Universidade do Minho
Escola de Ciências da Saúde

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Combining neuroprotective agents: effect of riluzole and magnesium in a rat model of thoracic spinal cord injury

Combinação de agentes neuroprotetores: efeito do riluzole e magnésio num modelo animal de lesão medular torácica

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DECLARAÇÃO

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The only way is forward

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RESUMO

Danos infligidos na medula espinal podem resultar em deficiências irreversíveis e perda total de funções motoras, sensoriais e autonómicas. Os fármacos riluzole e magnésio têm sido amplamente investigados como agentes neuroprotetores em modelos animais de lesão vertebro-medular. Dado que estes fármacos protegem a medula lesionada através de mecanismos diferentes, um estudo foi desenvolvido com o objetivo de determinar se a sua eficácia neuroprotetora poderia ser cumulativa. Um ensaio *in vivo* foi definido utilizando ratos fêmeas Wistar Han submetidos a uma contusão torácica da medula espinal (T8). Uma hora após a lesão, os animais foram distribuídos aleatoriamente para receber: 1) solução salina, 2) riluzole (2,50 mg / kg), 3) cloreto de magnésio (24,18 mg / kg) numa formulação de polietilenoglicol, ou 4) um tratamento combinado (riluzole e magnésio). Os tratamentos subsequentes foram dados em quatro injeções intraperitoneais (com espaçamento de 12 horas). Foram utilizados a escala de classificação locomotora Basso, Beattie e Bresnahan, um teste de campo aberto (open-field) e um teste de natação, para avaliar a recuperação comportamental/motora dos animais durante um período de quatro semanas. Foi também realizada uma análise histológica das medulas espinais de forma a medir a extensão do volume da lesão, preservação das fibras axonais, serotonérgicas e glutamatérgicas, sobrevivência de neurónios motores e inflamação. Os resultados mostraram que apenas o tratamento com riluzole melhorou significativamente a recuperação funcional, preservou tecidos e concomitantemente obteve volumes de lesão reduzidos, assim como aumentou a preservação das fibras serotonérgicas e axonais na parte caudal da medula espinal. O tratamento combinado, embora visando simultaneamente dois mecanismos relacionados com desequilíbrios iónicos e excitotoxicidade, não resultou em melhorias funcionais e histológicas, quando comparado com a administração isolada de riluzole.

Palavras-Chave: Lesões vertebro-medulares, citotoxicidade, neuroproteção, riluzole, magnésio

ABSTRACT

Damage to the spinal cord can result in irreversible impairments and complete loss of motor, sensory and autonomic functions. Riluzole and magnesium have been widely investigated as neuroprotective agents in animal models of spinal cord injury. As these drugs protect the injured spinal cord through different mechanisms we aimed to investigate if their neuroprotective efficacy could be cumulative. An *in vivo* experiment was set using female Wistar Han rats that underwent a thoracic spinal cord contusion (T8) using a weight drop method. An hour after injury, animals were randomly distributed to receive: 1) saline, 2) riluzole (2.50 mg/kg), 3) magnesium chloride (24.18 mg/kg) in a polyethylene glycol formulation, or 4) a combined treatment (riluzole and magnesium). Subsequent treatments were given in four intraperitoneal injections (spaced 12 h apart). The Basso, Beattie, and Bresnahan locomotor rating scale, an activity box test, and a swimming test were used to evaluate behavioral recovery for periods up to four weeks. Histological analysis of the spinal cords was performed to measure the extent and volume of the lesion, axonal preservation, serotonergic and glutamatergic fiber sparing, motor neuron survival, and inflammation. The results demonstrated that only the riluzole treatment significantly improved behavioral recovery, promoted tissue sparing, reduced lesion volume, while increasing serotonergic fiber sparing and axonal preservation in the caudal portion of the spinal cord. The combined treatment, although simultaneously targeting ionic and excitotoxic-related mechanisms, did not further improve behavioral and histological outcome, when compared with riluzole given alone.

Keywords: Spinal cord injury, cytotoxicity, neuroprotection, riluzole, magnesium

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LIST OF ABBREVIATIONS

°C - celsius
5-HT - 5-hydroxytryptamine
µm - micrometer
µmol - micromole

A

AIS - ASIA Impairment Scale
ALS - Amyotrophic Lateral Sclerosis
AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA - analysis of variance
ASIA - American Spinal Injury Association
ATP - adenosine-5'-triphosphate
ATPases - adenosine 5'-triphosphatases
AUC - area under the curve

B

BBB - Basso, Beattie and Bresnahan locomotor test
BDNF - brain-derived neurotrophic factor
BSCB - blood-spinal cord barrier

C

C - cervical vertebra
Ca^{2+} - calcium
cm - centimeter
C_{max} - peak concentration
CNS - central nervous system
CST - corticospinal tract
CT - computed tomography

D

d - day
DCX - doublecortin
DNA - deoxyribonucleic acid

E

EAA - excitatory amino acid
EAAT - EAA transporter

F

FDA - Food and Drug Administration
FL - forelimb

G

g - gram
GDNF - glial cell-derived neurotrophic factor

H

h - hour
H^+ - hydrogen
H&E - hematoxylin and eosin
HL - hindlimb

I

IL - interleukin
ip - intraperitoneal
iv - intravenous

K

K^+ - potassium
kdyn - kilodyne
Kg - kilogram

L

L - lumbar vertebra

M

MAP2 - microtubule associated protein 2
MDA - malondialdehyde
mg - milligram
Mg^{2+} - magnesium
$MgCl_2$ - magnesium chloride
$MgSO_4$ - magnesium sulfate
min - minute
ml - milliliters

mm - millimeter
mM - millimolar
MP - methylprednisolone
MRI - magnetic resonance imaging

N

n - total number of data points
Na^+ - sodium
NASCIS - National Acute Spinal Cord Injury Study
NeuN - neuronal marker
NMDA - N-methyl-D-aspartate
NMDAR - NMDA receptor
NGF - neurotrophin nerve growth factor
NF - neurofilament
NO - nitric oxide

O

OCT - optimal cutting temperature
OECs - olfactory ensheathing cells

P

PBS - phosphate-buffered saline
PEG - polyethylene glycol
PFA - paraformaldehyde
PNS - peripheral nervous system

R

ROS - reactive oxygen species
RT - room temperature

S

s - second
S - sacral vertebra
SC - subcutaneous
SCI - Spinal Cord Injury
SEM - standard error of the mean
SSEPs - somatosensory evoked potentials

T

T - thoracic vertebra
TBI - Traumatic Brain Injury
TGF- β - transforming growth factor-β
TNF-α - tumor necrosis factor-α
TTX - tetrodotoxin

U

USA - United States of America

V

VGLUT - marker for glutamatergic neurons
VGSC - voltage-gated sodium channels

W

w - week

CHAPTER 1 - INTRODUCTION

1. INTRODUCTION

Trauma to the spinal cord can cause severe damage to nervous tissue leading to temporary or permanent changes in the spinal cords normal motor, sensory, or autonomic function (Hagen, 2015). Spinal cord injury (SCI) patients usually suffer permanent neurologic deficits and disability that heavily impact their physiological, psychological, and social behavior (Craig et al., 2009; 2012; Krueger et al., 2013).

In developing countries, the annual incidence of SCI was estimated in 25.5 cases per million (Rahimi-Movaghar et al., 2013). In 2012, the estimated annual incidence of SCI in the United States of America (USA) was 54 cases per million, while in Portugal, the only epidemiological study was performed by Martins et al. and set the annual incidence rate at 57.8 new cases per million inhabitants (Jain et al., 2015; Martins et al., 1998). Although SCI incidence rates are not very high, it certainly places substantial financial burden on health care systems mainly due to the long-term effects of disability and handicap that persist throughout the patient's life. In 2006, costs related to SCI patients healthcare were estimated at 9.7 billion dollars per year in the USA alone (Thompson et al., 2015).

Injury to the spinal cord can result from traumatic events like contusion, compression and/or laceration, while non-traumatic injuries usually results from tumors, spinal stenosis and vascular events (New & Marshall, 2014; Thuret et al., 2006). Leading causes of injury include automobile crashes, falls, as well as sport related injuries, affecting mainly young adult males (Chen et al., 2013; Jain et al., 2015).

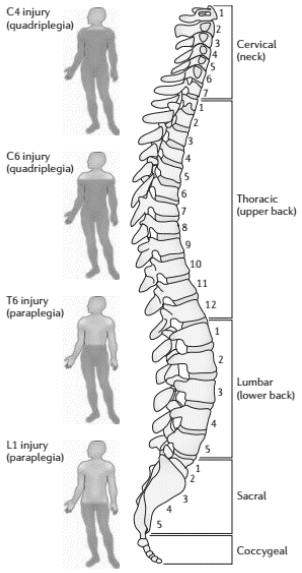
The neurological impairment of SCI is commonly classified in different grades of severity according to the American Spinal Injury Association (ASIA) Impairment Scale (Table I). Grading relies on sensory and motor evaluation of the SCI patient at the S4-S5 sacral segment, at 72 hours (h) post-injury, allowing to determine whether the injury is complete (AIS A: no sensation or motor function at S4-S5), or incomplete (AIS B to D: motor and/or sensory function is partially preserved at S4-S5) (Kirshblum et al., 2011; Scivoletto et al., 2014). Furthermore, the level of injury is an important factor concerning survival, with a more cephalad level associated with higher rates of mortality (Wilson et al., 2012). Respiratory failure and cardiovascular dysfunction are the main cause of early mortality in complete, high-cervical (C1-C4) injuries of SCI (Shao et al., 2011).

Methylprednisolone has been widely used as the only standard therapeutic agent for SCI treatment. However, its clinical utility and role in SCI recovery remains controversial (Bydon et al., 2014). In light of this, a recent survey revealed a significant decrease in the number of surgeons using high-dose

steroids in 2014 as compared with 2006, clearly revealing an urgent need to develop alternative therapeutic strategies (Schroeder et al., 2014).

Table I. Classification of spinal cord injury severity using the American Spinal Injury Association Impairment Scale.
Adapted from Thuret et al., 2006.

A (complete)	No motor or sensory function is preserved in the sacral segments S4–S5.
B (incomplete)	Sensory but not motor function is preserved below the neurological level and includes the sacral segments S4–S5.
C (incomplete)	Motor function is preserved below the neurological level, and more than a half of key muscles below the neurological level have a muscle grade of <3.
D (incomplete)	Motor function is preserved below the neurological level, and at least a half of key muscles below the neurological level have a muscle grade of ≥3.
E (normal)	Motor and sensory functions are normal.



Pathologic events following SCI can be divided into two distinct but highly interconnected phases. First, a primary injury resulting from the initial mechanical damage leads to the disruption of neural and vascular structures at the impact site. The secondary injury response follows the initial trauma and further compromises neurologic function. Several events take place during this secondary stage, ranging from ionic imbalances, free radical production, inflammation, to excitotoxic events (Oyinbo, 2011). Ultimately, these events lead to the formation of an astroglial scar that creates a physical barrier, as well as an inhibitory environment, leading to unsuccessful axonal regeneration at the lesion site (Cregg et al., 2014).

Collectively, these secondary events result in severe neuronal damage. However, they also pose as an opportunity for therapeutic intervention that promotes neuroprotection. Ionic imbalances and glutamate excitotoxicity noticeably exacerbate the functional problems encountered after SCI and are, therefore, targets of current SCI therapeutic strategies (Park et al., 2004).

Riluzole and magnesium have been extensively investigated as neuroprotective agents in animal models of SCI mainly because of their ability to modulate ionic imbalances and excitotoxic events that follow SCI. Furthermore, combining drugs may lead to improved efficacy over single drug treatments as

drug interactions may occur contributing to an additive or synergistic effect that further promotes neuroprotection. Having this in mind, we aimed to investigate the therapeutic efficacy of both individual and combined administration of riluzole and magnesium chloride in SCI.

1.1 Spinal cord: basic anatomy and physiology

Anatomically speaking, the nervous system is divided in two distinct, but highly interconnected parts: the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS is the processing/control center that correlates and integrates nervous information. It receives information from and sends information to the PNS enabling the connection between the CNS and the rest of the body. The CNS is composed by the brain and the spinal cord, and while the brain controls most functions of the body, this is only possible due to the existence of the spinal cord, a major information conduit (Watson et al., 2009). However, its purpose far exceeds the simple relay of information as the spinal cord is able to integrate and modify both afferent and efferent signals allowing for the precise control of sensory, autonomic and motor functions (Nielsen, 2004).

A major difference between the CNS and the PNS lies in their different capacity to regenerate after injury. Unlike the PNS, the CNS has an extremely limited capacity to regenerate when damaged (Illis, 2012). Failure of CNS neurons to regenerate is a consequence of a surrounding inhibitory environment in combination with low intrinsic growth properties of CNS axons (Horner & Gaje, 2012).

The spinal cord, which is usually 40 to 50 centimeters (cm) long, extends from the *foramen magnum,* continuing with the *medulla oblongata* to the level of the first or second lumbar vertebrae. It comprises 31 pairs of spinal nerves composed of both motor and sensory fibers, each pair connecting a different part of the body. There are 8 cervical (C) nerves, associated with the muscles of the neck, shoulders, arms, hands, and diaphragm; 12 thoracic (T) nerves, associated with the chest and abdominal walls; 5 lumbar (L) nerves, associated with the hip, leg, and foot; and 5 sacral (S) nerves, associated with the genitals and lower digestive tract. Each spinal nerve is attached to the spinal cord by a dorsal (sensory) and a ventral (motor) root (Silva et al., 2014).

Protection to the spinal cord is provided by the vertebral column, the protective tissue of the meninges (*dura, arachnoid* and *pia mater*), and the cerebrospinal fluid.

The spinal cord, similarly to the brain, is composed of gray and white matter. The grey matter, which is centrally located and shaped like a 'butterfly', consists mainly in neuronal cell bodies, glial cells, and unmyelinated axons (Thuret et al., 2006).

Grey matter is divided into four main columns: the dorsal horn, the intermediate column, the lateral horn and the ventral horn column. The neuronal bodies in the grey matter are organized in ten successive layers - laminae I-X from dorsal to ventral - depending on their cellular structure (Figure 1). Laminae I to VI are located in the dorsal horns and are involved in sensory input. Laminae VII to IX are found in the lateral and ventral grey matter and are related to autonomic and motor functions. Lamina X is located in the center, surrounding the central canal (Watson et al., 2009).

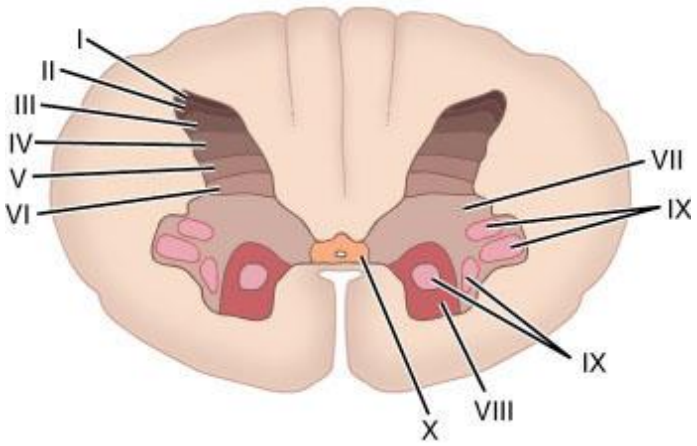


Figure 1. Organization of laminae in the spinal cord.
Obtained from: http://medicine.academic.ru/134796/Rexed_laminae

Surrounding grey matter we find white matter which consists mainly of longitudinally running myelinated axons as well as glial cells, the most abundant being oligodendrocytes. When a group of nerve fibers have the same origin and function, they are referred to as 'tract', while a group of tracts with a related function is referred to as 'pathway'. The course of spinal tracts and pathways can be either ascending (sensorial) or descending (motor) allowing for the adequate communication between the central and the peripheral nervous systems.

It is important to notice that differences in tract organization can be found in different species, such as rodents, cats, primates, and humans (Figure 2). This is particularly significant in spinal cord injury research because it relies on different animal models. For example rodents cortical spinal tract (associated with voluntary movement) is located dorsally, while in humans it can be found both lateral and ventrally (Watson et al., 2009).

