Marta Raquel Lima Ferreira

Combine baclofeno with pregabalin administration as a potential therapy for spinal cord injury

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Authorship Statement

I hereby declare to be the author of this work, which is original and unpublished. Authors and papers consulted are duly cited in the text and are included in the list references.

(Marta Raquel Lima Ferreira)

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Abstract

Spinal cord injury (SCI) is a neurological impairment that hampers the communication between the brain and the rest of the body, resulting in permanent loss of motor function and sensory perception. After the injury, it is initiated a cascade of biological and biochemical processes. No effective therapy for SCI has yet been developed, however several studies in distinct areas has been developed. The comorbidities that result from a SCI decrease the patient's quality of life. Nevertheless, some approved drug can help to control some of these problems, such as baclofen and pregabalin. Baclofen and pregabalin are effective in controlling spasticity and neuropathic pain in people with SCI, but recently they have also been used to as potential therapies to treat the consequences induced by SCI. In these studies, it was found that baclofen could improve the locomotor function and act as a neuroprotector. Regarding to pregabalin, it was observed that this drug improve motor function, but could also induce axonal regeneration, and protect the spinal cord after an insult. The aim of these study was to evaluate the effect of a combine baclofen with pregabalin administration after a transected SCI in mice. To do that , it was performed an *in vivo* experiment during six week, where it was assessed the animals´ behaviour and histological analysis. No motor improvements were observed, however treated animals achieved weight support. On this study one of the treated groups do not exhibit allodynia after SCI, confirmed by von Frey test. Through the spinal cord tissue analysis, it was observed that the treatment not only promotes a better neuroprotection and an axonal regeneration after SCI, but also appears to induce a more controlled systemic inflammatory response. Overall, our study suggests that combined administration of baclofen with pregabalin leads to a neuroprotection and a neuroregeneration after SCI, but further studies need to be done to a better understanding of these strategy.

Keywords: Spinal cord injury; Baclofen; Pregabalin; Neurodegeneration; Immune response modulation.

Resumo

A lesão traumática na medula espinhal é uma condição neurológica que impede a comunicação entre o cérebro e o resto do corpo. Esta neuropatologia desencadeia um conjunto de processos complexos. Esta cascata de eventos abrange processos bioquímicos, como a excitotoxidade, influxo de cálcio descontrolado e formação de radicais livres, e processos biológicos, como por exemplo a morte neuronal, disrupção dos vasos sanguíneos, desmielinização, e a ativação excessiva das células da microglia. As alterações que acontecem após a lesão na medula espinhal, alteram a normal fisiologia do corpo humano. Dependendo do local em acontece a lesão na medula espinhal, as consequências podem ser mais ou menos severas. A perda de locomoção, incapacidade de controlar a bexiga e os intestinos, disfunção sexual, problemas respiratórios, bradicardia, espasticidade e dor neuropática são as consequências mais frequentes que ocorrem após lesões medulares. Estas alterações drásticas afetam o quotidiano e a qualidade de vidas dos pacientes. Para além disso, pessoas que sofrem lesões medulares tendem a ter altos custos de vida no que toca à sua recuperação após o insulto medular.

Atualmente, ainda não foi desenvolvida uma terapia eficaz para que estes indivíduos possam recuperar as funções perdidas após a lesão na medula espinhal. Muitos estudos estão a decorrer para se encontrar uma terapia eficiente para lesões medulares. Esta procura é iminente pois, o número de casos de pessoas com lesões medulares tem aumentado no decorrer dos anos. Os projetos de investigação englobam diversas áreas, desde engenharias, eletrofisiologia, terapia celular, secretoma e farmacologia. Apesar da procura por uma terapia para lesões medulares, foram desenvolvidos e aceites pelas entidades responsáveis, fármacos que auxiliam a uma melhor qualidade de vida, uma vez que estes tratam dificuldades como a espasticidade e a dor neuropática. Estas duas comorbidades afetam o dia-a-dia dos pacientes lesionados, e para tal, estes já têm disponível o baclofeno e a pregabalina de forma a suportarem estas condicionantes nas suas vidas.

O baclofeno tem sido utilizado em estudos relacionados com lesões medulares, visto ter sido descoberto que, este fármaco, pode ter o potencial em melhorar a disfunção motora e ainda atuar como um neuroprotetor após lesões na medula espinhal. Estudos de traumas na espinhal medula não só estão a utilizar o baclofeno, como a pregabalina está a ser objeto de estudo. Neste caso, foi descoberto que, para além da pregabalina modular a dor neuropática, também parece

levar a melhorias na locomoção, proteger o tecido medular após lesão e ainda induzir a regeneração axonal.

Este projeto teve como objetivo desenvolver uma estratégia com potencial terapêutico, para lesões na medula espinhal, utilizando a administração combinada de baclofeno com pregabalina. De forma a se alcança este objetivo, foi conduzido um experimento *in vivo* durante seis semanas, em que se induziram lesões por transecção, nas medulas espinhais de ratinhos fêmeas. Posteriormente, foram criados quatro grupos experimentais distintos, para que se pudesse não só estudar as diferentes abordagens de administração, mas também para que se pudesse analisar os efeitos que os fármacos poderiam ter após a lesão das medulas. Estudos anteriormente conduzidos na nossa equipa demonstraram que, uma administração aguda de baclofeno com dosagem de 1mg/Kg conduziam a melhores resultados após lesão espinhal. Posto isto, a três dos quatro grupos experimentais foi administrado baclofeno com a dose referida anteriormente, após a indução da lesão. Ainda nestes três grupos experimentais, foi adicionalmente injetado pregabalina, a uma dose de 30mg/Kg, em diferentes momentos após a lesão. Um dos grupos a pregabalina foi dada 24 horas após a lesão e por um período de quatorze dias consecutivos, a outro grupo foi injetado pregabalina após a lesão, e ao terceiro grupo foi administrado pregabalina após a lesão medular e ainda por um período de quatorze dias consecutivos. Ao grupo restante, foi injetado uma solução salina após a lesão na medula espinhal, tendo sido este o grupo que serviu de controlo experimental.

Inicialmente, durante as seis semanas do *in vivo* foi estudado o comportamento dos animais, através de testes experimentais como o Basso Mouse Scale (BMS), ensaio da função da bexiga e ainda o von Frey (vF). Após o término do *in vivo*, procedeu-se à análise histológica dos tecidos das medulas espinhais lesionadas, nos quais foram analisados parâmetros como a matéria branca preservada, tecido fibrótico, número de neurónios motores, fenótipo das células microglia, quantificação do neurofilamento e ainda a que tipo de neurónios corresponderia a quantificação relacionada com o neurofilamento. No decorrer do in vivo, em diferentes momentos (1-, 7-, 14-, 42-dias após lesão), foram recolhidas amostras de sangue, para se isolar o plasma das mesmas, de forma a se analisar a resposta imunitária sistémica de cada grupo experimental.

Os resultados dos testes de comportamento, demonstraram que a administração combinada dos fármacos não leva a melhorias motoras significantes, apesar de que a maioria dos animais dos grupos tratados conseguiram atingir o suporte de peso. No seguimento de resultados negativos, após análise dos testes funcionais da bexiga, percebeu-se que não foram

induzidas melhorias no que toca ao controlo da bexiga nos grupos que foram tratados com baclofeno e pregabalina. Contudo, verificou-se que estes fármacos podem diminuir a hipersensibilidade resultante da lesão medular, quando são administrados com 24 horas de intervalo. A metabolização dos fármacos também foi verificada através da pesagem dos fígados, na qual se verificou que não foram induzidos efeitos negativos no metabolismo dos animais. Quanto à análise dos tecidos das espinhais medulas, percebeu-se que a administração aguda dos fármacos juntamente com a prolongação da administração da pregabalina leva a melhores resultados histológicos. O grupo experimental em questão, apesar de apresentar menos neurónios comparativamente com os restantes grupos experimentais, demonstrou ser o que mais projeções axonais teve, formou-se pouco tecido fibrótico e ainda teve uma resposta imunitária sistémica menos acentuada.

Este estudo sugere que, a administração combinada de baclofeno com pregabalina, pode efetivamente induzir a neuroprotecção, neuromodulação e a neuroregeneração. No entanto, é necessário perceber-se de uma forma mais detalhada os mecanismos que atuam em conjunto e de forma individual após administração destes fármacos. Além disso, seria interessante estudar-se a histologia da parte sensorial da medula espinhal. Por estas razões, o seguimento de estudos complementares seria crucial para a validação dos efeitos conduzidos pelo baclofeno e a pregabalina em lesões da medula espinhal.

Palavras-chave: Lesão medular; Baclofeno; Pregabalina; Neurodegeneração; Modelação resposta imune

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Abbreviations

A

AMPA: (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate)

ANOVA: Analysis of variance

ANS: Autonomic nervous system

ATPase: Adenosine triphosphatase

B

BBB: Blood brain barrier

BFA: Bladder function analysis

BMS: Basso Mouse Scale

C

cAMP: Cyclic adenosine monophosphate

c-Myc: proto-oncogene c-Myc

CNS: Central nervous system

CPG: Central pattern generator

CSF: Cerebrospinal fluid

D

DAPI: 4′,6-Diamidino-2-phenylindole dihydrochloride

DO: Detrusor overactivity

DPI: Days post-injury

DRG: Dorsal root ganglia

DSD: Detrusor sphincter dyssynergia

E

EMA: European Medicines Agency

ESCs: Embryonic stem cells

F

FES: Functional electric stimulation

FDA: Food and Drug Administration

FBS: Fetal bovine serum

G

GABA: Gamma-aminobutyric acid

GM-CSF: Granulocyte-macrophage colony-stimulating factor

H

HPI: Hours post-injury

5-HT: 5-hydroxytryptemine

I

Iba-1: Ionized calcium binding adaptor molecule 1

IFN-β: Interferon-beta

IFN-γ: Interferon-gamma

IgG: Immunoglobulin G

IH: Infinitive horizon

IHC: Immunohistochemistry

IL: Interleukin

iPSCs: Induced pluripotent stem cells

K

Klf4: Krüppel-like factor 4

L

LAT: L-type amino acid transporter

LOD: Limit of detection

LUT: Low urinary tract

LUTD: Low urinary tract dysfunction

M

M1: Classically activate microglia

M2: Alternatively activate microglia

MASCIS: Multicenter Animal Spinal Cord Injury Study

MCP-1: Monocyte chemoattractant protein-1

MP: Methylprednisolone

MSCs: Mesenchymal stem cells

N

NeuN: Neuronal Nuclei

NF-H: Neurofilament heavy polypeptide

NMDA: *N*-methyl-D-aspartate

NPC: Neural progenitor cell

O

OCT: Optimal cutting temperature

OECs: Olfactory ensheathing cells

OSU: Ohio State University

Oct4: Octamer binding transcription factor $^{3}/_{4}$

P

PBS: Phosphate-buffered saline

PDGFR: Platelet-derived growth factor receptor

PFA: Paraformaldehyde

PNS: Peripheral nervous system

R

RNS: Reactive nitrogen species

ROI: Region of interest

ROS: Reactive oxygen species

RT: Room temperature

S

SA-PE: Streptavidin-phycoerythrin

SCI: Spinal Cord Injury

SEM: Standard error of the mean

SOX 2: Sex determining region Y-box 2

T

TBI: Traumatic Brain Injury

TGFβ: Transforming growth factor beta

TH: Tyrosine hydroxylase

TNF-α: Tumor necrosis factor alpha

V

VDCC: Voltage-dependent calcium channel

vF: von Frey

W

WPI: Weeks post-injury

Symbols and units

Ca^{2+} : Calcium ion

Cl^{-} : Chloride ion

ºC: Degrees Celsius

g: Grams

K^{+} : Potassium

Kg: Kilogram

mg: Milligram

µg: Microgram

µL: Microliter

µm: Micrometer

% : Percentage

rpm: Rotations per minute

Na^+ : Sodium ion

mm^2 : Square millimeters

Chapter 1 - Introduction

1.1 Nervous system

The nervous system is a complex structure responsible for the control, communication, and coordination of the different body parts. The perception of sensation is also possible due to the nervous system. These abilities of nervous system are only possible due to a presence of a group of cells as neurons and glial cells. In humans, nervous system is composed of three different branches: (1) central nervous system (CNS), (2) peripheral nervous system (PNS) and (3) autonomic nervous system (ANS). The latter one is included in CNS and PNS and has the capacity of controlling the glandular organs. PNS is composed of nerves that connect to the CNS, transmitting information to and from different body parts. CNS is composed of brain and spinal cord. The brain is responsible to acquire, integrate and process information to trigger a response. The information and the response that go to and from the brain is conduct through the spinal cord (Barker et al., 2018; Seeley, 2003).

1.2 Spinal cord

The spinal cord is essential for the normal function of the central nervous system as it is responsible for carrying information between the brain and the rest of the body (Barker et al., 2018; Seeley et al., 2003).

1.2.1 Anatomy and physiology of spinal cord

Spinal cord is a structure composed of different types of cells, forming an organized network. For instance, astrocytes that are responsible for controlling the blood brain barrier permeability, provide nutrients to neurons, and also participate in cellular immunity and homeostasis. Oligodendrocytes are responsible for the production of the myelin sheath present in the neurons of the CNS. In turn, the neurons are capable to receive stimulus and generate an action potential. The cells in charge for immunity of the CNS are microglia (Purves, 2012). Additionally, being a complex structure, the spinal cord is also fragile, long, and tubelike structure that extends the rom brain stem, that is, the medulla oblongata, to the level of the second lumbar vertebrae (Barker et al., 2018; Seeley et al., 2003).

Spinal cord runs through the centre of vertebrae column to be protected. In addition, the spinal cord is also protected by three layers of connective tissue, called meninges. The dura mater is the layer of connective tissue more superficial and is also the thickest layer. The

connective tissue layer that follows is the arachnoid mater. This meninge is thin and similar to a spider web. The space between dura mater and arachnoid mater is designated by subdural cavity, which contains serous fluid. Lastly, the meninge that is in contact with the spinal cord is known as pia mater. Among arachnoid mater and pia mater also exist one cavity, the subarachnoid space, which consists of blood vessels and cerebrospinal fluid (CSF) (Cho, 2015).

Anatomically, the spinal cord is divided in 31 segments including 8 cervical, 12 thoracic, 5 lumbar, 5 sacral and 1 coccygeal (**Figure 1.1**). However, the levels of spinal cord do not correspond to the levels of vertebrae column (Bican et al., 2013; Silva et al., 2014). This difference is due to the different growth rates during embryonic development, as the vertebrae column has a faster growth compared to the spinal cord (Cho, 2015).

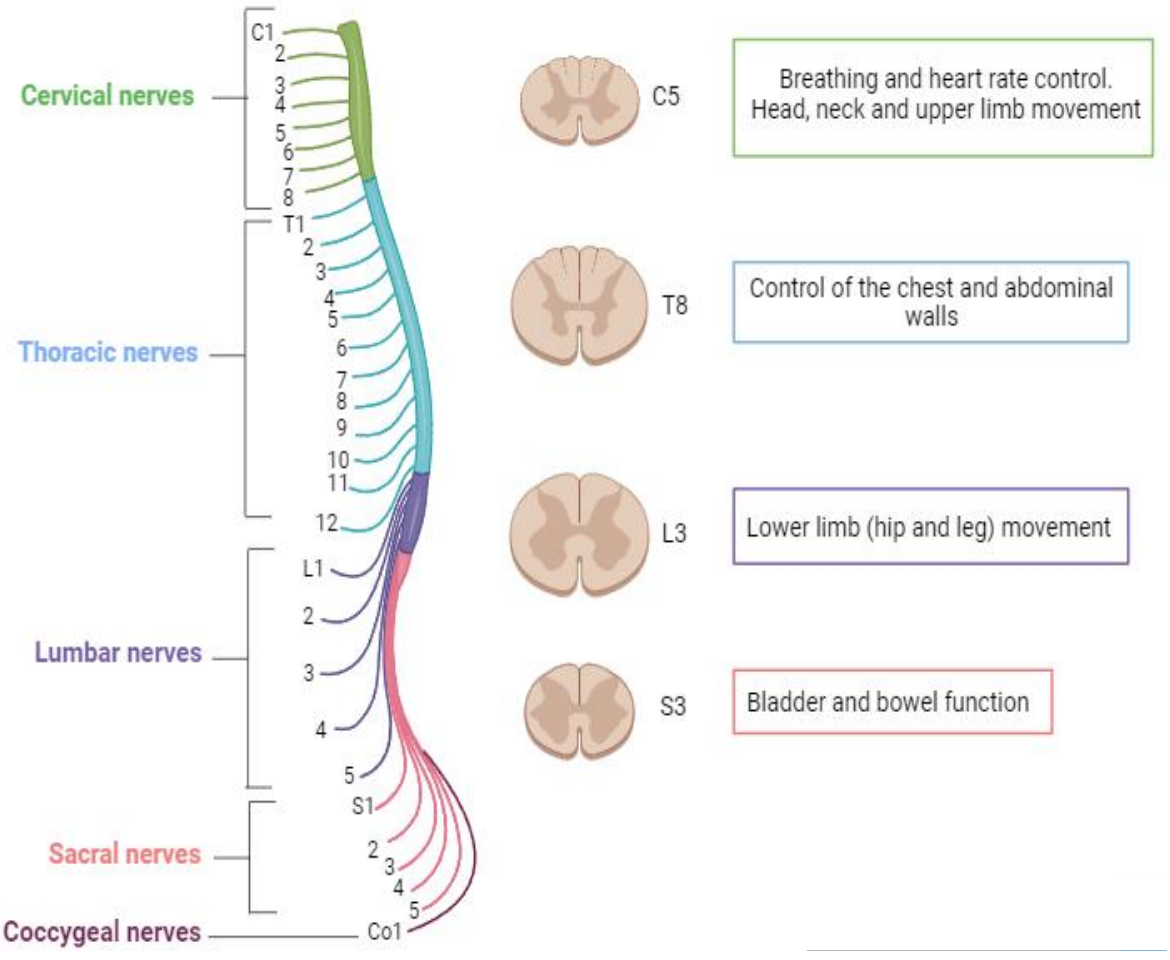


Figure 1.1 Spinal cord anatomy. Levels of the spinal cord, transversal section of the spinal cord representation, and the function of the spinal nerves. Image created in BioRender.

From each segment of spinal cord, one pair of spinal nerves is formed. Thus, in spinal cord there are a total of 31 pair of spinal nerves. These spinal nerves are responsible for carrying electrical signs between the brain and the rest of the body. The messages that spinal nerves transport allows the body to move and have sensations (Sheerin, 2004; Silva et al., 2014). The spinal nerves have two roots, the ventral and dorsal roots. This division is important owing to the function of each root. The varying widths of the spinal cord along its length can be attributed to the spinal nerves. The cervical and lumbar areas are the regions with the biggest width, because they have a greater number of spinal nerves (**Figure 1.1**). The cervical area is responsible for enervating the upper limb´s muscle (**Figure 1.1**). The enlargement, known as brachial plexus, goes from the fourth cervical segment to the second thoracic segment. The lumbar area supplies the lower limb´s muscle, and this enlargement begins in the second lumbar segment and ends in the third sacral segment. This enlargement is called as lumbosacral plexus (Khan & Lui, 2022).

The spinal cord is separated, partially by two sulci, the posterior (dorsal) median sulcus and the anterior (ventral) median sulcus. These two sulci are connected by white and grey commissures (Seeley et al., 2003). In the transversal section of spinal cord is possible to distinguish three structures, the central canal, white matter, and grey matter (**Figure 1.2**). The central canal is in the centre of the grey commissure and is the prolongation of the fourth ventricle present in the brain. Like the ventricle, the central canal is filled with CSF and surrounded by glial cells. The white matter is composed of myelinated axons. The white matter can be divided into three columns, posterior, lateral, and anterior column. The grey matter is constituted by dendrites and cell bodies of neurons. The grey matter, like the white matter, is organized in sections called horns, posterior (dorsal) horn, lateral horn, and anterior (ventral) horn. The lateral horn is only present in some segments of spinal cord, that are related to the autonomic nervous system (ANS), like thoracic areas (Cho, 2015; Silva et al., 2014).

Neurons in the spinal cord, as in the rest of the body, are organized. Regarding the white matter, the axons are distributed according to the signal they carry, that is, whether the messages go to the brain or whether the messages leave the brain. The pathways that carry electrical signs from the brain to the rest of the body, through spinal cord, are called descending spinal cord tracts. On the other hand, the pathways that transport the electrical signs to the brain are designated ascending spinal cord tracts. The ascending tracts are associated with sensations, like pain, proprioception, and temperature. In contrast, the descending spinal cord tracts are

predominantly motor, and the principal descending motor tract is the corticospinal tract (Cho, 2015; Silva et al., 2014).

According to the organization of neurons in spinal cord, is possible separate the neurons related to sensory and motor systems, respectively. The dorsal zone of spinal cord, both in the white matter and the grey matter, contains neurons responsible for sensations. The ventral area of spinal cord has motor neurons (**Figure 1.2**) (Khan & Lui, 2022).

Neurons, as mentioned before, are responsible for receive, interpret, and transmit information between them. For the communications between neurons, neurotransmitters are release and these are capable to bind to their specific receptor. This bind is responsible for allowing or inhibiting the transmission of electrical signs, that carried the information along the neuron, and once arrived at the dendrite's neurotransmitters are release, and the process repeats. Neurons can be divided in three different types, (a) the afferent neurons or sensory neurons that conduct information from periphery to the CNS, (b) the efferent or motor neurons transport messages from CNS to the periphery and (c) the interneurons that transmit information among afferent and efferent neurons (**Figure 1.2**) (Bican et al., 2013; Purves, 2012).

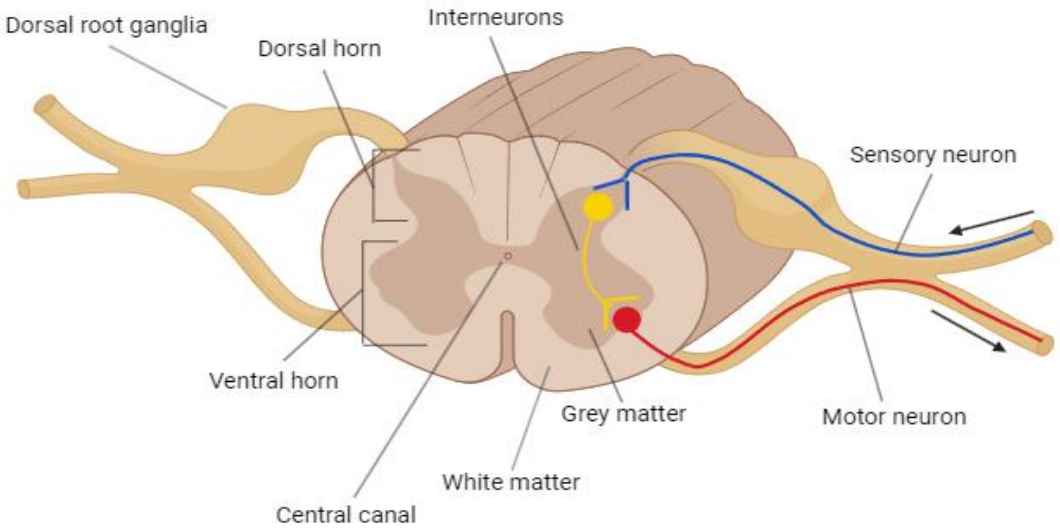


Figure 1.2 Transversal section of the spinal cord. Anatomy of the transversal section of the spinal cord, highlighting the white and grey matter, and the sensory (afferent) and motor (efferent) neurons. Image created in BioRender.

In spinal cord the transmission of information between neurons is made in the synaptic cleft. The neuron that releases the signals is called presynaptic neuron and the neuron that receive the signal is named postsynaptic neuron. The presynaptic neuron is excited by an action potential that run through the axon and induce the diffusion of vesicles in the presynaptic membrane. These result in a release of neurotransmitters that are present in the vesicles in the synaptic cleft. Later, the neurotransmitters will interact with the receptors in the postsynaptic membrane, causing a response that can be inhibitory or excitatory. When occur an inhibitory response it is called hyperpolarization and the excitatory effect corresponds to depolarization. There are different types of receptors, however the most common are the ionotropic receptors that correspond to fast responses (AMPA and NMDA) and metabotropic receptors responsible for slow responses (Purves, 2012).

The neuronal excitability of CNS is controlled by inhibitory and excitatory neurotransmitters. Glutamate is the most common excitatory neurotransmitter. Other example of neurotransmitter, but inhibitory, is the gamma aminobutyric acid, better known as GABA. The neurons are very sensible in alterations of ions, namely to sodium (Na^+) and calcium (Ca^{2+}). These two ions are mostly present in extracellular space and potassium (K^+), is found mostly in intracellular space. The ion balance is essential for the CNS stability and normal functioning (Purves, 2012).

1.3 Spinal cord injury

The spinal cord injury (SCI) is a serious damage that occurs in central nervous systems. This insult in spinal cord could results in a temporary or permanent change in motor, sensory or autonomic function, as it leads to the inability of spinal cord to carry the messages that are responsible for sensory and autonomic systems and motor movements (De Sousa et al., 2022; B. Fan et al., 2022)

1.3.1 Epidemiology

Over the last years, the incidence cases of SCI increased abruptly. It is estimated that, annually, between 250 thousand and 500 thousand people suffer from SCI. In 2019, there were 909 thousand cases of SCI worldwide in which 108 thousand occurred in Europe (Ding et al., 2022; Ridlen et al., 2022). SCI present a higher incidence in developed countries than in developing countries and mostly countries that are localized in North America and western

Europe (**Figure 1.3**) (James et al., 2019). Regarding the etiology, a smaller percentage of SCI cases can be the result of non-traumatic causes, like tumors, infections, spinal stenosis. The majority of the cases are related with physical trauma derived from falls, road accidents and sport activities. Overall, the SCI incidence is higher in man than female, mostly in elder people (> 70 years), although there is a peak of cases related with cervical lesions in young adults (20-40 years) (Ding et al., 2022; James et al., 2019). Moreover, the cause of SCI seems to be related with the age, since SCI in young adults are usually caused by traumatic situations, while in the children's and elderly SCI are mostly caused by non-traumatic causes. Besides these numbers, SCI cases require an expensive and lifelong healthcare, and patients have a significantly decreased quality of life as well as decreased life expectancy (Ding et al., 2022; Ridlen et al., 2022).

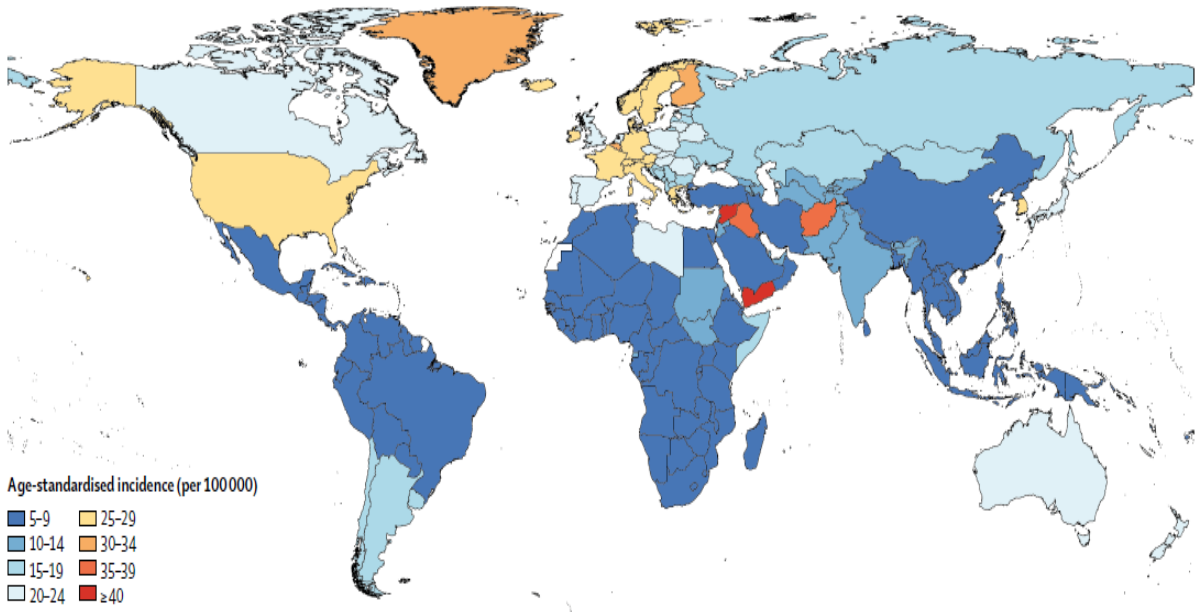


Figure 1.3 Epidemiology of SCI. Representation of a map, in which the countries are represented with distinct colours that correspond to a specific rage of age-standardised of SCI per 100 000 people. The data is normalized for both sexes. Image adapted from James *et al* (2019).

