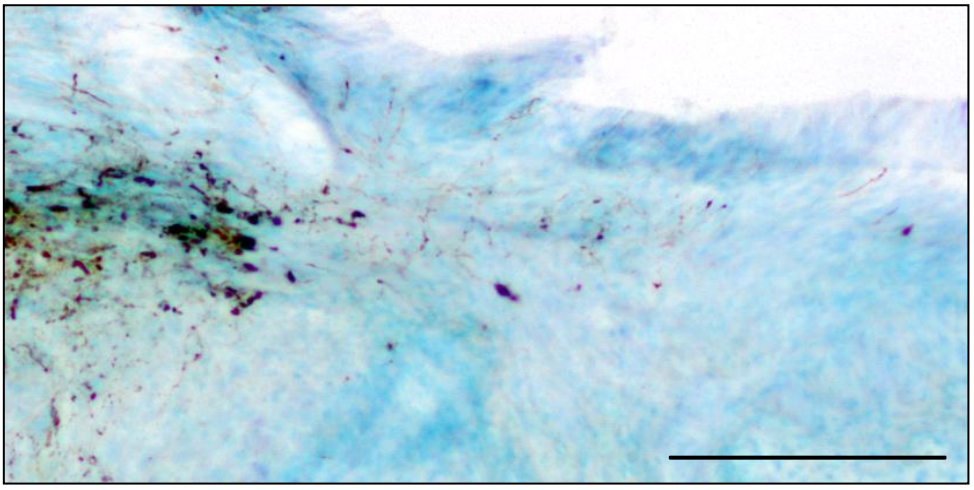


(B)

Figure 24 - Representative sagittal sections of the dorsal spinal cord, after spinal cord injury in a wild type mouse. Regenerating axons fail to cross the lesion site. (A) Spinal cord section where the injury site is represented. D – dorsal, V – ventral, C – caudal, R – rostral. (B) Zoom in on the lesion site. CTβ-immunolabeled axons are shown in dark brown. The arrow points to the lesion site. (Scale bar = 200 µm).



(A)



(B)

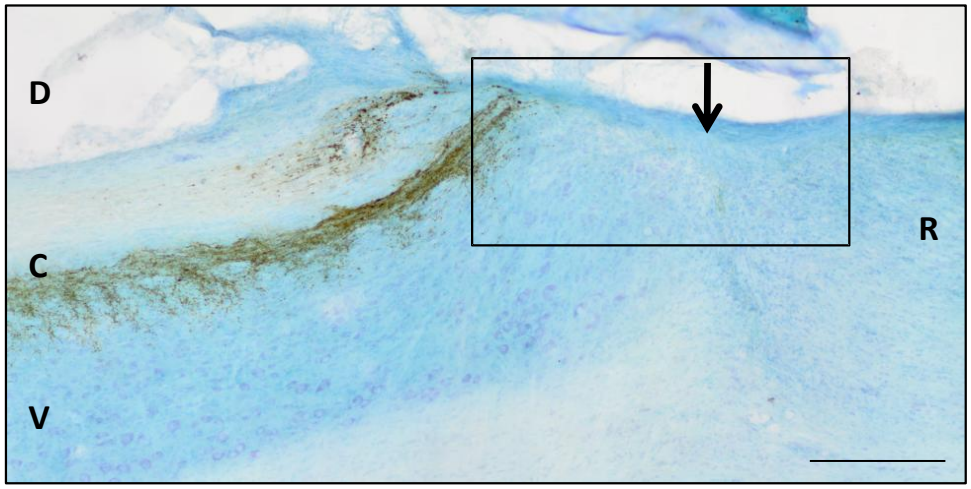
Figure 25 - Representative sagittal sections of the dorsal spinal cord, after conditioning lesion in a wild type mouse. Regenerating axons are able to cross the lesion site. (A) Spinal cord section where the injury site is represented. D – dorsal, V – ventral, C – caudal, R – rostral. (B) Zoom in on the lesion site. CTβ-immunolabeled axons are shown in dark brown. The arrow points to the lesion site. (Scale bar = 200 µm).