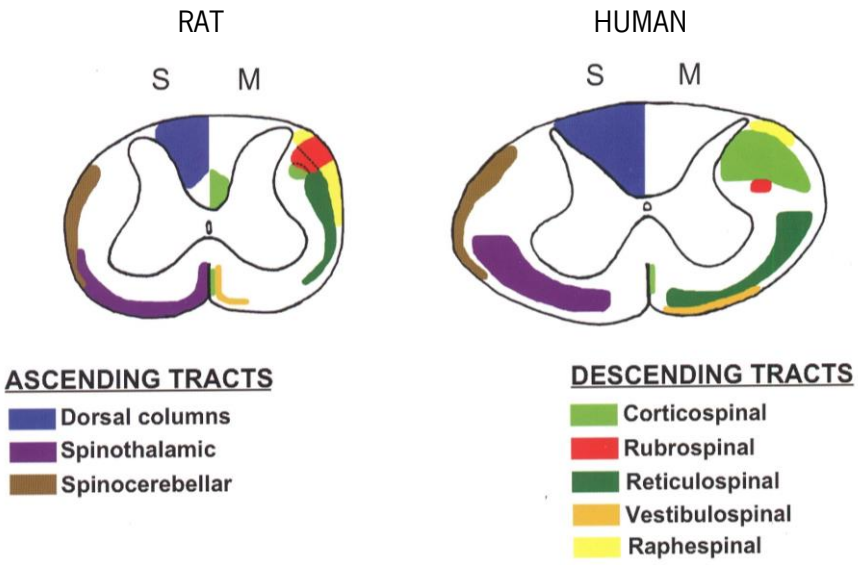


Figure 2. Location of the ascending and descending tracts of the spinal cord.
Differences in ascending sensory (S) and descending motor (M) tracts in rats and humans. Adapted from Watson et al., 2009.

At a cellular level the nervous tissue of the spinal cord is mainly comprised of neuronal and glial cells (Purves et al., 2001). It was in the late nineteenth century that Ramon y Cajal first proposed that neurons were the basic structural and functional units of the nervous system, thus paving the way for modern neuroscience (De Carlos & Borrell, 2007).

Although there are many classes of neurons, their basic structure remains the same: a cell body (soma) and two types of cell processes, axons and dendrites. The cell body contains the nucleus and is the processing center of the neuron. Dendrites receive information from other cells while the axon is the neuronal structure that allows communication between neurons. Neurons ability to generate and transmit electrochemical signals is essential for this communication to occur. The electrical signal, generated by an action potential (i.e. a transient alteration of the membrane voltage), is sent through the axon of a presynaptic neuron. Long axons, also known as nerve fibers, are insulated by fatty myelin sheets that act as electrical insulators, speeding nerve impulses.

Neurons communicate at structures called synapses. When an action potential reaches the synaptic button (the terminal end of an axon) it triggers calcium-regulated fusion of neurotransmitter-filled synaptic vesicles with the presynaptic cell membrane, ultimately releasing neurotransmitters in the synaptic cleft (Augustine, 2001). This release of neurotransmitters can result in inhibitory or excitatory signals that are conveyed to the postsynaptic neuron. Neurons, ultimately, are the tool by which the central nervous system communicates and processes information.

However, the tasks performed by neurons would not be possible without the support of glial cells (Figure 3). These cells differ both morphologically (lack of axons and dendrites) and functionally from neurons, and include astrocytes, microglia, and oligodendrocytes (Kriegstein & Alvarez-buylla, 2011).

Astrocytes provide trophic and metabolic support for neurons, control of extracellular osmotic pressure, local cerebral blood flow, and extracellular neurotransmitter concentration. They can be further divided in two types: protoplasmic astrocytes that can be found in the grey matter where their endfoot processes contact with blood capillaries and synapses, and fibrillary astrocytes that can be found in the white matter where their endfeet contact with blood capillaries and nodes of Ranvier (myelin sheath gaps) (Jukkola et al., 2013).

Although astrocytes are considered non-excitable cells, they are able to communicate with each other and interact with neurons through changes in intracellular calcium (Ca^{2+}) concentration (Navarrete et al., 2013). Typically ascribed supportive roles for proper neuronal function there is now numerous evidence that these cells play an important part in regulating synaptic transmission and plasticity, and thus are being interpreted as possible integral components of the neuronal networks and not mere bystanders (Araque & Navarrete, 2010).

It was Pío del Río Hortega in 1919, which first introduced the concept of microglia and oligodendrocytes as glial cells, a vision not shared by his mentor and employer Ramon y Cajal, that ultimately lead to his dismissal as a consequence for publishing his findings (McGeer & McGeer, 2011).

Microglia serve as the CNS resident immune competent cells, and are mobilized to present antigens and become phagocytes during injury, infection, or degenerative diseases (Ousman & Kubes, 2012). These cells usually display a 'resting' phenotype. However, upon sensing changes in the CNS microenvironment they become 'activated' and undergo morphological and functional changes. Activation of these cells can range from "classical" activation, characterized by a highly pro-inflammatory profile, to "alternative" activation, associated with a less inflammatory, neuroprotective profile (Derecki et al., 2013; Giunti et al., 2014).

Oligodendrocytes are the myelin-forming cells of the CNS, providing electrical insulation of axons and consequently accelerating action potential propagation by saltatory conduction from one node of Ranvier to the next, setting the speed up to 430 km/h instead of approximately 3.6 km/h for an unmyelinated axon (Káradóttir & Attwell, 2007). Other functions performed by these cells are associated to maintenance of axonal health and provision of trophic neuronal support (Czopka et al., 2013). Loss of myelin characterizes many CNS disorders such as multiple sclerosis, Traumatic Brain Injury (TBI), and

SCI, and determines the disruption of neuronal signaling and axonal integrity. Thus, preventing myelin loss may be an important target for therapeutic interventions.

Ependymal cells can also be considered a specialized form of glial cells. They are responsible for the ventricular lining of the brain and spinal cord and help move cerebrospinal fluid through the ventricular system cells. Research is now focusing on their neural stem cell potential following injury to the spinal cord (Lacroix et al., 2014).

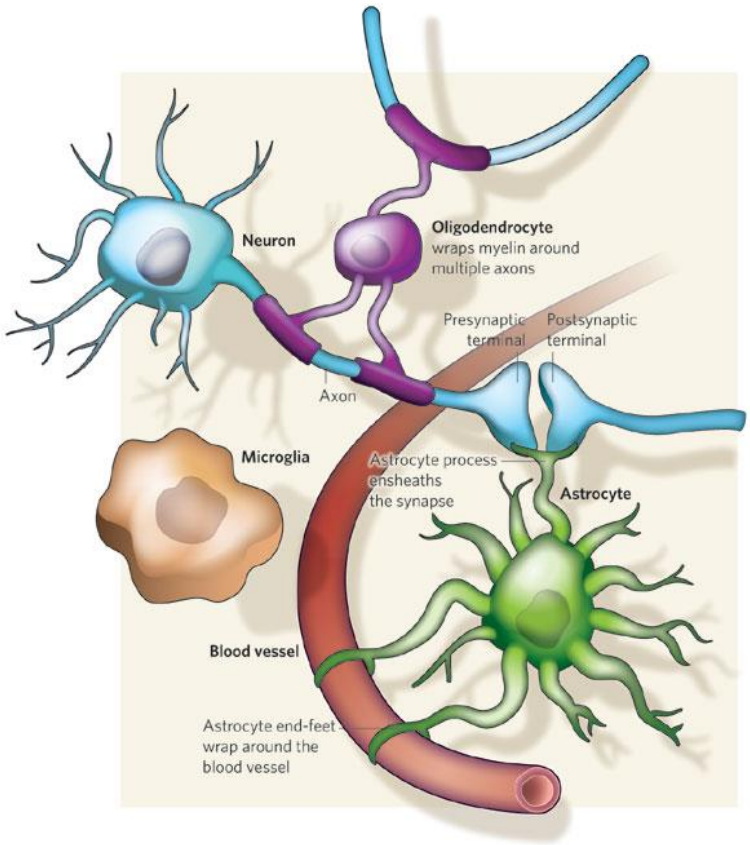


Figure 3. Glial-neuron interaction.
Neurons are responsible for the conduction of electrical currents and information relay. However, their function is highly dependent on glial cells. Oligodendrocytes myelinate CNS axons increasing the efficiency and velocity of nerve impulse conduction. Besides providing structural and chemical support neurons, astrocytes can also modulate neuronal dynamics. Microglia are the resident phagocytic cells in the brain. Reproduced from Allen & Barres, 2009.

1.2 The pathophysiology of spinal cord injury

As previously referred, injury to spinal cord can be caused by traumatic and non-traumatic events. For the purpose of this thesis we will focus on traumatic SCI as this is the most common cause for SCI and the most studied one.

Its pathophysiology follows a specific temporal pattern through different stages that are characterized by several specific biological and molecular events. The primary injury corresponds to the physical and mechanical trauma that occurs to the spinal cord, which is followed by a cascade of downstream events, termed secondary injury, that includes a sub-acute phase ranging from minutes to weeks, and a chronic phase, ranging from months to years after injury (Oyinbo, 2011). Furthermore, this secondary injury is not restricted to the initial lesion site and in fact it extends both radial and rostro-caudally, contributing to increased neurological dysfunction (Ek et al., 2010).

1.2.1 Primary injury

The primary injury is characterized by a mechanical insult, usually compressive, contusive and/or lacerative, that often causes vertebra fracture or disc displacement. Neuronal and endothelial cell membrane shearing occurs as a consequence of the mechanical insult, leading to tissue and blood-spinal cord barrier (BSCB) disruption, as well as vasospasm and edema (Tator & Fehlings, 1991). Hemorrhagic events immediately follow, first in the highly vascularized grey matter causing necrotic neuronal death and later expanding into the peripheral white matter (Profyris et al., 2004; Simon et al., 2009; Thuret et al., 2006).

Primary injury mechanisms that fully disrupt the anatomical continuity of the cord hardly ever occur and most often thinly myelinated or unmyelinated axons are spared and can be found in a subpial rim. However, axonal conduction of these spared axons is highly compromised (Nashmi & Fehlings, 2001; Radojicic et al., 2005).

This initial injury does not stand as promising therapeutic target as it is often uncontrollable and unpredictable. Nevertheless, primary injury highly determines the patient's neurologic grade on admission and is therefore a strong prognostic indicator (Dumont et al., 2001).

1.2.2 Secondary injury

Secondary pathophysiological cellular and molecular events that occur after the initial injury further magnify the damage to the spinal cord leading to extensive tissue loss around the injury site and in a

rostro-caudal manner. Secondary injury was first described in 1911 by Allen, when he observed improvement of neurological function after fluid removal from the lesion site (Allen, 1911).

Throughout this phase increase of the hemorrhagic area occurs. This expansion is characterized by the increase of petechial hemorrhages which progressively coalesce causing tissue ischemia and hypoxia. This leads to a disturbed supply of oxygen and nutrients to the damaged area and its surroundings resulting in immediate cellular death by necrosis and later on by apoptosis (Tator & Fehlings, 1991). Furthermore, the release of hemoglobin is highly toxic to CNS cells, catalyzing hydroxyl radicals production and lipid peroxidation (oxidative degradation of lipids), causing further injury to neural tissue (Gerzanich et al., 2009).

During ischemia, reactive oxygen species (ROS) including superoxide, hydroxyl radicals, and nitric oxide (NO) are generated via multiple cellular pathways including, but to limited to nitric oxide synthases, Ca^{2+}-mediated activation of phospholipases, and inflammatory cells (Bao et al., 2005; Jia et al., 2012). The CNS has limited antioxidant defense mechanisms as the brain and spinal cord exhibit low levels of catalase activity and only moderate levels of superoxide dismutase and glutathione peroxidase, Therefore, when oxidative stress exceeds the protective cellular antioxidant capacity, it causes oxidative damage to lipids, proteins, and nucleic acids ultimately resulting in cellular death (Christie et al., 2008; Mautes et al., 2000; Xu et al., 2005).

In turn, hypoxia and consequential oxygen deprivation hampers the cellular energy metabolism leading to adenosine triphosphate (ATP) depletion which in turn compromises cellular structural and functional integrity, triggering multiple necrotic mechanisms including loss of cell membrane permeability, release of lysosomal contents, and activation of a variety of Ca^{2+}-activated proteases including phospholipases, adenosine 5'-triphosphatases (ATPases), and endonucleases (Hagg & Oudega, 2006; Oudega, 2012). Furthermore, cells are driven towards anaerobic glycolysis, leading to lactic acidosis and consequential hindering of enzymatic function and deoxyribonucleic acid (DNA) damage.

Moreover, the relative immune privileged status of the spinal cord is also compromised due to BSCB disruption, as a consequence of direct mechanical disruption of the vasculature, and increased vascular permeability of endothelial cells caused by inflammatory mediators tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), allowing the infiltration of inflammatory cells. Permeability of the BSCB is further maintained up to two weeks post-injury by a number of vasoactive substances, such as ROS, NO, elastase, and histamines, that are released by glia and leukocytes (Donnelly & Popovich, 2008; Figley et al., 2014)

The immune response that follows SCI aims to remove damaged tissues and promote the healing responses of astrocytes through the release of pro-inflammatory cytokines, and is primarily mediated by resident microglia. Trauma to the spinal cord leads to sustained elevations of signaling molecules such as ATP, DNA, glutamate, growth factors and cytokines, that are released by injured neurons and glial cells, resulting in the activation of microglia and consequent release of signaling molecules such as TNF-α, IL-1β, and IL-6, which act to recruit circulating leukocytes to the injury site (Donnelly & Popovich, 2008; Loane & Byrnes, 2010; Pineau & Lacroix, 2007; Zhou et al., 2014).While the number of microglial cells remains relatively stable soon after the injury until several weeks later, neutrophils accumulate until they reach a peak at 24 h post-injury and then decline, while the number of T-lymphocytes and macrophages increases, peaking at 7 days (d) post-injury (Neirinckx et al., 2014; Rowland et al., 2008; Zhang et al., 2012).

Neutrophils have been described to promote neurotoxicity due to the activity of matrix metalloproteinase-9, generation of ROS, and secretion of TNF-α (Nguyen et al., 2007). However, their exclusively deleterious role in SCI role as been questioned by recent studies (Ghasemlou et al., 2010; Kurimoto et al., 2013; Stirling et al., 2009).

Neutrophil recruitment declines by 48 h and monocytes start to accumulate at the site of injury where they differentiate into macrophages. Several macrophage subsets have been identified, namely "classically activated" pro-inflammatory (M1) or "alternatively activated" anti-inflammatory (M2) macrophages (Kigerl et al., 2009). M1 macrophages produce pro-inflammatory cytokines such as TNF-α, IL-1, and IL-6, as well as ROS, contributing to tissue inflammation and damage. Additionally, NO generated by NO synthase activity of macrophages induces neuronal apoptosis (Satake et al., 2000; Tzekou & Fehlings, 2014). Alternatively, M2 macrophages produce anti-inflammatory factors (IL-10, transforming growth factor-β; TGF-β) contributing to wound healing and tissue-remodeling (Kigerl et al., 2009; Ren & Young, 2013).

There is still much debate on whether T-lymphocytes promote injury or recovery after SCI. While Popovich et al. have shown that myelin-reactive T-lymphocytes are activated by SCI and contribute to neurodegeneration, reports by Kipnis et al. and more recently Raposo et al. have proposed that these cells are able to mediate neuroprotection (Kipnis et al., 2002; Popovich & Jones, 2003; Raposo et al., 2014).

Overall, the inflammatory response following SCI paradoxically presents both detrimental and beneficial roles leading to the current view of inflammation as a "double-edged sword", where

inflammation may lead to enhanced damage and impaired regeneration, but also stands as a key regulator of tissue repair after injury (Cherry et al., 2014; Neirinckx et al., 2014).

Secondary injury is also characterized by extensive cellular death occurring as a consequence of ionic imbalances and glutamate excitotoxicity. Due to the relevant role of these mechanisms in the present thesis, they are further detailed in section 1.5.

1.2.3 From secondary injury to chronic phase

The loss of neurons and glial cells, as well as the clearance of debris by microglia and macrophages, results in the formation of a cystic cavity in the spinal cord.