1.3.2 Causes of Spinal Cord Injury

When an injury is made in the spinal cord, the interactions between the cells that constitute the spinal cord, are interrupted. The lack of communication among cells, result in an interruption in ascending and descending tracts, and consequently the functions associated with these tracts are not made (De Sousa et al., 2022; Silva et al., 2014).

Regarding the etiology, the SCI cases can be result of traumatic or non-traumatic event. Usually, the traumatic cause is a physical trauma such as falls, vehicle accidents, violent acts, sport activities, medical and surgical complications. While non-traumatic causes comprise infections, degeneration of spinal cord, cancer, inflammation, and congenital cases (Ge et al., 2018). Among all the causes that can lead a SCI, motor vehicle accidents and falls are the most common (James et al., 2019; Venkatesh et al., 2019).

1.3.3 Types of Spinal Cord Injury

Not all the lesions in spinal cord leads to a complete interruption in communication, and most lesions end up causing just a partial lack of interaction in cells of spinal cord. Therefore, damage in spinal cord can cause complete and incomplete SCI (Ge et al., 2018).

Complete SCI are the most severe because there is no signal transmission below the lesion. The condition of patients varies, depending on the segment of injury in spinal cord. In the case of the damage occurs in the thoracic or lumbar segments, the injured person becomes paraplegic. These situations have consequences, like the loss of sensibility and motor function from waist down. However, the person has ability to move and have sensations on the upper limbs. On the other hand, in cases where the lesion covers cervical segment of spinal cord, the death rate is higher and the patient is diagnosed as quadriplegic, that is considered the worst diagnosis. Quadriplegia is a clinic condition in which the person is no longer able to move both upper and lower limbs and have difficulties with bowel and bladder control. Furthermore, the sensory system is incapable to send signals to the brain and therefore the individual also lacks sensations in the upper and lower limbs (Ge et al., 2018; Sheerin, 2005; Silva et al., 2014).

Regarding incomplete SCI, the sensory system, even if it is faint, and motor system can convey messages to and from the brain, below the injury. Incomplete SCI can lead distinct clinic syndromes, as anterior cord syndrome, central cord syndrome and Brown-Sequard syndrome. The consequences of these clinic conditions are only related with the part of spinal cord that is damage. For example, the motor system stops functioning normally when the anterior part of spinal cord is damaged. In addition to motor and sensory loss, incomplete SCI can also result in an areflexic bowel and bladder (Ge et al., 2018; Sheerin, 2005; Silva et al., 2014).

1.3.4 Pathophysiology of Spinal Cord Injury

Several mechanisms are activated after injury, such as the overload of intracellular calcium, formation of reactive oxygen species (ROS), leukocyte activation, inflammatory response, and neuronal apoptosis. These alterations happen in a cascade and destabilize the microenvironment of spinal cord. The mechanisms of traumatic SCI are mainly divided in two phases. The initial phase after a trauma in spinal cord is defined as primary injury (**Figure 1.4**). Is the moment that happens right after the trauma, where bone fragments are still present. Secondary injury is triggered by the primary injury (**Figure 1.4**). In this phase happen the neuronal excitotoxicity that induce neurons death. Pathophysiology in the secondary injury can be further divided into three phases depending on the time elapsed after the injury. The acute phase is the first phase of secondary injury and occurs between two hours after lesion, up to forty-eight hours. The subacute phase take place between forty-eight hours and six months after lesion. The last phase in traumatic spinal cord injury is denominate as chronic phase and comprehends the rest of patient's life (Anjum et al., 2020; Donnelly & Popovich, 2008; B. Fan et al., 2022; Silva et al., 2014).

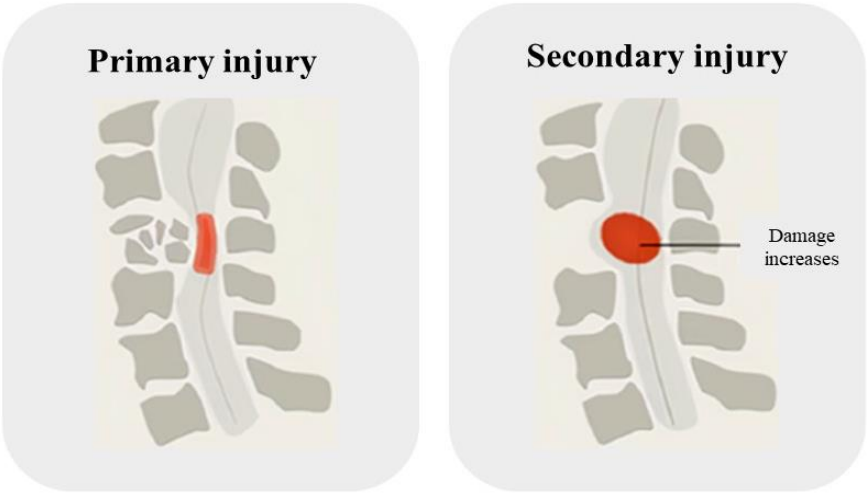


Figure 1.4 Phases of SCI. Representation of primary and secondary injury.
Image adapted from Anjum et al. (2020).

Primary injury. Right after the trauma in the spinal cord, blood vessels are disrupted, allowing blood cells to abandon blood vessels. In primary lesion also occurs the destruction of parenchymal cells, axonal network, and glial membrane. These changes have repercussions like haemorrhagic, cytokine release, and gaps between brain and body communication. The first

manifestations supress vascularization at the site of injury, loss of tone in the blood vessels and their dilation. This will cause hypotension. Many patients suffering from a traumatic SCI may also experience bradycardia (Ahuja et al., 2017; Anjum et al., 2020; Silva et al., 2014).

Acute phase. Blood cells release from blood vessels initiate the acute phase of secondary lesion. Overflowing immune system cells at the site of injury will create pressure on the injured tissue, causing disruption of blood circulation, creating vasospasm. Lack of blood supply at the site of injury causes ischemia in these tissues. Ischemia in spinal cord has consequences such as cytotoxicity, ionic oedema, and vasogenic oedema (**Figure 1.5 A**). Molecular and water balance in cells is disturbed, thus influx Na^+, Cl^- and water will promote cell swelling and loss of cytoskeletal integrity. Ionic edema is the increased permeability of blood barrier in the spinal cord. This will increase trans-endothelial ion transport, and the loss of ions and water from the interstitial space. Inflammation and endothelial damage lead to vasogenic edema, as they cause increased pores allowing large plasma-derived molecules to pass through cell membranes. Continuous haemorrhage, edema, and inflammation lead to substantial cell necrosis (**Figure 1.5 A**). This necrosis is characterised by an increase concentrations inflammatory molecules and the presence of structural biomarkers in the CSF. These processes result in the formation of free radicals, excitotoxicity, and neurotoxicity. Glutamate excitotoxicity is the last stage of the acute phase. Activation of glutamate receptors during spinal cord injury, considerably increases glutamate concentrations leading to excitotoxicity and ultimately to neurons death (**Figure 1.5 A**). Augmentation of glutamate excitation in neurons is provoked by mechanic stress, lipid peroxidation, formation of necrotic and apoptotic cells, and failure of ATPase Na^+/K^+ bombs in the axonal membrane. Glutamate release induces activation glutamate receptors, NMDA and AMPA, which rises influx of Na^+ and Ca^{2+} and decrease intracellular K^+ in neurons, promoting necrotic and apoptotic cell death (Ahuja et al., 2017; Anjum et al., 2020; Silva et al., 2014).

Subacute phase. Once the acute phase of secondary injury is complete, the subacute phase begins with neurotoxicity (**Figure 1.5 B**). Neurotoxicity is characterized by increased intracellular calcium that destabilises ion homeostasis, since inhibits mitochondrial respiration and causes energy depletion. There is an increase of intracellular calcium because of alteration in Na^+/K^+ ATPase bombs. These modifications facilitate axonal membrane depolarization and leads to excessive influx of Na^+ to axonal membrane of axons. This ionic dysregulation leads cytotoxic edema in cells, axonal acidosis, phospholipases activation, elevate production of ROS and reactive nitrogen species (RNS), mitochondrial disfunction, and increase calcium

permeabilization. The energy generated by mitochondria's is necessary for many functions, in particularly for being used by brain and for neuronal survival (Ahuja et al., 2016; Anjum et al., 2020; Silva et al., 2014).

Within the pathophysiology of the injury neuroinflammation also plays an essential role. Many cell types are involved in neuroinflammation such as astrocytes, dendritic cells, macrophages, microglia, neutrophils, and B- and T-lymphocytes. The complexity of inflammatory responses after lesion can produce a neurotoxic or neuroprotective effect, depending on the time and duration of inflammatory response (Anjum et al., 2020; Donnelly & Popovich, 2008; Silva et al., 2014).

Chronic phase. Once the sub-acute phase is over, the chronic phase begins, typically six months after the injury. In this last phase of secondary injury there are two predominant processes, apoptosis, and necrosis. Beyond the death of neurons, it also occurs axonal degeneration, axonal remodulation, and axonal demyelination. Lastly, in the chronic phase cell death and neurodegeneration create a cystic cavity, that is filled with extracellular fluid, connective tissue, and macrophages. Around this cavity it is formed a glial scar, constituted by glial cells (astrocytes, oligodendrocytes, and microglia) (**Figure 1.5 C**). The development of glial scar is a result of a set of events, as reactive gliosis, white matter demyelination, dissolution of grey matter and the deposition of connective tissue. Additionally, other cells (fibroblast, endothelial and inflammatory immune cells) migrate to the lesion core and occurs the formation of fibrotic scar. These events increase the complexity of the injury-induced environment, since the scars act like a barrier and prevent axonal formation through the core of the lesion (Ahuja et al., 2017; Anjum et al., 2020; Silva et al., 2014).

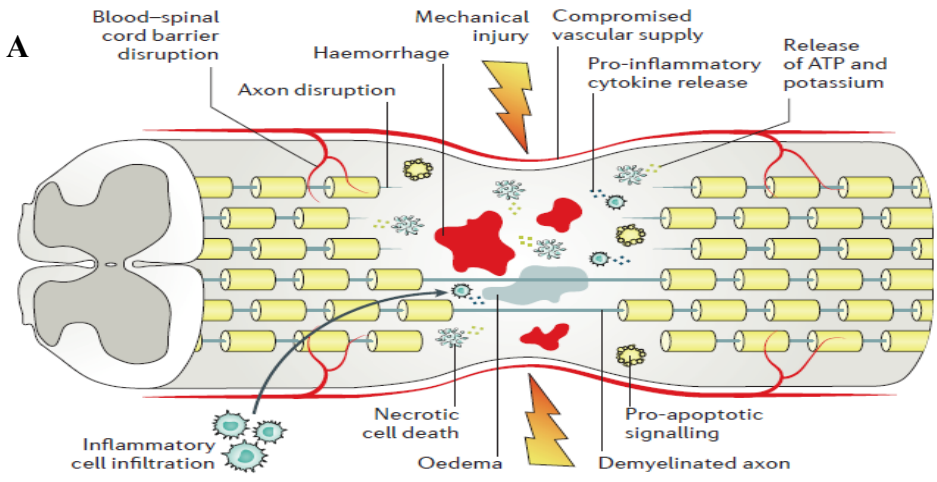


Figure 1.5 Events according to SCI phases. (Image continue below).

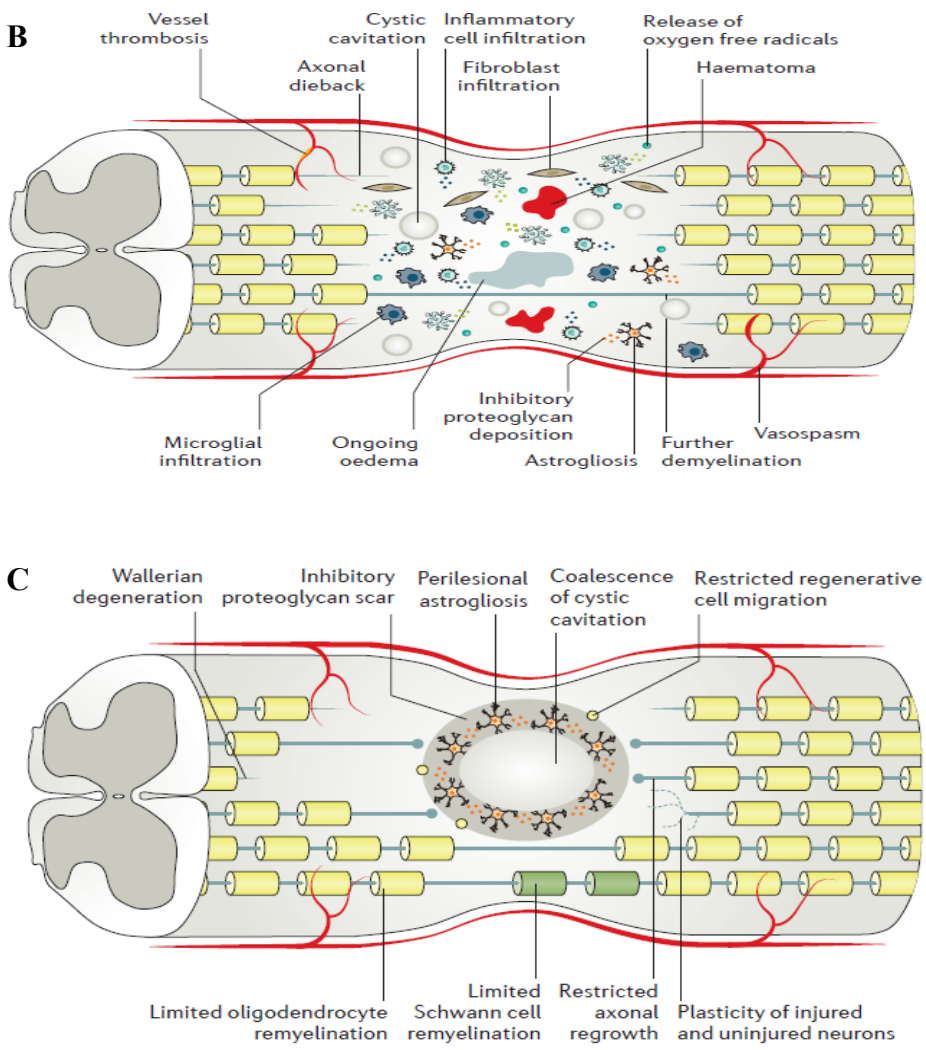


Figure 1.5 Events according to SCI phases. Pathophysiology of SCI in secondary injury. (A) Acute phase of SCI. (B) SCI subacute phage. (C) Chronic phage of SCI). Image adapted form Ahuja et al. (2017).

Throughout the phases of the injury, microglia cells present an important role, however it remains controversial if microglia are beneficial or harmful in SCI. After a lesion in spinal cord, injured neural cells release cytokines (INFγ, IL-6, IL-1β) that activate microglia. In acute and subacute phase, the classically active microglia (M1) are recruited, these microglia release inflammatory factors, exacerbating the inflammation (**Figure 1.6**). In the chronic phase, the alternatively activated microglia (M2) are recruited. Contrary to M1, the M2 is responsible to promote regeneration, secreting factors such as IL-10 and TGFβ (**Figure 1.6**) (B. Fan et al., 2022).

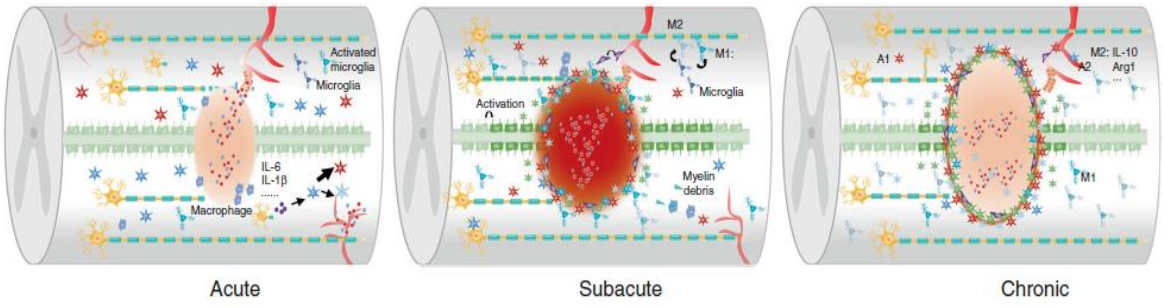


Figure 1.6 Changes of microglia in distinct phases of SCI. Classically active microglia (M1) is more prevalent in acute and subacute phage. During the chronic phase microglia is mainly alternative active microglia (M2). Image adapted from Fan *et al*. (2022).

1.3.5 Consequences of Spinal Cord Injury

All alterations that happen after a lesion in the spinal cord not only led to a loss of motor, and sensory function. Individuals that suffer from this clinic condition may have difficulty breathing, loss of bladder and bowel control, changes in sexual function, spasticity, and neuropathic pain (Kumru & Kofler, 2012; Silva et al., 2014).

Spasticity is a neurological impairment and is defined as being "a disordered sensorimotor control resulting from an upper motor neuron lesion, presenting as intermittent or sustained involuntary activation of muscles". Around 70% of patients with SCI suffer from spasticity. It is believed that spasticity and neuropathic pain are two sides of the same coin. Neuropathic pain covers 50-60% of people with SCI. This condition is characterized as "pain arising as a direct consequence of a lesion or disease affecting the somatosensory system". Neuropathic pain can be spontaneous, and patients described this pain as a burning, pinking, or tightening (Finnerup, 2017; Jensen et al., 2011; Pandyan et al., 2005).

These disabling conditions decrease quality of life and affect the execution of task. Not only people lose the ability of walk and control their bladder and bowel, as loss their independence in the first years, the sexual life became affected and depending on the job, people can find some barriers in developing the work. These changes in the life of people with SCI affect the mental health (Bouillon et al., 2002; Gurcay et al., 2010).

1.4 Potential therapies for spinal cord injury

Until today, there is not effective treatment for SCI, however the knowledge on the SCI field is increasing. Mostly studies are focus on understanding the neuroprotection and neuroregeneration, but with the technology developments, studies with cell therapies, secretome and electric stimulation are being more explored (Ahuja et al., 2016; Ashammakhi et al., 2019; Kabu et al., 2015; Pinho et al., 2020).

1.4.1 Cell therapy

Cell therapy consist in the transplantation cells, either from the own individual (autologous) or from a donor (allogenic), in order to treat disorder. The first experiments applying cell therapy in SCI came in 1980, with peripheral nerve grafts (Richardson et al., 1980). Nowadays, a wide range of cells are use in medicine regenerative for cell therapy in SCI, as mesenchymal stem cells (MSCs), induced pluripotent stem cells (iPSCs) and glial cells .

Regarding MSCs, have been demonstrated that these cells have the potential of modulating inflammation, secrete neuroprotective actors, stimulate angiogenesis and axonal growth in situations like SCI (Assunção-Silva et al., 2015; Fehlings et al., 2017; Kabu et al., 2015; Mothe & Tator, 2012).

In 2006, Yamanaka and colleagues found that is possible differentiate adult stem cells (fibroblast) into pluripotent stem cells (iPSCs) through the application of four transcription factors: octamer-binding transcription factor ¾ (Oct 4), sex determining region Y-box 2 (Sox2), proto-oncogene c-Myc (c-Myc) and Krüppel-like factor 4 (Klf4) (K. Takahashi & Yamanaka, n.d.). iPSCs are the alternative to embryonic stem cells (ESCs) since their overcome the ethical problems that surge with the application of ESCs. For application in SCI, iPSCs were reprogrammed to generate neural stem cells (iPSC-NPC). The results of these study demonstrated that occurs the migration of survival cells in the spinal cord, and their differentiation into neurons and glial cells, leading to locomotor improvements (Fujimoto et al., 2012).

Glial cells, namely olfactory ensheathing cells (OECs), have the ability to create a permissive environment for axonal remyelination and regrowth. Was found in 1991, that OECs contribute for the regeneration of peripheral olfactory neurons (Doucette, 1991). In 2000, a study conduct by Ramón-Cueto and colleagues, demonstrate that the transplantation of OECs

leads to an axonal regeneration and locomotor function in a complete transection model (Ramón-Cueto et al., 2000).

1.4.2 Secretome

In the beginnings of the 20th century, it was hypothesised that MSCs secret bioactive molecules with therapeutic effects (Gnecchi et al., 2005). The soluble compounds of cells secretome comprises proteins, pro- and anti-inflammatory cytokines, growth factor and other molecules. Besides, the secretome also contain extracellular vesicles, apoptotic bodies, exosomes and microparticles (Beer et al., 2017; Hoogduijn & Lombardo, 2019; Pires et al., 2016). Studies with secretome of MSCs from distinct origins shown that secretome promote neurite outgrowth improving the locomotor function, angiogenesis, axonal growth, and modulation of immune response (Hoogduijn & Lombardo, 2019; Pinho et al., 2020; Wallace et al., 2016; Xin et al., 2012).

1.4.3 Electric stimulation

Over the last years, electric stimulation has been intensively study and applied in SCI cases. Although this field is recent regarding SCI, the investigations made demonstrated that functional electric stimulation (FES) have beneficial effects, improving respiratory, circulation, mobility, adaptive plasticity, and neuronal network activation (Alojz Kralj et al., 2022; Hamid & Hayek, 2008; S. Luo et al., 2017).

Currently, there is no cure for SCI, although health care providers as decompression of spinal cord and the administration of methylprednisolone (MP-neuroprotective agent), are recurrent procedures to mitigate the effects of SCI (Ahuja et al., 2017; De Almeida et al., 2023). However, strategies to improve the quality of life of SCI patients have been developed. In order to manage spasticity and neuropathic pain, people with SCI can use GABAergic drugs and Pregabalin, to relieve the consequences provoked by the lesion.

1.5 Baclofen

1.5.1 History and function of Baclofen

Baclofen was initially developed in 1960 to treat epilepsy. As the results were not promising, baclofen was taken off the market. In 1971 it was discovered that baclofen had a positive effect in muscular spasticity. Nowadays, baclofen is used to manage spasticity in people with SCI and is prescribed for people with multiple (Romito et al., 2021). The main purpose of baclofen is to reduce the release of excitatory neurotransmitters, as glutamate, in pre-synaptic neurons, and stimulate inhibitory signals in post-synaptic neurons (Farmer & Mittal, 2022; Romito et al., 2021).

1.5.2 Baclofen and GABA receptors

Baclofen (beta-[4-chlorophenyl]-GABA) is an agonist of the $GABA_B$ receptor in mono and polysynaptic neurons present in the brain and spinal cord. GABA is the major inhibitory neurotransmitter in the CNS, modulating the neuronal activity. Action of GABA neurotransmitters is made by two classes of receptors, ionotropic receptors, $GABA_A$ and $GABA_C$, and metabotropic receptors, $GABA_B$. The first type of receptors is responsible for fast synaptic inhibition and the formation of chloride channels. In turn, $GABA_B$ receptors are associated with slow synaptic inhibition (Benarroch, 2012; Terunuma, 2018).

$GABA_B$ receptors are G protein-coupled receptors that are implicated in several neurologic disorders, namely spasticity, and psychiatric disorders. $GABA_B$ receptors are heterodimers constituted by seven transmembrane domains and two subunits, $GABA_{B1}$ and $GABA_{B2}$. The $GABA_{B1}$ subunit contain an extracellular binding site for GABA or GABA agonists, like baclofen. $GABA_{B2}$ subunit is coupled with G-protein. When GABA binds to the binding site occurs the activation of $GABA_B$ receptors that provoke the conformational changes of these two subunits. This results in activation of G-protein that trigger signalling pathways on potassium and calcium ionic channels, cyclic adenosine monophosphate (cAMP), and voltage-dependent calcium channels (VDCC) (Benarroch, 2012; Terunuma, 2018).

Baclofen interacts with subunit $GABA_{B1}$ of the GABA receptors activating them, mimicking GABA action. This binding will give rise to alterations at pre-synaptic and post-synaptic levels (**Figure 1.7**) (De Sousa et al., 2022).

Pre-synaptic action. $GABA_B$ receptors at pre-synaptic level are mostly detected in the extracellular membrane, over the pre-synaptic membrane specialization. Activation of these receptors by both GABA and baclofen will cause VDCC to close preventing Ca^{2+} influx. Therefore, the reduction of Ca^{2+} in the pre-synaptic neurons leads to its hyperpolarization and the release of excitatory neurotransmitter, as glutamate and norepinephrine, to the synaptic cleft is inhibited (**Figure 1.7**) (Benarroch, 2012; De Sousa et al., 2022).

Post-synaptic action. In the post-synaptic neurons, $GABA_B$ receptors are uniformly distributed on the dendritic spines. Baclofen binds to $GABA_B$ receptors and activates them leading to a cascade of signals that activate G-protein that cause the activation of K^+ ionic channels and allow the efflux of K^+ from the post-synaptic neurons. In addition, G-protein activation also close the Ca^{2+} ionic channels, so the influx of Ca^{2+} to post-synaptic neurons is inhibited (**Figure 1.7**) (Benarroch, 2012; De Sousa et al., 2022).

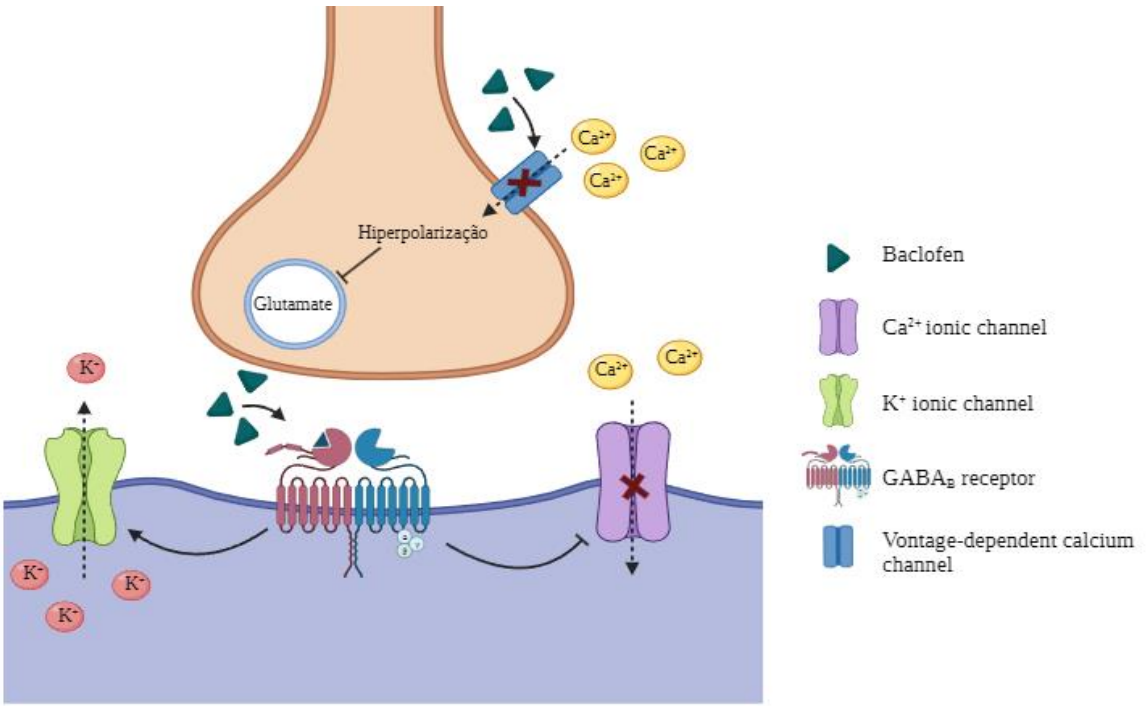


Figure 1.7 Mechanism of Baclofen. Interaction of baclofen in pre- and post-synaptic neuron. Image crated in BioRender.