3.4.3 Evaluation of the Role of GSK-3β in Axonal Regeneration

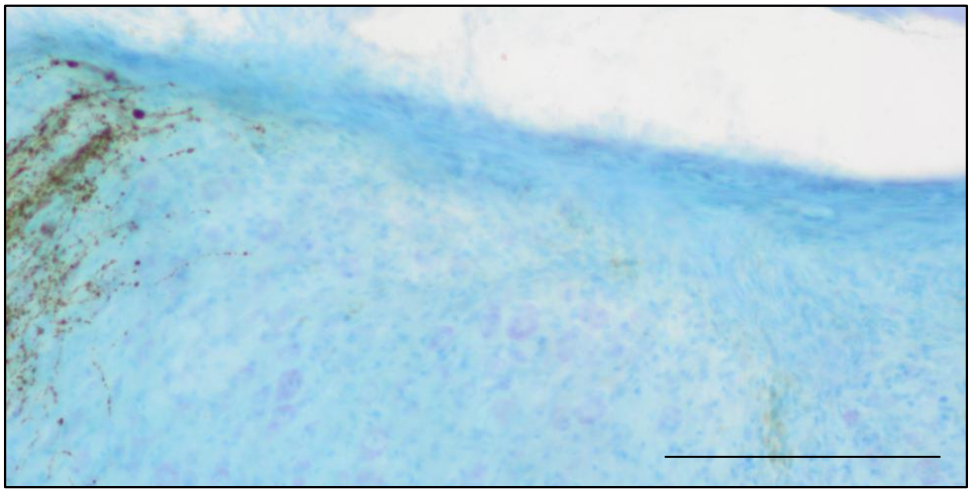
Several proteins belonging to the GSK-3β pathway were previously identified in our group as being modified in extracts of dorsal root ganglion, when compared in animals that suffered spinal cord injury or conditioning lesion. It was though that this group of proteins could be implicated in the gain of axonal regeneration capacity after conditioning lesion. To confirm the involvement of this kinase in axonal regeneration, animals with GSK-3β constitutively active (GSK-3β KI) were used and compared with animals where the kinase was not altered (wild type). Both genotypes were subjected to either spinal cord injury or conditioning lesion.

3.4.3.1 Constitutively active GSK-3β does not affect the increase in the intrinsic axonal regeneration capacity

After injury, axonal regeneration in the central nervous system is abortive, meaning that the ability of axons to regrow is diminished or even absent. The GSK-3β pathway is described as having major implications in the regulation of axonal growth. We observed that in GSK-3 KI mice after spinal cord injury, axons were not able to cross the glial scar (Figure 26). Additionally, after conditioning lesion, GSK-3 KI axons were still capable of growing through the glial scar (Figure 27).

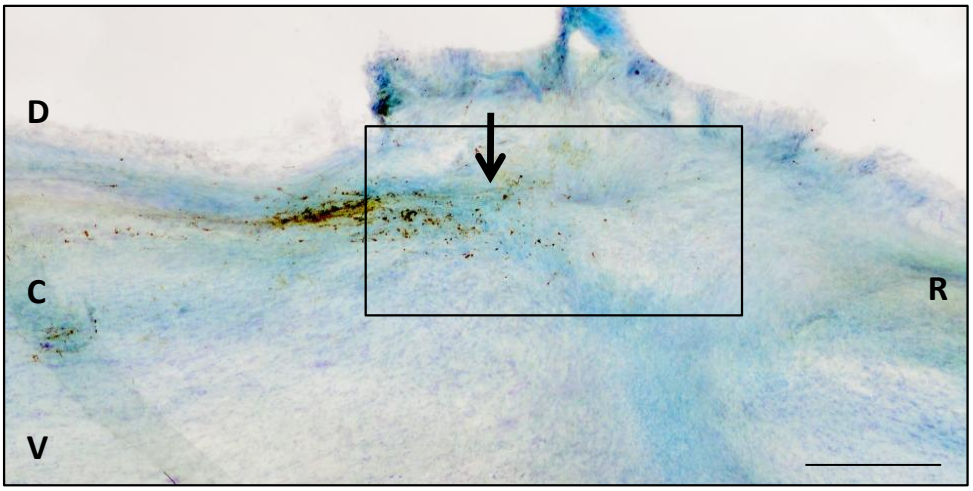


(A)