Furthermore, cytokines and growth factors such as IL-1, TGF-β, neurotransmitters such as glutamate, and proteins such as fibrinogen activate astrocytes leading to changes in their morphology and molecular expression, which culminates with the formation of a glial scar (Cregg et al., 2014). Besides representing a physical barrier, the glial scar is responsible for the development of an inhibitory environment at the lesion site caused mainly by the production of molecules, including tenascin, semaphorin 3A, ephrins, and chondroitin sulphate proteoglycans, which inhibit axonal growth (Kawano et al., 2012; Rolls et al., 2009; Yiu & He, 2006).

Nevertheless, the glial scar is also known to aid SCI repair. In fact, studies where astrogliosis was ablated lead to increased lesion volume and inflammation, as well as impaired functional recovery, and did not further promote axonal regeneration (Faulkner et al., 2004; Herrmann et al., 2008; Okada et al., 2006). Reactive astrocytes can in fact promote glutamate uptake, free radical scavenging, promote BSCB repair, limit the infiltration of inflammatory cells in healthy tissue, as well as release growth factors, therefore protecting the spinal cord from deleterious secondary events (Kawano et al., 2012).

Additionally, the chronic phase is also characterized by anterograde axonal degeneration, a process known as Wallerian degeneration, and progressive loss of myelin sheaths, occurring up to extended periods of time (Hagg & Oudega, 2006). Keeping in mind that spared axons that cross the injury site are the only connection left between the brain and caudal spinal neurons, inefficient and compromised communication through these axons is a significant clinical issue (Oyinbo, 2011).

Presently, several mechanisms involved in SCI pathophysiology have been identified and extensively studied (Figure 4). Despite this increasing knowledge the fact that these injury mechanisms are intrinsically intertwined and often have both beneficial and detrimental roles may be the reason why

there is still no effective treatment for SCI. Nevertheless, they also present an opportunity for injury modulation and therapeutic intervention.

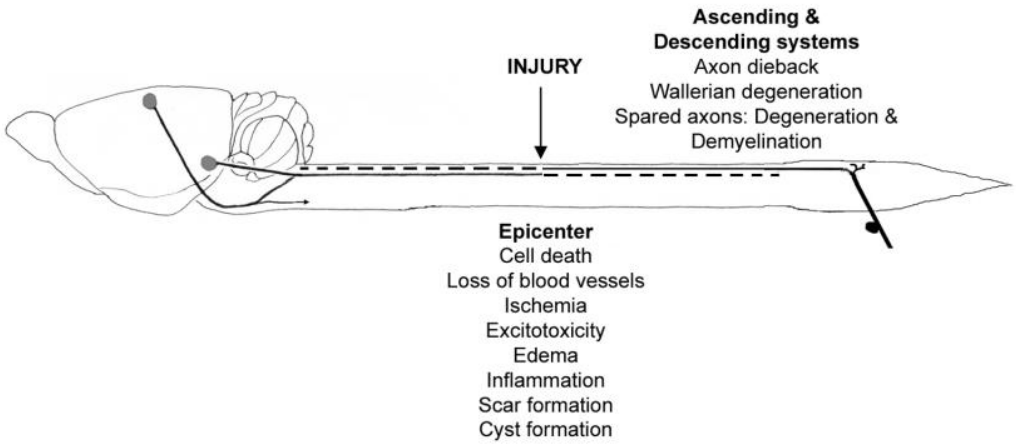


Figure 4. Schematic representation of the injured spinal cord.
The major degenerative processes that occur following an injury are indicated. Reproduced from Hagg & Oudega, 2006.

1.3 Management of spinal cord injury: current standard practice

The twentieth century came to revolutionize the standard medical care provided to SCI patients, namely with the implantation of SCI units and increasing focus on the importance of rehabilitation (Donovan, 2007). Currently, medical approaches to SCI aim to at minimize the progression of the initial injury and prevent secondary injury.

Patients that have suffered trauma to the spinal cord are usually immobilized during the pre-hospital setting generally resorting to a cervical collar, head immobilization, and a spinal board. On the clinical onset, imaging techniques, such as X-rays, computed tomography (CT) scans, and magnetic resonance imaging (MRI), allow for a more accurate diagnosis (Stroman et al., 2014). Peri- and postoperative airway management, namely following cervical injury, as well as hemodynamics and cardiovascular control are essential to avoid neurological compromise, morbidity, and death following SCI and are presently standard care procedures (Furlan & Fehlings, 2008; Martin et al., 2015).

Furthermore, spine stabilization and surgical decompression are performed. Decompression is associated with improved neurological outcomes when performed early (less than 24 h after injury), as supported by a recently conducted randomized multicenter trial, the Surgical Treatment of Acute Spinal Cord Injury Study. This study showed that decompressive surgery within the first 24 h confers a 2.83

times higher chance of a 2-grade AIS improvement when compared to surgery performed after 24 h. Additionally, differences in complication rates were not detected (Fehlings et al., 2012).

A limited number of pharmacological agents have been applied to human SCI patients including two corticosteroids (methylprednisolone and tirilazad mesylate), an opiate receptor antagonist (naloxone), and GM-1 ganglioside, However, and despite the fact that no clinical evidence exists to definitively recommend the use of any neuroprotective pharmacologic agents, methylprednisolone (MP) is still the most widely used drug therapy for acute SCI (Sharma, 2012).

The benefits of MP in SCI treatment are mainly attributed to the inhibition of lipid peroxidation, through neutralization of free-radicals, maintenance of the BSCB, and immune-modulation (Yılmaz & Kaptanoğlu, 2015). The immunomodulatory role of MP is linked to its ability to reduce microglia/macrophage activation, as well as a reduction in pro-inflammatory cytokine (TNFα, IL-6, interferon-γ) release, and increased anti-inflammatory cytokine (IL-10) secretion (Bowes & Yip, 2014). Furthermore, patients receiving high-dose MP (bolus 30 mg/kg and 5.4 mg/kg per hour over 24 h, initiated within 8 h of injury), showed decreased intramedullary spinal cord hemorrhage (Leypold et al., 2007).

The use of MP as a therapeutic strategy for SCI followed the reports of Bracken et al. in the National Acute Spinal Cord Injury Study (NASCIS) I and II. The latter revealed that a high dose administration (30 mg/kg), at time of admission and a 5.4 mg/kg/h infusion for the following 23 h, and within an 8 h range, lead to neurological improvements (light touch, pinprick sensation, and muscle strength) (Bracken et al., 1984; 1990). NASCIS III in turn, revealed that patients treated with a MP bolus administration of 30 mg/kg, followed by a 5.4 mg/kg/h infusion for a 48 h period showed improved motor recovery, namely when the first administration occurred between 3 and 8 h after SCI. However, increased adverse effects were also reported in this group, namely increased risk of severe sepsis, pneumonia and urinary tract infections (Bracken et al., 1997). The validity of these reports has been widely disputed, as well as the beneficial role of MP administration following SCI (Bydon et al., 2014; Markandaya et al., 2012; Miller, 2008).

In 2013 the American Association of Neurological Surgeons/Congress of Neurological Surgeons Guidelines for the Management of Acute Cervical Spine and Spinal Cord Injury issued a level 1 recommendation that MP not be used due to its side effects (Hurlbert et al., 2013). Nevertheless, there are also contradictory voices that state the need to carefully evaluate the administration of MP, as recent studies showed for example synergistic effects of early decompression and MP administration in patients undergoing surgical decompression after cervical SCI (Fehlings et al., 2014).

The systemic or local application of hypothermia has demonstrated to be neuroprotective after SCI, in both experimental and human studies, as it is able to mitigate several harmful effects caused by secondary injury (Cappuccino et al., 2010; Wang & Pearse, 2015). Therapeutic hypothermia drew significant clinical and general public notoriety as a treatment for SCI, following the recent report of an acute cervical spinal cord injury in a professional football player, Kevin Everett, who sustained a complete cervical SCI (AIS A) and received moderate systemic hypothermia for 36 h along with surgical decompression and MP administration, achieving rapid neurologic improvement that later translated into a neurological evaluation of AIS D (incomplete) (Cappuccino et al., 2010).

Rehabilitation therapies have also shown to improve recovery of locomotor function both in animal models as well as in human studies, most likely due to the reorganization of neuronal circuits that have been spared by the lesion (Edgerton et al., 2006; Engesser-Cesar et al., 2005). In an effort to maximize functional recovery after SCI, several rehabilitation strategies have been developed in order to elicit residual function bellow injury, such as over-ground training, body weight supported treadmill training, robotic-assisted step training, and functional electrical stimulation, and they are now being assessed for their efficacy in promoting functional ambulation following SCI (Lam et al., 2007).

SCI also increases the incidence of specific medical disorders such as neurogenic bladder, neuropathic pain, spasticity, and sexual dysfunction. However, progressively, this problems have been tackled and current therapies represent a huge increment in SCI patients' lives (Cardenas et al., 2013; Nomura et al., 2002; Schmid et al., 2000; Taricco et al., 2006).

1.4 Emerging therapeutic strategies for spinal cord injury

Although SCI medical care as greatly evolved over the last few years, largely improving the chances of patients' recovery, there is still no definitive therapeutic strategy to recover neurologic function.

Treatment strategies for SCI are mainly focused on events that follow the initial trauma, either by promoting neuroprotection or enabling neuroregeneration. The first essentially aims to prevent cellular dysfunction and death caused by secondary injury decreasing the progressive extension of the lesion and damage to the spinal cord, while the latter mainly aims to promote axonal growth and plasticity.

Several lines of strategies have been devised as possible therapies for SCI, ranging from cell transplantation, to pharmacological and combinatorial therapies, many of which showing valid potential in supporting spinal cord repair and thus standing as possible treatment strategies for SCI.

Several stem cells, such as neural stem cells, mesenchymal stem cells, embryonic stem cells, and induced pluripotent stem cells have been studied as possible therapeutic strategies in SCI. These cells are mainly characterized by self-renewal and the ability to differentiate in cells from different tissues. This last feature stands as an attractive feature in SCI as these cells can potentially be directed to differentiate into neurons or glia, replacing neural cells that are lost after injury, therefore supporting anatomical and functional recovery (Tewarie et al., 2009). Neuroprotective and axon regeneration-promoting effects have also been credited to transplanted stem cells through the secretion of growth factors and modulation of the inflammatory response (Baraniak & McDevitt, 2010).

A landmark stem cell clinical trial, the first in the USA involving human embryonic-derived oligodendrocyte progenitor cells, began in 2010 and was carried by the Geron Corporation. The companies' managing director, Thomas Okarma, enthusiastically stated that the trial represented "the dawn of a new era in medical therapeutics". However, the Geron Corporation abruptly discontinued the study in November 2011 for financial reasons, largely disappointing the scientific community (Scott & Magnus, 2014). Recently however, their embryonic stem cells technology was acquired by Asterias Biotherapeutics. After a Phase I trial (source: clinicaltrial.gov; clinical trial identifier NCT01217008) met its primary endpoints of safety and feasibility, a Phase I/IIA clinical trial (NCT02302157) has been cleared by the Food and Drug Administration (FDA), holding promise for stem cell therapies in SCI recovery.

Other cell types, such as olfactory ensheathing cells (OECs) and Schwann cells have also attracted the attention of investigators in the field. As the name suggests, OECs are glial cells that ensheath non-myelinated olfactory axons and are responsible for growth and regeneration of olfactory axons throughout the life of adult mammals. They have received the attention of the media and general population after reports of a patient that suffered a traumatic transection of the spinal cord improved from ASIA A to ASIA C, following OECs transplantation (Tabakow et al., 2014).

Schwann cells are also glial cells that are known for their roles in supporting nerve regeneration in the peripheral nervous system (Oudega & Xu, 2006). These cells are particularly interesting in SCI because of their ability to migrate to the injury site, express growth promoting factors, and myelinate regenerating axons. However, their ability to overcome the glial scar is limited revealing the need to develop combinatorial strategies, for example with biomaterials in order to maximize their regenerative potential (Assunção-Silva et al., 2015; Pêgo et al., 2012). In line with this thought, an interesting strategy is being developed by the company InVivo Therapeutics using both biomaterial scaffolds and neural stem cells with promising results obtained using a non-human primate model (Pritchard et al., 2010). The FDA has already approved a pilot study of clinical safety and feasibility of the scaffold (NCT02138110) and in

future trials the company plans to load the scaffold with neural stem cells in order to functionally bridge the injury site.

A number of promising molecular and pharmacological therapies are currently under investigation for neuroprotective and neuroregenerative abilities in animal models of SCI. Furthermore, several of these therapeutic agents are already in, or close to, clinical trials.

Pharmacological agents that aim to prevent secondary injury include, but are not limited to steroids (methylprednisolone, tirilazad mesylate), opiate blockers (naloxone), anti-inflammatory agents (indomethacin, ibuprofen), glutamate receptor antagonists (magnesium, MK-801), ion channel blockers (riluzole), apoptosis inhibitors (minocycline), cytokines (erythropoietin, granulocyte colony-stimulating factor), antioxidants/free radical scavengers (tirilazad mesylate, quercetin), and immune blockers (cyclosporine-A) (Kwon et al., 2011; Nagoshi & Fehlings, 2015; Priestley et al., 2012).

Furthermore, several agents have also been studied in order to promote axonal regeneration which is necessary for re-establishing connectivity across the injury site, either by targeting myelin-associated inhibitory molecules (anti-Nogo antibody), by blockade of the Rho pathway (cethrin), stimulation of axonal growth (neurotrophic factors), or by degradation of chondroitin sulfate proteoglycan inhibitory molecules (chondroitinase ABC) (Silva et al., 2014).

Albeit all the promising results of some of these therapies, when translated to a clinical context most of them have failed to prove their efficacy. Examples such as methylprednisolone and GM-1 ganglioside, are a reminder that although some of these therapies may in fact have inadequate potency there is a growing need to improve the rigor and conduct of human trials to ensure that the true effects of treatment, positive and negative, are accurately detected and reported (Lammertse, 2013).

Despite the increasing knowledge of important mechanisms regarding neuronal protection and regeneration, alongside the innumerous studies that have been, and are being performed, no gold standard therapy for SCI has yet been established and therefore identification of effective therapeutic interventions are urgently needed.

1.5 Targeting excitotoxicity and ionic imbalances to protect the spinal cord

Understanding the pathophysiology of SCI is the basis for the development of treatments for spinal cord repair. Excitotoxicity and ionic dysregulation are closely related processes that immediately follow SCI contributing to extensive neuronal damage and loss. Pharmacological protection of the spinal cord against these deleterious events is the main focus of this thesis, and therefore this section will center on

these mechanisms and how they individually and collectively contribute to SCI providing the rational for the use of riluzole and magnesium as therapeutic strategies.

The term 'excitotoxicity' was coined by Olney in 1969, when he observed that subcutaneous injection of monosodium glutamate lead to neuronal death, and refers to postsynaptic neuronal death produced by over-activation of glutamate receptors (Olney, 1969). Physiologically, the concentration of glutamate in the synaptic cleft is tightly regulated. Removal of glutamate mainly occurs by sodium (Na^+) dependent excitatory amino acid (EAA) transporters (EAAT), present in both neurons and glial cells, which maintain the extracellular concentration below toxic levels. In the spinal cord, the astrocyte glutamate transporter EAAT2 (glutamate transporter GLT-1 in rodents), is responsible for a significant percentage of extracellular glutamate functional uptake (Lepore et al., 2011; Shigeri et al., 2004).

Glutamate acts on specific postsynaptic receptors including ionotropic N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors, as well as metabotropic glutamate receptors. Ionotropic receptors act as ion channels (permeable to Na^+, potassium; K^+, and varying permeability to Ca^{2+}), while metabotropic receptors are G protein-coupled and act through signal transduction cascades (Stys & Lipton, 2007). Ionotropic receptors are further divided in NMDA and non NMDA-receptors, such as AMPA and kainate receptors, according to their affinity to the synthetic glutamic acid analogue N-methyl-D-aspartic acid. Non-NMDA receptors are not voltage dependent and are highly permeable to Na^+ (Shigeri et al., 2004). NMDA receptors (NMDARs), which are characterized by their voltage dependence and high permeability to Ca^{2+}, have gained particular interest in SCI pathology, due to their preponderant role in Ca^{2+}-mediated excitotoxicity (Park et al., 2004).