Adenylyl cyclase regulation. The activation of $GABA_B$ receptors coupled to G-proteins can induce either inhibitory or stimulatory responses. When occurs the activation of $GABA_B$ receptor that is coupled to a stimulatory G-protein, the activity of adenylyl cyclase is increased, which leads to an augmentation levels of cAMP. On the other hand, when $GABA_B$ receptors coupled to an inhibitory G-protein are activated, the opposite process occurs. $GABA_B$ receptors,

through these mechanisms, are capable to regulate a variety of cAMP-dependent mechanism in neurons, related to cellular metabolism, synaptic plasticity, and gene transcription (De Sousa et al., 2022).

Baclofen by interacting in these ways with neurons, prevents the signal propagation in neurons and thus the spasticity is relieved (Benarroch, 2012; Kent et al., 2020).

1.5.3 Administration and metabolism of Baclofen

Spasticity is a related SCI consequence that leads to patients' soreness and loss of life quality. To control spasticity on these patients, baclofen is still the first line therapy. Baclofen administration is available in two ways, as an oral medication or in an intrathecal pump. Oral baclofen is rapidly absorbed from gastrointestinal tract. However, about 85%, is eliminated through the kidneys and excreted in the urine unchanged. The remaining 15% are metabolised by the liver as an inactive metabolite. The concentration of baclofen in plasma reaches a peak two hours after oral administration, and the half-life its approximately 3-4 hours. Although baclofen can cross blood-brain barrier (BBB), the efflux of baclofen is higher than its influx. Consequently, baclofen distribution to the brain and spinal cord is inefficient. Considering the half-life, dependency on transport mechanisms, and the rapid metabolism, patients should take 3-4 daily doses of baclofen. As result, the high concentrations of baclofen in plasma may result in unwanted side effects such as drowsiness, ataxia, and respiratory and cardiovascular depression. Intrathecal administration of baclofen is only recommended for patients with severe spasticity whose maximum oral medication has no effect to manage spasticity, or patients of which the spasticity is of cerebral origin. In this type of administration, baclofen is injected directly in CSF and delivered directly in the CNS. As baclofen is not metabolized as in oral administration, its half-life is higher, around five hours, and has an effect in spasticity 0,5-1 hour after administration. Baclofen doses for intrathecal administration are lower compared to oral medication, thus the side effects are less. However, intrathecal administration requires an invasive surgery to place a pump that injects baclofen continuously into the intrathecal space. Additionally, this approach is considerably more expensive, since there are possible complications associated, as the leak of CSF, fistula formation, malfunction of pump and infections (De Sousa et al., 2022; Kent et al., 2020; Romito et al., 2021).

1.5.4 Side effects of Baclofen

As it was mentioned, the baclofen oral administration causes more side effects compared to intrathecal pump administration. This difference is due to the dose of oral administration being higher than the dose of intrathecal administration. Dizziness, ataxia, sedation, weakness, respiratory and cardiovascular depression are the collateral symptoms most common as regards to oral medication. Of all the side effects, dizziness affects the highest percentage of people taking oral baclofen. These effects are less frequent in the intrathecal pump administration, but if the pump is injecting the wrong dosage, the person may be subject to an overdose or an underdose. Pump malfunction can provoke symptoms as dizziness, headaches, nauseas, and muscle weakness. In the cases that pump injects excessive doses of baclofen, or the patients mistakenly take high oral doses, an overdose of baclofen occurs. As result of overdose, the individual may experience weakness, areflexia, hypotonia, seizures, respiratory depression, and in rare cases, dead. Overdose incidence related to intrathecal pump is less than 7% (Kent et al., 2020; Romito et al., 2021).

1.5.5 Precautions with Baclofen

Beyond the side effects, baclofen can cause toxicity in some patients. Considering the way that baclofen is eliminated, mostly by the kidneys, people who suffer renal deficiencies should avoid baclofen, as otherwise there is the risk of toxicity. In cases that toxicity happens, people may experience symptoms like hypotension, bradycardia, blurred vision, hypothermia, and mental changes (Kent et al., 2020; Romito et al., 2021).

Another factor to be considered when taking baclofen is drugs combination. As baclofen is a drug that depresses CNS, should be avoid taking other medications, which have the same effect on the CNS, simultaneously (Kent et al., 2020; Romito et al., 2021).

Furthermore, baclofen in patients with pre-existent psychiatric disorders can induce adverse effects. The same applies to people who have seizures, since baclofen may reduce the seizures threshold (Kent et al., 2020; Romito et al., 2021).

The abrupt withdrawal of baclofen is not recommended, because it can provoke headaches, insomnia, fever, hallucinations, motor hyperactivity, and return of spasticity. Conversely, tolerance to baclofen covers about 3-20% of the cases. In order to work around this problem, there “off” periods in which patients are treated with another anti-spasticity drug for a few weeks, instead (De Sousa et al., 2022; Kent et al., 2020; Romito et al., 2021).

1.5.6 Baclofen doses

The doses of baclofen vary from person to person and depends on the type of drug administration. In the oral medication, usually, the first dose is 5-30 mg/day, divided in 2-3 takes per day. The baclofen dose may be increased, as recommended by the physician, until the required daily dose is reached. In administration by intrathecal pump, the doses are low, but also vary from patient to patient. The daily doses of baclofen in this type of administration are 50-900 µg, with controlled dose increases in the first year (Farmer & Mittal, 2022; Romito et al., 2021).

For the right dosage recommendation, the physician should analyse the clinical history of each patient and check the existence of seizures, psychiatric disorders, and liver and renal disfunctions. It is also important to consider the degree of severity and the origin of spasms for the best choice of type administration and dose of baclofen (Kent et al., 2020).

1.5.7 Baclofen as a potential therapy for SCI

Baclofen plays a key role in the management of spasticity. Beyond this function, recent studies in lampreys demonstrated that baclofen may have a positive effect in neuroprotection and neuroregeneration after a SCI (Cragg et al., 2019; de Sousa et al., 2023; Romaus-Sanjurjo et al., 2018). Moreover, studies with repeat administration of baclofen in rat until the sub-acute phase of SCI promote an improvement in motor impairment (de Sousa et al., 2023; Kucharíková et al., 2014).

As stated before, microglia are important during the SCI pathophysiology, on both inflammation and axonal growth. In agreement with this, baclofen have shown a putative role in the modulation of microglia phenotype in a mice SCI model and improved locomotor performance (de Sousa et al., 2023).

Taking these studies in consideration, baclofen could not only attenuate spasticity but could also have a positive effect regarding motor recovery, neuroprotection and neuroregeneration after a SCI. Although more studies should be conducted to consolidate these facts, baclofen may have potential as a possible treatment for SCI in humans (De Sousa et al., 2022; de Sousa et al., 2023; Kucharíková et al., 2014; Romaus-Sanjurjo et al., 2018).

1.6 Pregabalin

1.6.1 History and function of Pregabalin

Pregabalin was discovered 20 years after baclofen, in 1990. Only in 2004 pregabalin was approved by Food and Drug Administration (FDA) and European Medicines Agency (EMA) with the commercial brand name Lyrica (Barenie et al., 2021).

Pregabalin is a gabapentinoid drug, that belongs to class of antiepileptic. Currently, pregabalin is used to treat neuropathic pain associated to SCI. Beyond this, pregabalin is also recommended for people who have fibromyalgia, neuropathic pain derived from post-herpetic neuralgia, and diabetic peripheral neuropathy, and is used as adjuvanted therapy for seizures in adults with epilepsy (Cross et al., 2022; Taylor et al., 2007).

1.6.2 Pregabalin and alpha$_2$-delta subunit

Pregabalin, (S)-3-(aminomethyl)-5-methylhexanoic acid), is the new generation of gabapentinoid and acts similarly to gabapentin, also a gabapentinoid. Gabapentinoids are antiepileptic drugs derived from inhibitory neurotransmitter GABA. Therefore, pregabalin is an analogue of GABA. Structurally, it is necessary to add an aliphatic side chain at the position three to the chemical backbone of the GABA, to obtain pharmacological activity of pregabalin (Faingold & Blumenfeld, 2014; Taylor et al., 2007).

Pregabalin has a high affinity to alpha$_2$-delta ($\alpha_2\delta$) subunit, that is present in VDCC. There are four different $\alpha_2\delta$ subunits in mammals, but pregabalin does not present affinity for all four subunit types. The α_2-δ subunits are characterized as type 1 ($\alpha_2\delta$-1), type 2 ($\alpha_2\delta$-2), type 3 ($\alpha_2\delta$-3), and type 4 ($\alpha_2\delta$-4). These are encoded by different genes, CACNAD2D1, CACNAD2D2, CACNAD2D3, and CACNAD2D4, respectively. The different type of subunits are expressed at different locations. The type 1 is highly expressed in skeletal muscle but also are present in smooth and cardiac muscle. Beyond the muscle, the type 1 is also in the brain, more specifically in the neocortex, amygdala, hippocampus, striatum and in the dorsal horn of spinal cord. This subunit, in general, is expressed in excitable cells, being higher in excitatory neurons than inhibitory neurons. The type 2 is less expressed when compared to type 1. Type 2 is in the habenula and molecular layer of cerebellum, being highly expressed in the Purkinje cells. The types 3 and 4 are fewer expressed in the brain than the previous two, with type 3 being in the stratum, neocortex, thalamus, and skeletal muscle. The type 4 is located in pituitary, adrenal gland, intestine, and retina. Genetic mutation in this $\alpha_2\delta$ subunits leads to night

blindless. Although the amino acid sequences of subtypes 1 and 2 are similarly as subtypes 3 and 4 these subunits play distinct roles. Due to the structural difference of the $\alpha_2\delta$ subunits, pregabalin only can bind to the type 1 and type 2 of $\alpha_2\delta$ subunits (Dolphin, 2013; Taylor et al., 2007).

After pregabalin administration, this drug will bind to VDCC in pre-synaptic neurons, in the $\alpha_2\delta$-1 and $\alpha_2\delta$-2 subunits. This interaction will provoke the close of VDCC and consequently a reduction of the Ca^{2+} influx to the pre-synaptic neuron. The lower concentration of Ca^{2+} leads to hyperpolarization of pre-synaptic neurons and there is no release of neurotransmitter for synaptic cleft (**Figure 1.8**). In the absence of release of neurotransmitters, as glutamate, noradrenaline and substance P, the transmission of signal is prevented. Thus, the pathways responsible for neuropathic pain are inhibited (Taylor et al., 2007; Verma et al., 2014).

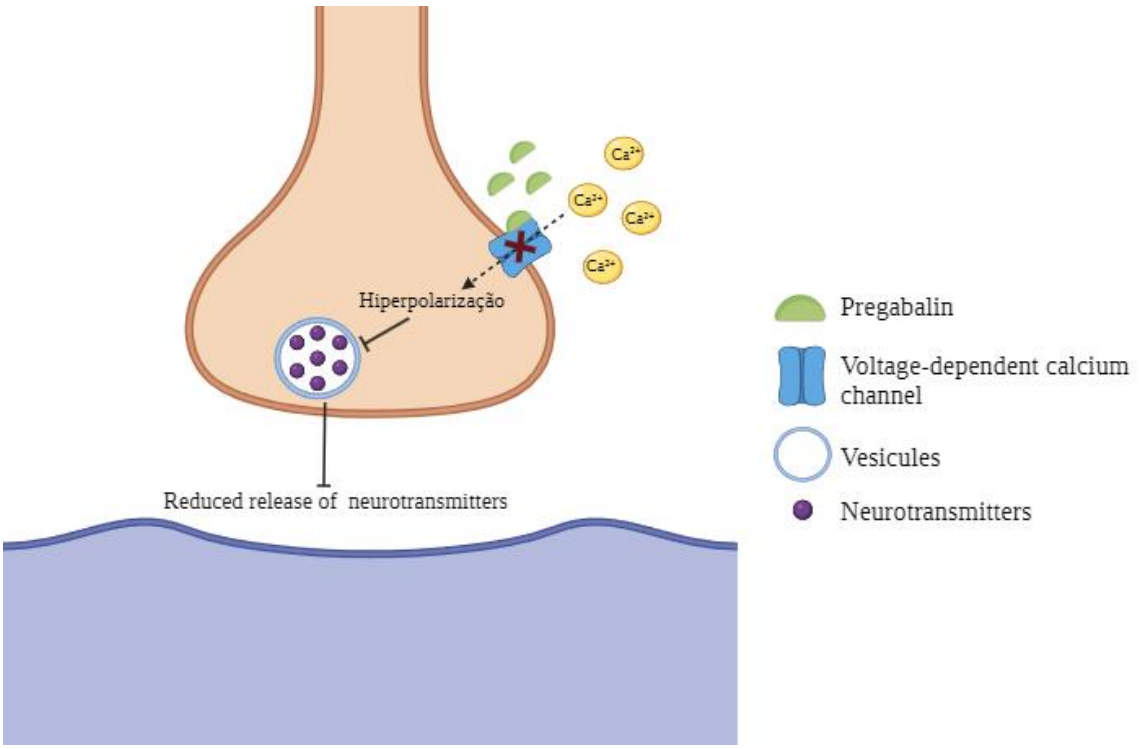


Figure 1.8 Mechanism of Pregabalin. Interaction of pregabalin in pre-synaptic neuron and mechanisms associated with pregabalin voltage-dependent calcium channel binding. Image crated in BioRender.

1.6.3 Administration and doses of Pregabalin

Unlike baclofen, pregabalin can be administrated only by oral medication. The patient may take doses between 150mg and 600mg per day. These doses are divided in two or three

daily doses. Initially, an individual with neuropathic pain starts pregabalin with a dose of 150mg/day. Depending on the patient´s reaction, this dose may be increased, if necessary, to 300mg/day after an interval of one week with the initial dose. If the pain persists and the patient tolerates well the 300mg/day dose for 2-3 weeks, this dose can be increased to 600mg/day. This is the maximum dose a person can take a day for neuropathic pain. If discontinuation of pregabalin taking is recommended, should be done gradually, for at least one week to avoid side effects (Cross et al., 2022; Toth, 2014).

1.6.4 Metabolism of Pregabalin

After oral administration pregabalin is rapidly absorbed in the gastrointestinal tract. Pregabalin reach its peak concentration in the plasm within one hour of taking it, and the half-life is around 6.3 hours. Only after two hours, approximately, pregabalin reaches the CSF and reach its higher concentration eight hours after taking. Pregabalin is hydrophilic and double charged at neutral pH, therefore is insoluble in lipids, namely cell membranes. Beyond to this particularly of pregabalin, this drug is transported for a specific transport system, the L-type amino acid transporter (LAT). This system comprises different types according to the substrate (LAT1, LAT2, LAT3 and LAT4). Since pregabalin is a neutral amino acid, the LAT responsible for transport this amino acid is the LAT1. Once pregabalin arrived at the circulation, is incapable to cross the BBB, and LAT1 is the responsible to induce its passage through the BBB. LAT1 also transport amino acids like phenylalanine, leucine, isoleucine, and valine (Errasti-Murugarren et al., n.d.; Liu & He, 2017; Y. Takahashi et al., n.d.).

Three other advantages of pregabalin and its transport mechanism are the fact that this drug does not bind to plasma protein, can rapidly penetrate blood-brain barrier, and in humans the metabolism of pregabalin is negligible, less than 2%. Pregabalin is excreted, from the body, by the kidneys in an unchanged form (Ben-Menachem, 2004; Bockbrader et al., n.d.).

1.6.5 Side effects of Pregabalin

Pregabalin secondary effects can vary depending on the dose of the drug. Regarding low doses of pregabalin, the most common symptoms are dizziness, drowsiness, headache, weight gain, dry mouth e blurred vision. With the increasing dose, usually the maximum dose (600mg/day), symptoms such as headache, blurred vision and dizziness are more frequent. These secondary effects arise, because pregabalin acts in several VDCC that are present in

different parts of the brain. Pregabalin induces the decrease of the activity of these channels and for this reason, the patient can experience the mentioned symptoms. In addition to VDCC in the CNS, these channels are present in the heart and thus people with heart problems must be cautious. As pregabalin is excreted by the kidneys, people with kidney deficiencies should also be cautious about taking this drug. Another aspect to consider when taking pregabalin is patient drug dependency, like alcohol or benzodiazepines. Pregabalin cause disturbance in CNS, and the administration of pregabalin with alcohol or benzodiazepines causes a synergistic effect and depress neuronal conduction. Due to these situations, pregabalin administration must have a continuous monitoring, and before the prescription of this drug the physician must analyse the clinical history of each patient (Onakpoya et al., 2019; Verma et al., 2014).

1.6.6 Other effects of Pregabalin

Pregabalin is known to have a positive effect on neuropathic pain. However, this drug was used in studies with other pathologies, like traumatic brain injury (TBI), that demonstrate that pregabalin in acute TBI can relieve the edema and control the inflammation (Calikoglu ABEF et al., 2015; Shamsi Meymandi et al., 2018). Nevertheless, emerging studies have been showed that this drug can influence other aspects. For instance, pregabalin interaction with the VDCC, more specifically the α_2-δ subunit, appears to be beneficial to axonal regeneration, according to the study developed by Andrea Tedeschi (Tedeschi et al., 2016). Other study performed on rats with SCI, in which pregabalin was administered, demonstrated that this drug may act as a neuroprotector after an injury in spinal cord (Ha et al., 2008). In addition to the neuroprotective role of pregabalin, there is also other study supporting that early administration of pregabalin may influence motor recovery in humans (Warner et al., 2017).

1.7 Models to study Spinal Cord Injuries

Due to the complexity of SCI, several studies have been carried out to understand what happens at the pathophysiologic level after an injury in spinal cord. Moreover, multiple studies are focus on discovering possible therapies for definitive treatments for SCI. However, for an efficient study is necessary a suitable and reliable model that mimic as much as possible the process that happens in human spinal cord when it is injured. The models that most closely resemble to what occurs in spinal cord injuries, and that provide better information for scientific research, are the animals models (Ridlen et al., 2022; Zhang et al., 2014).

The SCI models must follow a set of conditions, that is:

- Simulate a damage similar to what happens in humans with SCI;
- Be controlled, reproducible and stable;
- Involve a simple technique;
- The equipment used for lesions is straightforward and quick to reproduce.

1.7.1 Types of models of SCI

Currently, there are three SCI animals models available, the contusion model, the compression model, and the transection model (Zhang et al., 2014).

Contusion model. The contusion model is used to induce an injury through a force applied against spinal cord. This model usually uses an impactor to induce to the lesion. There are different types of impactors, Multicenter Animal Spinal Cord Injury Study (MASCIS) impactor, infinite horizon (IH) impactor, Ohio State University (OSU) impactor and air gun impactor (Cheriyan et al., 2014; Ridlen et al., 2022).

In this model, a laminectomy is performed on the animal to have access to the spinal cord. After the spinal cord exposure, a weight (30-50g) is dropped against spinal cord (B. Fan et al., 2022; Ridlen et al., 2022). The injury takes seconds to minutes to be perform. The contusion model can only do an injury at the thoracic level of the spinal cord, and the animal's models in which this lesion can be performed are rodents and pigs (Cheriyan et al., 2014; Ridlen et al., 2022).

As disadvantage, this model requires a laminectomy, there is a variability in the applied force, the pressure exerted is release instantly, and it is difficult to identify regenerated and residual nerves. On the other hand, the contusion model has advantage of being able to easily control the lesion parameters, such as the pressure, duration, and the exerted force. Furthermore, the pathophysiologic characteristics of this model are similar to the humans (Cheriyan et al., 2014; Ridlen et al., 2022).

Compression model. The compression model has as main goal to compress the spinal cord to obtain the most frequent injury in humans, the traumatic spinal cord injury (Cheriyan et al., 2014; Ridlen et al., 2022).

This model was established by Tator and Rivlin (Rivlin & Tator, 1978) who used a modified aneurism clip to induce the lesion in spinal cord in an animal model. Although

compression model was initially performed using a compression clip, currently are approved various equipment that can generate this model like the balloon compression, solid spacer compression, expanding polymer, forceps compression, screw compression, spinal cord strapping and remote compression. Some of these methods require laminectomy and other do not. Despite these methods induce a spinal cord compression injury, they are distinct in different parameters, namely in the level at which the lesion can be induce, the type of animal model that can be used, and the type of lesion (traumatic or non-traumatic SCI) (Cheriyan et al., 2014; Ridlen et al., 2022).

The clip and forceps compression are two methods that can provoke a lesion at cervical and thoracic level. This lesion takes seconds to minutes to be made and laminectomy is necessary. Both methods simulate a traumatic injury and can only be performed in rodents. Like the two methods mentioned above, the balloon compression also mimics a traumatic SCI, takes seconds to minutes to be execute, and a laminectomy is needed, but the balloon only can induce an injury at the thoracic level. However, the balloon compression can be used in different animal's models, such as rodents, rabbits, dogs, and goats (Cheriyan et al., 2014; Ridlen et al., 2022).

The solid spacer compression and the expanding polymer are used in rodents, and to induce the lesion by these two methods it is necessary to do a laminectomy. Nevertheless, the solid spacer can provoke an injury at thoracic level in spinal cord and the expanding polymer induce an injury at the cervical or thoracic level in rodent's spinal cord (Cheriyan et al., 2014; Ridlen et al., 2022).

As opposite to the methods present so far, spinal cord strapping, screw compression and remote compression, they do not require laminectomy. The spinal cord strapping lesion the thoracic segment of spinal cord and are done only in rodents. The screw compression can cause thoracic and cervical lesions in animal´s models, such as rodents and goats. Last of all, the remote compression is the only methods that can induce lesions just at the cervical level of spinal cord and is used in sheep (Cheriyan et al., 2014; Ridlen et al., 2022).

In general, the compression model has as disadvantage the difficulty to identify possible regenerated and residual nerves, and the most of methods of compression models require laminectomy. Nevertheless, within its advantages, it induces injuries consistently and the evolution of the pathophysiology in this model is very similar to the evolution of SCI in humans (B. Fan et al., 2022; Ridlen et al., 2022).

Transection model. The transection model is executed through the resources of microscopic instruments, to perform a cut in spinal cord or removal of a part of it. This model is useful when the objective of study is tissue engineer. This injury model is the most severe among the three, due to induction of a defect in spinal cord tissue in a certain area. There are two types of transections, complete and incomplete (Cheriyan et al., 2014; Ridlen et al., 2022).

Complete transection occur when exist a total dissociation between rostral and caudal segments. While in an incomplete transection only a part of spinal cord is injured. The major disadvantage of this model is that the transection injury is not so clinically relevant as other models. However, transection model is useful in studies that the main goal is to analyse neuronal regeneration. Furthermore, lesions in this model are very consistent, therefore there is less heterogeneity in lesion between animals (Cheriyan et al., 2014; B. Fan et al., 2022; Kwon et al., 2002).

1.7.2 Choice of animal model and type of model of SCI

To study such a complex mechanism as SCI there is no perfect model. However, the animal model choice and the type of lesion, must be the one that most closely resembles what occurs in the clinic. Moreover, it is also important to take into account the purpose and the main goals of the study.

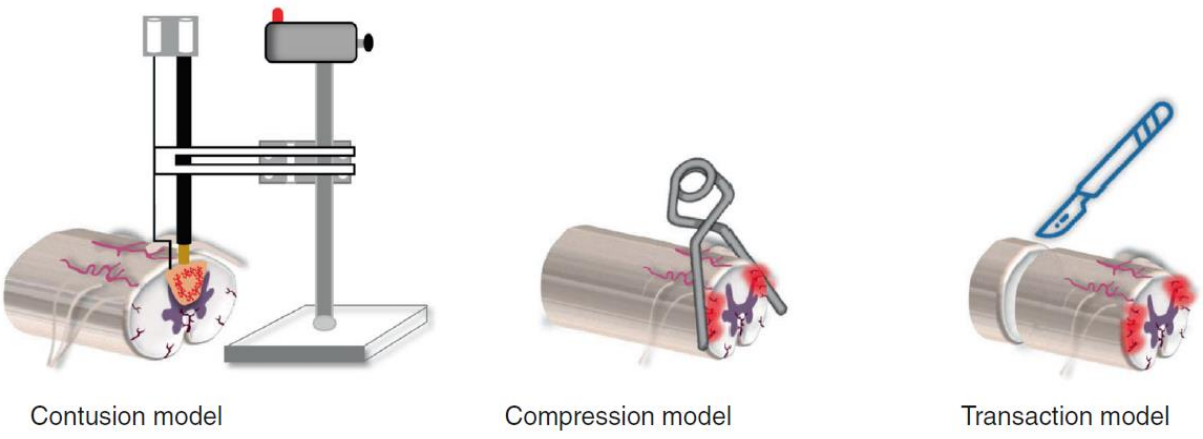


Figure 1.9 Models of SCI. Mechanism to induce three different types of SCI in animal model. Image adapted from Fan *et al.* (2022).

1.7.2.1 Animal model

The animal species most widely used for SCI are rodents. Mice and rats when compared to other animal models are low cost, require less stringent regulations and fewer ethical considerations. The rat is used because its well-known anatomy and physiology. It rarely has infections due to the surgery, and the techniques to work with these animals are well established. Moreover, as it happens to humans after an injury, rats usually develop a large cystic cavity at the site of injury. However, as rats are quadrupeds and not bipeds, their corticospinal tracts are dorsal while in human corticospinal tracts are ventral (Sharif-Alhoseini et al., 2017).

Contrary to what happens in rats and humans, in mice there is no formation of cystic cavity, instead there is a formation of a dense layer of cells, which decrease over the time due to the absence of the cyst. On the other hand, mouse models are increasingly being used, since they are ideal when the objective it to study the cellular and molecular biology after SCI, since mice have a genome similar to humans and the inflammatory response is well characterized with available anti-bodies for immune cellular characterization. Besides, mice have a hight reproductive rates and they are easier to handle (Sharif-Alhoseini et al., 2017).

1.7.2.2 Type of model of SCI

Like the animal model, the type of model of SCI, must meet what is intended to be studied. In clinic, lesions in spinal cord, mostly, are caused by traumatic events, for example road accidents and falls. Only a small part of injury has non-traumatic causes. For this reason, it is important to prioritize the purpose of the study and choose the methods that better mimic the lesion that researchers want to study (B. Fan et al., 2022; Sharif-Alhoseini et al., 2017).

If the objective of the study is to analyse the pathophysiology of a spinal cord, the recommended models are contusion and compression, since they are what happens in most patients with SCI. However, it is necessary to be aware, because within the compression models there are methods that cause injuries similar to traumatic injuries and other methods that mimic non-traumatic injuries. To study regeneration, degeneration, neuroplasticity or tissue engineer, the model more indicated is the transection model (B. Fan et al., 2022; Kwon et al., 2002; Sharif-Alhoseini et al., 2017).

1.7.3 Limitations of animal models

As mentioned before, it is hard to find the perfect model to study. Even the closest model that better resemble what happens in SCI in humans has its limitations. In the most studies of SCI, adult animals are used. After the injury there is scar formation, and this is one of the main reasons why there is no axonal regeneration. In some animal's models, like zebra fish and amphibians, after an injury there is no scar formation allowing the axonal regeneration and reestablishment of the neuronal circuit. However, these animal models do not mimic what happen in human SCI (B. Fan et al., 2022; Zhang et al., 2014). Studies conducted by the team of Zhigang He et al. showed that in neonatal mice (P2) the scar formation does not proceed SCI. For these reasons, animal models of SCI with different ages should be studied (Li et al., 2020).