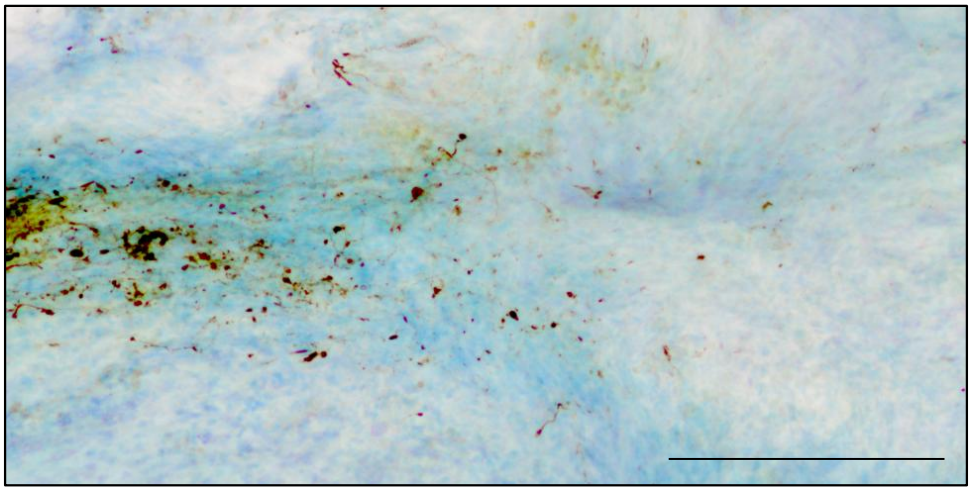


(B)

Figure 26 - Representative sagittal sections of dorsal spinal cord, after spinal cord injury in a GSK-3 KI mouse. Regenerating axons fail to cross the lesion site. (A) Spinal cord section where the injury site is represented. D – dorsal, V – ventral, C – caudal, R – rostral. (B) Zoom in on the lesion site. CTβ-immunolabeled axons are shown in dark brown. The arrow points to the lesion site. (Scale bar = 200 µm).



(A)



(B)

Figure 27 - Representative sagittal sections of the dorsal spinal cord after conditioning lesion in a GSK-3 KI mouse. Regenerating axons are able to cross the lesion site. (A) Spinal cord section where the injury site is represented. D – dorsal, V – ventral, C – caudal, R – rostral. (B) Zoom in on the lesion site. CTβ-immunolabeled axons are shown in dark brown. The arrow points to the lesion site. (Scale bar = 200 µm).

3.5 DISCUSSION

To elucidate the role that inactivation of GSK-3β plays in the promotion of central nervous system axonal regeneration, we tested spinal cord injury and conditioning lesion in GSK-3β KI mice where the Ser21 and Ser9 phosphorylation sites were changed to Ala [69], thus disabling the inactivation of GSK-3 through the phosphorylation in these residues. Furthermore, to understand if constitutively active GSK-3β would affect the functional recovery in these animals, daily records of their movement and bladder control recovery were assessed, as well as their body weight loss.

From the 31 animals initially subjected to surgical procedures, 25 survived until the end of the study with higher incidence among GSK-3 KI animals. Moreover, 75% of these deaths occurred after conditioning lesion. The fact that only males died can be justified by their urethra anatomical differences that introduce difficulties in the bladder voiding and, consequently, may result in easier bladder rupture that was in fact the major cause of death. The fact that the majority of these animals died after conditioning lesion can be a consequence of the increasing number of surgical procedures and not a consequence of the initial injury itself.

After injury in the spinal cord, although all the animals lost their motor function, also all recovered it until the end of the study. As such, the fact that in GSK-3 KI, GSK-3β is constitutively active has no effect on recovery. Although Dill *et al* suggested that pharmacological inhibition of GSK-3 would promote locomotor function recovery, we were not able to confirm these results in our study.

The recovery of bladder control was also very similar between GSK-3 KI and wild type animals (8.3 and 7.7% respectively). However only animals subjected to conditioning lesion have recovered this function.

After spinal cord injury body weight loss is a frequent consequence [42]. In mice, 20% of body weight loss is the maximum acceptable. During our study, none of the animals had this body weight loss. Moreover, the body weight loss rate was equally distributed between GSK-3 KI and wild type animals subjected to either spinal cord injury or conditioning lesion. Moreover, 64% of the animals did not lose more than 10% body weight and 20% of the animals have even gained weight, which is a good indication of the animal's welfare after these surgical procedures.