NMDARs form a heterotetramer assembled by combination of two GluN1 subunits with two GluN2A-D or GluN3A-B subunits. These receptors require dual agonists, glutamate and glycine, for activation, and are blocked by physiological levels of magnesium (Mg^{2+}) in a voltage dependent manner. The sensitivity to the Mg^{2+} block as well as Ca^{2+} permeability depends on the subunit constitution, with subunit GluN2A and GluN2B, mainly present in neurons being highly sensitive to magnesium block and Ca^{2+} permeability, while the subunits GluN2C, GluN2D and GluN3, mainly present in oligodendrocytes and astrocytes having lower sensitivity to the magnesium block and Ca^{2+} permeability (Paoletti et al., 2013). Subunit diversity also provides distinct pharmacological properties to NMDARs, enabling the development of specific antagonists and selective modulation of these receptors. Toxic levels of glutamate are reached within 15 min after SCI in rats at local and regional tissue surrounding the injury epicenter (McAdoo et al., 1999). The disruption of cellular membranes following SCI causes an acute burst of EAA, namely glutamate, the main excitatory neurotransmitter of the CNS (Liu et al., 1991; McAdoo et al., 1999)

Glutamate accumulation also occurs due to the reversal of Na^+-dependent glutamate transporters leading to synaptic glutamate release (Li et al., 1999; Li & Stys, 2001) Oxidative stress is also known to impair glutamate uptake contributing also to extracellular glutamate accumulation (Barger et al., 2007). Furthermore, activated microglial cells are also known to release glutamate. In neurons, Ca^{2+}-dependent postsynaptic exocytosis also leads to release of glutamate. All of these mechanisms serve as self-amplifying glutamatergic loops. Excessive activation of NMDARs due to increased extracellular glutamate accumulation plays a significant role in Ca^{2+}-mediated excitotoxicity (Cao & Dong, 2013; Matute et al., 2007). Pathological increases in intracellular Na^+ and Ca^{2+} concentrations lead to excitotoxic cellular death, via the activation of protein kinases, phospholipases, proteases, mitochondrial dysfunction, production of ROS, intracellular acidosis, edema, and cell lyses (Dong et al., 2009; Dumont et al., 2001; Matute et al., 2006; Oyinbo, 2011). Additionally, the recent studies by Salter and Micu et al. reported that NMDAR are expressed in developing oligodendrocytes processes as well as in myelin sheaths, providing compelling evidence for the involvement of these receptors in white matter injury (Micu et al., 2006; Salter & Fern, 2005).

However, it is now known that NMDARs may also play an important role in supporting neuronal survival. Furthermore, this protective role of NMDAR activity has been proposed as a reason for the failure of several clinical trials which evaluated NMDAR antagonists as potential neuroprotective drugs. NMDAR location (synaptic or extrasynaptic) and subtype composition have been suggested as two factors that may indicate its pro-survival or pro-excitotoxic profile (Besancon et al., 2008; Hetman & Kharebava, 2006).

Tightly related to excitotoxic events, severe ionic imbalances also occur after SCI. Intracellular concentrations of Na^+, Ca^{2+} increase, while K^+ and Mg^{2+} levels severely decrease (LoPachin et al., 1999). Ionic imbalances mainly occur due to widespread shearing and destruction of cellular membranes occurring at the injury site, alongside energetic depletion and consequential energy-dependent electrolytic transport failure. The outcome of this deregulation is dysfunction and inappropriate propagation of action potentials along damaged axons. In particular, intracellular accumulation of Na^+ has several deleterious effects, in which voltage-gated Na^+ channels (VGSC) play a pivotal role. The characteristics and function of these channels were unraveled by work developed by Hodgkin and Huxley (Hodgkin & Huxley, 1952). Physiologically, VGSC mediate membrane depolarization and are responsible for propagating action potentials along the axonal membrane. These channels are heteromeric assemblies of one α subunit ($Na_v1.1$ through $Na_v1.9$) and one or more β subunits. Only five of these isoforms are expressed in the CNS, namely $Na_v1.1$, $Na_v1.2$, $Na_v1.3$, and $Na_v1.6$. While expression of the α subunit alone is sufficient to

produce a functional channel, β subunits are responsible for most channel functions such as voltage sensing, gating, ion permeation, and inactivation (Theile & Cummins, 2011). Additionally, the α subunit expresses six distinct neurotoxin-binding sites that when activated can lead to either pore occlusion and concomitant inhibition of Na^+ conductance, or modification of gating leading to altered gating kinetics and voltage-dependence of these channels (Caldwell et al., 2000; Stevens et al., 2011).

Following trauma, such as SCI, persistent activation of VGSC occurs due to neuronal membrane dysfunction, resulting in an increase of intracellular Na^+ concentration ($[Na^+]_i$) (Stirling & Stys, 2010). Alongside, the activity of Na^+/K^+ pumps is compromised due to energetic deficits, leading to a cutback in Na^+ efflux. Several pathological effects occur as a consequence of pathological $[Na^+]_i$ such as cytotoxic edema and increased acidosis (via the Na^+/H^+ exchanger). Furthermore, increased $[Na^+]_i$ is known to promote Ca^{2+}-mediated cellular death. This occurs mainly due to the reverse operation of Na^+/Ca^{2+} exchangers, causing Ca^{2+} to be pumped in and Na^+ extruded, as well as activation of voltage-dependent Ca^{2+} channels (Agrawal & Fehlings, 1996; Ates et al., 2007; Hains et al., 2004; Rosenberg et al., 1999; Schwartz & Fehlings, 2001). Calcium overload in turn leads to mitochondrial damage, activation of Ca^{2+}-dependent cell death proteases (such as caspases), as well as formation of free radicals, resulting in apoptotic cell death of neurons. Additionally, membrane depolarization leads to glutamate release, through the reversal of Na^+-dependent glutamate transporters and vesicular glutamate release and further contributes to excitotoxicity (Li & Stys, 2001; Stys, 2005). In white matter, high intracellular Ca^{2+} levels result in the activation of ubiquitous proteases, specifically calpains, which contribute to neurodegeneration and compromised axonal integrity through cytoskeletal degradation of neurofilaments, as well as microtubule associated protein 2 (MAP2).

Experimental evidences concerning the role of Na^+ channels in secondary injury arose mainly from studies in which the use of Na^+ channel blockers, such as tetrodotoxin (TTX), a potent neurotoxin, lead to reduced apoptotic neuronal death, while the application of veratridine, a voltage gated Na^+ channel activator, induced neuronal apoptosis and caspase-3 activation (Banasiak et al., 2004; Stys et al., 1992).

Furthermore, recent studies have found that voltage-gated Na^+ channels are also present on immune cells and that these channels contribute to the activation and phagocytic function of microglia and macrophages raising the possibility that Na^+ channel blockade may attenuate the inflammatory response (Jung et al., 2013; Pappalardo et al., 2014).

In SCI both ionic imbalances and glutamate excitotoxicity significantly contribute to the secondary injury (Figure 5). However, pharmacological treatments, if applied early, may interrupt or modulate this injurious cascade of events and consequentially improve tissue preservation and neurological outcome

following SCI. In this thesis we will focus on two promising neuroprotective agents that can attenuate secondary pathophysiology and reduce functional deficits, namely, the Na^+ channel blocker riluzole, as well as the NMDA receptor antagonist magnesium.

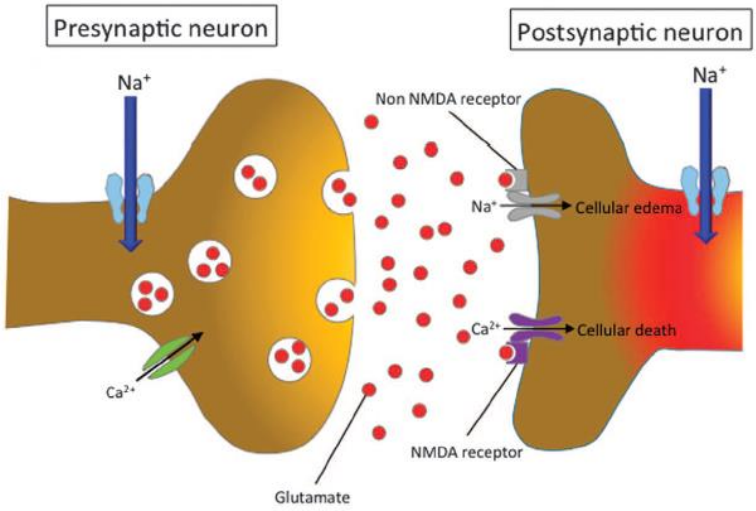


Figure 5. Representation of neuronal injury following ionic imbalances and excitotoxicity. Neuronal ionic balance is disrupted following the primary injury and the intracellular Na^+ concentration increases as a result of trauma-induced activation of voltage-sensitive Na^+ channels promoting concomitant influx of Ca^{2+} ions. The excessive influx of Na^+ and Ca^{2+} triggers pathologic extracellular release of excitatory neurotransmitter glutamate. In the postsynaptic neuron, sodium and calcium influx through NMDAR leads to cellular death and axonal edema. Adapted from (Fehlings et al., 2015).

1.5.1 Riluzole

Riluzole (2-amino-6-trifluoromethoxybenzothiazole) is a Na^+ channel blocker agent approved in 1995 by the FDA for the treatment of Amyotrophic Lateral Sclerosis (ALS), a progressive neurodegenerative disorder characterized by motor neuron and corticospinal tract degeneration (Miller et al., 2012). The analysis of four placebo-controlled, randomized trials, has concluded that the administration of 100 mg daily is safe and improves overall survival of ALS patients in two or three months (Miller et al., 2012). Because neurological dysfunction due to loss of spinal motor neurons and axonal degeneration are also common in SCI, numerous preclinical studies used riluzole to demonstrate that its administration following injury improved both functional and histological outcomes in injured animals.

As previously stated, neuronal ionic balance is disrupted after SCI giving rise to an increase in intracellular Na^+ concentration, resulting from constitutively activation of VGSC. This accumulation is further amplified due to dysfunction of membrane-bound Na^+/K^+ pump. Downstream effects of Na^+

deregulation include cytotoxic edema, acidosis, and Ca^{2+}-dependent cellular death. Riluzole exerts neuroprotection in SCI via blocking of persistent Na^+ currents, as it specifically blocks inactivated Na^+ channels thus preventing the above stated pathological processes (Table II). Furthermore, the Na^+ channel blockade may also reduce energetic demands, for example through the reduction of Na^+/K^+ pump, resulting in an improved resistance of cells (Theiss et al., 2007; Urbani & Belluzzi, 2000; Wahl & Stutzmann, 1999). Riluzole has also an important role as an anti-glutamatergic agent, through inhibition Ca^{2+}-dependent release of glutamate from presynaptic terminals as well as promotion of glutamate reuptake (Fumagalli et al., 2008; Wang et al., 2004). Additionally, riluzole is thought to preserve spinal cord white matter by preventing the disruption of the axonal Na^+/H^+ exchanger system (Nagoshi & Fehlings, 2015).

Table II. Mechanism of action for riluzole in preventing secondary injury following SCI.
Adapted from Wilson & Fehlings, 2014.

PRIMARY MECHANISM	SECONDARY EVENTS	END RESULT
Blockade of continuous posttraumatic activation of neuronal voltage gated Na^+ channels	Prevents increase in neuronal cytosolic Na^+ concentrations	Prevents development of neuronal acidosis and swelling
	Prevents excessive neuronal entry of H^+ through Na^+/H^+ exchanger	
	Prevents excessive neuronal entry of Ca^{2+} through Na^+/Ca^{2+} exchanger	Prevents Ca^{2+}-induced release of glutamate and excitotoxicity

Neuroprotection is also thought to be exerted by riluzole resulting from its ability to stimulate astrocyte expression of several neurotrophic factors, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and glial cell-derived neurotrophic factor (GDNF) (Mizuta et al., 2001). Additionally, increased levels of BDNF and TGF-1β have also been detected in the serum of Huntington's patients treated with riluzole (Squitieri et al., 2009).

When reviewing the preclinical evidence published using riluzole in traumatic models of SCI (Table III), we find that although all eleven studies were performed using rats as animal models, several strains were used (Wistar, Long-Evans, and Sprague-Dawley) in a variety of thoracic and cervical injuries, including weight drop, clip compression, and balloon compression. Riluzole was most commonly administered intraperitoneally in doses ranging from 5 to 8 mg/kg, with higher doses associated with lethargy and locomotor ataxia as side effects. Therapeutic neuroprotective efficacy was achieved up to a

clinical relevant time of 4 h post-injury. Behavioral outcomes included improved Basso, Beattie and Bresnahan (BBB) locomotor scores, greater inclined plane angles, as well as beam balance and rotarod scores, while non-behavioral outcomes included increased tissue sparing, reduced MAP2 loss, decreased lipid peroxidation, capillary fragmentation, apoptosis, and inflammation, as well as improved electrophysiological recordings.

Only the study performed by Mu et al. (2000b) reported the absence of improved behavioral and non-behavioral outcomes when riluzole was administered alone. However, when combined with methylprednisolone, both motor and histological outcome was improved.

The study of Kitzman et al. employed a transection model and riluzole administration was performed 4 weeks post-injury (chronic phase). However, this was to examine the effect on established tail spasticity and not acute local tissue protection or locomotor behavior.

Based on these preclinical evidences the administration of riluzole following SCI naturally progressed on to clinical trials. A Phase I safety trial of riluzole in acute cervical SCI, Riluzole in Spinal Cord Injury Study, in which thirty-six patients with ASIA grades A-C were enrolled (28 cervical and 8 thoracic), has already established the absence of serious adverse effects and that the administration of riluzole (within 12 h of injury, 50 mg every 12 h for 28 doses) may have a beneficial effect in motor outcome, particularly for cervical SCI patients (NCT00876889) (Grossman et al., 2014). Furthermore, clinical pharmacokinetics of riluzole in patients with SCI was also studied revealing that the peak concentration (C_{max}) and the 12-h area under the plasma concentration curve $(AUC)_{(0-12h)}$ achieved in SCI patients were lower than those in ALS patients (on the same dose basis) due to a higher clearance and larger volume of distribution in SCI patients. Furthermore, SCI patients presented large interpatient variability in plasma concentration and an increase in the clearance and distribution of riluzole between the 3rd and 14th day after SCI, with a lower plasma concentration of riluzole on the 14th day, stressing the importance of monitoring changes in drug metabolism after SCI (Chow et al., 2012).

On the basis of these results, a multi-center, randomized, placebo controlled, double-blinded, Phase IIB/III trial of efficacy and safety of riluzole is currently recruiting (NCT01597518) (Fehlings et al., 2015).

Table III. Summary of *in vivo* studies performed in animal models of traumatic SCI using systemically administered riluzole.