In the clinic, the most common type of SCI in patients occurs at the cervical level, yet in the studies the most frequent SCI is induced at the thoracic level. Thoracic SCI models are more used because it is easier to induce the lesion, the complications associated to the injury are less frequent at thoracic level, and the survival rate is bigger compared to the lesions in the cervical levels. Although thoracic SCI models present advantages do not represent what occurs in the great majority of patients with SCI (B. Fan et al., 2022; Sharif-Alhoseini et al., 2017).

One of the major limitations and quite difficult to overcome, is the performance of laminectomy. Laminectomy is necessary to remove the spinal lamina to have access to the spinal cord. When execute laminectomy, the dynamic of CSF will change. Beyond that, laminectomy can cause alterations in the degree of the injury in the compression and contusion models. The closer to the injury site, the greater the biomechanical alteration of the injury induced. In clinical practice, the laminectomy is used as a treatment for compression SCI caused by cervical myelopathy in spinal cord. Although researchers could choose the methods that not require laminectomy, for instance the screw compression, solid spacer strapping and remote compression, these methods only mimic SCI non-traumatic, and this lesion is not the most common in patients with SCI (Ridlen et al., 2022).

Although there is no perfect animal model and lesion method for studying spinal cord injuries, the analysis of the pros and cons of each of the parameters should allow the selection of the one that best suits the study purpose of each project. Using a serious criteria is essential to make that possible therapies can be translated into the clinic.

Chapter 2 – Research purpose

Lesions in spinal cord cause an entropy in the microenvironment, leading to spinal cord damage and motor, sensory and physiologic consequences in the human organism.

SCI affects millions of people every year all over the world, and although the knowledge about this pathology is gradually increasing from year to year there is still no cure available. Although there are already drugs to control symptoms trigger by the SCI, like baclofen and pregabalin to manage spasticity and neuropathic pain, respectively, there is no definitive treatment that improve the quality of life of these individuals.

Currently, there are some ongoing studies in the field of SCI that try to find potential therapies. These studies use different approaches, from stem cells, tissue engineer, gene therapy, rehabilitation, cellular reprogramming, and pharmacological approaches.

Previous investigations, in which baclofen and pregabalin were used, found that these drugs may have the potential to promote neuroprotection, neuroregeneration, and locomotor improvement in different animal models.

Due to this previous data, the present study aims to analyse whether the combined administration of baclofen with pregabalin has a synergistic effect, enhancing both motor recovery and bladder control and whether neuroregeneration can occur after administration of these two drugs using a transected SCI mouse model.

Chapter 3 – Materials and Methods

3.1 Animals and housing

All experiments performed were previously approved by the Ethical Subcommittee in Life and Health Sciences (SECVS; ID:018/2019, University of Minho) and the Portuguese Authority (DGAV; ID:005453). Experimentation, animal care and local regulations (European Union Directive 2010/63/EU) were respected. Female C57BL/6J mice with 10 weeks old (Charles River, USA), were maintained ate the animal facilities of the Institute of Life and Health Sciences (ICVS, Braga, Portugal) under sterile conditions and in light, humidity, and temperature-controlled rooms. Animals had food and water provided ad libitum.

In this study, 37 animals with 10 weeks old were used. All the 37 animals were maintained in standard conditions, 12 hours light/12 hours dark cycles, 22ºC, relative humidity of 55%, ad libitum access to standard food and water. The number of the animals used in this study was determined by G-power software that use a medium effect size of 0.5, a confidence coefficient of 0.05 and a statistical power pf 0.9 (G*power) (University of Kiel, Germany

3.2 Surgery preparation

A week before surgeries, animals were handled once a day, to reduce their stress level and habituation to the human presence (**Figure 3.3**). One day prior surgery, animals were weighted and were identified with marks in the tail for the administration of corrected doses of anesthesia and analgesic.

3.3 SCI surgery

C57BL/6J mice were prepared for surgery. Initially was used a mixture containing ketamine (Imalgene, 75 mg/Kg, Merial, France) and medetomidine (Dormitor, 1 mg/Kg, Pfizer, USA) to anesthetize the animals via intraperitoneal injection. It was also administrated buprenorphine (Bupaq, 0.05 mg/Kg), subcutaneously, for analgesia. The hypothermia and the loss of reflex blink are consequences of the anesthesia, so before and after surgery, mice were placed under a warm lamp, preventing a drop in body temperature. During surgery, a warm surgical blanket was also use for the same purpose. Saline solution (Salin Solution, 0.9%, B. Braun, SKU 06063042) was applied in the eyes, to prevent drying and corneal damage.

The dorsal thoracic area of the animals was shaved and disinfected with chlorhexidine. Also, all the materials used in the surgeries were subjected to sterilization processes. To confirm the surgical plane of anesthesia, mice were pinched on the lower limb toes. A midline incision in the skin on the back of the animal was made at the level of the thoracic spine T5 toT12. Then the paravertebral muscles were exposures and retracted for the remotion of the spinous and the laminar arc of T8-T9 vertebral body, allowing the access to spinal cord. With microdissection scissor, the spinal cord was completely cut (transection). Lesions were performed consistently with the same microdissection scissor and always by the same experimenter, in order to reduce variability. After SCI, the muscles and skin were closed with surgical staples (MikRon, Clay Adams, USA). To reverse anesthesia, it was administered subcutaneously Atipamezole (Antisedan/Pfizer), and animals were put to recover from the anesthesia under the warm lamp. After complete recovery, animals were rehoused in individuals cages with moisturized food pellets on the floor to allow easy access to water and food.

3.4 Post-operative care

SCI is considered a sever surgery, so these animals require an intensive post-operative care, mainly in the first five days post-injury. Post-operative care in these days included buprenorphine (Bupaq, 0.05 mg/Kg), vitamins (Duphalyte, Pfizer, New York, NY, USA), saline (Salin Solution, 0.9%, B. Braun, SKU 06063042), and enrofloxacin (Bayer, Leverkusen, Germany) administration, twice a day. After SCI, animals' loss their ability to urinate, so manual voiding of the bladders was performed twice a day, in the first five days, and then once a day until the end of the *in vivo.* Parameter like body weight, temperature, behaviour, activity, nesting and urine and faeces were monitored to evaluate the animal welfare.

Mice were put together in mice cages 7 days after surgeries, in order to avoid behavior changes due to isolation. The maximum number of animals per cage was determined following the rules of the Ethical Subcommittee in Life and Health Science.

3.5 Treatment

After SCI, all the animals were randomly and blinded distributed into 4 experimental groups. The treatment was injected intraperitoneally, within 10 minutes after injury. Animals assigned to the group 1 were administered with 100 µL of saline (Salin Solution, 0.9%, B. Braun, SKU 06063042), whereas animals that belong to 2, 3 and 4 group receive Baclofen ((±)-

β-Aminomethyl-4-chlorobenzenepropanoic acid, Lioresal, (±)-Baclofen, Sigma-Aldrich, CAS number 1134470) to the concentration of 1mg/Kg, and Pregabalin ((S)-3-(Aminomethyl)-5-methylhexanoic acid, Sigma-Aldrich, Merck, CAS number 148553-50-8) to the concentration of 30mg/Kg within 10 minutes after injury. Thus, the animals were distributed by the following experimental groups: Vehicle (Vh), SCI mice control group treated with saline solution within 10 minutes after injury; Baclofen acute + Pregabalin 24 hours Delay + Chronic (Ba+P24c), SCI mice receive Baclofen acutely after lesion and Pregabalin 24 hours after lesion and then for 14 consecutive days; Baclofen acute + Pregabalin acute (Ba+Pa), SCI mice were treated with Baclofen and Pregabalin after injury; Baclofen and Pregabalin acute + Pregabalin chronic (Ba+Pac), SCI mice treated group with Baclofen and Pregabalin after injury and with Pregabalin for 14 consecutive days (**Table 3.1**).

Baclofen and Pregabalin concentrations were determined based on a previous studies (de Sousa et al., 2023; Ha et al., 2008). Through the experiment, animals´ survival rate was analysed. In order to exclude the cage input environment, animals from the same cage received different treatments.

Table 3.1: Experimental group and treatments

Experimental groups	Treatment
Vehicle (Vh)	Saline solution
Baclofen acute + Pregabalin 24h delay and chronic (Ba+P24c)	Baclofen acutely after injury and Pregabalin 24 hours after injury and then for 14 consecutive days
Baclofen acute + Pregabalin acute (Ba+Pa)	Baclofen and Pregabalin acutely after injury
Baclofen acute + Pregabalin acute and chronic (Ba+Pac)	Baclofen and Pregabalin acutely after injury and then with Pregabalin for 14 consecutive days

3.6 Motor, sensory and bladder function analysis

3.6.1 Basso Mouse Scale

Basso Mouse Scale (BMS) it is a standard test to evaluate the locomotor function of SCI mice. This test is based on a scale from 0 to 9, where 0 represent an animal that its incapable

to move voluntarily , and a score of 9 correspond to an animal with normal plantar stepping with coordination and trunk stability (Basso et al., 2006). Several features are analysed in this teste: stepping (plantar or dorsal), ankle movement, weight support, trunk stability and rotation of hindlimbs (**Annex 1**).

To performed BMS, animals were placed in an open arena for 4 minutes and were scored by two independent trained observers that evaluated their locomotor function according to the BMS scale. The researchers were blinded to the experimental group.

As a quality control to exclude eventual partial lesion, locomotor behavior (BMS score) was assessed at 48 hours post-injury (hpi). Besides, BMS was performed once a week for 6 weeks (**Figure 3.3**). Body weight support was considered when animals´ reach BMS score ≥4.

3.6.2 Bladder function analysis

The experimental design was made in order to analyse the bladder function, since animal´s with SCI loss the ability to control their bladder. After the second week post-injury, animals were placed individually in a cage for 4 hours, once a week until the end of the experiment (**Figure 3.3**). Before carrying out the test, cages were lined with Wattman paper, and mice bladders were completely void. After that, 800 µL of saline (Salin Solution, 0.9%, B. Braun, SKU 06063042) was injected subcutaneously. During this test, animals were deprived of water and food. At the end of 4 hours, bladders were voided to a beaker and the urine was weight, and the Wattman paper was collected for analysis. In addition to the injured animals, 3 uninjured animals were placed to perform this test every week (Hill et al., 2018; Wegner et al., 2018).

The urine weight was analysed per experimental group, and through the Wattman paper the area and the spots number were analysed (**Figure 3.1**). The ratio between these two data was performed to assess the average area. During the weeks of bladder trail, were also assigned scores to the Wattman paper, according to the animal´s urine pattern (**Figure 3.2**). To avoid variability, it was the same researcher to perform the injection of saline.

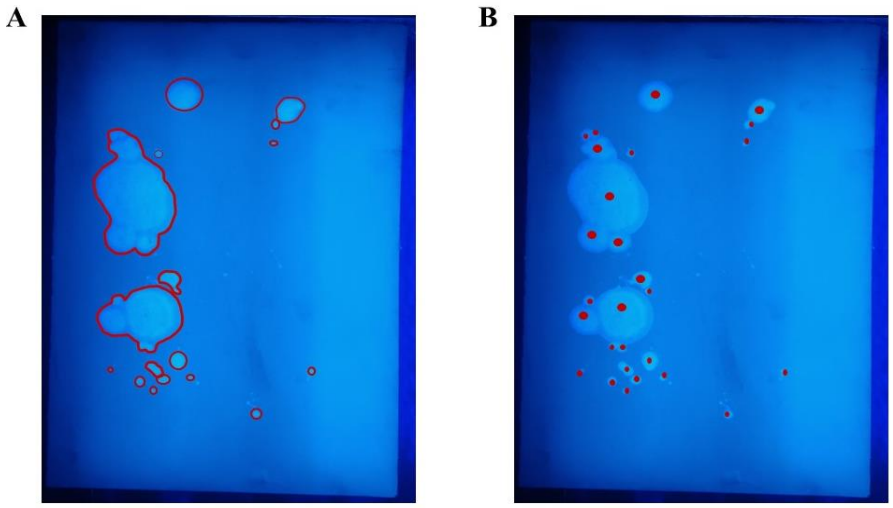


Figure 3.1 Average area of urine. Analysis of urine through Wattman paper after 4 hours. (A) Area of urine. (B) Number of urine spots.

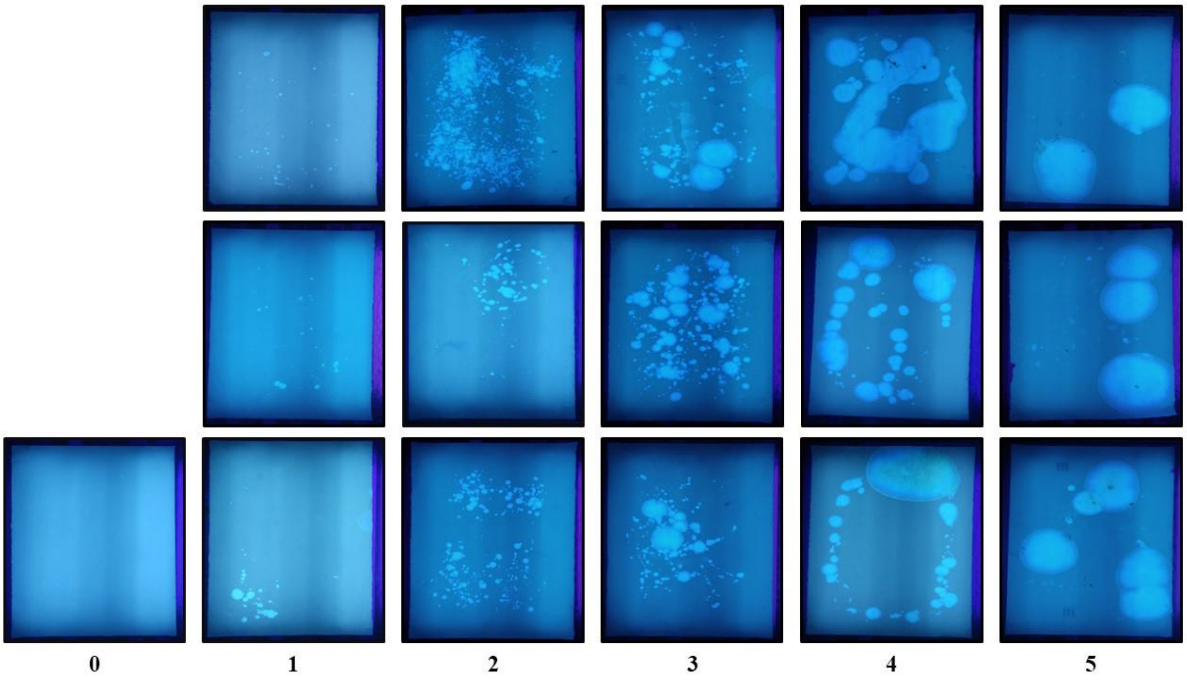


Figure 3.2 Urine scores. Urine pattern representation with the respective scores.

3.6.3 von Frey test

Manual von Frey test (vF) is used to evaluate the mechanical allodynia in rodents, which is the pain induced by a stimulus that normally does not provoke pain (Deuis et al., 2017). To analyse the response of the mice to this test, were used the "up-down method" for approximation of the 50% withdrawal threshold (Chaplan et al., 1994).

In the last week of the *in vivo*, vF was performed (**Figure 3.3**). For that, animals were immobilized in a small cage, on a grid, to allow the passage of the von Frey filaments. These filaments are made of nylon threads , and this test consist of application of these filaments under the paw of the mice. In the case of a mouse, are used 10 filaments with different thickness, which vary between 0.008g and 1.4g. The test start with the middle filament of 0.16g, and then the next filament used change according to the mice response to the stimulus. For instance, if the mouse reacts to the stimulus induced by the filament, the next filament used is of lesser thickness. On the other hand, if there is non-response to the stimulus, a thicker filament is applied. The paw withdrawal threshold was defined after three readings.

To determine the 50% withdrawal threshold, the pattern of responses (O- non response and X- responsive to stimulus) as the last filament were registered. These data were applied in the formula 50% g threshold = $10^{x+k\delta}/10^4$, was used, where x represent the number of the last filament, k is the value obtained by the response sequence, and the δ is the mean difference between stimuli (Chaplan et al., 1994).

3.7 Blood sample collection

In order to analyse the immune response during the *in vivo*, blood samples were collected at 1-, 7-,14- and 42- days after injury (**Figure 3.3**). The blood was obtained from the mice tail vein to an Eppendorf. Then the blood was allowed to clot for 30 minutes and centrifugated for 15 minutes at 1500 rotations per minute (rpm). The serum was collected for new Eppendorf's and store at -80 ºC until the day of the experiment.

3.8 Euthanasia and spinal cord collection

In order to proceed to the collection of spinal cord for posterior histological analysis, mice were euthanized six weeks post-injury (**Figure 3.3**). For that, was administered a lethal dose of anaesthetic containing Ketamine (Imalgene, 150mg/Kg, Merial, France), Medetomidine

hydrochloride (Dormitor, 2 mg/Kg, Pfizer, USA) and saline (Salin Solution, 0.9%, B. Braun, SKU 06063042). After being anesthetized and verifying that the animals do not response to the paws pinching, was made a ventral incision and mice were perfused transcardially with 20 mL of cold buffered saline (PBS) to remove the blood from the circulation, and then with 20 mL 4% paraformaldehyde/PBS (PFA) to initiate the tissue preservation. To remove the vertebral columns, it was made a dorsal incision.

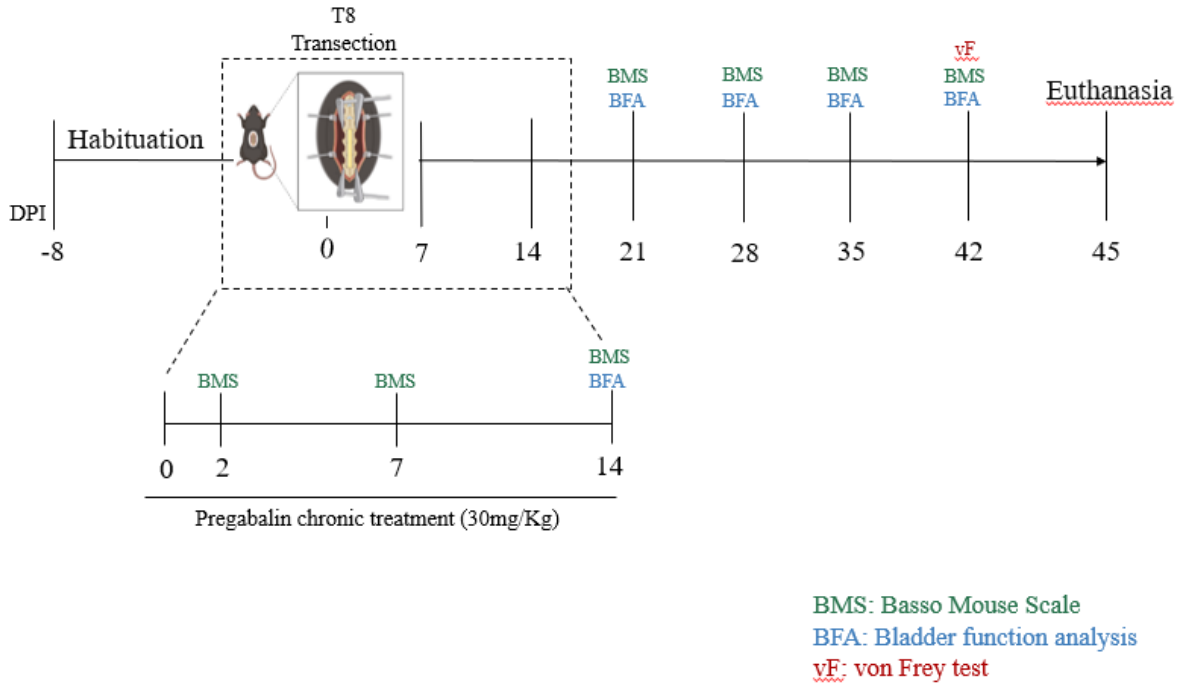


Figure 3.3 Experimental design. Representative scheme with chronic treatment administration, behavior test and duration of *in vivo*.

3.9 Tissue cryoprotection and sectioning

The vertebral columns that were removed from the mice, were fixed with PFA (4%) for 24 hours at 4 ºC. Then, the spinal cords were dissected and dehydrated in a sucrose solution (30% sucrose in PBS 1x) for 48 hours at 4ºC, in order to avoid ice crystal formation in the tissue after freezing. Posteriorly, spinal cord was removed from sucrose solution and then was cut 1 cm around the lesion site. These segments were cryoprotected in an optimal cutting temperature compound (OCT, ThermoFisher, Scientific, Massachusetts, USA) and frozen in a liquid nitrogen, and stored in the freezer at -20 ºC.

The spinal cords were sectioning in a transversal cuts of 20 µm using a Cryostat (Leica CM 1950, Nussloch, Germany). Tissue sections were collected with charged microscope slides (Superfrost Plus, Thermo Fisher Scientific, Massachusetts, USA), to allow the binding of frozen tissue to reduce tissue loss. All the samples were stored at -20 ºC.

3.10 White matter sparing

To evaluate the myelin preserved in spinal cord transversal cross- sections, the FluoroMyelin green fluorescent myelin staining protocol was performed with FluoroMyelinTMGrenn (F34651, Thermo Fisher Scientific, Massachusetts, USA) for 1 hour at room temperature (RT). In the end, the slides were mounted with Immu-Mount® (Thermo Fisher Scientific, Massachusetts, USA) (Paramos-de-Carvalho et al., 2021). The slides were storage ate 4ºC.

3.11 Immunohistochemistry

In order to study alterations that occurs in spinal cord injury with the treatments administered, the immunohistochemistry protocol (IHC) was performed. For that, spinal cord sections were analysed by collecting photomicrographs every 1000 µm, both rostrally and caudally from the lesion epicenter, at T8 lever. The area ranging from -200 to 200 µm around the epicenter was considered the lesion epicenter. The rostral and caudal analysed extended from -2000 to -200 µm and from 200 to 2000 µm from the epicenter, respectively. Initially, the slides are thawed at RT for 20 minutes. Then, the slides are covered, for 10 minutes at RT, with 0,2% Triton X-100 in PBS 1x, for permeabilization of the cellular membrane. Before the antibody addition, was used a blocking solution containing 5% Fetal Bovine Serum (FBS) in 0.2% Triton X-100, for 30 minutes. Afterwards, the spinal cords sections were incubated overnight at RT, with the primary antibodies, namely mouse anti-NeuN (1:200; Millipore, Darmstadt, German), rabbit anti-PDGFR (1:500; ABCAM, Cambridge, UK), rabbit anti-Iba-1 (1:1000; Wako®, Osaka, Japan), mouse anti-NF-H (1:400; Millipore, Darmstadt, German), goat anti-5-HT (1:500; Millipore, Darmstadt, German) and rabbit anti-TH (1:700; Millipore, Darmstadt, German). On the next day, slides were washed 3 times in PBS for 3 minutes, following an incubation, for 2 hours at RT, with a solution containing the secondary antibodies with specific fluorophore: Alexa FluorTM 594 goat anti-mouse IgG (1:1000; Invitrogen, Paisley, UK), Alexa FluorTM 488 goat anti-rabbit IgG (1:1000; Invitrogen, Paisley, UK), Alexa

FluorTM 594 goat anti-rabbit IgG (1:1000; Invitrogen, Paisley, UK), Alexa FluorTM 488 goat anti-mouse IgG (1:1000; Invitrogen, Paisley, UK) and Alexa FluorTM 488 donkey anti-goat IgG (1:1000; Invitrogen, Paisley, UK). After the 2 hours of incubation, a counterstaining of spinal cord sections with 4′,6-diamidino-2-phenylindole (DAPI) during 10 minutes at RT (1:1000; Sigma-Aldrich, Missouri, USA). In the end, tissues sections were washed 3 times for 3 minutes and mounted with Immu-Mount® (Thermo Fisher Scientific, Massachusetts, USA). All the samples were storage at 4ºC.

3.11.1 Quantification of fibrotic tissue

Quantification of fibrotic tissue was performed to evaluate the difference of fibrotic scar between the experimental groups. Tissue sections were stained with the primary antibody rabbit anti-PDGFR (1:500; ABCAM, Cambridge, UK) and with the secondary antibody Alexa FluorTM 488 goat anti-rabbit IgG (1:1000; Invitrogen, Paisley, UK).

3.11.2 Quantification of motor neurons

The motor neurons quantification was made using the staining with the primary antibody mouse anti-NeuN (1:200; Millipore, Darmstadt, German) and the secondary antibody Alexa FluorTM 594 goat anti-mouse IgG (1:1000; Invitrogen, Paisley, UK) (Lima et al., 2021). Analysis of motor neuron was made in the ventral horns because this is where the motor neurons are located.

3.11.3 Quantification of axonal fibers

A region of interested (ROI-ventral zone of spinal cord that encompasses white and grey matter) was used to quantify the axonal fibers. For that, the spinal cord transversal cross-sections were stained with the primary antibodies mouse anti-NF-H (1:400; Millipore, Darmstadt, German), goat anti-5-HT (1:500; Millipore, Darmstadt, German) and rabbit anti-TH(1:700; Millipore, Darmstadt, German), and with the secondary antibodies Alexa FluorTM 488 goat anti-mouse IgG (1:1000; Invitrogen, Paisley, UK), Alexa FluorTM 488 donkey anti-goat IgG (1:1000; Invitrogen, Paisley, UK) and Alexa FluorTM 594 anti-rabbit IgG(1:1000; Invitrogen, Paisley, UK), respectively.

3.11.4 Quantification of microglia

In order to analyse the microglia phenotype in a region of interested (ROI-ventral zone of spinal cord that encompasses white and grey matter), the slides were stained with the antibodies rabbit anti-Iba-1 (1:1000; Wako®, Osaka, Japan) and Alexa FluorTM 594 goat anti-rabbit IgG (1:1000; Invitrogen, Paisley, UK), as primary and secondary antibodies, respectively (Zanier et al., 2015).

3.12 Imaging

The stained spinal cord transversal cross-sections photos were acquired using the Olympus Widefield Inverted Microscope IX81, Tokyo, Japan. In the case of FluoroMyelin green fluorescent myelin staining, the photomicrographs were obtained every 200 µm from -1000 µm rostrally to the lesion epicenter (considered -200 to 200 µm around the epicenter) and 1000 µm to the lesion site. For this staining was used a 20x objective. Regarding to the immunohistochemistry, the photomicrographs were collected every 1000 µm, for -2000 µm rostrally and 2000 µm caudally to the lesion epicenter (considered -200 to 200 µm around the epicenter). The photos of fibrotic tissue and motor neurons were obtained with a 20x objective, while microglia e NF-H were obtained with a 40x objective.

Image J software was used to process all the images. Also, was applied brightness/contrast and color balance adjustment to the images.

3.13 LegendPlex

Serum´s from the blood samples that were collected at 1-, 7-, 14- and 42-days after lesion from the mice tail vein to an Eppendorf, were assayed through LEGENDplexTM Mouse Inflammation Panel kit (Biolegend, Cat. No. 740446) according to the manufacturer´s instructions. This kit includes analytes pro-inflammatory and anti-inflammatory, such as IL-1α, IL-1β, IL-6, IL-10, IL-12p70, IL-17A, IL-23, IL-27, MCP-1, IFN-β, IFN-γ, TNF-α and GM-CSF.

Reagents were prepared previously, as well as standard serial dilutions to generate a standard curve. All the compounds are provided by the kit. After the reagents and standard samples preparations, 25 µL of samples and standards were placed in the respective wells of the V-bottom plate. Next, were add to each well 25 µL of assay buffer and then 25 µL mixed

beads. The plate was sealed and placed in a plate shaker for 2 hours at 500 rpm at RT. Posteriorly, the plate was washed with 1x Wash Buffer (200 µL) and detection antibody (25 µL) was add. Plate was again placed in continuous rotation for 1 hour at 500 rpm at RT. After that, Streptavidin-phycoerythrin (SA-PE) (25 µL) was added directly to the previous solutions. Plate was incubated at RT, wrap in aluminium foil, for 30 minutes at 500 rpm. Once washing with 1x Wash Buffer (150 µL) and resuspend the beads, samples were ready to be read in the flow cytometer. The data (300 beads per analyte) was acquired using a BD LSR II Flow Cytometer (RRID:SCR_002159) (**Annex 2**).