After spinal cord injury, regenerating axons are not able to cross the glial scar formed at the lesion site. However, after conditioning lesion, axons within the central nervous system are expected to gain regenerative capacity and to be able to cross the glial scar. In our study we confirmed the enhanced intrinsic capacity of axonal regeneration after a conditioning lesion also in mouse models. However, using the constitutively active GSK-3β KI mice, the same qualitative results were obtained. After spinal cord injury, axons remained unable to cross the glial scar, while after conditioning lesion axons were able to cross it. Moreover, these results were also confirmed in our group by *in vitro* cultures of GSK-3 KI dorsal root ganglion neurons, where an increase in neurite outgrowth was observed in conditioned dorsal root ganglion neurons from both GSK-3 KI and wild type animals (data not published). According to Dill *et al*, inhibition of GSK-3β trough Ser9 phosphorylation promotes axonal regeneration and functional recovery *in vivo* after central nervous system injuries [66]. On the other hand, Alabed *et al* reported that GSK-3β inactivation through Ser9 phosphorylation contributes to the failure of regeneration in the central nervous system [68]. However, our results show that the modulation of Ser9 phosphorylation does not affect axonal regeneration in the adult central nervous system, neither after spinal cord injury nor after conditioning lesion. Thus, our results show that the conditioning effect may occur independently of GSK-3β inactivation through Ser9 phosphorylation.

To further confirm the role of GSK-3β in central nervous system regeneration, we will use other transgenic GSK-3β mouse models, namely GSK-3β knockout (KO) heterozygous mice [70] (where GSK-3β activity is partially reduced) and the floxed GSK-3β mice [71] crossed to Thy1-cre mice [72], where GSK-3β activity in neurons is ablated. With these animals we will determine if a diminished GSK-3β activity has a central role in the conditioning lesion effect.

PART 4

GENERAL CONCLUSIONS

"…the facts have not yet been sufficiently ascertained.
And if at any future time they are ascertained,
then credence must be given to the direct evidence rather
than to the theories; and to the theories also, provided that
the results which they show agree with what is observed".

(Aristotle)

GENERAL CONCLUSIONS

Spinal cord injury is a devastating condition affecting the life of millions of people worldwide. Due to the number of societal and economical implications that central nervous system injuries have on society, there has been an increasing interest to find therapeutic approaches able to revert the abortive axonal regeneration in the adult vertebrate central nervous system.

In our study, several animal models were used to evaluate and characterize a conditioning lesion model. Our strategy resulted in an improvement in the intrinsic capacity to promote axonal regeneration in the central nervous system. Moreover, we established this procedure very successfully as our animals had a very small mortality rate. As such, we can now widely apply this model to test new therapeutic approaches to identify additional molecules responsible for the gain of regenerative capacity. Nonetheless, the higher number of males dying during the recovery process can justify a preference to use females in these studies whenever possible.

Moreover, we also established retrograde labeling of regenerating dorsal column axons as a good approach to assess, *in vivo*, axonal regeneration.

Daily, there are new evidences that the regenerative ability of adult central nervous system axons is not totally lost. With the current advances in modern medicine and the increased interest in central nervous system axonal regeneration, we hope to contribute to transform a previously assumed "*ailment not to be treated*" in a treatable one.

PART 5

REFERENCES

"In science the credit goes to the man who convinces the world, not to the man to whom the idea first occurs".

(William Osler)