PUBLICATION	ANIMAL & INJURY MODEL	DOSE, ROUTE & FREQUENCY	MAIN OUTCOMES
Stutzmann *Neuroreport,* 1996(Stutzmann et al., 1996)	Male Wistar rats T10-T12 compression (Fogarty catheter)	2 mg/kg IV, 30 min PI and then twice daily for 10 d	Non-behavioral: Decreased damage to grey and white matter recovery of SSEPs (amplitude, duration and latency Behavioral: Animals were able to use their paws to sit upright
Springer *J Neurochem*, 1997(Springer et al., 1997)	Rats (sex and strain not specified) T10 impactor	8 mg/kg IP, 15 min prior to injury and 2 h PI	Non-behavioral: Reduced loss of MAP2 Behavioral: Not reported
Mu *J Neurotrauma,* 2000(Mu et al., 2000a)	Female Long-Evans rats T10 impactor	8 mg/kg IP, 2 and 4 h PI and then daily for 7 d	Non-behavioral: riluzole alone did not improve spinal cord cavitation. Improvement was only achieved in combination with MP (30 mg/kg) Behavioral: Only the combination of riluzole and MP resulted in improved locomotor scores (BBB scale)
Mu *Brain Res,* 2000(Mu et al., 2000b)	Female Long-Evans rats T10 impactor	8 mg/kg IP, 15 min and 2 h PI	Non-behavioral: Five measures of oxidative stress were used (mitochondrial function; ROS levels; thiobarbituric acid reactive product levels; glutamate and glucose levels). Riluzole improved mitochondrial function and enhanced glucose uptake. Riluzole and MP (30 mg/kg) combined treatment improved all five measures of oxidative stress Behavioral: not reported
Schwartz *J Neurosurg,* 2001(Schwartz & Fehlings, 2001)	Female Wistar rats C7-T1 clip compression	5 mg/kg IP, 0 h PI*	Non-behavioral: Counts of red nuclei neurons were significantly increased in the riluzole-treated group as well as reduced cavitation Behavioral: Improved BBB scores and inclined plane testing (greater inclined plane angles)
McAddo *Brain Res*, 2005(McAdoo et al., 2005)	Male Sprague-Dawley rats T9-T10 impactor	2 mM via microdialysis fiber, 0 h PI*	Non-behavioral: Riluzole did not decrease trauma-induced glutamate release following SCI Behavioral: Not reported
Ates *J Clin Neurosci,* 2007(Ates et al., 2007)	Male Wistar albino rats T7-T10 weight drop contusion	8 mg/kg IP, 0 h PI*	Non-behavioral: Increased white and gray matter sparing as well as smaller lesion areas. Lower MDA levels and spinal cord edema were also reported Behavioral: Improved motor function scores and higher inclined plane angles were achieved

Kitzman *Neurosci Lett,* 2009(Kitzman, 2009)	Female Sprague-Dawley rats S2 transection	8 mg/kg IP, 4 w PI and then daily for 3 d; 10 mg/kg IP, 4 w PI and then daily for 3 d	Non-behavioral: Not reported Behavioral: 8 mg/kg administration resulted in diminished tail spasticity (1 and 3 h post-administration). 10 mg/kg administration also resulted in diminished tail spasticity but also resulted in lethargy and locomotor ataxia in two of three animals
Simard *Exp Neurol,* 2012(Simard et al., 2012)	Female Long-Evans rats C7 weight drop hemicontusion	2.5 mg/kg IP, 2 min PI; 2.5 mg/kg IP, 3 h PI and then twice daily for 7 d*	Non-behavioral: Riluzole (2.5 mg/kg, 2 min PI) reduced capillary fragmentation, neuronal death and progressive haemorrhagic necrosis Behavioral: BBB scores revealed that riluzole treatment improved motor function
Wu J *Neurotrauma,* 2013(Wu et al., 2013)	Female Wistar rats C7-T1 clip compression	8 mg/kg IP, 1 h PI and then 6mg/kg every 12 h for 7 d; 8 mg/kg IP, 3 h PI and then 6 mg/kg every 12 h for 7 d	Non-behavioral: Delayed administration of riluzole (1 and 3 h PI) preserved axonal integrity and function, lead to tissue preservation, reduced inflammation and apoptosis Behavioral: Riluzole administration improved locomotor and sensory-motor function. Mechanical allodynia was not increased
Hosier *J. Neurotrauma*, 2014(Hosier et al., 2015)	Female Long-Evans rats C7 weight drop hemicontusion	5 mg/kg IP, 4 h PI and then twice daily for 7 d*	Non-behavioral: Riluzole treatment lead to decreased lesion volumes and concomitantly increased spared tissue. Behavioral: Overall motor function was increased by riluzole treatment as assessed by improved BBB scores, as well as beam balance and rotarod scores

T = thoracic vertebra; C = cervical vertebra; S = sacral vertebra; mg= milligram; g = gram; kg = kilogram; mm = millimeter; mM = millimolar; min = minute; h = hour; d = day; w = week; IV = intravenous; IP = intraperitoneal; PI = post-injury; SSEPs = somatosensory evoked potentials; MAP2 = microtubule associated protein 2; MP = methylprednisolone; ROS = reactive oxygen species; BBB = Basso, Beattie and Bresnahan locomotor test; MDA = malondialdehyde

* Authors compare other neuroprotective agents (see reference for details)

1.5.2 Magnesium chloride

Intravenously administered Mg^{2+} has been extensively investigated as a neuroprotective agent namely in preclinical studies of TBI and stroke. Furthermore, it has already been evaluated in clinical trials revealing no significant adverse events, although no consistent beneficial effects have been reported (Saver et al., 2015; Temkin et al., 2007).

Excessive release of glutamate occurs following SCI causing overstimulation of the postsynaptic NMDA receptors and resulting in excitotoxic neuronal death. During normal physiological processes, Mg^{2+} is a noncompetitive inhibitor of the NMDA receptors. However, following SCI Mg^{2+} depletion occurs (Lemke et al., 1987; Vink et al., 1989). Moreover, the decrease in Mg^{2+} is correlated with increased neurologic

deficits (Stippler et al., 2007). Magnesium administration therefore allows for replacement of depleted levels occurring after injury.

The systematic review of Mg^{2+} in acute traumatic models of SCI resulted in thirteen studies (table IV). While most studies employed Wistar and Sprague-Dawley rats as animal models, Ozdemir et al. used a rabbit model. Almost all studies used a thoracic injury model with the exception of Lee et al. in which a cervical model was used. Cord injury was generally achieved with weight drop or clip compression.

Systemic administration of magnesium was most commonly administered intraperitoneally and intravenously in doses ranging from 100 to 600 mg/kg. However, these dosages exceed human tolerability (300-600 mg/kg). Ditor, as well as Kwon et al. were able to surpass this limitation by combining magnesium in a polyethylene glycol (PEG) formulation, a widely used excipient that has also shown some degree of neuroprotection in experimental SCI models.

Therapeutic neuroprotective efficacy of both behavioral and non-behavioral outcomes was achieved up to a clinical relevant time of 8 h post-injury. A delay of 24 h, and to a lesser extent 48 h, achieved non-behavioral improvement, which was not translated to functional recovery.

Behavioral outcomes revealed improvements on incline plane testing, as well as increased Tarlov and BBB scores, and reduced mechanical allodynia. However, the studies performed by Lee and Muradov et al. reported no relevant functional recovery after Mg^{2+} treatment, only ascribing histological sparing to the treatment.

Non-behavioral outcomes included increased protection of vascular function, improved tissue sparing and electrophysiological recordings, reduced apoptosis, lipid peroxidation and neutrophil infiltration, and restoration of BSCB integrity.

Once again, and similarly to riluzole, the strong preclinical evidence towards increased functional and histological recovery lead this treatment to clinical trials in SCI. Currently, a proprietary form of Mg^{2+} with PEG (AC105) has been approved for a Phase II double-blinded, randomized, placebo-controlled clinical trial (NCT01750684), to determine the safety, tolerability, and pharmacokinetics of the drug in patients that have sustained SCI (Nagoshi & Fehlings, 2015).

Table IV. Summary of *in vivo* studies performed in animal models of traumatic SCI using systemically administered magnesium.

PUBLICATION	ANIMAL & INJURY MODEL	DOSE, ROUTE & FREQUENCY	MAIN OUTCOMES
Suzer *Spinal Cord,* 1999(Suzer et al., 1999)	Male albino rats T9 aneurysm clip compression (50 g x 30 s)	300 mg/kg $MgSO_4$ SC, 1 h PI; 600 mg/kg $MgSO_4$ SC, 1 h PI	Non-behavioral: High-dose magnesium improved the amplitudes of SSEPs and decreased MDA levels Behavioral: Not reported
Kaptanoglu *J Clin Neurosci,* 2003(Kaptanoglu, Beskonakli, Okutan, et al., 2003)	Female Sprague-Dawley rats T7-T8 weight drop contusion (50 g x cm)	100 mg/kg $MgSO_4$ IP, 0 h PI; 600 mg/kg $MgSO_4$ IP, 0 h PI	Non-behavioral: High-dose magnesium preserved spinal cord ultrastructure (electron microscopic evaluation of edema, nucleus damage, axonal and vascular changes) Behavioral: Better results in inclined plane, Tarlov and BBB tests were achieved by the high-dose treated group
Kaptanoglu *Neurosurg Rev,* 2003(Kaptanoglu, Beskonakli, Solaroglu, et al., 2003)	Female Sprague-Dawley rats T8 weight drop contusion (50 g x cm)	600 mg/kg $MgSO_4$ IP, 0 h PI	Non-behavioral: $MgSO_4$ protected the blood-spinal cord barrier at 2 and 24 h after SCI Behavioral: Locomotor function improved according to inclined plane testing, BBB and Tarlov scale scores
Ozdemir *Magnes Res,* 2005(Ozdemir et al., 2005)	New Zealand rabbits Injury model not reported	100 mg/kg $MgSO_4$ IV, 5 min PI	Non-behavioral: Treatment normalized lactate levels and significantly decreased MDA levels Behavioral: Not reported
Solaroglu *Surg Neurol,* 2005(Solaroglu et al., 2005)	Female Wistar rats T8 weight drop contusion (40 g x cm)	600 mg/kg $MgSO_4$ IP, 0 h PI*	Non-behavioral: $MgSO_4$ treatment decreased caspase-3 activity Behavioral: Not reported
Gok *Chin J Physiol,* 2007(Gok et al., 2007)	Female Wistar rats T7-T9 weight drop contusion (40 g x cm)	600 mg/kg $MgSO_4$ IP, 0 h PI*	Non-behavioral: $MgSO_4$ treatment decreased neutrophil infiltration Behavioral: Improvement of early functional scores of inclined plane testing

Ditor *J Neurosci Res,* 2007(Ditor et al., 2007)	Male Wistar rats T4 clip compression (50 g x 1 min)	300 mg/kg $MgSO_4$ IV, 15 min PI and then 6 h PI; 300 mg/kg $MgSO_4$ in PEG IV, 15 min PI and then 6 h PI*	Non-behavioral: The combination of PEG and $MgSO_4$ resulted in a significant reduction in lesion volume. Dorsal compact myelin sparing was also improved with $MgSO_4$ in PEG and $MgSO_4$ alone Behavioral: $MgSO_4$ and the combination of $MgSO_4$ in PEG reduced mechanical allodynia and provided significantly better locomotor recovery, although the latter added no benefit when compared with $MgSO_4$ administration alone. None of the treatments improved autonomic dysreflexia
Wiseman *J Neurosurg Spine,* 2009(Wiseman et al., 2009)	Female Sprague-Dawley rats T9-T10 NYU impactor (10 g x 25 mm: severe) (10 g x 12.5 mm: moderate)	600 mg/kg $MgSO_4$ IP, 10 min PI or 8, 12, 24 h PI*	Non-behavioral: Histological analysis of animals that suffered severe injury indicated that $MgSO_4$ increased white matter sparing Behavioral: For the severe injury the mean scores of BBB were significantly better for the $MgSO_4$-treated group. Severe autophagy was reported in 15% of $MgSO_4$-treated animals. For the moderate injury the BBB scores were significantly improved at 8, but not 12 or 24 h PI
Kwon *J Neurotrauma,* 2009(Kwon et al., 2009)	Female Sprague-Dawley rats T10 IH impactor (150 kdyn)	60 mg/kg $MgSO_4$ or 60 mg/kg $MgSO_4$ in PEG IV, 15 min and 6 h PI; 127 µmol/kg or 254 µmol/kg $MgCl_2$ in PEG IV, 2 h PI and then 1,3, or 5 infusions 6 or 8 h apart; 190 µmol/kg $MgCl_2$ in PEG IV, 15 min, 2, 4, or 8 h PI and then 4 infusions 8 h apart*	Non-behavioral: 60 mg/kg $MgSO_4$ in PEG significantly decreased lesion volumes. Different magnesium salts ($MgSO_4$ *vs.* $MgCl_2$) provided similar neuroprotective effects. 254 µmol/kg $MgCl_2$ in PEG conferred increased neuroprotection when compared to 127 µmol/kg. Animals treated with 3 and 5 infusions 127 µmol/kg $MgCl_2$ presented reduced lesion volume. Both 6 and 8 h interval between infusions significantly reduced lesion volumes. 190 µmol/kg $MgCl_2$ in PEG decreased lesion volumes with 5 infusions (with a delay up to 8 h PI) Behavioral: 60 mg/kg $MgSO_4$ in PEG improved locomotor scores (BBB scale with 12-point Fergusson transformation). When comparing different magnesium salts $MgCl_2$ provided earlier locomotor recovery. 254 µmol/kg $MgCl_2$ in PEG conferred significantly increased locomotor recovery when compared to 127 µmol/kg. Animals treated with 3 and 5 infusions 127 µmol/kg $MgCl_2$ had better motor recovery. Shorter time infusions (6 h) provided significantly improved locomotor recovery. 190 µmol/kg $MgCl_2$ in PEG significantly improved locomotor scores with 5 infusions (with a delay up to 4 h PI)
Lee *Spine,* 2010(Lee et al., 2010)	Male Sprague-Dawley rats C4-C5 OSU impactor hemicontusion (200 kdyn)	190 µmol/kg $MgCl_2$ in PEG IV, 2 h PI and 5 subsequent infusions 6 h apart	Non-behavioral: Treatment provided significantly higher cumulative white matter sparing. Grey matter sparing was also increased Behavioral: The horizontal ladder test revealed that $MgCl_2$ in PEG-treatment was associated with significantly less forelimb errors. Cylinder Rearing Test and Catwalk Gait Analysis revealed no significant difference between the treated animals. There were also no significant differences in the force or latency to forepaw withdrawal (sensory testing)

Muradov *J Neurotrauma,*2013(Muradov & Hagg, 2013)	Female Sprague-Dawley rats T9 IH impactor (150 kdyn)	760 µmol/kg/day $MgCl_2$ continuous IV, 0 h PI and for 24, 48 h and 7 d	Non-behavioral: 24 and 48 h $MgCl_2$ treatment increased microvascular perfusion without increasing hemorrhage following SCI, although the effect is lesser at 48 h. White matter sparing, oligodendrocyte survival and microvessel quantity is not affected by the 7 d treatment Behavioral: No improvement in locomotor function was verified when assessed with the BBB locomotor scale and gridwalk test.
Sencer *T J Neurotrauma,* 2013(Sencer et al., 2013)	Male Sprague-Dawley rats T9 clip compression (110 g x 30 s)	100 mg/kg $MgSO_4$ IP, 0 h PI and then daily for 5 d*	Non-behavioral: $MgSO_4$ treatment reduced the number of inflammatory cells, necrosis and apoptosis Behavioral: Inclined plane testing revealed increased functional recovery. However, Tarlov motor grading scale revealed no differences in locomotor recovery
Farsi *Acta Medica Iranica*, 2014(Farsi et al., 2015)	Male rats T9 clip compression (60 s)	600 mg/kg MgSO4 IP, 30 min PI*	Non-behavioral: Not reported Behavioral: Improvement of locomotor function (increased BBB scores) was achieved with magnesium treatment alone and in combination with MP (30 mg/kg), as well as decreased thermal hyperalgesia and cold allodynia

$MgSO_4$ = magnesium sulfate; $MgCl_2$ = magnesium chloride; PEG = polyethylene glycol; T = thoracic vertebra; C = cervical vertebra; IV = intravenous; µmol = micromole; mg= milligram; g = gram; kg = kilogram; mm = millimeter; cm = centimeter; s = second; min = minute; h = hour; d = day; w = week; IP = intraperitoneal; SC = subcutaneous; PI = post-injury; kdyn = kilodyne; SSEPs = somatosensory evoked potentials; MDA = malondialdehyde; MP = Methylprednisolone; BBB = Basso, Beattie and Bresnahan locomotor test

* Authors compare other neuroprotective agents (see reference for details).

CHAPTER 2 – AIMS

2. AIMS

Trauma to the spinal cord is a major cause of disability worldwide. In a clinical context, stabilization and decompressing surgical procedures combined with the administration of pharmacological agents such as methylprednisolone are currently the main therapeutic approaches to SCI. However, the clinical outcomes upon methylprednisolone therapy are low and shaded by the controversial side effects.

The use of pharmacological drugs to modulate secondary events in SCI such as ionic imbalances and excitotoxicity play an essential role in promoting neuroprotection. For instance, riluzole promotes neuroprotection by inhibition of Na^+ voltage-sensitive channels and regulation of glutamate release and uptake. Magnesium in turn protects against excitotoxic cell death, through inhibition of NMDA receptors. Furthermore, both drugs have been extensively studied and shown efficacy in preclinical SCI studies and are consequently being analyzed in human clinical trials.

Because riluzole and magnesium have independent mechanisms of action and target distinct aspects of secondary injury, it is reasonable to assume that when simultaneously administered these drugs may increase neuroprotection and promote increased neurological outcome (Figure 6).