Biolegend´s LEGENDplexTM data analysis software suite was used to analyse the FCS file generated by the cytometer. The concentration of detected analyte was presented in pg/mL, and analytes with concentrations below the limit of detection (LOD) were excluded from the analysis.

3.14 Statistical analysis

GraphPad Prim version 8.0.1 for windows (GraphPad software) was used to perform the statistical analysis. Data from the behavior analysis, BMS and Bladder function, histological analysis, and LegendPlex was assessed by a repeated measure Two-way ANOVA test. For multiple comparison between groups, was used the post hoc Bonferroni test. Unpaired Student´s t-test was applied to analyse the Von Frey test.

Statistical significance was considered when *p-value* < 0.05 (95% confidence interval). All the data presented on text were expressed as group mean ± standard error of the mean (SEM). Details regarding to statistical parameters, for instance sample numbers and precision measures, are described in the figure legend or in the main text.

All the procedures, behaviour analysis, tissue processing, serum and the histological analysis were performed and evaluated blindly to the treatment group.

Chapter 4 - Results

4.1 *In vivo* study: administration of baclofen and pregabalin drugs combination does not change the survival rate neither animal's behaviour

4.1.1 Survival rate

Our study began with 38 female C57BL/6J mice and ended with 28 female mice. The majority of the deaths occurred up to the second week of the in vivo, due to the difficulty of the animals in thermoregulating their body temperature, present cold urine, and low activity (**Figure 4.1**). One of the cases was according to a humane endpoint, since the mouse lost more than twenty percent of the body weigh because of a genetic disease.

The animals' deaths are distributed throughout all experimental groups, therefore, these death appears to be unrelated with the combined administration of the drugs.

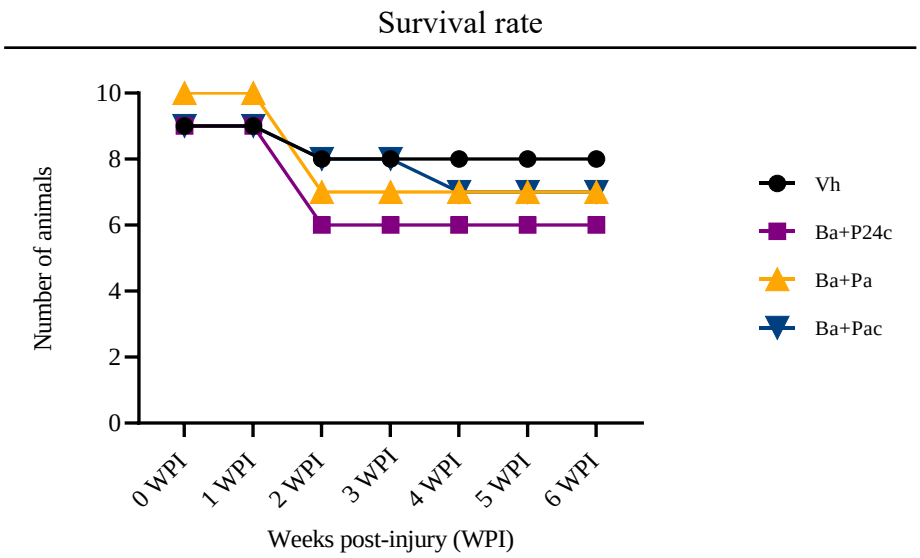


Figure 4.1 Survival rate of the animals after combined administration of baclofen with pregabalin. After surgery, was administered different treatments to the animals: control group, treated with saline solution (Vh), group treated with baclofen acute and pregabalin 24 hours delay and for 14 consecutive days (Ba+P24c), group treated with baclofen and pregabalin acute (Ba+Pa), group treated with baclofen acute and pregabalin acute and for 14 consecutive days (Ba+Pac).

4.1.2 BMS test and weight support

Following the surgeries, a saline solution to the controls or a combination of baclofen and pregabalin (experimental), were intraperitonially administered. For the evaluation of locomotor recovery in SCI mice, it was performed the BMS test, two days after injury and then once a week for six weeks. In this test, the locomotor behaviour was assessed according to the

score table (Annex 1). Before the lesion all mice presented a normal locomotor behaviour. After the injury, all mice, except four, presented a complete paralysis of the hindlimbs. The animals that two days after injury did not present a total paralysis of the hindlimbs were discarded from the study.

Two days post-injury total paralysis was confirmed once mice did not present any movement of the hindlimb. One week after injury, animals treated with Ba+P24c and Ba+Pac were able to slightly move the ankle. In the following weeks, all the groups present a recovery after SCI. During the *in vivo* experiment, the BMS scores of the treated group were slightly higher than those of the vehicle group (**Figure 4.2 A**). However, no statistical difference between the treated and the vehicle groups was observed in the BMS test. Although there were no statistical differences in the BMS score, the percentage of mice that reach weigh support (BMS score >4) was analysed. In the last week of the trial, 50% of mice treated with Ba+Pa and more than half of the animals treated with Ba+P24c and Ba+Pac reach weight support, while none of the animals belonging to the vehicle group reaching weight support, throughout the *in vivo* (**Figure 4.2 B**). Also, during the weeks no statistical difference was found between the treated groups.

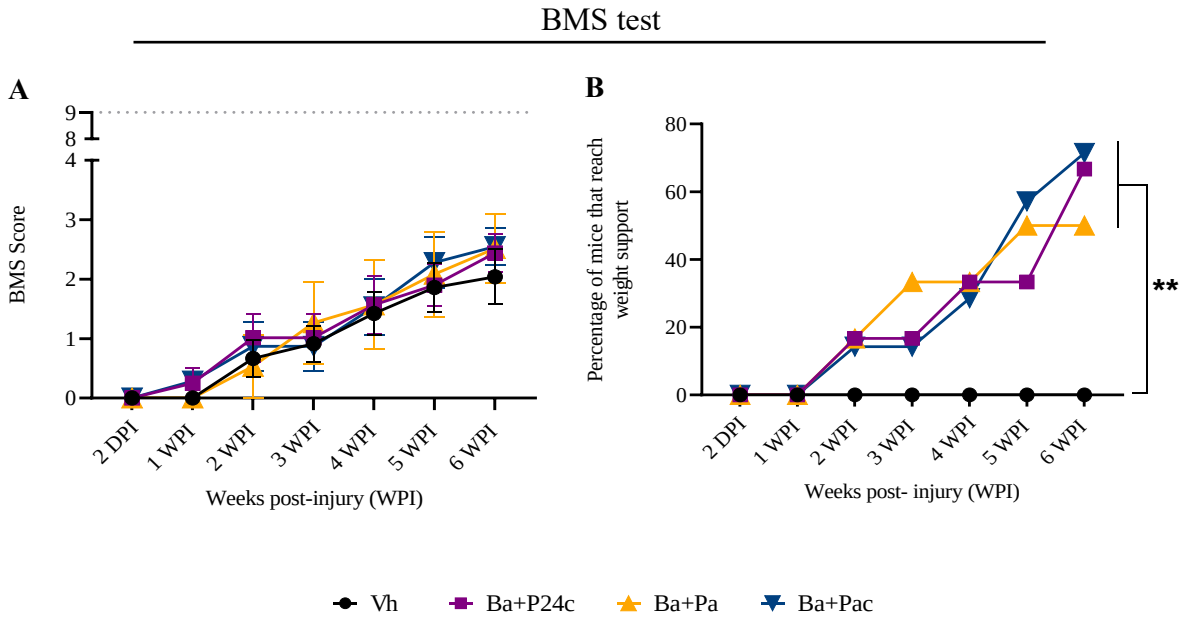


Figure 4.2 Locomotor behaviour evolution after combined administration of baclofen with pregabalin. (A) BMS test of SCI mice. Uninjured animals present a BMS score =9. (B) Percentage of mice that reach weight support (BMS >4). Animals were treated with saline solution (Vh, n=6), treated with baclofen acute and pregabalin 24 hours delay and for fourteen consecutive days (Ba+P24c, n=6) treated with baclofen and pregabalin post-injury (Ba+Pa, n=5), and treated with baclofen acute and, pregabalin acute and for fourteen consecutive days (Ba+Pac, n=7). Values shown as mean ± SEM. *p-value<0.05, **p-value<0.01, according to a repeat measure Two-way ANOVA test followed by a post hoc Bonferroni test (A, B).

4.1.3 Bladder function

A lesion in spinal cord does not only led to a loss of motor movements but also cause a loss of bladder control. To evaluate the impact of the treatments in bladder control of the animals it was performed a bladder trial. The following parameter were analysed: number of spots, urine area, average area, urine weight and score of the bladder control. To understand the normal mice bladder function, three uninjured animals were used. At the end of each bladder trial, urine was weighed, but no difference was observed among the experimental groups (**Figure 4.3**). To assess de average area, the ratio between urine area and urine spots number was calculated. For instance, an uninjured animal has a small number of spots and a large area. No statistical differences between the different experimental groups were found in this analysis (**Figure 4.4**). The urine information collected through the Wattman paper, was useful to assigning scores according to bladder control. The scores range from zero to five, where five represent a pattern of an uninjured animal. Similar to what happens in previous analysis, the score analysis did not present statistical differences between the experimental groups (**Figure 4.5**). Importantly, in none of the analysis the experimental groups show a urine control pattern similar to that of an uninjured animal.

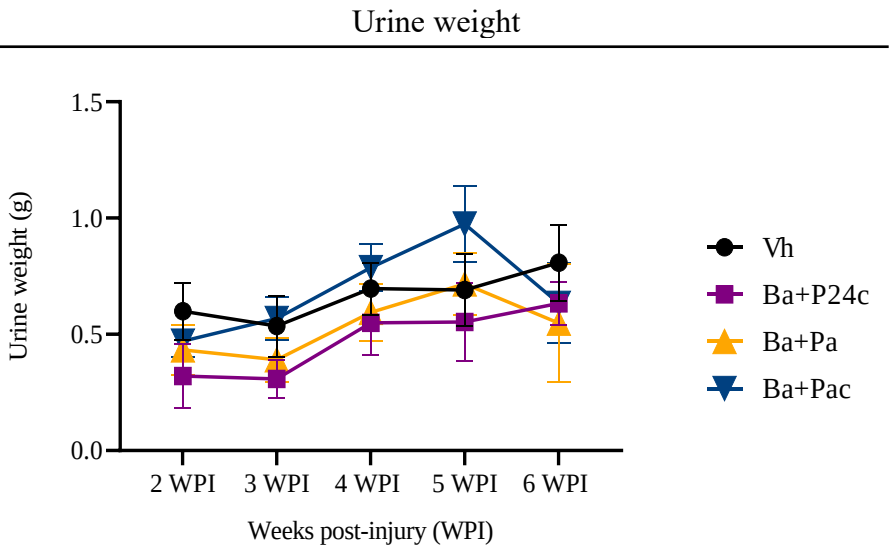


Figure 4.3 Urine weight. The animals were placed in a cage for four hours, at the end the urine was collected and weighted. Animals were treated with saline solution (Vh, n=6), treated with baclofen acute and pregabalin 24 hours delay and for fourteen consecutive days (Ba+P24c, n=6), treated with baclofen and pregabalin post-injury (Ba+Pa, n=5), and treated with baclofen acute and pregabalin acute and for fourteen consecutive days (Ba+Pac, n=7). Values shown as mean ± SEM. Repeat measure Two-way ANOVA test followed by a post hoc Bonferroni test.

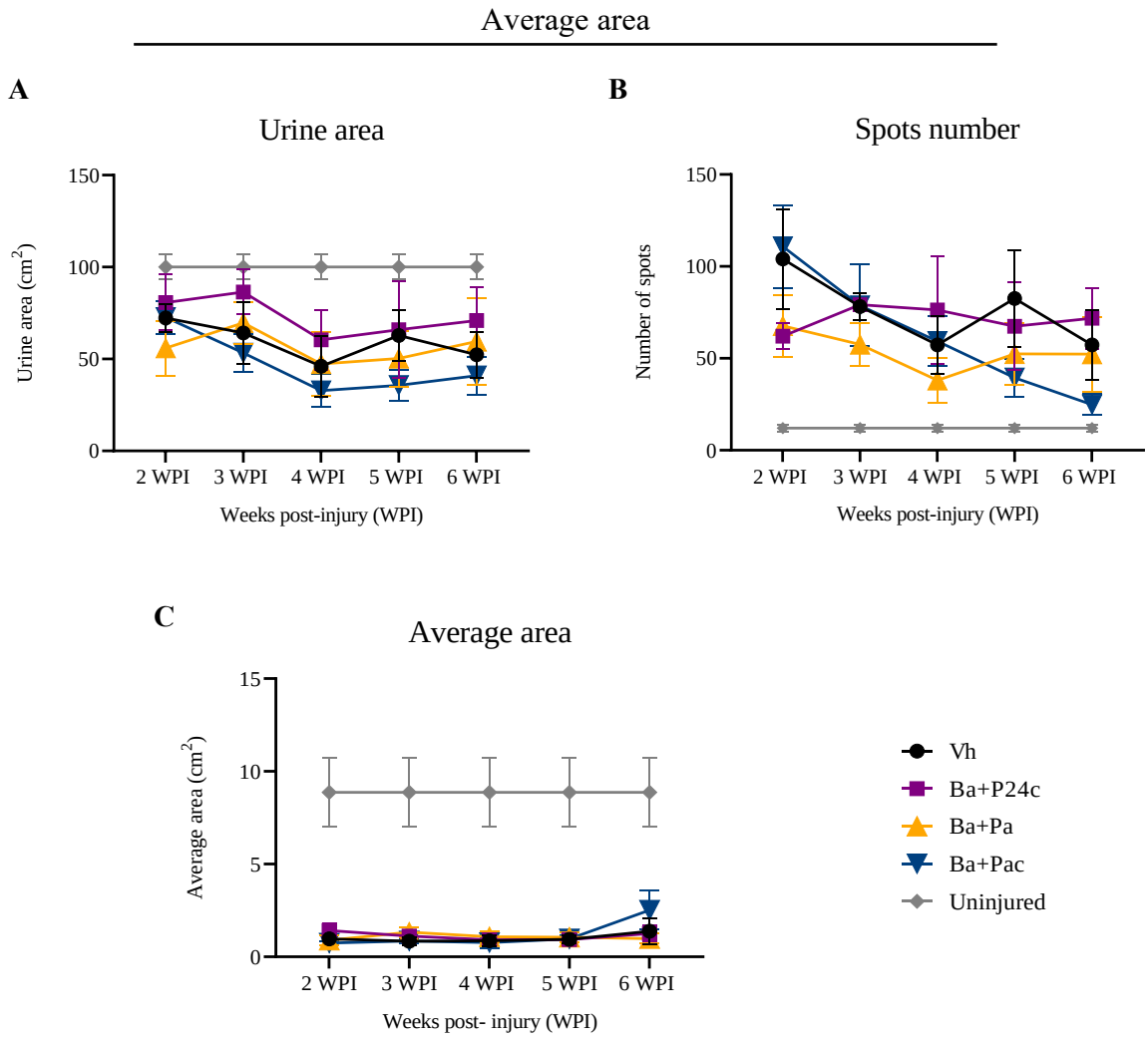


Figure 4.4 Analysis of the urine area. (A) Urine area of the experimental groups. (B) Number of urine spots of the experimental groups. (C) Ratio between the urine area and the number of spots. Animals were treated with saline solution (Vh, n=6), treated with baclofen acute and pregabalin 24 hours delay and for fourteen consecutive days (Ba+P24c, n=6), treated with baclofen and pregabalin post-injury (Ba+Pa, n=5), treated with baclofen acute and pregabalin acute and for fourteen consecutive days (Ba+Pac, n=7) and animals that were not lesioned (uninjured, n=3). Values shown as mean ± SEM. Repeat measure Two-way ANOVA test followed by a post hoc Bonferroni test (A,B e C).

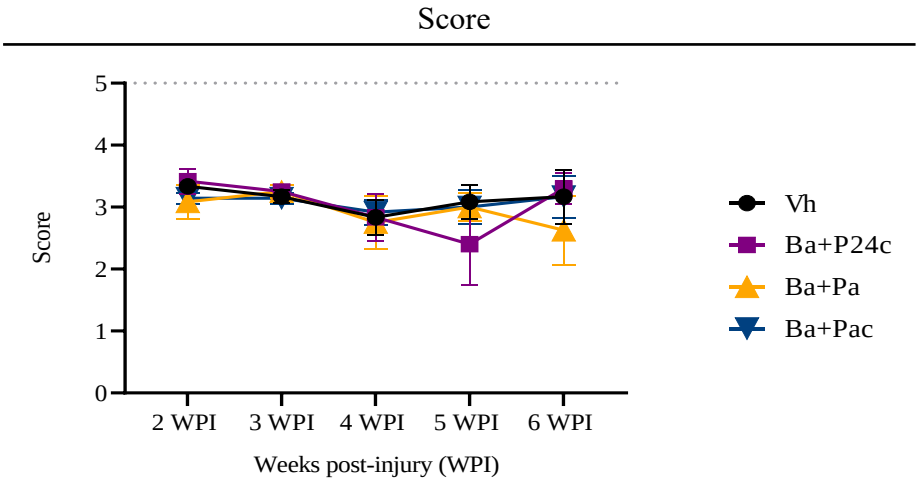


Figure 4.5 Score of the urine pattern. Score attributed to urine pattern presented in the Wattman paper. Animals were treated with saline solution (Vh, n=6), treated with baclofen acute and pregabalin 24 hours delay and for fourteen consecutive days (Ba+P24c, n=6), treated with baclofen and pregabalin post-injury (Ba+Pa, n=5), and treated with baclofen acute and pregabalin acute and for fourteen consecutive days (Ba+Pac, n=7). Uninjured animals present a score=5. Values shown as mean ± SEM. Repeat measure Two-way ANOVA test followed by a post hoc Bonferroni test.

4.1.4 von Frey test

Six weeks post-injury, animal´s hypersensitivity and allodynia were assessed using vF. In this test, paw withdrawals were registered through a code that was used to calculate the 50% threshold. Statistical differences were found between group vehicle and Ba+P24c. However, there is no statistical differences among Ba+Pa and Ba+Pac groups compared to vehicle group. Nevertheless, it is interesting that Ba+Pa group present a similar vF to Ba+P24c group, and Ba+Pac and vehicle groups have approximated 50% threshold (**Figure 4.6**).

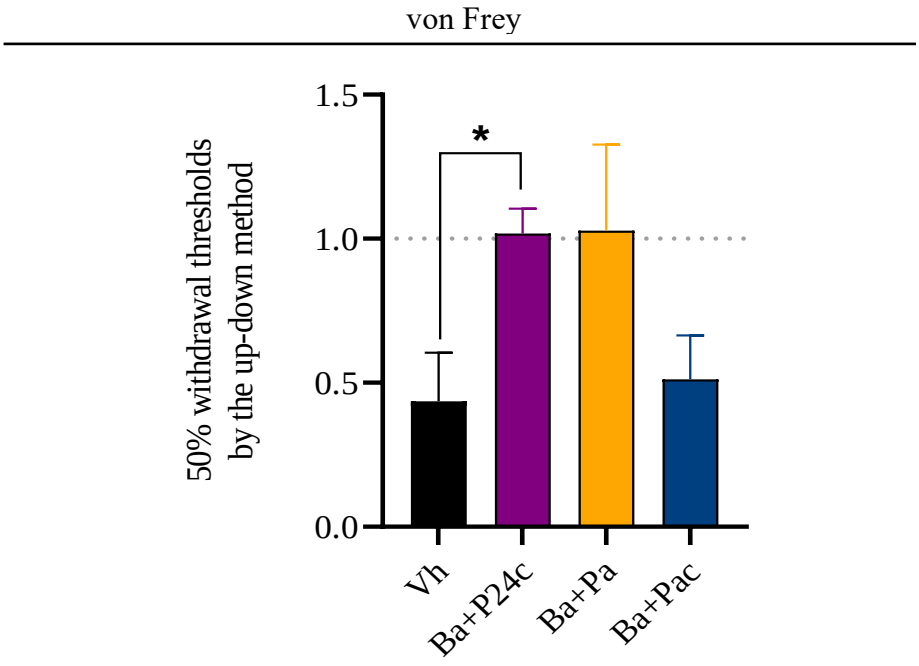


Figure 4.6 von Frey test. Calculation of 50% withdrawal threshold of SCI mice of different treatment groups, using the "up-down method", to determine the weight of stimulus that elicits a response 50% of the time it is applied. Animals were treated with saline solution (Vh, n=6), treated with baclofen acute and pregabalin 24 hours delay and for fourteen consecutive days (Ba+P24c, n=6), treated with baclofen and pregabalin post-injury (Ba+Pa, n=5), and treated with baclofen acute and pregabalin acute and for fourteen consecutive days (Ba+Pac, n=7). Values shown as mean ± SEM. *p-value<0.05 according to an unpair Student´s t-test.

4.1.5 Liver weight

To understand whether the combined administration of baclofen with pregabalin could negatively affect the animal´s organism, the livers were weighted. One day before euthanasia, animals were weighted to normalise the collected data. After de euthanasia procedure (perfusion with PBS and PFA) the livers were removed, and weight collected. With animal´s and livers weight data, was calculated the ration between liver weight and body weight. The results show that no statistical difference was found among ratios of the experimental groups (**Figure 4.7**).

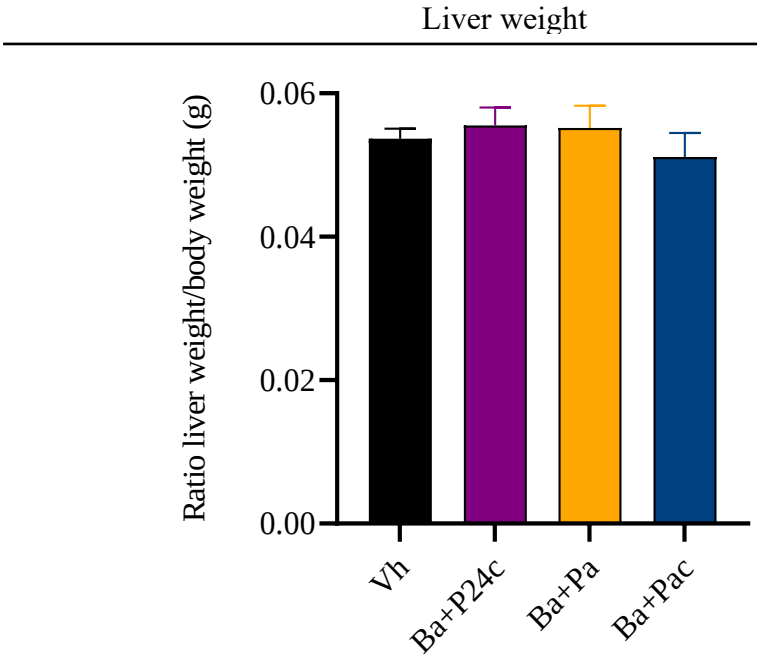


Figure 4.7 Ratio liver weight/body weight. Ratio between liver weight/body weight of the animals, after six weeks of in vivo. Animals were treated with saline solution (Vh, n=6), treated with baclofen acute and pregabalin 24 hours delay and for fourteen consecutive days (Ba+P24c, n=6), treated with baclofen and pregabalin post-injury (Ba+Pa, n=5), and treated with baclofen acute and pregabalin acute and for fourteen consecutive days (Ba+Pac, n=7). Values shown as mean ± SEM. Unpair Student´s t-test.

4.2 Baclofen and pregabalin drug combination does not promote white matter sparing after SCI

Myelin is a structure that surround axons and is important to maintain the proper neuronal functions. One of the SCI consequences is demyelination. In order to assess if the combined baclofen with pregabalin administration could prevent the demyelination, a staining with FluoroMyelin green fluorescence, was used. The white matter sparing was evaluated, and the quantification was made in the total cross-section area if the spinal cord tissue, 1000 µm rostrally and 1000 µm caudally to the lesion epicenter (**Figure 4.8 A**). White matter sparing analysis did not reveal statistical differences between the treated groups and the vehicle groups (**Figure 4.8 B**).

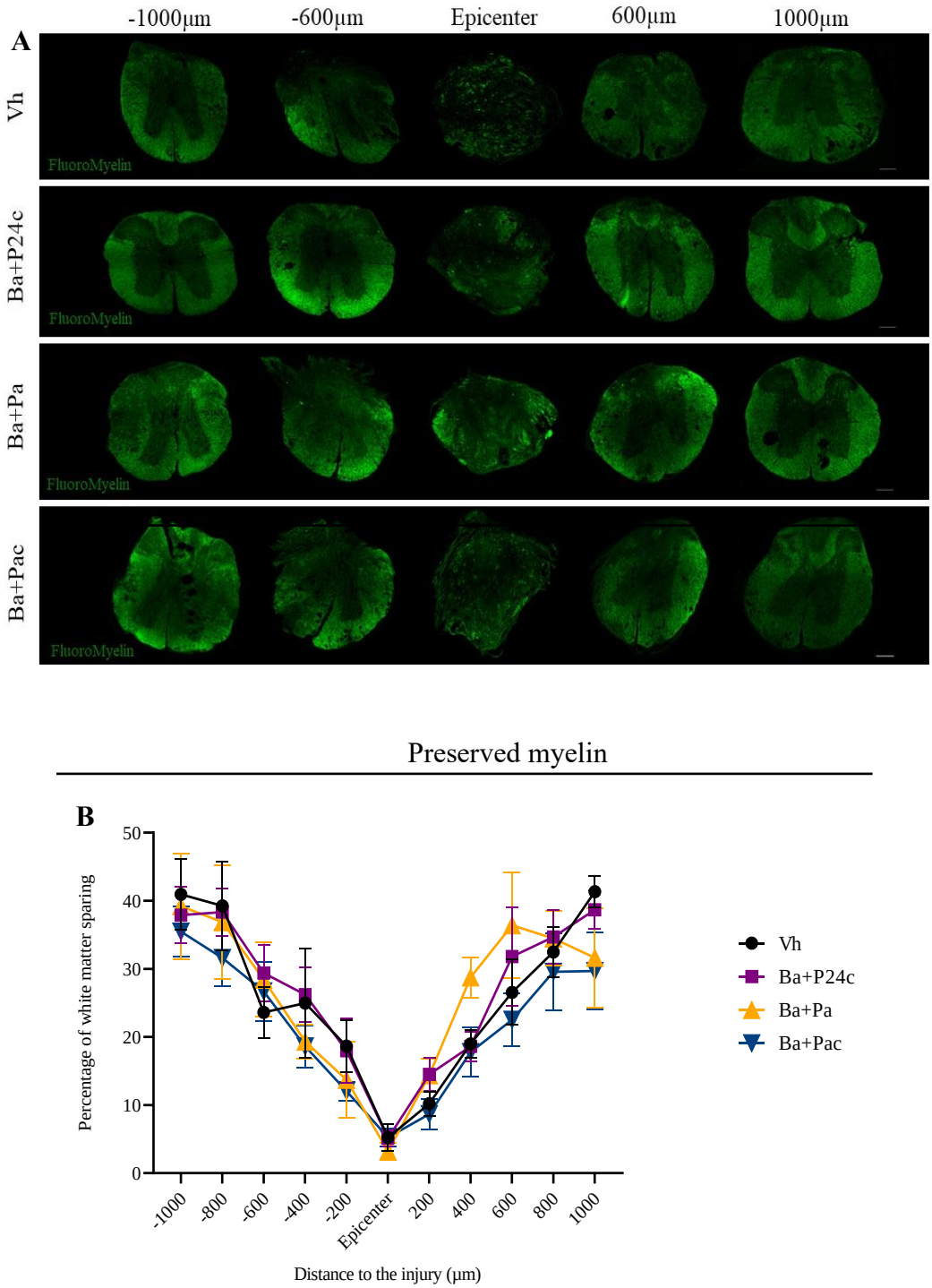
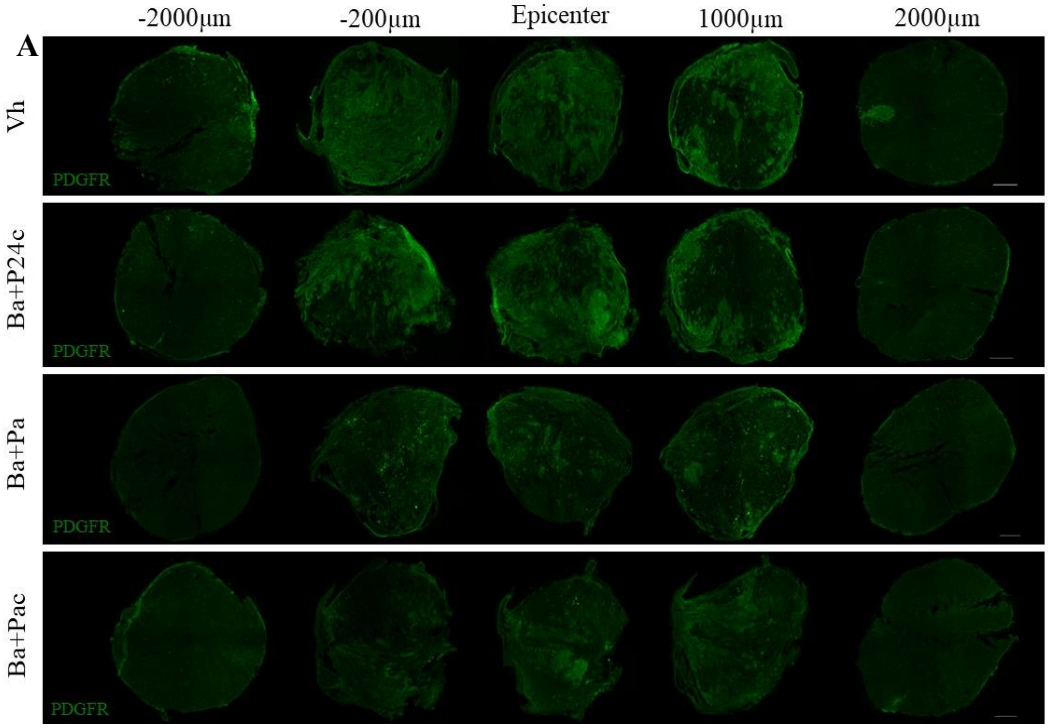


Figure 4.8 Preserved myelin. (A) Myelin fluorescence microscope images of spinal cord section. Magnification 20x. Scale bar: 200 µm. (B) Percentage of white matter sparing was calculated every 200 µm, at the distance 1000 µm rostrally and 1000 µm caudally to the lesion site. Vehicle treated group (Vh, n=6), group treated with baclofen acute and pregabalin 24 hours delay and for fourteen consecutive days (Ba+P24c, n=6), treated with baclofen and pregabalin post-injury (Ba+Pa, n=5), and treated with baclofen acute and pregabalin acute and for fourteen consecutive days (Ba+Pac, n=7). Values shown as mean ± SEM. Repeat measure Two-way ANOVA test followed by a post hoc Bonferroni test.