REFERENCES

1. Anders, H. and L. Richard, *Spinal Cord Injury*, I. Oxford University Press, Editor 2010, Oxford University Press, Inc.: Oxford New York. p. 3-4.
2. van Middendorp, J.J., G.M. Sanchez, and A.L. Burridge, *The Edwin Smith papyrus: a clinical reappraisal of the oldest known document on spinal injuries.* Eur Spine J, 2010. **19**(11): p. 1815-23.
3. Lifshutz, J. and A. Colohan, *A brief history of therapy for traumatic spinal cord injury.* Neurosurg Focus, 2004. **16**(1): p. E5.
4. Eltorat, I.M., *History of Spinal Cord Medicine.* Spinal Cord Medicine: principles and practice, 2003(I): p. 5-10.
5. Nieuwenhuys, R., J. Voogd, and C. van Huijzen, *The Human Central Nervous System.* 4th Edition. ed2007: Springer. 177-178.
6. Hanigan, W.C. and C. Sloffer, *Nelson's wound: treatment of spinal cord injury in 19th and early 20th century military conflicts.* Neurosurg Focus, 2004. **16**(1): p. E4.
7. Kao, C.C., *Comparison of healing process in transected spinal cords grafted with autogenous brain tissue, sciatic nerve, and nodose ganglion.* Exp Neurol, 1974. **44**(3): p. 424-39.
8. Ragnarsdóttir. *The treatment and cure of spinal injury*. Parliamentary Assembly 2001 [cited 2011 June 20]; Available from: http://assembly.coe.int/Main.asp?link=/Documents/WorkingDocs/Doc01/EDOC9154.htm.
9. Cripps, R.A., et al., *A global map for traumatic spinal cord injury epidemiology: towards a living data repository for injury prevention.* Spinal Cord, 2011. **49**(4): p. 493-501.
10. *Spinal Cord Injury Facts and Figures at a Glance*. National Spinal Cord Injury Statistical Center 2011 [cited 2011 June 20]; Available from: https://www.nscisc.uab.edu/public_content/pdf/Facts%202011%20Feb%20Final.pdf.
11. *Spinal Cord Injury Statistics*. BrainandSpinalCord.org 2011 [cited 2011 June 20]; Available from: http://www.brainandspinalcord.org/spinal-cordinjury/statistics.htm.
12. Nolte, J., *The Human Brain. An Introduction to Its Functional Anatomy.* 6th Edition. ed2009., Philadelphia.: Mosby Elsevier. 1-2.
13. Watson, C. and e. all., *The Spinal Cord.*, in *A Christopher and Dana Reeve Foundation Text and Atlas.*2009., Elsevier. p. 1-2.
14. Herbrandson, C. *Nervous tissue*. Learning the Nervous System 2005 [cited 2011 July 20]; Available from: http://academic.kellogg.edu/herbrandsonc/bio201_mckinley/Nervous%20System.htm.
15. *Peripheral Nervous System*. Students Corner [cited 2011 July 17]; Available from:http://sciencecity.oupchina.com.hk/biology/student/glossary/img/peripheral_nervous_system.jpg.
16. Purves, D., et al., *Neuroscience*, 2004, Sinauer Associates, Inc.: U.S.A.
17. Thuret, S., L.D. Moon, and F.H. Gage, *Therapeutic interventions after spinal cord injury.* Nat Rev Neurosci, 2006. **7**(8): p. 628-43.
18. McDonald, J.W. and C. Sadowsky, *Spinal-cord injury.* Lancet, 2002. **359**(9304): p. 417-25.