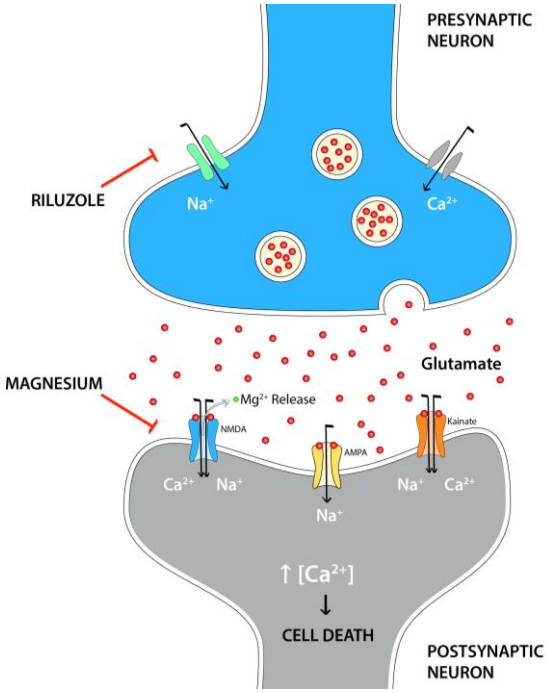


Figure 6. Ionic imbalance and glutamate excitotoxicity following SCI lead to neuronal and glial death, further aggravating the initial damage.
The neuroprotective effects of riluzole results mainly from the blockade of Na^+ channels while magnesium blocks NMDA receptors.

Therefore, with this study we aimed to investigate the efficacy of both individual and combined systemic administration of riluzole and magnesium chloride using an experimental animal model of thoracic spinal cord contusion that closely mimics the pathophysiology of human SCI.

In order to determine if neurological recovery was achieved we used several behavioral tests to assess functional recovery (BBB locomotor rating scale, activity box test, and swimming test), as well as a detailed histological evaluation comprising inflammation, axonal preservation, motor neuron survival, lesion volumes, neurogenesis/neural progenitor cells and serotonergic and glutamatergic fiber sparing.

Chapters 3, 4 and 5 are based on the following publication:

Vasconcelos NL, Gomes ED, Oliveira EP, Silva CJ, Lima R, Sousa, N, Salgado AJ and Silva NA, "Combing neuroprotective agents: effect of riluzole and magnesium in a rat model of thoracic spinal cord injury", Submitted.

CHAPTER 3 – METHODS

3. METHODS

3.1 Spinal cord injury model

All procedures were carried out in accordance to EU directive 2010/63/EU and Portuguese national authority for animal experimentation, Direção Geral de Veterinária (ID: DGV9457), guidelines on animal care and experimentation.

Nineteen, in-house bred, Wistar Han female rats (14 weeks old, weighing 210-260 g) were used for the study. Animals were kept under standard laboratory conditions (12 h light: 12 h dark cycles, 22°C, relative humidity of 55%, *ad libitum* access to standard food and water), and pair housed. Animal handling was carried out three days prior to surgery.

General anesthesia was induced by an intraperitoneal injection of a ketamine (100 mg/ml, Imalgene/Merial, Georgia, USA) and medetomidine hydrochloride (1 mg/ml, Dormitor/Pfizer, New York, USA) mixture, at a volume ratio of 1:1. Once anesthetized, animals received subcutaneous injections of the analgesic butorphanol (10 mg/ml, Butomidor/Richter Pharma AG, Wels, Austria), and the antibiotic enrofloxacin (5 mg/ml, Baytril/Bayer, Leverkusen, Germany). The fur was shaved from the surgical site and the skin disinfected with chlorhexidine. Surgical procedures were performed under sterile conditions.

The animals were placed in a prone position and a dorsal midline incision was made at the level of thoracic spine (T5-T12). The paravertebral muscles were retracted and the spinous processes and laminar arc of T8 was removed, and the spinal cord exposed. The *dura* was left intact. A weight drop trauma model was used, that consisted in dropping a 10 g weight rod from a 20 cm height on to the exposed spinal cord (Allen, 1911). The rod was guided through a stabilized tube that was positioned perpendicularly to the center of the spinal cord. After the trauma, the muscles were sutured with Vicryl suture (Johnson and Johnson, New Jersey, USA) and the incision closed with surgical staples (Fine Science Tools, Heidelberg, Germany). Anesthesia was reversed using atipamezole (5mg/ml, Antisedan/Pfizer). Rats received subcutaneous injections of vitamins (Duphalyte/Pfizer) and 0.9% NaCl, and were kept under heat lamps until recovery.

Post-operative care included butorphanol administration twice a day, for a five day period as well as vitamins, saline, and enrofloxacin, twice a day for a seven day period. Manual expression of bladders was performed twice a day until animals recovered spontaneous voiding. Body weight was monitored weekly as a parameter of general health of the animals. If a weight loss over 10% of body weight was

detected, a high-calorie oral supplement (Nutri-Cal®) was administered daily. If needed, rats were individually housed to avoid removal of surgical staples by their cage-mates.

3.2 Drug preparation

Drug concentrations were chosen based on previous studies reporting the neuroprotective properties of systemically administered riluzole (Sigma, Missouri, USA) and magnesium in a PEG 1000 formulation (Sigma), when individually administered.

Riluzole was solubilized in DMSO at high concentration and then diluted in saline and administered at a dose of 2.50 mg/kg, pH≅8. $MgCl_2$ was prepared in a PEG formulation (1 g/kg; 30% w/w in sterile saline) and administered at a dose of 24.18 mg/kg, pH≅8. All solutions were filtered through a 0.45 µm filter.

3.3 Experimental groups

One hour after induced SCI, rats we randomly assigned to receive one of four different treatments: 1) saline (n=5), 2) riluzole (n=4), 3) magnesium chloride in a PEG formulation (n=5), or 4) a combined treatment (n=5). Treatment regimen consisted of five intraperitoneal injections, 1 h post-injury and the following 12 h apart (Figure 7).

An animal from group 3 died nineteen days post-injury. Two animals from group 1 died, thirteen and eighteen days post-injury. This was probably due to urinary complications as the animals continuously presented an enlarged bladder and difficulty in bladder expression.

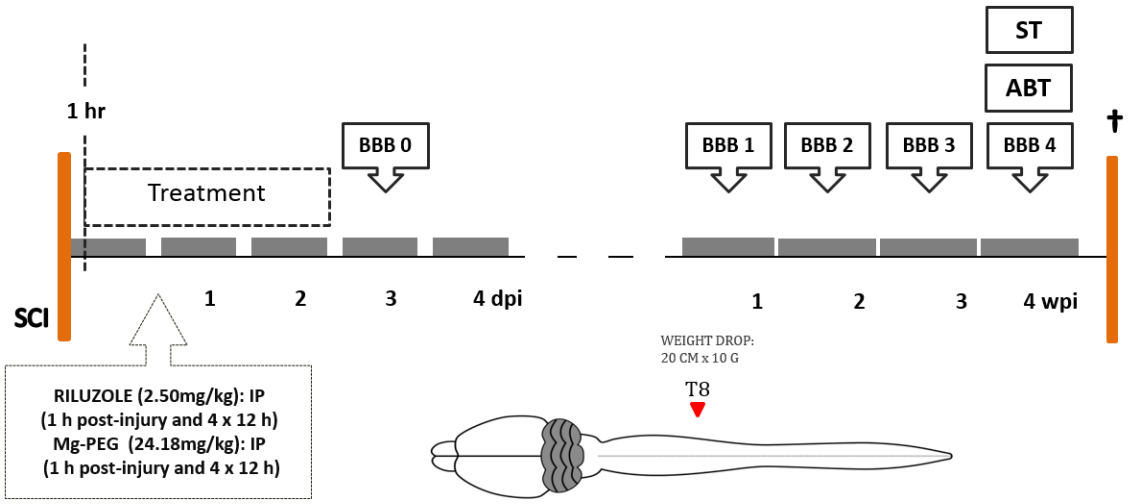


Figure 7. Schematic representation of the approach used to explore the therapeutic efficacy of both individual and combined administration of riluzole and MgCl2.
BBB= Basso, Beattie and Bresnahan test; ABT= activity box test; ST= swimming test; dpi= days post-injury; wpi= weeks post-injury.

3.4 Behavioral assessment

The BBB locomotor rating scale, the activity box test, and a swimming test were used to evaluate functional recovery. The BBB test was performed three days post-injury and thereafter weekly for a 4 week period. The activity box test was performed on day 29 and the swimming test on day 31 post-injury. All behavioral tests were performed blindly to the treatment groups.

3.4.1 BBB locomotor rating scale

Locomotive recovery was evaluated using an open-field test based on grading hindlimb locomotion in rats. Briefly, two examiners independently registered movements in hindlimb joints, paw placement during stepping, weight support, and forelimb-hindlimb coordination performed by animals, on an open field arena over the course of 4 minutes, and assessed motor function based on a 21 point scoring system (Table V) (Basso et al., 1995). The left and right hindlimb scores were averaged to obtain a single value. A BBB score of 0 indicates no hindlimb movement. A BBB score of 1 through 8 indicates joint movement, but no weight support. A BBB score of 9 through 20 indicates an ability to support weight and use the limb for locomotion but with some degree of abnormality. A BBB score of 21 corresponds to the locomotion of a normal rat.

Table V. Basso, Beattie, Bresnahan Locomotor Rating Scale.
Adapted from Basso et al., 1995.

0	No observable hindlimb movement (HL)
1	Slight movement of one or two joints, usually the hip and/or knee
2	Extensive movement of one joint or extensive movement of one joint and slight movement of one other joint
3	Extensive movement of two joints
4	Slight movement of all three joints of the HL
5	Slight movement of two joints and extensive movement of the third
6	Extensive movement of two joints and slight movement of the third
7	Extensive movement of all three joints of the HL
8	Sweeping with no weight support or plantar placement of the paw with no weight support
9	Plantar placement of the paw with weight support in stance only (i.e. when stationary) or occasional, frequent or consistent weight-supported dorsal stepping and no plantar stepping
10	Occasional weight-supported plantar steps; no forelimb (FL)/HL coordination
11	Frequent to consistent weight-supported plantar steps and no FL/HL coordination
12	Frequent to consistent weight-supported plantar steps and occasional FL/HL coordination
13	Frequent to consistent weight-supported plantar steps and frequent FL/HL coordination
14	Consistent weight-supported plantar steps; consistent FL/HL coordination, and predominant paw position during locomotion is rotated (internally or externally) when it makes initial contact with the surface as well as just before it is lifted off at the end of stance; or frequent plantar stepping, consistent FL/HL coordination, and occasional dorsal stepping
15	Consistent plantar stepping and consistent FL/HL coordination and no toe clearance or occasional toe clearance during forward limb advancement; predominant paw position is parallel to the body at initial contact
16	Consistent plantar stepping and consistent FL/HL coordination during gait and toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift-off
17	Consistent plantar stepping and consistent FL/HL coordination during gait and toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel at initial contact and lift-off
18	Consistent plantar stepping and consistent FL/HL coordination during gait and toe clearance occurs consistently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift-off
19	Consistent plantar stepping and consistent FL/HL coordination during gait, toe clearance occurs consistently during forward limb advancement, predominant paw position is parallel at initial contact and lift-off, and tail is down part or all of the time
20	Consistent plantar stepping and consistent coordinated gait, consistent toe clearance, predominant paw position is parallel at initial contact and lift-off, and trunk instability; tail consistently up
21	Consistent plantar stepping and consistent gait, consistent toe clearance, predominant paw position is parallel throughout stance, and consistent trunk stability; tail consistently up

3.4.2 Activity box

The activity box test allows the assessment of gross motor behavior by measuring the total distance travelled by the animals (Sousa et al., 2006). The test was performed in an open arena (43.2 cm × 43.2 cm) with transparent acrylic walls (MedAssociates Inc., Vermont, USA). Animals started the test at the arena's center and were given 5 minutes to explore it. Data were collected using the activity monitor software. Distance travelled was used as a measure of locomotor activity.

3.4.3 Swimming test

This test provides a major advantage as animals constantly need to locomote in order to stay afloat. The swimming test allows for an overall assessment of locomotor behavior by measuring swimming velocity. Animals were allowed to swim freely in a circular pool with a diameter of 170 cm and a depth of 50 cm for 2 minutes (water temperature 24 - 25 °C). Each animal performed three trials. Data was collected by a fixed camera placed in the ceiling and connected to a video-tracking system (Viewpoint).

3.5 Histological assessment

Five weeks after injury animals were deeply anesthetized by an intraperitoneal injection of sodium pentobarbital (200 mg/ml, Eutasil/Ceva Sante Animale, Libourne, France) and transcardially perfused with 100 ml of cold 0.9% saline followed by 300 ml of 4% paraformaldehyde (PFA) in 1X phosphate-buffered saline (PBS). A rough dissection of the spine and spinal cord was performed and tissues were fixed in a solution of 4% PFA for 24 h (4 °C). The spinal cord was then dissected from the vertebral column and immersed in a 30% sucrose solution serving as a cryoprotectant until they equilibrated (≅48 h at 4 °C). Afterwards, 2 cm length of spinal cord tissues, centered on the lesion, were submerged in optimal cutting temperature (OCT) embedding medium, frozen on dry ice, and stored at -20 °C. Cross-sections of 20 µm thickness were performed using a cryostat (Leica CM1900, LeicaBiosystems, Nussloch, Germany) and thaw-mounted onto charged microscope slides (Superfrost Plus, Thermo Scientific, Massachusetts, USA).

All histological procedures and evaluation were performed blindly to the treatment groups.

3.5.1 Hematoxylin-Eosin staining (H & E)

For histochemical analysis, H & E staining of spinal cord cross-sections was performed using an automatic processor (Leica TP1020-1). Basically, the slides were immersed in hematoxylin and eosin solutions and then washed in distilled water, dehydrated in increasing concentrations of ethanol and finally cleared in xylene substitute. In the end, slides were mounted using Microscopy Entellan® (Merk & Co., Inc., New Jersey, USA).

Images were then captured with a stereology microscope (Zeiss Axioplan 2 Imaging, Jena, Germany) with a 2.5x objective. Damaged tissue evaluation (lesion) was performed on transverse sections (200 µm apart). The areas were manually traced and then quantified using Image J (NIH) software.

The extent of the lesion was assessed along the rostrocaudal lesion. The injured area was manually traced and then quantified (% injured tissue = injured area/total cross-sectional area × 100 %). The epicenter was defined as the cross-section with the highest amount of damaged tissue. The lesion volume was obtained by the sum of total lesion area multiplied by distance between the sections.

3.5.2 Immunofluorescence

For immunofluorescence staining slices were washed with PBS, permeabilized with 0,2% Triton X-100 for 10 min and blocked with 5% fetal calf serum in 0.2% Triton X-100 for 30 min. Afterwards, the following primary antibodies were incubated overnight at room temperature (RT): mouse anti-neuronal nuclei for neuronal cells (NeuN; 1:200; Millipore, Darmstadt, Germany); mouse anti-CD11b/c for microglia and macrophages (1:100; Pharmingen, California, USA); mouse anti-neurofilament for axons (NF; 1:200; Millipore); rabbit anti-5-hydroxytryptamine for serotonergic axons (5-HT;1:2000; Millipore); rabbit anti-vesicular glutamate transporter 2 for glutamatergic neurons (VGLUT2; 1:200; Millipore); mouse anti-nestin for progenitor cells (nestin; 1:200; Millipore); and rabbit anti-doublecortin for neuronal precursor cells and immature neurons (DCX; 1:500; Millipore).

The following day primary antibodies were then probed (2 h incubation) with the appropriate Alexa 594- or Alexa 488-conjugated secondary antibodies (1:1000; Invitrogen, Paisley, UK). Sections were counterstained with DAPI for 30 min (1:1000; Sigma) and mounted with Immu-Mount® (Thermo Scientific). Between steps, 5 washes with PBS (1x) were performed.

For all immunofluorescence procedures, the appropriate controls were obtained by omission of the relevant primary antibody.

For the assessment of motor neuron survival, coronal sections ranging from rostral to caudal segments (centered at the injury site) were sequentially chosen from each animal for quantification. Large neurons with a clearly identifiable nucleus and a cell soma were then counted in both ventral horns, namely in lamina VIII and IX, for an estimation of $NeuN^+$ cells.