4.3 The acute administration of pregabalin in combination with baclofen seems to reduce the fibrotic tissue in the SCI epicenter

In a chronic phase of SCI, it is formed a fibrotic scar. This is one of the causes responsible for the inability of axon projection. To understand if the combined treatment modulates the formation of fibrotic scar after a lesion, it was performed an immunostaining with anti-PDGFR antibody that labels fibroblasts. In the quantification, it was taken into account the total cross-sectional area of the spinal cord tissue, 200 µm, 1000 µm and 2000 µm from the epicenter of the lesion, both rostrally and caudally (**Figure 4.9 A**). In the distances 2000 µm and 1000 µm rostrally and caudally, no statistical differences between the experimental groups were found. However, at 200 µm rostrally from the lesion epicenter, both treated groups with Ba+Pa and Ba+Pac show significant less fibrotic tissue when compared to the vehicle group (*p-value*< 0.0001). At the epicenter of the lesion, the same pattern occurs, treated groups with Ba+Pa and Ba+Pac have less fibrotic tissue than vehicle group, being this difference statistically significant (*p-value*= 0.0002 and *p-value*= 0.008, respectively). At 200µm caudally, is only found statistical differences between Ba+Pa group and vehicle group (*p-value*= 0.0318). No differences in fibrotic tissue among experimental groups Ba+P24c and vehicle, are found at any distance from the epicenter of the lesion (**Figure 4.9 B**).



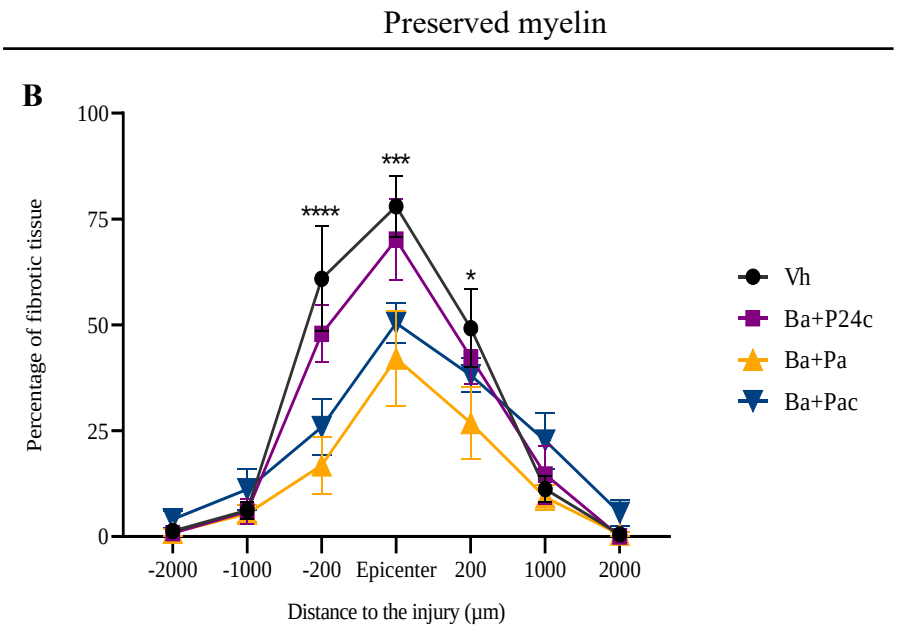


Figure 4.9 Fibrotic tissue. (A) Fibrotic tissue fluorescence microscope images of spinal cord section. Magnification 20x. Scale bar: 200 µm. (B) Analysis of fibrotic tissue at the epicenter, 200 µm, 1000 µm and 2000 µm from the lesion epicenter, rostrally and caudally. Experimental groups treated with saline solution, vehicle (Vh, n=6), treated with baclofen acute and pregabalin 24 hours delay and for fourteen consecutive days (Ba+P24c, n=6), treated with baclofen and pregabalin post-injury (Ba+Pa, n=5), and treated with baclofen acute and pregabalin acute and for fourteen consecutive days (Ba+Pac, n=7). Values shown as mean ± SEM. *p-value<0.05, ***p-value<0.001, p-value<0.0001, according to a repeat measure Two-way ANOVA test followed by a post hoc Bonferroni test.

4.4 Pregabalin change the number of α-motor neurons depending on the administration time-point after SCI.

Although the results of locomotor function did not show differences between the experimental group, the number of motor neurons was analysed, to understand if the combined administration of baclofen with pregabalin could lead to a higher preservation of α-motor neurons. To access this information, an immunostaining with anti-Neu-N antibody was performed. The α-motor neurons were quantified every 2000 µm and 1000 µm both rostrally and caudally to the lesion site, in the ventral horn of the spinal tissue (**Figure 4.10 A**). In all distances that were analysed, experimental group treated with Ba+P24c have a greater number of α-motor neurons than vehicle group, while Ba+Pa group have less α-motor neurons, however none of these results are statistically significant. On the other hand, treated group with Ba+Pac exhibited less α-motor neurons when compared with the vehicle group, in all considered

distances, being these data statistically significantly at 2000 µm rostrally (*p-value*= 0.0351) and 2000µm caudally (*p-value*= 0.0333) to the epicenter of the lesion (**Figure 4.10 B**).

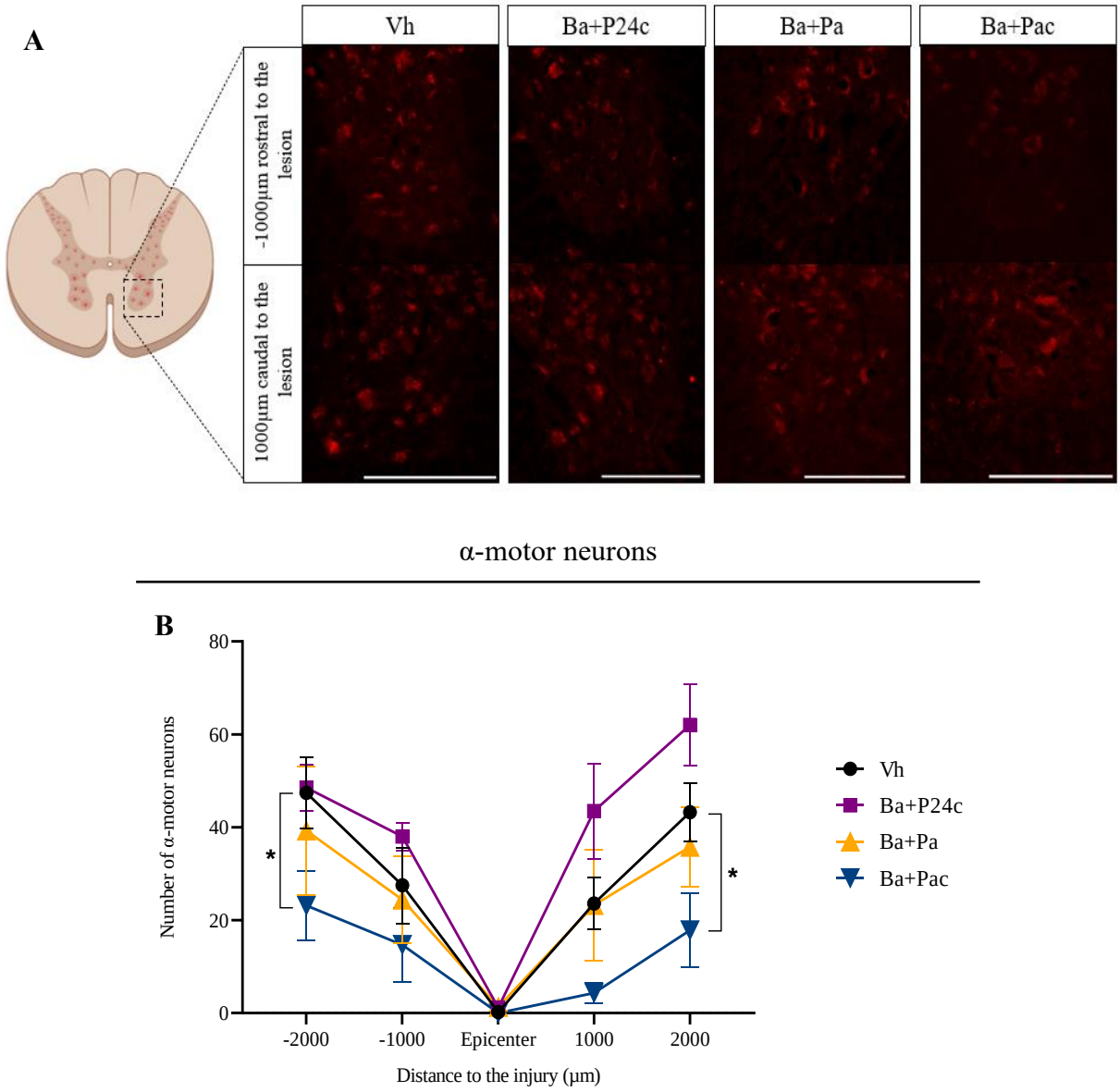


Figure 4.10 α-motor neurons. (A) Motor neurons fluorescence microscope images from ventral horn spinal cord section. Magnification 20x. Scale bar 200 µm. (B) Quantification of α- motor neurons at 2000 µm and 1000 µm rostrally and caudally to the lesion epicenter (considered -200 µm, epicenter and 200 µm). Vehicle treated group (Vh, n=6), group treated with baclofen acute and pregabalin 24 hours delay and for fourteen consecutive days (Ba+P24c, n=6), treated with baclofen and pregabalin post-injury (Ba+Pa, n=5), and treated with baclofen acute and pregabalin acute and for fourteen consecutive days (Ba+Pac, n=7). Values shown as mean ± SEM. *p-value<0.05, according to according to a repeat measure Two-way ANOVA test followed by a post hoc Bonferroni test.

4.5 Pregabalin treatment acutely and chronic increases axonal projections after SCI.

Besides the results of quantification of α-motor neurons, immunostaining with anti-NF-H antibody was carried out to understand the quality of the connection between neurons. NF-H is responsible to mark neurofilament heavy protein, present in the cytoplasm of neurons. More NF-H means more projections between neurons. The analysis of NF-H was performed in the ventral zone of the spinal cord, using a ROI that spans white and grey matter, at 2000 µm and 1000 µm from the lesion site, both rostrally and caudally (**Figure 4.11 A**). When compared to the vehicle group, treated groups show a tendency to have more signal intensity of NF-H. However, just Ba+Pac group present statistically differences with the vehicle group at 1000 µm rostrally and caudally (*p-value*= 0.0285 and *p-value*= 0.0212, respectively) (**Figure 4.11 B**).

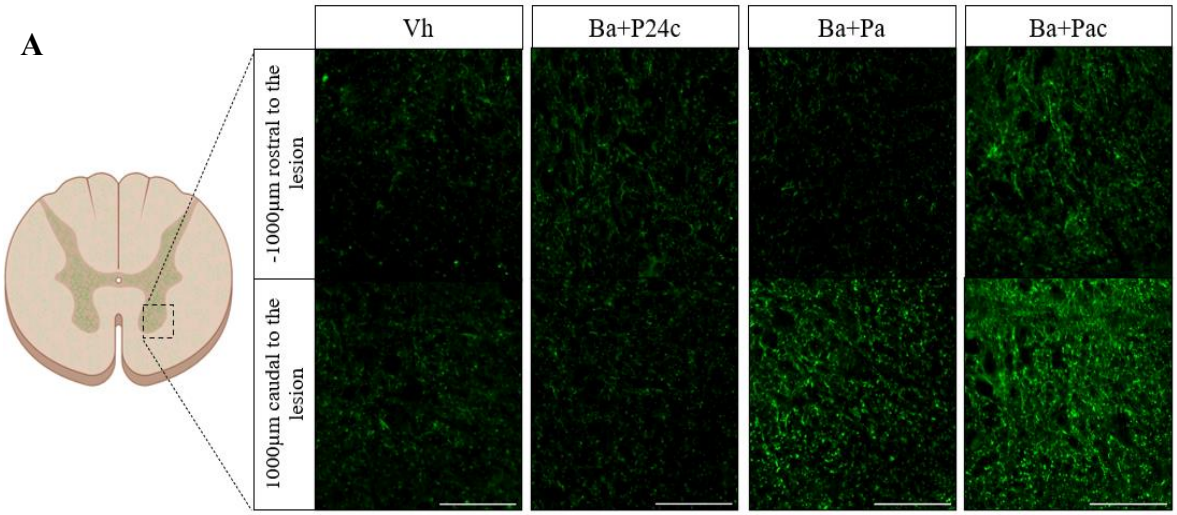


Figure 4.11 NF-H fibers. (Image continue below).

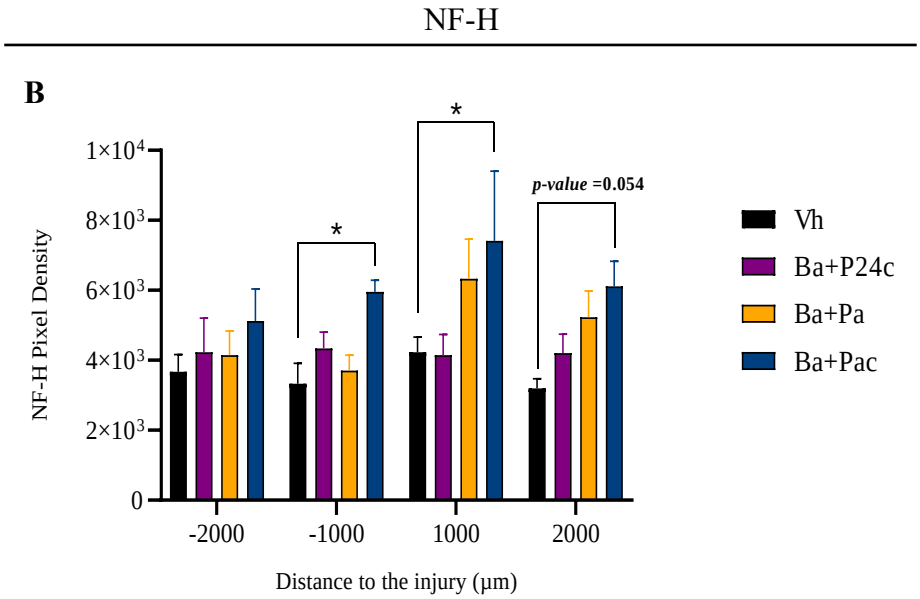


Figure 4.11 NF-H fibers. (A) Neurofilament fluorescence microscope images from region of interest (ROI). Magnification 40x. Scale bar: 200 µm. (B) Quantification of NF-H-positive fibers in the ventral zone of spinal cord tissue, using a ROI (white and grey matter). at 2000 µm and 1000 µm rostrally and caudally to the lesion epicenter. Vehicle treated group (Vh, n=6), group treated with baclofen acute and pregabalin 24 hours delay and for fourteen consecutive days (Ba+P24c, n=6), treated with baclofen and pregabalin post-injury (Ba+Pa, n=5), and treated with baclofen acute and pregabalin acute and for fourteen consecutive days (Ba+Pac, n=7). *p-value<0.05, according to according to a repeat measure Two-way ANOVA test followed by a post hoc Bonferroni test.

4.6 Pregabalin administration seems to play a role in the serotonergic axonal projections.

The analysis of immunostaining for NF-H show differences between vehicle and Ba+Pac groups (**Figure 4.11**). Since NF-H is present in every type of neurons, two different immunostainings were performed to found out which type of neurons the combined administration of the drugs has an effect on. One of the immunostainings was with anti-5-HT antibody to identify serotonergic neurons and other with anti-TH antibody to mark catecholaminergic neurons. The data was analysed using the same parameters as for immunostaining for NF-H.

Serotonergic projections: Rostrally to the lesion epicenter all the treated groups present a higher tendency of signal intensity than vehicle group, but caudally just Ba+Pa and Ba+Pac groups show a higher signal intensity than the vehicle. Besides these tendencies, only Ba+Pac treated group present a statistically different when compared to the vehicle group, at

the distances 1000 µm from the lesion site, rostrally and caudally (*p-value*= 0.0168 and *p-value*= 0.0321, respectively) (**Figure 4.12 A**).

Catecholaminergic projections: Regarding to immunostaining with anti-TH antibody, tendencies are also visible. Rostrally to the epicenter, treated groups with pregabalin acute exhibit mor signal intensity of TH than vehicle group. Caudally to the lesion site all treated groups show more signal intensity when compared to vehicle group. Despite these tendencies, no major differences are found between experimental group (**Figure 4.12 B**).

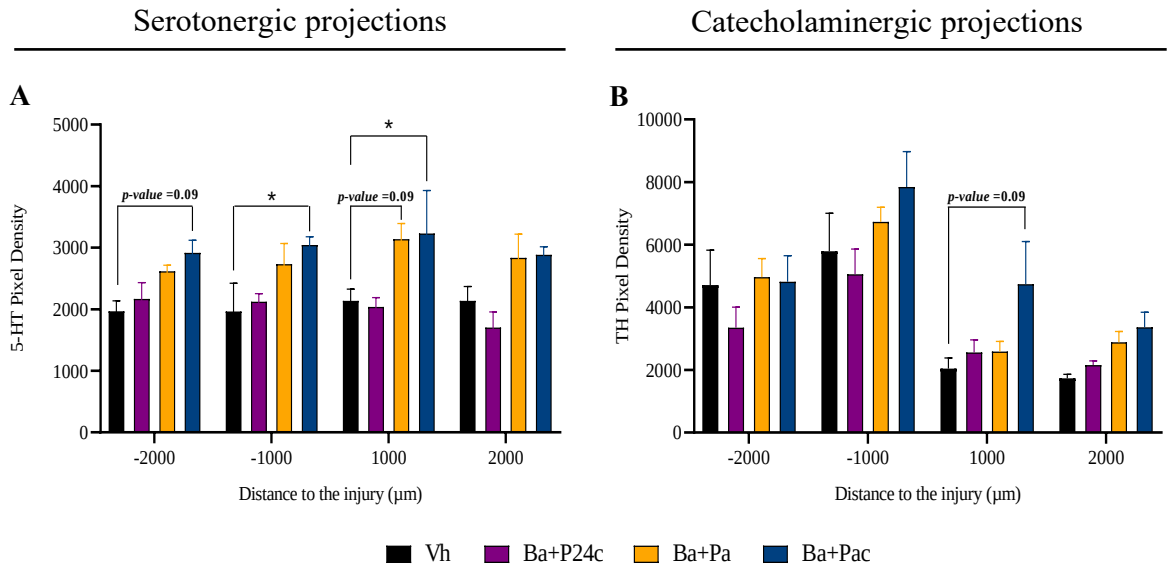


Figure 4.12 Serotonergic and dopaminergic neurons. (A) Quantification of serotonergic neurons (5-HT). (B) Quantification of dopaminergic neurons (TH). Analysis was made in the ventral zone of spinal cord tissue, using a ROI (white and grey matter), at 2000 µm and 1000 µm rostrally and caudally to the lesion epicenter. Vehicle treated group (Vh, n=6), group treated with baclofen acute and pregabalin 24 hours delay and for fourteen consecutive days (Ba+P24c, n=6), treated with baclofen and pregabalin post-injury (Ba+Pa, n=5), and treated with baclofen acute and pregabalin acute and for fourteen consecutive days (Ba+Pac, n=7). *p-value<0.05, according to according to a repeat measure Two-way ANOVA test followed by a post hoc Bonferroni test.

4.7 The combination of baclofen and pregabalin modulates the microglia phenotype after SCI.

Inflammation response is exacerbated in cases of SCI, mostly in the acute and subacute phases. To analyse the role of combined baclofen with pregabalin administration in inflammatory response, a protocol of immunostaining with anti-Iba-1 antibody that labels

microglia cells was executed. The analysis was performed at the lesion epicenter, 2000 µm and 1000 µm from the lesion site, using a (region of interest) ROI in the ventral zone of the spinal cord tissue cross-sectioned (**Figure 4.13**). The purpose of the assay was mark microglia cells to analyse their phenotype (number of cells per mm^2, average size, perimeter, circularity, feret and solidity) (Zanier et al., 2015).

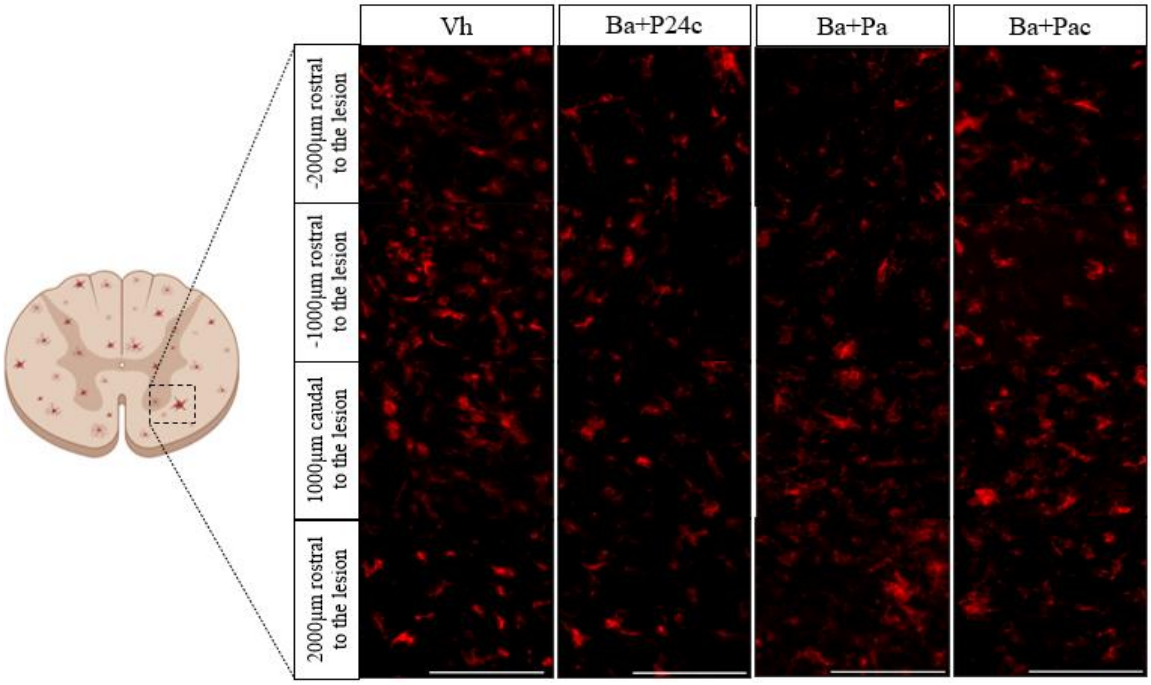


Figure 4.13 Analysis of microglia cells. Microglia fluorescence microscope images from region of interest (ROI). Ampliation 40x. Scale bar: 200 µm.

Number of cells per mm^2. The number of microglia cells were analysed through the ROI used (**Figure 4.13**). The higher is the number of cells more intense is the immune response. At all distance, vehicle group presented a higher number of microglia cells compared to treated groups. At 2000 µm rostrally from the epicenter, vehicle group show differences when compared to Ba+Pa (*p-value*= 0.001) and to Ba+Pac (*p-value*= 0.007). At 1000 µm rostrally, were found statistical differences between the vehicle group and all the treated groups (*p-value*< 0.0001). Interestingly, from the epicenter of lesion, only the chronically treated groups present statistical differences compared to the vehicle group. At the epicenter site exist differences between vehicle and Ba+P24c (*p-value*= 0.0109), at 1000 µm caudally vehicle present considered differences with Ba+P24c (*p-value*< 0.0001) and with Ba+Pac (*p-value*= 0.0002).

At the distance 2000 μm were found differences between vehicle and Ba+P24c (*p-value*= 0.0032) (**Figure 4.14 A**).

Average size. The average size of microglia corresponds to the total number of pixels present in the total cells in the ROI used (**Figure 4.13**). The largest is the average size, less reactive are the microglia cells. Treated group with Ba+Pa presents a smallest average size compared to vehicle group, while Ba+P24c and Ba+Pac treated groups show a largest average size than vehicle group in almost all analysed distance. However no statistical differences between the experimental groups are found (**Figure 4.14 B**).

Perimeter. Perimeter is calculated based on the outline length of each microglia cell. This parameter is higher in cells ramified, which means that are less reactive microglia. In the present study, vehicle group exhibit a higher perimeter than treated group caudally to the lesion epicenter. Also, Ba+P24c treated group present a smallest perimeter than vehicle group in all distances analysed, but significantly differences are found only at 1000 μm (*p-value*= 0.0205) rostrally to the lesion site. At 2000 μm from the lesion epicenter, vehicle group and Ba+Pac group show statistical differences (*p-value*= 0.0339), with Ba+Pac presenting a smaller perimeter than vehicle group (**Figure 4.14 C**).

Circularity. When microglia are in homeostasis, their phenotype are more extending with more ramification, but when they are active-like present a round form, almost like a perfect circle. To analyse the microglia circularity was applied a formula ($4\pi\times(\text{area}/\text{primeter}^2)$). The result data comprise values between zero (linear polygon, resting-like) and one (perfect circle, active-like). Although Ba+Pa treated group show less circularity in the course of the distances, are not found statistical differences between the experimental group (**Figure 4.14 D**).