19. Tetzlaff, W., K. Fouad, and B. Kwon, *Be careful what you train for.* Nat Neurosci, 2009. **12**(9): p. 1077-9.
20. He, Z. *Intrinsic control of axon regeneration*. Journal of Biomedical Research, 2010. **24**, 2-5.
21. Horner, P.J. and F.H. Gage, *Regenerating the damaged central nervous system.* Nature, 2000. **407**(6807): p. 963-70.
22. Webb, A.A., S. Ngan, and J.D. Fowler, *Spinal cord injury I: A synopsis of the basic science.* Can Vet J, 2010. **51**(5): p. 485-92.
23. Giger, R.J., E.R. Hollis, and M.H. Tuszynski, *Guidance molecules in axon regeneration.* Cold Spring Harb Perspect Biol, 2010. **2**(7): p. a001867.
24. Zurn, A.D. and C.E. Bandtlow, *Regeneration failure in the CNs: cellular and molecular mechanisms.* Adv Exp Med Biol, 2006. **557**: p. 54-76.
25. Sun, F. and Z. He, *Neuronal intrinsic barriers for axon regeneration in the adult CNS.* Curr Opin Neurobiol, 2010. **20**(4): p. 510-8.
26. Vargas, M.E. and B.A. Barres, *Why is Wallerian degeneration in the CNS so slow?* Annu Rev Neurosci, 2007. **30**: p. 153-79.
27. Fenrich, K. and T. Gordon, *Canadian Association of Neuroscience review: axonal regeneration in the peripheral and central nervous systems--current issues and advances.* Can J Neurol Sci, 2004. **31**(2): p. 142-56.
28. Fitch, M.T. and J. Silver, *CNS injury, glial scars, and inflammation: Inhibitory extracellular matrices and regeneration failure.* Exp Neurol, 2008. **209**(2): p. 294-301.
29. Liu, L., M. Rudin, and E.N. Kozlova, *Glial cell proliferation in the spinal cord after dorsal rhizotomy or sciatic nerve transection in the adult rat.* Exp Brain Res, 2000. **131**(1): p. 64-73.
30. Fawcett, J.W. and R.A. Asher, *The glial scar and central nervous system repair.* Brain Res Bull, 1999. **49**(6): p. 377-91.
31. Kimura-Kuroda, J., et al., *An in vitro model of the inhibition of axon growth in the lesion scar formed after central nervous system injury.* Mol Cell Neurosci, 2010. **43**(2): p. 177-87.
32. Silver, J. and J.H. Miller, *Regeneration beyond the glial scar.* Nat Rev Neurosci, 2004. **5**(2): p. 146-56.
33. Cao, Z., et al., *The cytokine interleukin-6 is sufficient but not necessary to mimic the peripheral conditioning lesion effect on axonal growth.* J Neurosci, 2006. **26**(20): p. 5565-73.
34. Bareyre, F.M., et al., *The injured spinal cord spontaneously forms a new intraspinal circuit in adult rats.* Nat Neurosci, 2004. **7**(3): p. 269-77.
35. Abe, N. and V. Cavalli, *Nerve injury signaling.* Curr Opin Neurobiol, 2008. **18**(3): p. 276-83.
36. Silver, J., *CNS regeneration: only on one condition.* Curr Biol, 2009. **19**(11): p. R444-6.
37. Taoka, Y. and K. Okajima, *Spinal cord injury in the rat.* Prog Neurobiol, 1998. **56**(3): p. 341-58.
38. Carlstedt, T., *Perspectives on the treatment of the longitudinal spinal cord injury.* Front Neurol, 2010. **1**: p. 11.
39. Aguilar, J., et al., *Spinal cord injury immediately changes the state of the brain.* J Neurosci, 2010. **30**(22): p. 7528-37.

40. Raineteau, O., et al., *Reorganization of descending motor tracts in the rat spinal cord.* Eur J Neurosci, 2002. **16**(9): p. 1761-71.
41. Wu, L.K. and X. Wang. *Stem cell treatment for SCI*. Wu Stem Cells Medical Centre 2011 [cited 2011 August 30].
42. Primeaux, S.D., M. Tong, and G.M. Holmes, *Effects of chronic spinal cord injury on body weight and body composition in rats fed a standard chow diet.* Am J Physiol Regul Integr Comp Physiol, 2007. **293**(3): p. R1102-9.
43. Leung, P.Y., C.S. Johnson, and J.R. Wrathall, *Comparison of the effects of complete and incomplete spinal cord injury on lower urinary tract function as evaluated in unanesthetized rats.* Exp Neurol, 2007. **208**(1): p. 80-91.
44. Yoshimura, N., *Bladder afferent pathway and spinal cord injury: possible mechanisms inducing hyperreflexia of the urinary bladder.* Prog Neurobiol, 1999. **57**(6): p. 583-606.
45. de Groat, W.C., *A neurologic basis for the overactive bladder.* Urology, 1997. **50**(6A Suppl): p. 36-52; discussion 53-6.
46. de Groat, W.C., et al., *Developmental and injury induced plasticity in the micturition reflex pathway.* Behav Brain Res, 1998. **92**(2): p. 127-40.
47. Yu, X., et al., *Effect of spinal cord injury on urinary bladder spinal neural pathway: a retrograde transneuronal tracing study with pseudorabies virus.* Urology, 2003. **62**(4): p. 755-9.
48. Apodaca, G., et al., *Disruption of bladder epithelium barrier function after spinal cord injury.* Am J Physiol Renal Physiol, 2003. **284**(5): p. F966-76.
49. Rossignol, S., *Locomotion and its recovery after spinal injury.* Curr Opin Neurobiol, 2000. **10**(6): p. 708-16.
50. Onifer, S.M., A.G. Rabchevsky, and S.W. Scheff, *Rat models of traumatic spinal cord injury to assess motor recovery.* ILAR J, 2007. **48**(4): p. 385-95.
51. Griffin, J.W., et al., *Measuring nerve regeneration in the mouse.* Exp Neurol, 2010. **223**(1): p. 60-71.
52. Neumann, S. and C.J. Woolf, *Regeneration of dorsal column fibers into and beyond the lesion site following adult spinal cord injury.* Neuron, 1999. **23**(1): p. 83-91.
53. Enes, J., et al., *Electrical activity suppresses axon growth through Ca(v)1.2 channels in adult primary sensory neurons.* Curr Biol, 2010. **20**(13): p. 1154-64.
54. Ylera, B., et al., *Chronically CNS-injured adult sensory neurons gain regenerative competence upon a lesion of their peripheral axon.* Curr Biol, 2009. **19**(11): p. 930-6.
55. Plunet, W., B.K. Kwon, and W. Tetzlaff, *Promoting axonal regeneration in the central nervous system by enhancing the cell body response to axotomy.* J Neurosci Res, 2002. **68**(1): p. 1-6.
56. Hoffman, P.N., *A conditioning lesion induces changes in gene expression and axonal transport that enhance regeneration by increasing the intrinsic growth state of axons.* Exp Neurol, 2010. **223**(1): p. 11-8.
57. Buchser, W.J., et al., *Kinase/phosphatase overexpression reveals pathways regulating hippocampal neuron morphology.* Mol Syst Biol, 2010. **6**: p. 391.
58. Kaytor, M.D. and H.T. Orr, *The GSK3 beta signaling cascade and neurodegenerative disease.* Curr Opin Neurobiol, 2002. **12**(3): p. 275-8.