For the assessment of inflammation (CD11b/c), axonal preservation (NF), serotonergic and glutamatergic fiber sparing (5-HT/VGLUT2), coronal sections ranging from rostral to caudal segments (centered at the injury site) were sequentially chosen from each animal for quantification. Images were acquired using a fluorescence microscope (Olympus BX61, Hamburg, Germany), except for VGLUT2 images which were acquired by confocal microscopy using a confocal point-scanning microscope (Olympus FV1200), and then opened using Image J (NIH) software. The images were then converted to

8-bit black and white pictures. Regions of meninges and roots were excluded for quantification. Inflammation was assessed as the area occupied by CD11b/c$^+$ cells in each coronal section.

To analyze axonal preservation, a region of interest was selected (dorsal corticospinal tract) which was then normalized in all sections analyzed. Serotonergic fiber sparing analysis was performed in a pre-determined area (39474 μm^2), adjacent to the ventral horns. Glutamatergic fiber sparing was analyzed as the density of fibers exiting the ventral horn. In order to do that, the area occupied by the ventral horn was subtracted to the total image area and the area occupied by VGLUT$^+$ fibers was analyzed and normalized in all sections.

3.6 Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.00 software for Windows. Data from SCI volume was analysed using Student's t-test. Data from the activity box, swimming test, SCI volume, and immunofluorescence analysis of nestin$^+$ and DCX$^+$ cells were assessed by one-way analysis of variance (ANOVA). Differences between groups were compared with the *post hoc* Bonferroni test. Data from the BBB test, lesion percentage and immunofluorescence analysis were assessed by a two-way ANOVA test. Differences between groups were compared with the *post hoc* Bonferroni test. Statistical significance was defined for $p<0.05$ (95% confidence level). Data are shown as mean + standard error of the mean (SEM).

CHAPTER 4 – RESULTS

4. RESULTS

4.1 Behavioral assessment

4.1.1 BBB locomotor rating scale

Motor evaluation using the BBB scale began 3 days after surgery and was subsequently performed weekly during a four week period to generate a trend of functional recovery over time (Figure 8). Three days after a severe contusive injury, all animals' demonstrated decreased levels of locomotor function (group mean = 1.3±0.9), meaning that animals' hindlimbs movements were severely impaired as only slight to moderate movement of one or two joints, or extensive movement of one joint was achieved.

The saline-treated group demonstrated spontaneous recovery over time, however the BBB scores apparently stabilized at week 3 post-injury reaching a score of 6.2±3.2. In the group treated with riluzole, BBB scores reached 9.1±2.4 at week 4 post-injury. Moreover, there was a significant difference between this group and control animals (saline-treated group) at weeks 1 and 2. Further statistical analysis of the riluzole treatment at these specific time points, using *Cohen's d* effect size (week 1: $t_{(7)} = 2.666$; $p = 0.0322$; $d = 1.79$; week 2: $t_{(6)} = 1.922$; $p = 0.1030$; $d = 1.36$), revealing a strong effect size caused by riluzole treatment at early time points when compared to saline controls. Additionally, the riluzole-treated group achieved weight-supported stepping from week 2 up to week 4 post-injury, a functional difference with clinical relevance (Kwon et al., 2011).

In the magnesium-treated group, BBB scores reached 6.9±2.4 at week 4 post-injury. At the same time point, the group treated with the combination of riluzole and magnesium scored 5.7±4.8.

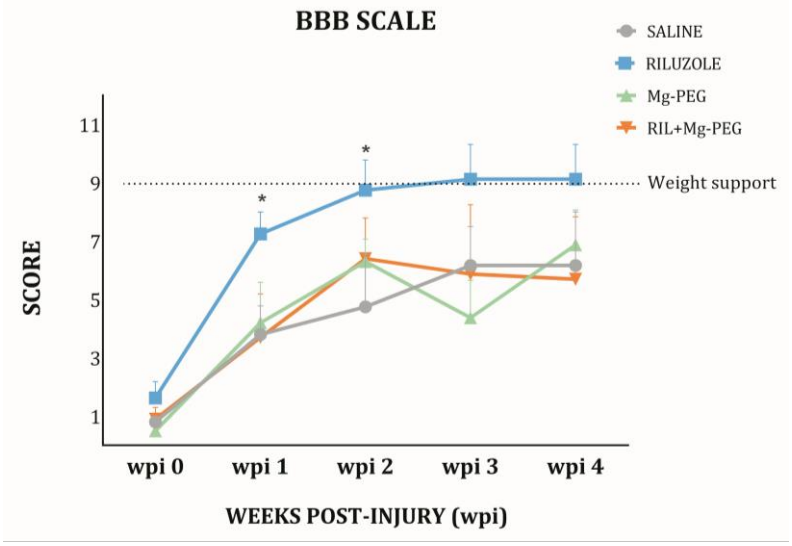


Figure 8. Functional hind limb recovery determined by the BBB test in rats that received either riluzole, magnesium, a combination of either drugs, or saline.
At week 1 and 2 post-injury, BBB score of riluzole-treated group is significantly higher when compared to saline-treated group. Only the riluzole-treated group achieved plantar weight support (BBB=9), from week 3 post-injury. Values shown as mean + SEM; * P<0.05.

4.1.2 Activity box

General motor behavior was assessed on day 29 (week 4) post-injury. Animals were placed on an open arena (43.2 cm × 43.2 cm) with transparent walls and allowed to explore it for 5 minutes.

Results showed an overall significant increase in total distance travelled of riluzole-treated when compared to the saline-treated group (2023±11 *vs.* 1450±22 cm). Both the magnesium-treated group (1909±40 cm), and to the combination-treated group (1873±55 cm) presented values similar to the riluzole-treated group, however without significant differences when compared to controls, revealing just a trend for an increase in the distance travelled (Figure 9A).

4.1.3 Swimming test

Swimming velocity was assessed on day 31 (week 4) post-injury. Animals were allowed to swim freely in a circular pool with a diameter of 170 cm and a depth of 50 cm for 2 minutes. Velocity was recorded using a video-tracking system.

Animals from the combination-treated group (25.7±5.7 cm/s) presented higher swimming velocity when compared to animals that received magnesium (23.1±2.6 cm/s), riluzole (21.6±2.6 cm/s) or saline (24.0±1.8 cm/s). However, no significant differences were detected between the groups (Figure 9B).

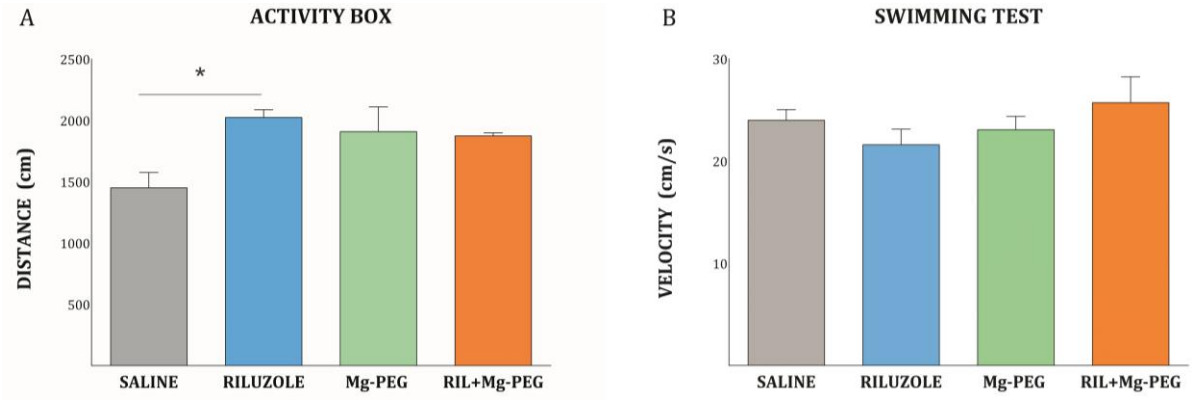


Figure 9. Measurement of general motor behavior using: A) an activity box test (time=5min) and B) a swimming test (time=2min).
The swimming test revealed no difference between treated groups and control (saline), while the activity box test showed an overall increase in total distance travelled of riluzole-treated group when compared to the saline group. Values shown as mean + SEM; * P<0.05.

4.2 Histological assessment

4.2.1 Percentage of injured tissue and lesion volume

Measurements of the injured tissue revealed decreased loss of tissue, namely in the caudal region of the spinal cord, in the riluzole-treated group (Figure 10A). The differences achieved were significant from 200 to 600 μm caudally to the lesion epicenter ($p<0.05$).

Total lesion volume estimation revealed no significant differences between groups when a one-way ANOVA test was performed, however a t-test analysis further revealed that the riluzole-treated group presented a significantly smaller lesion volume (3.23±0.52 mm^3) when compared to the saline-treated group (4.74±1.39 mm^3). The volumes of the riluzole-treated group, the magnesium-treated group (4.37±0.87 mm^3), and the combination-treatment group (4.45±1.23 mm^3) represent a 32%, 8% and 6% reduction in lesion size, respectively, when compared to saline-controls (Figure 10B).

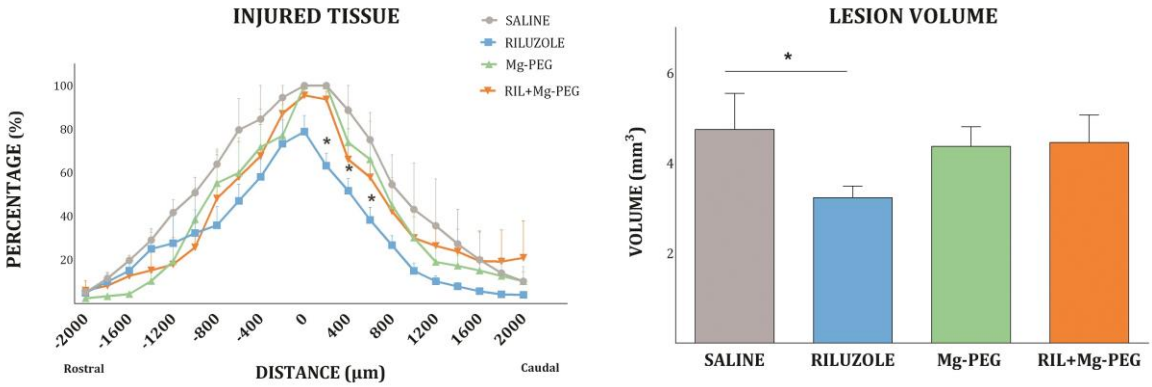


Figure 10. Histological recovery evaluation.
Hematoxylin-Eosin staining revealed: A) a decreased loss of tissue, namely in the caudal areas of injury (200 to 600 µm), in the riluzole-treated group. B) The riluzole-treated group (3.23±0.52 mm^3) presents a smaller lesion volume when compared to the saline-treated group (4.74±1.39 mm^3). Values shown as mean + SEM; * P<0.05.

4.2.2 Axonal preservation

Neurofilament immunoreactivity was used to determine axonal preservation in the corticospinal tract (CST). The results indicate that the riluzole treatment induced significant caudal axonal preservation of the CST when compared to saline controls at 1600 µm (0.4±0.5 % vs. 1.1±0.4 %, p<0.01) and 2000 µm (0.0±0.0 % vs. 1.5±0.5 %, p<0.001) distance from the epicenter (Figure 11A). However, this effect is not present in the rostral portion of the spinal cord.

The combined treatment group also presented an effect at 2000 µm distance from epicenter when compared to saline-controls (0.8±1.1 % vs. 0.0±0.0 %, p<0.05).

4.2.3 Serotonergic and glutamatergic fiber sparing

Only the riluzole treatment led to a higher plasticity/spared serotonergic fibers when compared to saline-controls (Figure 11B), which was translated in an increased area of serotonergic fibers. However, this effect is only present in the caudal portion of the spinal cord, and is lost after 1600 µm (p<0.01) from the epicenter. Concerning glutamatergic fiber sparing, a similar trend for increased percentage of fiber sparing in the riluzole-treated group was seen, once again in the caudal portion of the spinal cord (Figure 11C). At 1200 µm this difference is significantly different when comparing riluzole to the magnesium-treated group (p<0.05). Oppositely, the combinatorial treatment seems to promote fiber sparing in the rostral portion of the spinal cord.

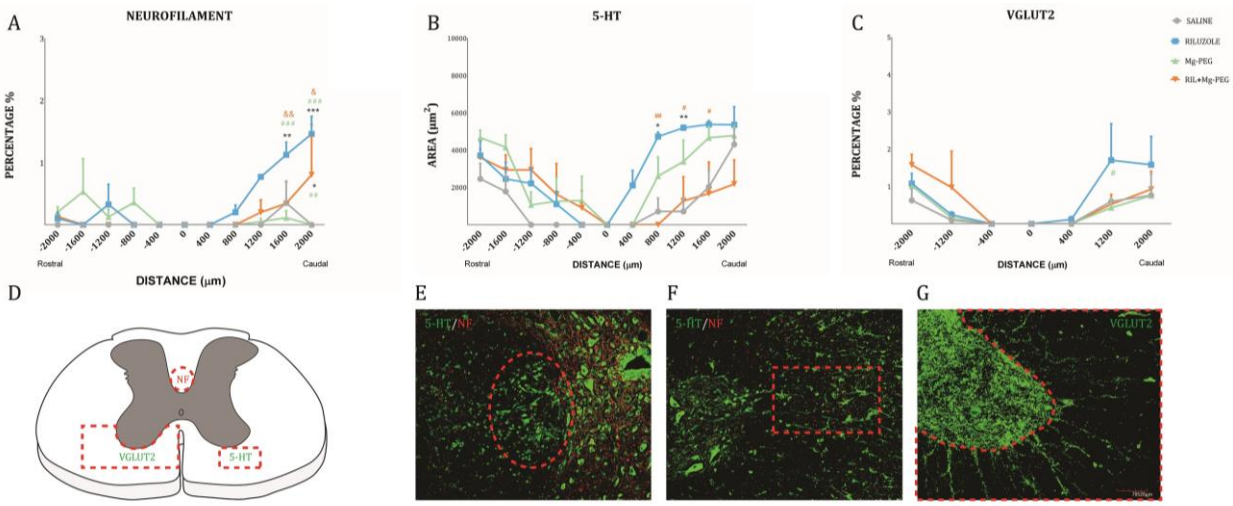


Figure 11. Axonal preservation analysis.
A) Neurofilament immunofluorescence labeling revealed increased axonal preservation in the riluzole-treated group, namely in the caudal portion of the spinal cord (1600 to 2000 µm). Quantification was performed in the corticospinal tract area. B) 5-HT immunofluorescence labeling revealed that riluzole treatment increased serotonergic fiber sparing in the caudal portion of the spinal cord (800 to 1600 µm)**.** C) VGLUT2 immunofluorescence labeling revealed a tendency for increased glutamatergic fiber sparing in the caudal portion of the spinal cord. At 1200 µm this difference is significantly different when compared to the magnesium-treated group. D) Schematic representation of the regions of interest were immunofluorescence was analyzed. E); F) and G) are representative micrographs. Values shown as mean + SEM; * P<0.05; **P0.01; ***P0.001.

4.2.4 Motor neuron survival

Motor neuronal death is characteristic of SCI and further contributes to permanent functional deficits (Xu et al., 2005). Apparently, neither treatment appeared to influence motor neuron survival (motor interneurons and alpha motor neurons located in lamina VIII and IX), both rostral and caudally, as the number of $NeuN^+$ cells spared following SCI was not affected by either treatment when compared to saline-controls (Figure 12).