Feret diameter. This parameter is analysed through the longest distance between two parallel lines. The more branched are microglia cells, the greater the feret diameter value, and more homeostatic are the microglia cells. Treated group with Ba+P24c present a largest feret diameter than vehicle group, yet statistical differences are not found. A similar pattern occurs with Ba+Pa group, but these one shows a smaller feret diameter than vehicle group with no statistical differences. However, statistical differences are found, when comparing the Ba+Pac treated group with vehicle group, at the 1000 μm rostrally to the lesion site and at the epicenter (*p-value*= 0.0275 and *p-value*= 0.0425, respectively). In this comparison Ba+Pac group exhibit a smallest feret diameter than vehicle group (**Figure 4.14 E**).

Solidity. Solidity is calculated based on the area and the convex area. The convex area corresponds to the smallest polygon around the microglia cell. The more branched microglia, the larger the convex area and therefore the smaller the solidity. Rostrally to the lesion epicenter treated group with Ba+P24c and Ba+Pa present a smaller solidity than vehicle group but Ba+Pac treated group show a larger solidity than vehicle group. This treated group and Ba+Pa group keeps the same pattern caudally to the lesion site, but Ba+P24c group caudally to the lesion epicenter present a larger solidity than vehicle group. Statistical differences are only found between vehicle and Ba+P24c group at 1000 µm rostrally to the epicenter (*p-value*= 0.0148) (**Figure 4.14 F**).

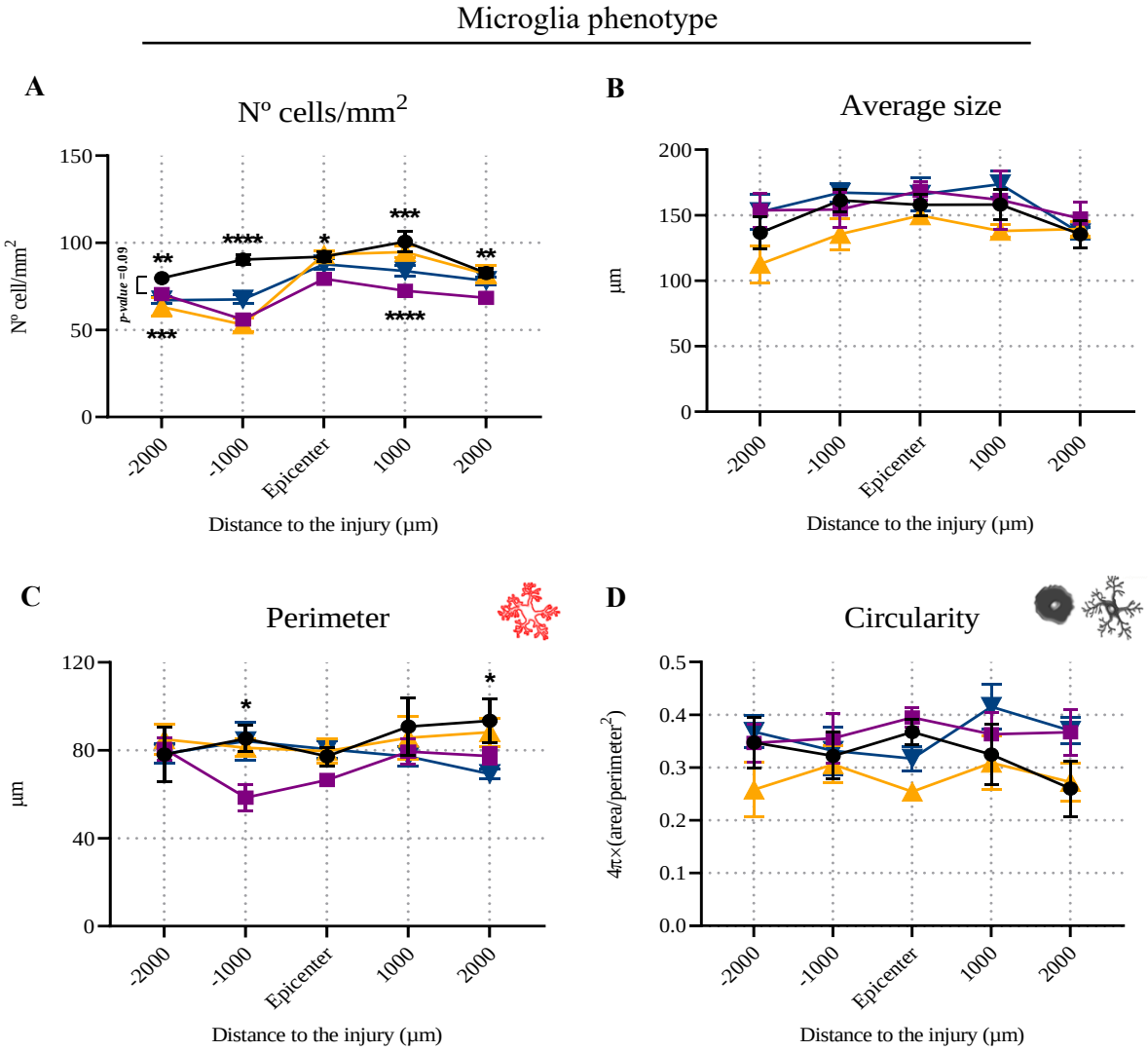


Figure 4.14 Microglia phenotype. (Image continue below).

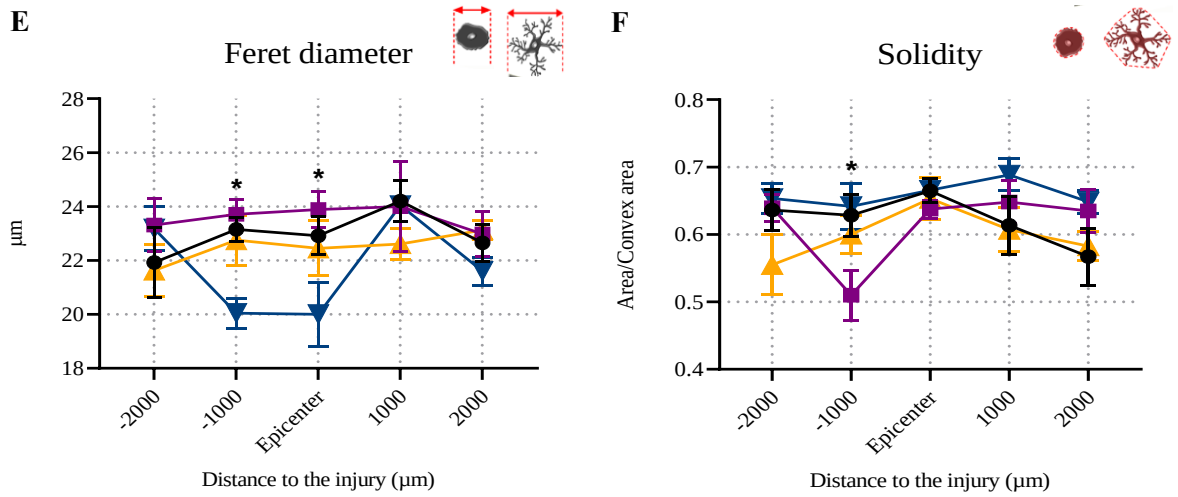


Figure 4.14 Microglia phenotype. Analysis of microglia cells was performed at ventral zone of spinal cord, using a ROI (white and grey matter) at 2000 µm and 1000 µm rostrally and caudally to the lesion epicenter (considered -200 µm, epicenter and 200 µm). Vehicle treated group (Vh, n=6), group treated with baclofen acute and pregabalin 24 hours delay and for fourteen consecutive days (Ba+P24c, n=6), treated with baclofen and pregabalin post-injury (Ba+Pa, n=5), and treated with baclofen acute and pregabalin acute and for fourteen consecutive days (Ba+Pac, n=7). Values shown as mean ± SEM. *p-value<0.05, **p-value<0.01, ***p-value<0.001, ****p-value<0.0001, according to according to a repeat measure Two-way ANOVA test followed by a post hoc Bonferroni test.

4.8 Combinatory administration of baclofen and pregabalin modulates the immune response after SCI

To study in deepth the effect of combined baclofen with pregabalin administration in immune response after SCI, it was design an experimental protocol using a LegendPlex kit to study a panel with thirteen different cytokines (**Table 4.1**). To perform this analysis, it was used serum from different time points of the *in vivo* (1-, 7-, 14-, and 42- days post-injury).

The pro-inflammatory cytokine IL-23, in all time points, assumes higher values in the groups that were treated with pregabalin acute, while in the vehicle and Ba+P24c groups the values are almost zero. Beside these tendencies, no major difference was found between experimental groups (**Figure 4.15 A**). The values of MCP-1, in groups with chronic treatment, are similar to vehicle group. However, group treated with acute treatment exhibit higher value of MCP-1. However, no statistical differences were found in this analysis (**Figure 4.15 B**). Two weeks post-injury, Il-12p70 is reduce in the Ba+Pa group. In all time-points treated group with Ba+Pac present lower values of this pro-inflammatory cytokine. Like the previously cytokines, this also do not show statistically differences (**Figure 4.15 C**). Ba+Pac treated group until the

second week present reduced values of IL-6 and GM-CSF, after the second week post-injury suffer a smaller increase. Regarding to IL-6, one day after injury Ba+Pac and vehicle present statistical differences (*p-value*= 0.0269), while GM-CSF show major differences two weeks after injury, also between Ba+Pac treated group and vehicle group (*p-value*= 0.0220) (**Figure 4.15 D, E**).Interferons β and γ present different patterns among experimental groups. IFN-β suffers an increase during the time in the Ba+P24c group, although there are no major differences (**Figure 4.15 F**). IFN-γ is always reduce in the Ba+Pac group and present statistical differences when compared to vehicle group at two weeks and six weeks post-injury (*p-value*= 0.0114 and *p-value*= 0.0036, respectively) (**Figure 4.15 G**). In both pro-inflammatory cytokines, IL-1α and IL-1β, in the sixth week post-injury, Ba+P24c group is increased when compared to the others experimental groups. IL-1β during the time points considered is reduce in the treated group with Ba+Pac, and statistical differences are found between this group and vehicle group at two weeks post-injury (*p-value*= 0.0496). Besides this difference, no other was found between experimental groups (**Figure 4.15 H, I**) . The only ant-inflammatory cytokine analysed, Il-10, did not exhibited statistical differences, yet some tendencies are visible. The treated group shows an increase of IL-10 after the first week post-injury (**Figure 4.15 J**). Neither tendencies nor statistical differences were observed in concentrations of TNF-α, IL-27, and IL-17A (**Figure 4.15 K, L, M**).

Interestingly, the majority of the differences occur between vehicle and Ba+Pac groups. It is also curiously that in some cytokines, vehicle group and treated group with Ba+P24c present a similar modulation pattern, mainly in the six-week post-injury (IL-23, IL-6, GM-CSF, IL-12P70 AND IFN-γ).

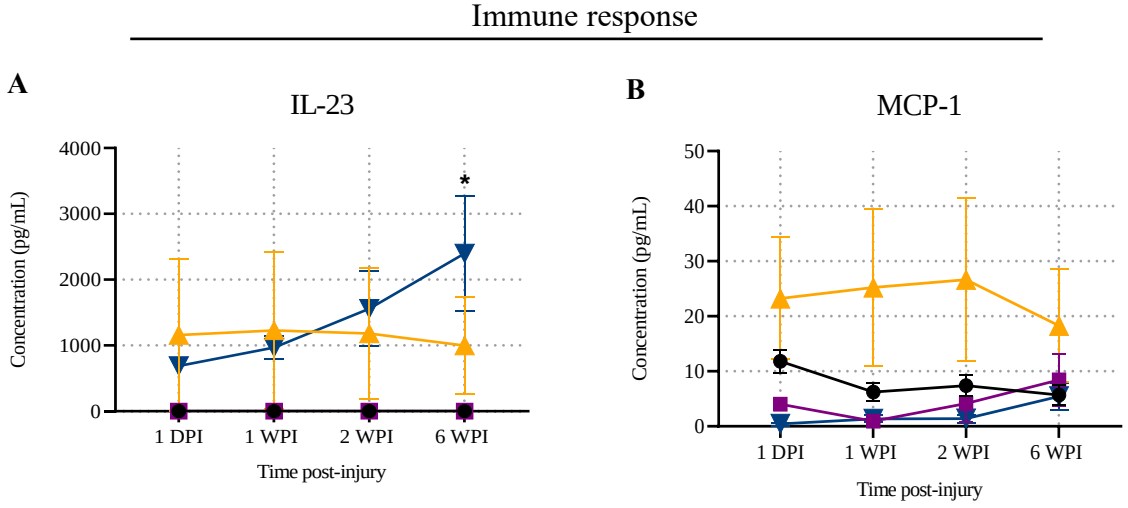


Figure 4.15 Inflammatory profile after SCI. (Image continue below).

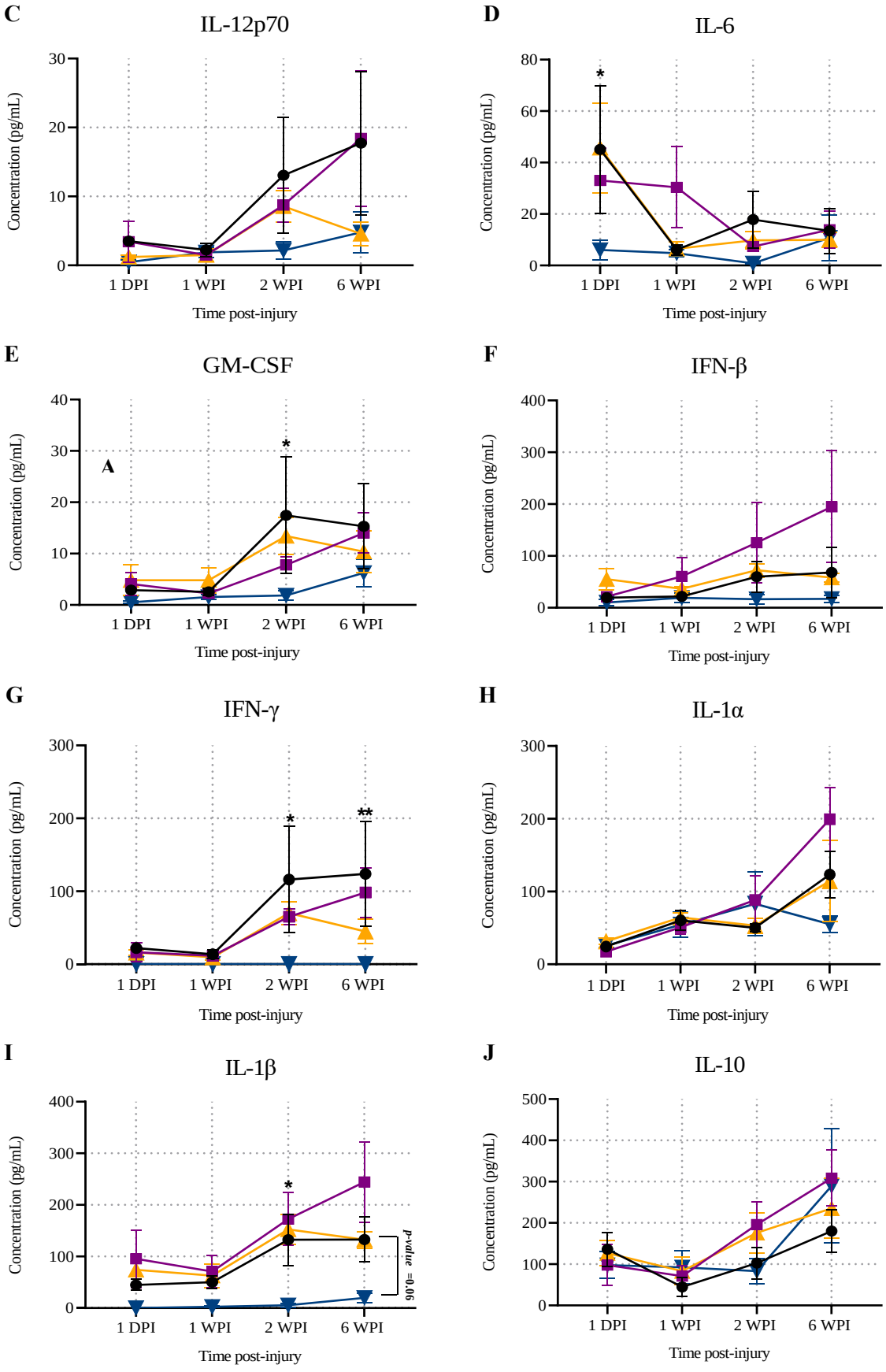


Figure 4.15 Inflammatory profile after SCI. (Image continue below).

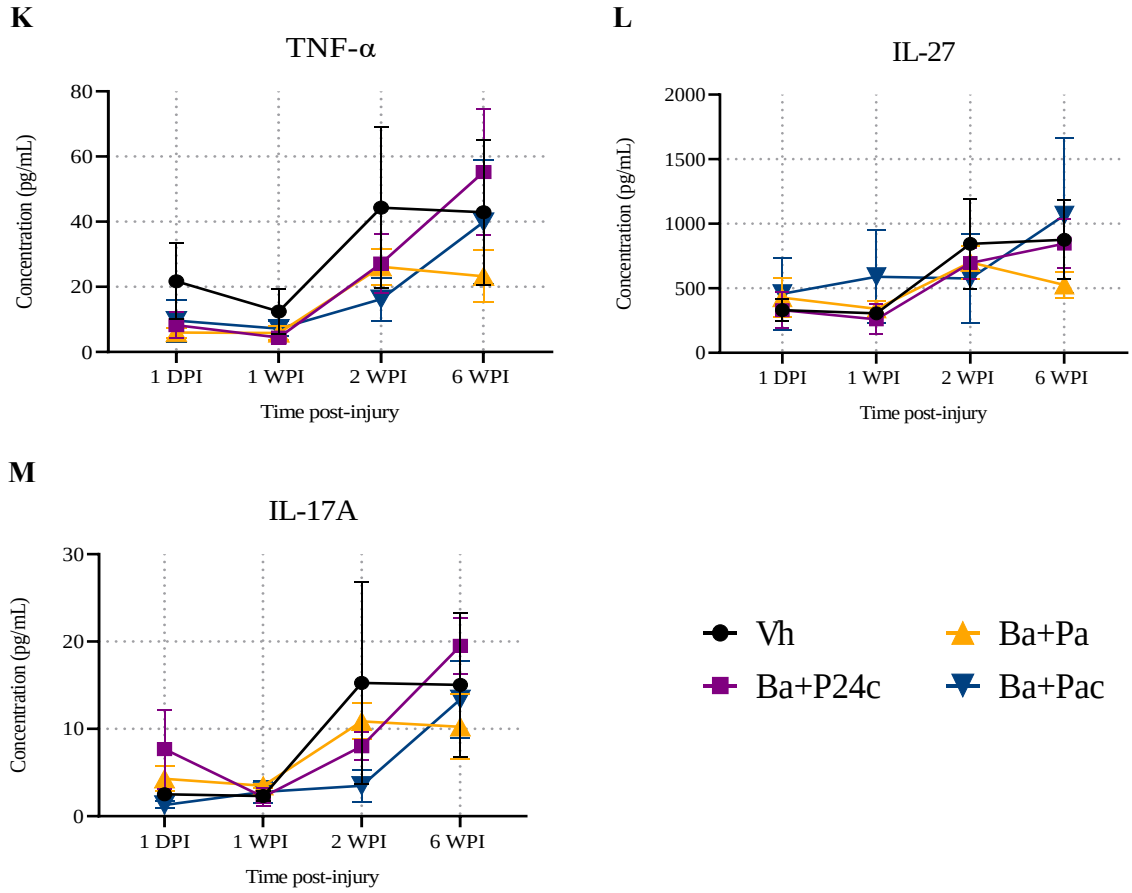


Figure 4.15 Inflammatory profile after SCI. Quantification of different cytokines at 1-, 7-, 14-, and 42-days post-injury, using a LegendPlex commercial kit. Vehicle group (Vh, n=6), group treated with baclofen acute and pregabalin 24 hours delay and for fourteen consecutive days (Ba+P24c, n=6), treated with baclofen and pregabalin post-injury (Ba+Pa, n=5), and treated with baclofen acute and pregabalin acute and for fourteen consecutive days (Ba+Pac, n=7). *p-value<0.05, ** p-value<0.01, according to according to a repeat measure Two-way ANOVA test followed by a post hoc Bonferroni test.

Table 4.1: Cytokines involving in inflammation and their role after SCI

Cytokine	Inflammatory role	Cell that secretes the cytokine	Effects after SCI
IL-23	Pro-inflammatory	Secreted by microglia, astrocytes, and dendritic cells	It is part of the IL-12 family and promotes the production of IL-17A.
MCP-1	Pro-inflammatory	Active T-cells, astrocytes, microglia, and monocytes	Activates and recruits mononuclear phagocytes, T-cells, and B-cells
IL-12p70	Pro-inflammatory	Produces by macrophages, dendritic cells, monocytes, neutrophiles, microglia and B-cells	Synthase of nitric oxide, and TNF-α in microglia . Belong to IL-12 family
IL-6	Pro-inflammatory	Secreted by astrocytes, microglia, and neurons	Activates inflammation, recruiting immune cells
GM-CSF	Pro-inflammatory	Is produced in response to immune activation, being produced by macrophages, mast cells, T-cells, fibroblasts, and endothelial cells	Stimulate the proliferation of bone marrow stem cells and microglia, changing their profile.
IFN-β	Pro-inflammatory	Secreted by T-cells, macrophages, natural killer cells, and monocytes	Appear to reduce the lipid peroxidation and oxidative reactions after SCI
IFN-γ	Pro-inflammatory	Produced by T-cells that activates macrophages	Promotes the secretion of IL-10 from microglia and can act with GM-CSF to induce the production of cytokines
IL-1α	Pro-inflammatory	Release in response to disease, infection, or inflammatory events by microglia.	Is necessary for neutrophil recruitment during sterile inflammation induced by cell death
IL-1β	Pro-inflammatory	Produced by microglia	Stimulate inflammatory mediators (prostaglandins, cyclooxygenase 2, and phospholipase A2). It plays a more important role than IL-1α
IL-10	Anti-inflammatory	Secreted by monocytes, B-cells, natural killer cells, and T-cells	Downregulates pro-inflammatory cytokines
TNF-α	Pro-inflammatory	Release by microglia, astrocytes, oligodendrocytes, monocytes, and neurons	Recruits' macrophages to injured site and affects cells proliferation, differentiation, and inflammation
IL-27	Pro-inflammatory	Secreted by macrophages and dendritic cells, microglia, monocytes, and B-cells	Belongs to IL-12 family, that increase the expression of brain-derived neurotrophic factor, which seems induce neurogenesis and remyelination after SCI
IL-17A	Pro-inflammatory	Produced by T-cells, dendritic cells, and macrophages	The upregulation of IL-17A appear to be correlated with the degeneration on SCI recovery

Chapter 5 - Discussion

Spinal cord injury (SCI) is a devasting neurological state that cause a cascade of alterations at the physical, chemical, cellular, and immunological levels. The biochemical processes that occur after an injury contribute negatively to the progression of the injury, which difficult the discovery of an effective treatment. In a way to try diminishing the damage provoked by a SCI, approaches as stabilization and decompression of spinal cord combined with methylprednisolone (MP) are used. However, these methods are not enough to a complete recovery after SCI. Besides the loss of sensory and motor function, SCI also leads to tissue damage and an overresponsive inflammation, which compromise the regeneration and functional recovery (Anjum et al., 2020; Silva et al., 2014). Neuropathic pain and spasticity are other two SCI consequences that affect the quality of life and the execution of tasks (Finnerup, 2017). Nevertheless, there are treatments approved by Food and Drug Administration (FDA) and European Medicines Agency (EMA) to manage neuropathic pain and spasticity, such as pregabalin and baclofen, respectively (Davari et al., 2020; De Sousa et al., 2022). The latter has been study by our group, and was found that baclofen promotes functional recovery after SCI when is acutely administered (de Sousa et al., 2023). Moreover, pregabalin studies demonstrated that this drug can act as a neuroprotector and improve motor recovery after SCI (Ha et al., 2008; Warner et al., 2017). However, no study was conducted to verify the effect of the combined administration of baclofen with pregabalin after SCI, in both physical recovery and tissue protection and regeneration. In this sense, we design an *in vivo* experiment, to understand the capacity of combined administration of baclofen with pregabalin to induce the motor and sensory recovery, and their ability to protect the spinal tissue and promote its regeneration, using a spinal cord transection mouse model. Furthermore, we analysed the inflammatory profile of different cytokines and the phenotype of microglia cells.

5.1 *In vivo* evaluation: the potential of combined administration of baclofen with pregabalin in behaviour analysis

A transection SCI mouse model was used because the complete disruption of the spinal cord facilitates the analysis of the tissue, namely the axonal regeneration and preservation. This SCI model is also most suitable to evaluate the treatment efficacy since functional recovery is due to the axonal regeneration and not to spinal cord native circuits. The animals with ten weeks old were subject to a SCI surgery, where was made a complete transection at T8 level. After the injury, the intraperitoneal administration of drugs was made according to the experimental

design, previous present. During the critical period of recovery from surgery (first five days) none of the animals died, yet from the 37 animals used, eight did not recover from the surgery and die in the first weeks. These deaths are distributed for the experimental groups which suggest that the combined administration of the drugs was not correlated to the deaths. In the third week, one animal was euthanized according to a humane endpoint due to a genetic condition.

To evaluate the locomotor recovery, it was performed Basso Mouse Scale (BMS) two days after injury and then once a week, until the end of the *in vivo* experiment. In this test it was analysed the movement of the hindlimb and was attributed a specific score according to the movement (Basso et al., 2006). Complete paralysis it was confirmed two days after the injury since animals presented a score of zero. One-week later, groups treated with chronic treatment improved. After the first week, all experimental groups present an improvement in the locomotor function, with the treated group presenting a slightly higher score than vehicle group. Besides these results, no statistical differences were found between treated group and vehicle group. These data suggest that the combined treatment did not induce a motor gains. Based on BMS data, it was calculated the percentage of the animals that reached weigh support (BMS≥4), that is, the animals were able to elevate the hindlimbs in way that the knees do not touch the ground. Until the first week, none of the animals was able to reach the weight support, but after the second week animals treated with baclofen and pregabalin began to support their weight. In the end of the experiment, 50% of Ba+Pa treated group reach weigh support while more than 60% of the animals with chronic treatment reach weight support. Although the BMS did not present motor gains induced by the treatments, the results of the percentage of the animals that reach weight support suggest that the drug treatment is promoting the difference between an animal be able to support their weight and walk and an animal that crawls. The majority of the methods available for evaluate the locomotor function were design for studies in rats (Pajoohesh-Ganji et al., 2010; Shigyo et al., 2014). The method most currently used to analyse the locomotor function in mice is BMS (Basso et al., 2006). Although BMS is reliable, the scale used leads to loss of sensitivity in the locomotor recovery. For example, BMS des not distinguish the weight support according to the functional improvement and neither detect the locomotor differences in the capacity of weight support. Besides this, BMS classified a range of functionalities as a single score. So, the best strategy to evaluate the locomotor recovery in mice is combined BMS with others behaviour test in order to obtain the best output of the effects induced by the therapy used (Pajoohesh-Ganji et al., 2010; Shigyo et al., 2014).