59. Rayasam, G.V., et al., *Glycogen synthase kinase 3: more than a namesake.* Br J Pharmacol, 2009. **156**(6): p. 885-98.
60. Prickaerts, J., et al., *Transgenic mice overexpressing glycogen synthase kinase 3beta: a putative model of hyperactivity and mania.* J Neurosci, 2006. **26**(35): p. 9022-9.
61. Simón, D., et al., *Pharmacological inhibition of GSK-3 is not strictly correlated with a decrease in tyrosine phosphorylation of residues 216/279.* J Neurosci Res, 2008. **86**(3): p. 668-74.
62. Phukan, S., et al., *GSK3beta: role in therapeutic landscape and development of modulators.* Br J Pharmacol, 2010. **160**(1): p. 1-19.
63. Salcedo-Tello, P., A. Ortiz-Matamoros, and C. Arias, *GSK3 Function in the Brain during Development, Neuronal Plasticity, and Neurodegeneration.* Int J Alzheimers Dis, 2011. **2011**: p. 189728.
64. Cohen, P. and M. Goedert, *GSK3 inhibitors: development and therapeutic potential.* Nat Rev Drug Discov, 2004. **3**(6): p. 479-87.
65. Zhou, F.Q. and W.D. Snider, *CELL BIOLOGY: GSK-3beta and Microtubule Assembly in Axons.* Science, 2005. **308**(5719): p. 211-214.
66. Dill, J., et al., *Inactivation of glycogen synthase kinase 3 promotes axonal growth and recovery in the CNS.* J Neurosci, 2008. **28**(36): p. 8914-28.
67. Stoll, G. and H.W. Müller, *Nerve injury, axonal degeneration and neural regeneration: basic insights.* Brain Pathol, 1999. **9**(2): p. 313-25.
68. Alabed, Y.Z., et al., *GSK3 beta regulates myelin-dependent axon outgrowth inhibition through CRMP4.* J Neurosci, 2010. **30**(16): p. 5635-43.
69. McManus, E.J., et al., *Role that phosphorylation of GSK3 plays in insulin and Wnt signalling defined by knockin analysis.* EMBO J, 2005. **24**(8): p. 1571-83.
70. Hoeflich, K.P., et al., *Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation.* Nature, 2000. **406**(6791): p. 86-90.
71. Tanabe, K., et al., *Genetic deficiency of glycogen synthase kinase-3beta corrects diabetes in mouse models of insulin resistance.* PLoS Biol, 2008. **6**(2): p. e37.
72. Young, P., et al., *Single-neuron labeling with inducible Cre-mediated knockout in transgenic mice.* Nat Neurosci, 2008. **11**(6): p. 721-8.