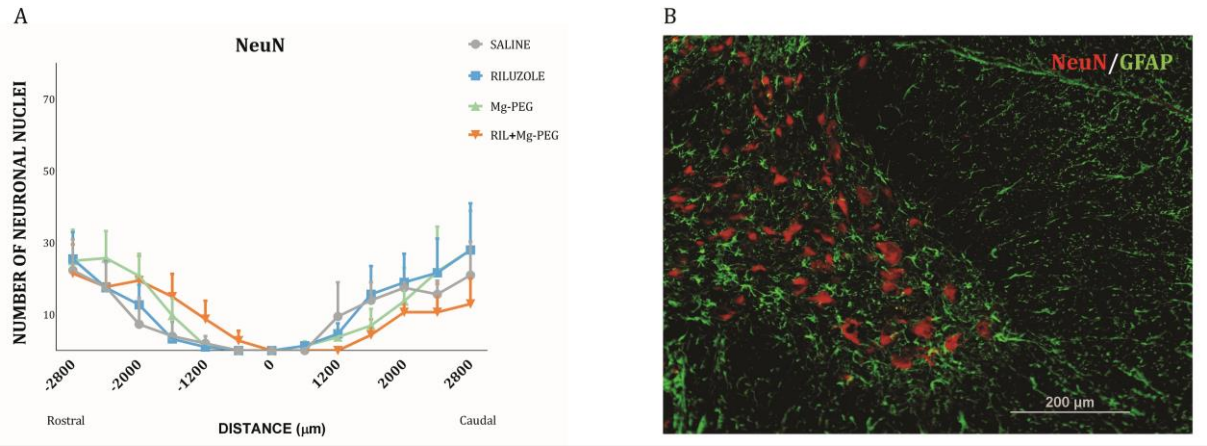


Figure 12. Motor neuron survival.
A) NeuN immunofluorescence labeling of surviving motor neurons (NeuN$^+$ cells) in the ventral horns revealed that there were no significant differences in motor neuronal loss between the groups. B) Representative micrograph. Values shown as mean + SEM.

4.2.5 Inflammation

SCI is followed by a severe inflammatory reaction that translates in activation of microglia, the resident central nervous system immune cells, and macrophage infiltration (Donnelly & Popovich, 2008).

When compared to the saline-treated group, a significant decrease in the area occupied by CD11b/c$^+$ immune cells was only observed in the combination-treated group (Figure 13). This effect seems circumscribed to areas close to the lesion epicenter (400 μm both rostral and caudally; $p<0.05$) and the epicenter itself ($p<0.01$). The magnesium-treated group also significantly decreased the area occupied by CD11b/c$^+$ immune cells, when compared to saline-controls, but only at the epicenter of the lesion ($p<0.05$).

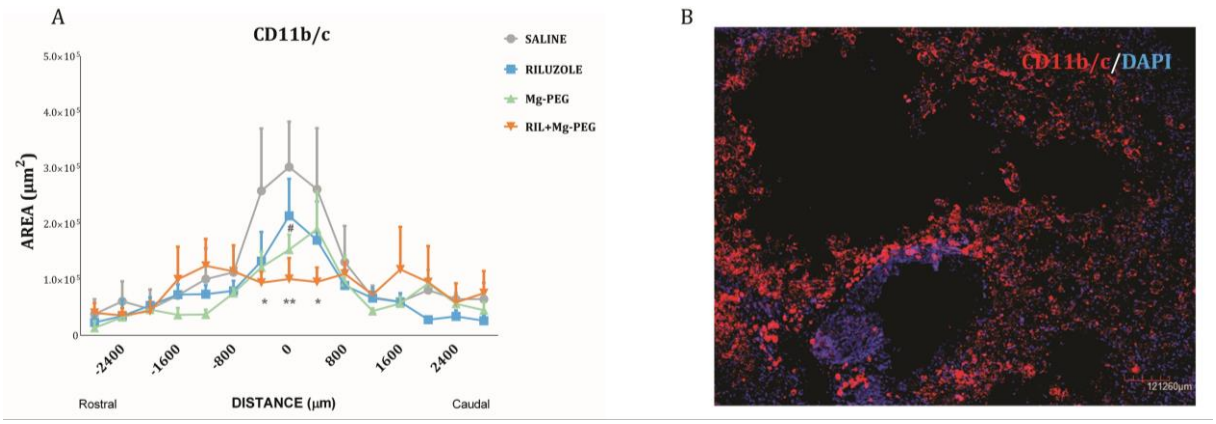


Figure 13. Inflammation assessment.
A) The combined treatment and the magnesium treatment to a lesser extent, significantly decreased inflammatory response after SCI, namely around the epicenter, as revealed by CD11b/c immunofluorescence analysis. B) Representative micrograph. Values shown as mean + SEM; * P<0.05; **P0.001.

4.2.6 Progenitor cells and immature neurons

The presence of progenitor neural stem cells (nestin$^+$ cells) and immature neurons (DCX$^+$ cells) was investigated in the ependymal lining of the central canal in the lumbar region. Using immunohistochemistry it was possible to verify the presence of both progenitor cells and immature neurons in the central canal (Figure 14C). Statistical analysis did not reveal significant differences in the number of nestin$^+$ (Figure 14A) and DCX$^+$ (Figure 14B) cells present in the central canal of the different treated groups. In the thoracic region of the spinal cord (injury zone) it was possible to detect a substantial positive staining for both progenitor cells and immature neurons (Figure 14D). DCX$^+$ cells were not detected inside the cavity formed by the injury, stopping its migration at the glial scar. Contrarily, nestin$^+$ cells were able to bypass the glial scar and were found both inside and outside the cavity.

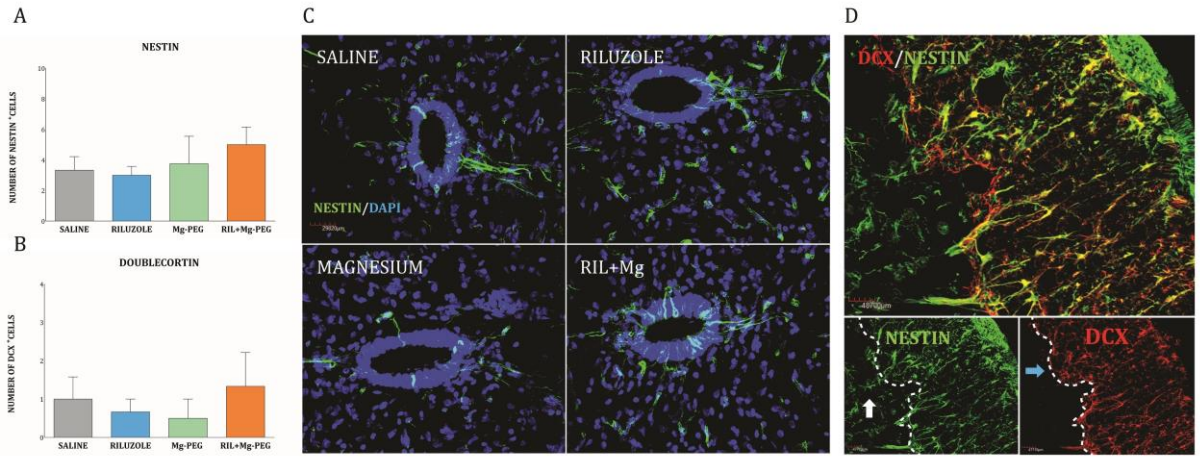


Figure 14. A) Nestin and B) doublecortin immunofluorescence labeling of neural stem cells and immature neurons in the central canal of the spinal cord revealed that there are no differences in the number of cells between groups.

C) Representative micrographs of neural stem cells in the different groups. Values shown as mean + SEM. D) DCX^+ cells were not detected inside the cavity formed by the injury, stopping their migration on the glial scar (blue arrow). Contrarily, $nestin^+$ cells were able to bypass the scar (white arrow) and were found both inside and outside the cavity.

CHAPTER 5 – DISCUSSION

5. DISCUSSION

Electrolytic imbalance and glutamate toxicity occur early after SCI and stand as neuroprotective targets following the injury itself. In this study we determined the effects of single and combined drug treatments in a rat model of thoracic SCI, using the neuroprotective agents' riluzole and magnesium chloride.

A 2.50 mg dose of riluzole was chosen as previous reports found that doses ranging from 5 to 8 mg/kg, repeated twice daily intraperitoneally (IP), left the animals in a comatose or moribund state (Kitzman, 2009; Mantz et al., 1992; Wu et al., 2013). Contrarily, a dose of 2.5 mg/kg IP every 12 h was found to be beneficial in terms of outcome after SCI (Simard et al., 2012; Wahl & Stutzmann, 1999).

Clinically relevant motor recovery was only achieved with the riluzole treatment. This was translated into weight supporting ability in injured rats, occurring as early as two weeks after injury. Additionally, despite the low number of animals, we found that riluzole treatment had a strong effect size (Week 1: d = 1.79; week 2: d = 1.36), during the acute phase of treatment (week 1 and 2). The statistical strength of riluzole treatment was calculated using the *Cohen's d* equation. Jacob Cohen demonstrated that the difference between two means with a "d value" smaller than 0.2 represent a small effect size of the intervention tested, while around 0.5 represent a medium effect size and higher than 0.8 is a large effect size (Cohen, 1977).

Furthermore, general locomotor behavior was also significantly improved in the riluzole-treated group, as observed in the activity box test. These results are in line with the BBB test. Riluzole-treated rats presented higher motor abilities compared to the other rats (weight support), and so they were able to travel an increased distance in the activity box test. However, in the swimming test we were unable to detect any significant difference between groups. This is probably related to the fact that water provides buoyancy, which allows rats to perform locomotor movements without having to support their own body weight (Smith et al., 2010).

Riluzole treatment also improved histological recovery, as shown by a decreased percentage of injured area, namely in the caudal portion of the spinal cord, and a decreased lesion volume. Additionally, riluzole also promoted significant axonal preservation in the corticospinal tract, a major descending pathway contributing to the initiation and control of voluntary movement in mammals (Carmel & Martin, 2014). Sparing of serotonergic and glutamatergic fibers was also increased with riluzole treatment, which was also detected in the caudal portion of the spinal cord. Descending serotonergic pathways (raphespinal tract) are highly important for posture maintenance, initiation of locomotion, and modulation of neuronal

circuits within the spinal cord (central pattern generators)(Ciranna, 2006; Jordan et al., 2008). Tracts that use fast glutamatergic synaptic transmission (corticospinal and reticulospinal tracts) are mainly responsible for voluntary initiation of movement (Rekling et al., 2000). The effect observed is most probably related to an increase in tissue preservation promoted by riluzole treatment. However, riluzole may also have enhanced fiber regeneration, fiber sparing, and/or fiber sprouting, resulting in improved functional recovery.

Regarding the fact that riluzole mainly caused histological improvements in the caudal portion of the spinal cord, this can be attributed to the fact that there appears to be a rostro-caudal asymmetry in SCI as described by some studies employing non-invasive imaging techniques (Deo et al., 2006; Narayana et al., 2004). These studies have reported an increase in white matter and grey matter sparing in the caudal sections of the spinal cord. Therefore, it is possible that the riluzole treatment further amplified this effect. Narayana et al. attribute the improvement of grey matter to increased caudal angiogenesis (Narayana et al., 2004). Supporting this idea is the fact that an asymmetric increase in expression of an angiogenic gene, osteopontin, was also shown in the caudal portion of the spinal cord, at 24 hours and 35 days post-injury (Aimone et al., 2004). Localized revascularization events following SCI can therefore be responsible for an increased delivery of riluzole in the caudal portion of the spinal cord and the resulting increase in histological recovery.

Contrary to other study reports, we did not establish a significant anti-inflammatory effect of riluzole treatment (Wu et al., 2013). However, a trend to decreased inflammation was observed. Furthermore, riluzole treatment did not confer increased motoneuron protection, an effect that has also been previously reported (Lang-Lazdunski et al., 1999; Nogradi et al., 2007).

Magnesium's neuroprotective effect has been extensively studied in animal models of stroke and TBI (Ginsberg, 2008; Temkin et al., 2007). Current clinical applications include preeclampsia and acute myocardial infarction (Shechter, 2010; Stocks, 2014). Furthermore, PEG is known to repair membrane damage, restore normal ion permeability, and allow the use of a decreased dosage of magnesium, which is adequate for human clinical applications. Unexpectedly though, we were not able to detect an overall significant effect of magnesium chloride treatment after SCI, both histologically and behaviorally, contrary to previous reports by Kwon and Lee et al. (Kwon et al., 2009; Lee et al., 2010). This decreased neuroprotective effect can be attributed to the use of a different SCI model. While both authors used a contusive injury model, Kwon et al. used an Infinite Horizon Impactor (150 kdyn) to establish a thoracic (T10) model of SCI, while Lee et al. established a cervical (C4-C5) hemicontusive model using the Ohio State University Impactor (200 kdyn), we use a weight drop model to establish a T8 contusive injury.

Furthermore, it has been reported that there are considerable differences in SCI outcome when using different rat strains (Mestre et al., 2015; Mills et al., 2001). Thus, it is important to note that both authors used Sprague-Dawley strain while we used Wistar Han rats as animal models which can also account for the different outcomes.

We further designed a combination treatment that simultaneously engaged two molecular targets in order to achieve a higher neuroprotective effect against excitotoxicity. However, the combination of riluzole and magnesium did not achieve superior effects when compared to the individual target-specific treatment, for both behavioral and histological outcomes. The only exception was the positive effect on inflammation. Indeed, the combined treatment and to a lesser extent the magnesium treatment, lead to significant decrease of the area occupied by microglia. Previous studies have found that NMDA receptor antagonists agents such as memantine and MK-801 are able to decrease microglial activation (Wu et al., 2009; Thomas & Kuhn, 2005). Thus, it possible that the NMDA blocking effect of the magnesium treatment may be responsible for the decrease in the inflammatory profile. Furthermore, voltage-gated sodium channel currents also elicit microglial activation (Jung et al., 2013). Consequently, we can deem that the blocking of these channels by riluzole treatment also plays a role in neuroinflammatory modulation and that the combination of both these drugs may have had a potentially additive effect.

Nevertheless, taking into account the poor overall effects of the co-administration of these two drugs, we can admit that there is the possibility of altered pharmacokinetics (drugs distribution, metabolism, and excretion). Furthermore, the co-treatment may be interfering with physiological functions due to the exacerbated disruption of ionic intracellular and extracellular levels, which is possibly amplified by the blocking of sodium channels and the rapid increase of magnesium levels.

Combinatorial strategies have been employed in multiple SCI studies with contrasting results. Positive outcomes in using combined treatments have been reported, namely using neutrophins and cyclic adenosine monophosphate as well as combined strategies with cell transplantation (Kubasak et al., 2008; Lu et al., 2004; Pearse et al., 2007). However, lack of additive or synergistic results have also been reported (Maier et al., 2009; Mountney et al., 2013; Streijger et al., 2014). Interestingly enough, concerning the combined administration of riluzole and magnesium to our knowledge, there was only one study performed. Lang-Lazdunski et al. used a rabbit model of spinal cord ischemia, and also found that there were no additive neuroprotective effects when these two drugs were combined, while their individual administration afforded significant spinal cord protection in a setting of severe ischemia (Lang-Lazdunski et al., 1999).

Although the present report does not favor the use of neuroprotective drugs riluzole and magnesium in combination (at this drug ratio), we believe that the complex nature of the injury will most likely be tackled by integration of multiple therapies in order to provide enhanced functional recovery.

CHAPTER 6 – CONCLUSION AND FUTURE PERSPECTIVES

6. CONCLUSION AND FUTURE PERSPECTIVES

The aim of this thesis was to investigate the use of a combined pharmacological approach using the clinical available drugs magnesium and riluzole to target excitotoxic events that follow the initial injury. Both drugs have been extensively studied in preclinical models of SCI, accomplishing promising results that are translated in both behavioral and histological recovery.

We demonstrate that only the riluzole treatment significantly improved behavioral recovery (both on BBB scale and activity box test). The combined treatment, although simultaneously targeting two excitotoxic-related mechanisms, did not improve locomotion.

A detailed histological evaluation comprising inflammation, axonal preservation, motor neuron survival, lesion volumes, neurogenesis/neural progenitor cells and serotonergic and glutamatergic fiber sparing was then performed. Overall, riluzole-treated rats presented once more improved outcomes. Collectively, these results provide further evidence for the neuroprotective effect of riluzole.

Moreover, although our findings do not favor the use of a combined treatment, we believe that reporting this data will surely add to the field of spinal cord injury research. However, it is important to keep in mind that beneficial/detrimental effects of specific pharmacological agents are not always consistently reproduced in preclinical SCI research. Aspects ranging from animal strain, sex, routes of drug administration, type of injury to severity of injury may result in altered neurological outcomes.

Lastly, we believe that due to the complex nature of SCI successful functional recovery will be achieved using a combined therapeutic approach that targets both the acute and chronic injury, proving the necessary environment for axonal regeneration and ultimately promoting functional recovery. Nevertheless, additional studies are warranted to find an optimal therapeutic regimen.

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