The test of bladder function provides information about the ability of the animals control their bladder, since SCI leads to a loss of bladder control. In mice the low urinary tract (LUT) has the function of urine storage and voiding by two functional units: (1) urinary bladder and (2) an outlet (bladder neck, urethra, and striated muscles of the pelvic floor). After SCI, the afferent communications are eliminated, leading to an areflexia bladder. Posteriorly, occurs the detrusor overactivity (DO) and detrusor sphincter dyssynergia (DSD). This results in an incontrollable voiding and a bladder hypertrophy. For a recovery of reflex bladder activity after a SCI is necessary the reorganization of reflex pathways in the spinal cord and the proprieties reorganization of the afferent's neurons. The urine storage and voiding are dependent on coordinate bladder activity. The mechanism in the brain and in spinal cord that control the coordinate bladder activity are regulated by C-fibers and Aδ-fibers (afferents fibers). The activation of the latter on, induces the normal voiding of the bladder, on the other hand the activation of C-fibers is correlated with the lower urinary tract dysfunction (LUTD) (Kadekawa et al., 2017; Wada et al., 2022).

In the protocol performed to evaluate the bladder function after SCI four parameters were analysed: score of the voiding, urine weight, spots numbers of urine and average area. The bladder function trial was performed based on previous studies (Hill et al., 2018; Wegner et al., 2018). Nevertheless, some alterations were made in the trial design. To normalize the experimental conditions in all experimental groups, the bladder was voided before test and it was given the same quantity of saline solution at all animals. In this way, the limitation of the water intake per animals was discarded from the study. To a detail analysis, our group create a score that is related with the pattern of the urine using the Wattman paper, where score of zero is an animal that is incapable to release any quantity of urine, and a score of five represents a pattern of an uninjured animal. Additionally, based in the urine area and the number of urine spots the average area of each experimental group was calculated. These alterations are not standardized. Our data from the bladder function analysis did not reveal that the combined administration of baclofen with pregabalin could improve the bladder function. However, a previous study demonstrated that the intrathecal baclofen could supress the afferent C-fibers bladder and improve both storage and voiding dysfunction (Wada et al., 2022). The present study does not show bladder control improvements, even with the baclofen administration. This result disparity could be explained by the method of drug administration. Moreover, studies demonstrated that the transected model is the model most difficult to recovery. Since the communication of the total afferents fibers is eliminated in these cases, the projection of these

fibers for lumbosacral region is more complicated. In addition to these, was suggested in these studies that the pharmacologic treatments could induce different effects according to the type of lesion (Leung et al., 2007; Mitsui et al., 2014).

Apart of motor impairments and bladder areflexia, animals with SCI developed neuropathic pain after SCI. This pain is spontaneous and is characterized or as an exaggerated responses to innocuous stimulus designated allodynia or as an excessive painful result from a painful stimulus defined as hyperalgesia (Chaplan et al., 1994; Deuis et al., 2017; Thomas Cheng, 2010). Neuropathic pain is a problem in ascending pain transmission and descending pain inhibitory response, both in spinal dorsal horn. Peripheral nerves, such as C-fibers and Aδ-fibers, conduct by dorsal root ganglia (DRG) transmit nociceptor information from the periphery to CNS through spinal cord. After these ascending pathways transmit the information, descending pathways (noradrenergic and serotonergic fibers) are responsible for conduct a pain-inhibitory response, also through spinal cord. This response is mediated by noradrenergic fibers with the release of norepinephrine and by 5-HT fibers with the release of GABA (Noristani, 2022; Pang & Rudd-Barnard, 2021; Ueda, 2008). When occurs an injury in spinal cord, these fibres become compromised, i.e., depolarization of afferent fibers to dorsal horn synapses, when occurs the release of excitatory neurotransmitters. Since spinal cord is injured, descending pain-inhibitory pathways cannot response to stimulus. Allodynia evaluation in SCI mice was evaluated through von Frey (vF) test. This test consists in the application of a set of microfilaments with different thicknesses, that do not cause pain in uninjured animals. Normal animals do not respond to the filaments, yet injured animals with allodynia withdraw their paws in response to the filaments. The threshold of a normal animal in vF test is one, so experimental groups with values below one experience pain (Chaplan et al., 1994; Deuis et al., 2017). In the present study, vF results show that two experimental groups reach the threshold of a normal animal, namely Ba+P24c and Ba+Pa. Despite that, only Ba+P24c group present significant statistical differences when compared to vehicle groups. Curiously, Ba+Pac treated group seems to response to vF similarly to vehicle group. These results could be justified taking into account a serious of previous studies. For instance, the experimental group Ba+P24c was initially treated with baclofen, that was described to supress afferent C-fibers (Wada et al., 2022). Moreover, baclofen is a GABA agonist, which means that its administration will inhibit the propagation of nociceptor signs induced by afferent fibers (Joosten & Franken, 2020; Kent et al., 2020). Twenty-four hours after baclofen administration, pregabalin was administered. This drug has the ability to binding to the subunit α2-δ of calcium channels, providing the

axonal regeneration (Taylor et al., 2007; Tedeschi et al., 2016). Our hypothesis is that with the 24 hours administration delay of pregabalin, the CNS has the time to originate a response more controlled and organized. Ba+Pa had similar results to Ba+P24c, probably because the drug administration was only acute, which also provide time to organize the neuronal network. On the other hand, Ba+Pac treated group had no time between drugs administration neither after the acute administration, since was given baclofen and pregabalin acutely and pregabalin for fourteen days.

As every studies with drugs administration, this one also has the interest to understand if the baclofen and pregabalin administration could negatively affect the animals. To evaluate the metabolism response to drug administration the mice livers were weighted, after their euthanasia. It was performed the ratio between liver weight and body weight to normalize the data. Our results showed that treated group do not present any liver anomaly when compared to vehicle group. Although additional studies such as histological analysis could be done to have a deeper understanding, our data suggest that combined baclofen with pregabalin administration do not induce harmful effects in the animals.

5.2 The combinatory treatment of baclofen and pregabalin administration seems to be modulating important SCI milestones

The main goal of the immunohistochemistry (IHC) analysis of spinal cord tissue section was to evaluate the potential effects that the combined administration of baclofen with pregabalin could induce relatively to neuroprotection, neuroregeneration and neuroinflammation in tissue of injured spinal cord. The distance among spinal cord cross-section is about 200 μm from the lesion epicenter.

The demyelination is one of the consequences after SCI, so, to evaluate the percentage of conserved white matter a staining of FluoroMyelin green fluorescence was performed (Paramos-de-Carvalho et al., 2021). In this analysis considered the distance 1000 μm rostrally and caudally from the lesion site. Our results did not show the differences in the white matter sparing between experimental group, suggesting that the combined administration of baclofen with pregabalin did not prevent demyelination. However, a previous study demonstrated that agonist of $GABA_B$ receptor, such as baclofen, could promote remyelination of CNS. Contrarily to our project, the study start to inject the baclofen only a few days after induced the

demyelination (Serrano-Regal et al., 2022). Since baclofen is rapidly metabolized, the difference between the lesion and injection could be crucial to induce axon remyelination.

In a chronic phase of SCI, it is formed a scar in tissue, that is characterized by a set of cells, as fibroblast, endothelial and inflammatory immune cells. This fibrotic scar is one of the causes that prevents the axonal projection through the lesion. In this work, it was performed an immunostaining with anti-PDGFR that mark fibroblasts. It was observed that groups treated with baclofen and pregabalin after injury presented less percentage of fibrotic tissue when compared with vehicle and treated group with acute baclofen and pregabalin 24 hours after injury. These observations agree with previous studies using baclofen (Guyon et al., 2013). Besides that, the data obtained also suggested that acute administration of baclofen combined with pregabalin decrease the fibrotic tissue resulted from SCI, since treated groups Ba+Pa and Ba+Pac showed less fibrotic tissue than Ba+P24c in the lesion epicenter.

Previous studies performed in lampreys described baclofen has a protector of motor neuronal death, however, is not described that pregabalin could also prevent the motor neurons death (Romaus-Sanjurjo et al., 2018, 2019). In order to evaluate the effect of baclofen with pregabalin administration the number of motor neurons was quantified, namely the neurons located in the ventral horn of the spinal cord tissue. The quantification was assessed through the immunostaining with Neu-N, which is used to identify all neurons present in the spinal cord tissue (Lima et al., 2021). Intriguingly, the major differences obtained in relation to vehicle group were with animals treated with pregabalin chronic. Rostrally and caudally to the lesion site Ba+P24c group has a tendency to present more motor neurons when compared to vehicle group. The opposite occurs with Ba+Pac treated group, that showed a smaller number of α-motor neurons than vehicle group. The main difference between these two groups, is the time at which the administration of pregabalin was started. However, when comparing the number of α-motor neurons with the results of BMS and the percentage of mice that reach weight support, is possible to observe that treated group with chronic pregabalin has similar results regarding to motor behavior. Besides the quantity of motor neurons, the output of motor recovery was very alike between chronic treated groups comparing to vehicle group.

Afterwards, the quality of the axonal projections was evaluated. To perform this analysis, it was used neurofilament heavy (NF-H) antibody, and quantifications were made at 2000 µm and 1000 µm rostrally and caudally from the lesion epicenter, in the ventral zone of spinal cord tissue-section. Treated group with Ba+Pac was the only that showed a significant higher number of axonal projections, both rostrally and caudally to the lesion site, when

compared to vehicle group. This result suggests that although no differences were observed in the number of motor neurons, pregabalin administration acutely and chronic, promotes a higher axonal projection after SCI. These observations are in agreement with previous studies, showing that pregabalin by blocking the calcium channel subunit α2-δ promotes axonal regeneration (Kugler et al., 2022; Taylor et al., 2007; Tedeschi et al., 2016). Since we found differences in NF-H between experimental groups, it was conducted an immunostaining to characterize the neurons type of which differences in NF-H correspond to. To perform that assay, it was used 5-hydroxytryptamine (5-HT) and tyrosine hydroxylase (TH) antibodies to quantify serotonergic and catecholaminergic neurons, respectively. No major differences were found between experimental groups regarding the catecholaminergic neurons, yet differences were found in the serotonergic neuron's quantification. Experimental group treated with Ba+Pac showed more intensity of 5-HT signal comparing to vehicle group 1000 µm rostrally and caudally to the epicenter. These results are in agreement with the NF-H quantification, since we also observed differences between vehicle and Ba+Pac groups at the same distances to 5-HT. Based on these data, it is possible to suggest that acute and chronic administration of pregabalin combined with acute baclofen, promotes axonal projections of serotonergic neurons. Furthermore, these data could also explain the behaviour results. TH is a marker for dopamine, norepinephrine, and epinephrine containing neurons and endocrine cells. Neurons that express TH are involving in controlling the autonomic systems, more specifically sympathetic system (heart rate, blood pressure and bladder reflex circuitry) (Weihe et al., 2006). Bladder trial results did not show an improvement in bladder function, which could be correlated with the lack of significant results in the TH analysis. Serotonergic neurons are responsible to control the central pattern generator (CPG), an important central for locomotor output. The CPG is a neural network contained in thoracic and lumbar segments of spinal cord. 5-HT is known as a neuromodulator of CPG activity (Ghosh & Pearse, 2015; Sławińska et al., 2013). Since Ba+Pac experimental group presented a significant higher signal of 5-HT, this could be the reason why this group has the highest percentage of animals that reach weight support.

SCI is characterized as a devastating neurological disorder that induce molecular and cellular alterations, namely those ones that are related to neuroinflammation such as microglia cells (Freyermuth-Trujillo et al., 2022; Hellenbrand et al., 2021). To understand the role of combined administration of baclofen with pregabalin in microglia modulation, it was conducted an assay with Iba-1 antibody, which marks microglia cells. The phenotype of these cells (number of cells/mm^2, average size, circularity, feret diameter and solidity) was analysed based

on a previous work (Zanier et al., 2015). Our data showed that the drugs administration does not affect parameters as microglia average size, solidity, and circularity. However, major differences were observed in the other parameters, especially in comparisons between chronic treated groups and vehicle group. Regarding to perimeter, the higher is the value more homeostatic-like are the microglia cells. Ba+P24c group presented a smaller perimeter than vehicle group at 1000 μm from the lesion site, and Ba+Pac group also showed a smaller perimeter than vehicle group at 2000 μm from the lesion core. This could indicate that at this distance from the epicentre, the treatment does not modulate the inflammatory response. As Feret diameter, the higher is the value more homeostatic are the microglia. In this case, only Ba+Pac treated group exhibit major differences comparing to vehicle group, at 1000μm rostrally to the lesion site and at the lesion epicenter. This data suggests that Ba+Pac group does not present a modulation in the microglia cells phenotype. Interestingly, counting the number of microglia cells resulted in a lower number of these cells in experimental treated groups. It is important to note that the major differences are located at 1000μm rostrally and caudally from the lesion core. Based on this data it was possible to understand that the administration of chronic pregabalin did not mitigate immune response. Although the lower number of microglia cells, our results showed that experimental groups treated with chronic pregabalin present a smaller perimeter and Feret's diameter, features normally associated with less homeostatic microglia. The treated group with acute drugs administration exhibit a similar data to vehicle group. So, despite the lower number of microglia cells, these experimental groups presented phenotypes related to less homeostatic microglia, which could indicate that treatment reduced the number of microglia cells, attenuating the immune response, but with these cells being less homeostatic in Ba+P24c and Ba+Pac groups, than those one of vehicle group.

Additionally, to the local immune response analysis, we also studied the systemic immune response in this experiment. For that, it was collected blood samples at different time points (1-, 7-, 14-, and 42-days post injury) and used a LegendPlex commercial kit, where thirteen distinct cytokines (IL-23, MCP-1, IL-6, GM-CSF, IL-12p70, IFN-β, IFN-γ, IL-1α, IL-1β, TNF-α, IL-10, IL-27, and IL-17A) were analysed. All the procedures were made following the kit indications (Annex 2). Data from the circulating cytokines showed that Ba+Pac group exhibit lower concentration of pro-inflammatory cytokines such as MCP-1, IL-6, GM-CSF, IL-12p70, IFN-β, IFN-γ and IL-1β, yet not all these cytokines present statistical results between Ba+Pac group and vehicle group. The major differences were found at two- and six-weeks post-injury, in a subacute and chronic phase of SCI. Based on these data it is possible to suggest that

the administration of baclofen acute with pregabalin acute and chronic induce a systemic immunomodulatory response in a subacute and chronic phase after SCI. Curiously, group treated with Ba+Pa stands out in the MCP-1 result, since is the only experimental group with higher concentration of this cytokine in all time points, although these differences between Ba+Pa are not statistically significant. MCP-1 is characterized for being secreted by T-cells, astrocytes, microglia, and monocytes (Hellenbrand et al., 2021). Taking into consideration that microglia analysis does not show differences in the Ba+Pa group comparing to vehicle group, the results of this cytokine concentration could be derived by the other cells that secret MCP-1. Moreover, IL-23 also present an interestingly result. Both treated groups with acute baclofen and pregabalin acutely shown a higher concentration of IL-23, with the Ba+Pac group presenting significant results when compared to vehicle group. IL-23 is a pro-inflammatory cytokine of IL-12 family, secreted by microglia, astrocytes, and dendritic cells. The secretion of IL-23 induces the production of IL-17A that consequently induce the production of IL-6 (Bian et al., 2014; X. Luo et al., 2021). In fact, the concentration of IL-23 in the Ba+Pac treated group begins to increase from the second week post-injury, and the concentration of IL-6 and IL-17A also increases from the second week, although these differences are not statistically significant. Furthermore, previous studies discovered that IL-23 has an essential role in induce chronic pain in rats but specially in female mice through C-fibers (Bian et al., 2014; Ji et al., 2021; X. Luo et al., 2021). These data are in concordance with vF results, since Ba+Pac was the group with the higher concentration of IL-23 and was the treated group with vF results similar to vehicle group, that is, with more allodynia. It is important to note that vF test was perform at six weeks post-injury, the same that point that IL-23 reach the higher concentration.

Overall, results suggest that the combined administration of baclofen with pregabalin could induce beneficial effects regarding pain management and tissue protection and regeneration. Despite no motor behavior and bladder improvements were observed, it was important to understand that the combined treatment could improve neuropathic pain. Besides, histological analysis showed an important role of the drugs administration in protect and regenerate the spinal tissue after transected spinal cord. The immunological response, especially the systemic response, demonstrate a strong association with the vF results which is exciting to design future experiments.

For these reasons, the combined baclofen with pregabalin administration contribute for the management of neuropathic pain as for the protection and axonal regeneration of spinal cord tissue, which is important for future recovery from SCI. Despite that, more research needs to

be performed to fully understand all the potential of this drugs combination as a target therapy for SCI.

Chapter 6 -Conclusion and Future perspectives

The spinal cord is one of the most complex structures in the human body and even the smallest change can result in an alteration in function of the human body. Affecting millions of people worldwide, SCI disturbs the patient's quality of life. Currently, there is no effective treatment available for SCI due to the its complexity, that involves a series of mechanisms and different pathophysiology stages. For these reasons, therapies should aim to cover multiple pathology mechanisms as possible, which is difficult to achieve.

Different therapies are being studied, but one possible approach is to combine two drugs that are already in the clinical for SCI people, namely baclofen and pregabalin. Previous studies demonstrated that baclofen has a role in manage spasticity, a consequence derived from a lesion in spinal cord. Moreover, it was also shown that baclofen could also improve locomotor and bladder function and modulate the microglia cells. Regarding pregabalin, this drug is indicated for control neuropathic pain that occurs after SCI. Nevertheless, previous studies demonstrated that the mechanism of action of pregabalin leads to a neuroprotection and axonal regrowth.

The work hereby presented had the objective of exploring the effects of combined administration of baclofen with pregabalin as a potential therapy for SCI, through neuroprotection, neuroregeneration and immunomodulation. For that, it was conducted an in vivo with four experimental groups (Vh, Ba+P24c, Ba+Pa and Ba+Pac), during six weeks. Firstly, it was performed analysis of behaviour tests and then it was studying the effect of the drugs in the spinal tissue of these animals.

The data from the first task suggests that the combined baclofen with pregabalin administration does not leads to a gain of motor improvements, yet treated animals had the ability to reach their own weigh support, in contrast to vehicle group. Moreover, the combined drugs administration does not seem to improve the bladder control. However, a major difference was found in vF test, Ba+P24c treated group presented data like an uninjured animals, which could indicate that acute administration of baclofen with pregabalin administration after 24 hours post-injury and for fourteen consecutive days, reorganized the sensorial neural network after SCI. On the other hand, the acute administration of baclofen and pregabalin with chronic administration of pregabalin does not improve the allodynia in animals with SCI.

The second aim consisted on understanding the effect of combined administration of baclofen with pregabalin behind the behavior analysis. In fact, the histological analysis was surprisingly. Groups treated with acute baclofen and pregabalin present a smaller percentage of fibrotic tissue in comparison with the control group, which suggest that acute administration of

baclofen with pregabalin could result in a neuroprotection response. Quantification of motor neurons demonstrate that Ba+P24c show a higher number of motor neurons while Ba+Pac present a smaller number of motor neurons when comparing to vehicle group. However, the quality of neurons connection is the most important for the communication of CNS. Indeed, this affirmation was verified by the analysis of NF-H, that result in a higher signal of axonal projections in the Ba+Pac treated group, that present a smaller number of motor neurons, Furthermore, it was study to which type of neurons those axonal projection corresponds to and it was found that those axonal projections belongs mainly to serotonergic neurons. The immune response was also analysed, both systemic and local response. Data from microglia analysis do not demonstrate that the combined drugs administration modulate the phenotype of microglia, yet modulate the number of microglia cells, decreasing its number. Regarding to systemic immune response, that was analysed in four different time points (1-, 7-, 14-, and 42-days post-injury). Our data showed that Ba+Pac treated group presented low concentration in many of the cytokines studied, mostly after the second week post-injury, which suggest that acute administration with baclofen and pregabalin plus chronic administration of pregabalin reduces the inflammatory systemic response. Furthermore, the cytokine that Ba+Pac show with higher concentration was the IL-23, that is related with neuropathic pain, which justified the vF data of these experimental group.

Taking this into consideration, the combined baclofen with pregabalin administration has a potential as possible therapy for SCI. However, more in depth studies needs to be done. Since BMS present limitations, an additional locomotor test could give a better output of the combined treatment. Since VF test data of Ba+P24c treated group present improvements in allodynia it is important to understand what type of pain filaments are correlated with these results. Moreover, in the future, it would be interesting to analyse the dorsal horn of spinal tissue, that is correlated with sensorial pathways. Trying to understand the mechanism that the combined administration of baclofen with pregabalin could induce in SCI tissue.

For all the reasons and data here present, the work produced contribute to the progression of studies made in the SCI field, although it is necessary to perform more research to a better knowledge about the potential therapy here implemented.

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Annexes

Annex 1: Scoring system for the BMS (Basso *et al.*, 2006)

Score	
0	No ankle movement
1	Slight ankle movement
2	Extensive ankle movement
3	Plantar placing of the paw with or without weight support -OR- Occasional, frequent or consistent dorsal stepping but no plantar stepping
4	Occasional plantar stepping
5	Frequent or consistent plantar stepping, no coordination -OR- Frequent or consistent plantar stepping, *some* coordination, paws *rotated* at initial contact and lift off (R/R)
6	Frequent or consistent plantar stepping, *some* coordination, paws *parallel* at initial contact (P/R, P/P) -OR- Frequent or consistent plantar stepping, *mostly* coordinated, paws *rotated* at initial contact and lift off (R/R)
7	Frequent or consistent plantar stepping, *mostly* coordinated, paws *parallel* at initial contact and *rotated* at lift off (P/R) -OR- Frequent or consistent plantar stepping, *mostly* coordinated, paws *parallel* at initial contact and lift off (P/P), and *severe* trunk instability
8	Frequent or consistent plantar stepping, *mostly* coordinated, paws *parallel* at initial contact and lift off (P/P), and *mild* trunk instability -OR- Frequent or consistent plantar stepping, *mostly* coordinated, paws *parallel* at initial contact and lift off (P/P), and *normal* trunk stability and tail *down or up & down*
9	Frequent or consistent plantar stepping, *mostly* coordinated, paws *parallel* at initial contact and lift off (P/P), and *normal* trunk stability and tail *always* up.

Slight: Moves less than half of the ankle joint excursion.
Extensive: Moves more than half of the ankle joint excursion.
Plantar placing: Paw is actively placed with both the thumb and the last toe of the paw touching the ground.
Weight support: (dorsal or plantar): The hindquarters must be elevated enough that the hind end near the base of the tail is raised off of the surface and the knees do not touch the ground during the step cycle.
Stepping: (dorsal or plantar): Weight support at lift off, forward limb advancement and re-establishment of weight support at initial contact.
Occasional: Stepping less than or equal to half of the time moving forward.
Frequent: Stepping more than half the time moving forward.
Consistent: Plantar stepping all of the time moving forward with less than 5 missed steps (due to medial placement at initial contact, butt down, knee down, skiing, scoliosis, spasms or dragging) or dorsal steps.
Coordination: For every forelimb step a hindlimb step is taken and the hindlimbs alternate during an assessable pass. For a pass to be assessable, a mouse must move at a consistent speed and a distance of at least 3 body lengths. Short or halting bouts are not assessable for coordination. At least 3 assessable passes must occur in order to evaluate coordination. If less than 3 passes occur then the mouse is scored as having no coordination.
Some coordination: Of all assessable passes (a minimum of 3), most of them are *not* coordinated.
Most coordination: Of all assessable passes (a minimum of 3), most of them *are* coordinated.
Paw position: *Digits* of the paw are parallel to the body (P), turned out away from the body (external rotation: E) or turned inward toward midline (internal rotation; I).
Severe trunk instability: Severe trunk instability occurs in two ways.

(1) The hindquarters show severe postural deficits such as extreme lean, pronounced waddle and/or near collapse of the hindquarters predominantly during the test.

or

(2) Five or more of any of the following *events* stop stepping of one or both hindlimbs
- Haunch hit: the side of hindquarters rapidly contacts the ground
- Spasms: sustained muscle contraction of the hindlimb which appears to immobilize the limb in a flexed or extended position
- Scoliosis: lateral deviation of the spinal column to appear "C" shaped instead of straight

Mild trunk instability: Less than 5 events listed above and some sway in the hindquarters. Mild trunk instability is scored when the pelvis and haunches predominantly dip, rock, or tilt from side-to-side (tilt). If the tail is up, the swaying of the pelvis and/or haunches produces side-to-side movements of the distal third of the tail which also indicates mild trunk instability (side tail).
Normal trunk stability: No lean or sway of the trunk, and the distal third of the tail is steady and unwavering during locomotion. No severe postural deficits or events and less than 5 instances of mild instability.

Annex 2: LegendPlex protocol

Performing the Assay Using a V-bottom Plate

- Allow all reagents to warm to room temperature (20-25°C) before use.
- Keep the plate upright during the entire assay procedure, including the washing steps, to avoid losing beads.
- The plate should be placed in the dark or wrapped with aluminum foil for all incubation steps.
- Standards and samples should be run in duplicate and arranged on the plate in a vertical configuration convenient for data acquisition and analysis (as shown in attached PLATE MAP, page 31). Be sure to load standards in the first two columns. If an automation device is used for reading, the orientation and reading sequence should be carefully planned.

1. **For measuring cell culture supernatant samples:**
   - Add 25 µL of Assay Buffer to all wells.
   - Add 25 µL of each standard to the standard wells.
   - Add 25 µL of each sample to the sample wells (See **Sample Dilution**).

   For measuring serum or plasma samples:
   - Add 25 µL of Matrix C to the standard wells.
   - Add 25 µL of Assay Buffer to sample wells.
   - Add 25 µL of each standard to standard wells.
   - Add 25 µL of each diluted serum or plasma sample to sample wells (See **Sample Dilution**).
2. Vortex mixed beads for 30 seconds. Add 25 µL of mixed beads to each well. The total volume should be 75 µL in each well after beads addition. (Note: During beads addition, shake mixed beads bottle intermittently to avoid bead settling).
3. Seal the plate with a plate sealer. Cover the entire plate with aluminum foil to protect the plate from light. Shake at 800 rpm on a plate shaker for 2 hours at room temperature **(Depending on the shaker, the speed may need to be adjusted. The optimal speed is one that is high enough to keep beads in suspension during incubation, but not too high so it causes spill from the wells)**.
4. Centrifuge the plate at 1050 rpm (~250 g) for 5 minutes, using a swinging bucket rotor (G.H 3.8) with microplate adaptor (Please refer to **Materials to be Provided by the End-User, page 8).** Do not exceed centrifugation speed as it can affect beads resuspension in later steps. **Make sure the timer of the centrifuge works properly and standby to make sure the centrifuge reaches preset speed.**

5. Immediately after centrifugation, dump the supernatant into a sink by quickly inverting and flicking the plate **in one continuous and forceful motion**. Do not worry about losing beads even if the pellet is not visible. The beads will stay in the tip of the well nicely. Blot the plate only once on a stack of clean paper towel and drain the remaining liquid from the well as much as possible. Be careful not to disturb the bead pellet.

   Alternatively, removal of the supernatant may be completed using a multichannel pipette set at 75 µL. Try to remove as much liquid as possible without removing any beads. Be sure to change pipette tips between each row or column.

6. Wash the plate once by dispensing 200 µL of washing buffer into each well. Shake the plate at 800 rpm for 1 minute and repeat step 4 and 5.

7. Add 25 µL Detection Antibodies to each well.

8. Seal the plate with a new plate sealer. Cover the entire plate with aluminum foil to protect the plate from light. Shake at 800 rpm on a plate shaker for 1 hour at room temperature.

9. **Do not wash the plate!** Add 25 µL of SA-PE to each well directly.

10. Seal the plate with a new plate sealer. Wrap the entire plate with aluminum foil and shake the plate on a plate shaker at approximate 800 rpm for 30 minutes at room temperature.

11. Repeat steps 4 - 6.

12. Add 150 µL of 1X Wash Buffer to each well. Resuspend the beads by pipetting.

13. Read samples on a flow cytometer, preferably within the same day of the assay (Note: Prolonged sample storage can lead to reduced signal).

    If the flow cytometer is equipped with an autosampler, the samples can be read directly. **Please be sure to program the autosampler to resuspend beads in the well immediately before taking samples. The probe height may need to be adjusted when using an autosampler**.

    If an autosampler is not available, the samples can be transferred from the plate to micro FACS (or FACS) tubes and read manually.