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GRAPE STALK AND COFFEE POLYPHENOLIC EXTRACTS ADMINISTRATION AS NEW PHARMACOLOGICAL STRATEGIES TO MODULATE SPINAL CORD INJURY-INDUCED NEUROPATHIC PAIN IN MICE

Anna Bagó Mas

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Doctoral Thesis

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2022

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Doctoral Programme in Molecular Biology, Biomedicine and Health

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Certify:

That the thesis entitled "Grape Stalk and Coffee polyphenolic extracts administration as new pharmacological strategies to modulate spinal cord injury-induced neuropathic pain in mice", presented by Anna Bagó Mas to obtain a doctoral degree with recognition as an international doctorate, has been completed under our supervision and meets the requirements to opt for an International Doctorate.

For all intents and purposes, we hereby sign this document.

Dr. Pere Boadas Vaello

Dra. Núria Fiol Santalo

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per tot el seu suport i amor incondicional

It takes curiosity to learn.

It takes courage to unlearn.

Learning requires the humility to admit what you don't know today.

Unlearning requires the integrity to admit that you were wrong yesterday.

Learning is how you evolve.

Unlearning is how you keep up as the world evolves.

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LIST OF ABBREVIATIONS

5-HT	Serotonin
ACC	Anterior cingulate cortex
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
AMG	Amygdala
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
ATF-2	Activating transcription factor 2
ATP	Adenosine triphosphate
BBB	Blood–brain barrier
BDNF	Brain-derived neurotrophic factor
BLA	Basolateral amygdala
BMS	Basso Mouse Scale
BTX-A	Botulinum toxin A
CaMKII	Ca_{2+}/calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
CatS	Cathepsin S
CCI	Chronic constriction injury
CCKB	Cholecystokinin B
CCL2	Chemokine (C-C motif) ligand 2
CCL21	Chemokine (C-C motif) ligand 21
CCR2	Chemokine (C-C motif) ligand 2 receptor
CeA	Central amygdala
CFA	Complete Freund's adjuvant
CGRP	Calcitonin-gene related peptide
CGRPR	Calcitonin-gene related peptide receptor
CNP	Central neuropathic pain
CNS	Central nervous system
COX-2	Cyclooxygenase 2
CREB	cAMP response element binding protein

CSF	Cerebrospinal fluid
CX3CL1	Chemokine (C-X3-C motif) ligand 1
CXCL1	Chemokine (C-X-C motif) ligand 1
CX3CR1	Chemokine (C-X3-C motif) ligand 1 receptor
Da	Daltons
dpi	Days post injury
DRG	Dorsal root ganglia
e.g.	Exempli gratia; from Latin, “for example”
EGCG	Epigallocatechin-3-gallate
EGFR	Epithelial growth factor receptor
Elk-1	ETS Like-1 protein
ERK	Extracellular signal-regulated kinase
FASN	Fatty acid synthase
FBS	Foetal Bovine Serum
G	Gram
GABA	γ-aminobutyric acid
GC	Guanylate cyclase
GDNF	Glial-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GPCR	G Protein Coupled Receptors
GRADE	Grading of Recommendations Assessment, Development, and Evaluation
GSH	Glutathione
i.p.	Intraperitoneal
IASP	International Association for the Study of Pain
IHC	Immunohistochemistry
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-10	Interleukin-10
ISF	Interstitial fluid
JNK	c-Jun N-terminal Kinase
KCC2	Potassium chloride cotransporter 2

LTP	Long-term potentiation
MANOVA	Multivariate analysis of variance
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein 1
MDA	Malondialdehyde
mg/Kg	milligrams/kilograms
mm	millimetre
MMP9	Matrix metallopeptidase 9
mPFC	Medial prefrontal cortex
MS	Mass spectrometry
NA	Noradrenalin
Na+	Sodium
NeuPSIG	IASP Special Interest Group of Neuropathic Pain
NF-κβ	Nuclear factor-κβ
NGF	Nerve growth factor
NK-1	Neurokinin 1
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NP	Neuropathic pain
NS	Nociceptive specific neurons
P2X	Purinergic receptor P2X subclass
P2X3	P2X purinoceptor 3
PAG	Periaqueductal grey
PBQ	Phenylbenzoquinone
PBS	Phosphate buffer saline
PFC	Prefrontal cortex
PGE2	Prostaglandin E2
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PNS	Peripheral nervous system
RNS	Reactive nitrogen species

ROS	Reactive oxygen species
RVM	Rostral ventral medulla
SC	Spinal cord
SCI	Spinal cord injury
SEM	Standard deviation of the mean
SNL	Spinal nerve ligation
SNRI	Serotonin-norepinephrine reuptake inhibitors
SNS	Somatosensory nervous system
SOD	Superoxide dismutase
SP	Substance P
STZ	Streptozotocin
TCAs	Tricyclic antidepressants
TNF-α	Tumour necrosis factor-α
TrkA	Tropomyosin receptor kinase A
TrkB	Tropomyosin receptor kinase B
TRP	Transient receptor potential cation channel
TRPA1	Transient receptor potential ankyrin 1
TRPM8	Transient receptor potential cation channel subfamily M member 8
TRPV1	Transient receptor potential cation channel subfamily V member 1
TRPV2	Transient receptor potential cation channel subfamily V member 2
TRPV3	Transient receptor potential cation channel subfamily V member 3
VGCCs	Voltage-gated calcium channels
VTA	Ventrotegmental area
WDR	Wide dynamic range
wpi	Weeks post injury

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MATERIALS AND METHODS

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ABSTRACT

Central neuropathic pain (CNP) following spinal cord injury (SCI) is developed in more than half of patients and around one-third of those report the pain to be severe. CNP after SCI has been associated with impairments in a variety of areas, including physical functioning and mobility, mental and cognitive functioning, social functioning and community reintegration, sleep, employment, and quality of life. Moreover, people who experience pain after SCI show poorer health, lower life satisfaction and a higher risk of depression. To address this health concern, several pharmacological treatments have been used to alleviate CNP. However, current treatments are often ineffective because they target only one or two of the several mechanisms that comprise CNP. Nowadays, to design a personalized therapeutic approach, treatment consists in a trial-and-error of different strategies, including antidepressants, antiepileptics, GABA-agonists, local anaesthetics, NMDA-antagonists, cannabinoids and opioids. However, these drugs are usually inadequate and only one third of patients respond to pharmacological treatments when compared with placebo. Moreover, the best pharmacological strategy results in a reduction of only 20–30% in pain intensity, frequently accompanied by severe side effects. Hence, given the lack of effective treatments, it is necessary to develop new pharmacological strategies not only for the neuropathic pain relief but also for the prevention of its chronification. Among the potential pharmacological strategies aimed at modulating pathological pain could be highlighted the use of polyphenols, since preclinical evidence of their antinociceptive effects can be found in the scientific literature. The properties that can be attributed to polyphenols that may explain pathological pain modulation are free radical scavenging/antioxidant, immunomodulatory, neuroprotective, anti-apoptotic and autophagy-regulating activities. However, although several studies have been specifically aimed at elucidating the effects of polyphenolic treatments on the neuropathic pain development, most of them have been conducted in preclinical models unrelated to SCI, such as peripheral neuropathic pain, diabetic neuropathic pain, or alcoholic neuropathy, among others. Furthermore, although studies on polyphenol treatment after SCI are available, most of them have focused on motor recovery or spinal cord regeneration, leading to a lack of information despite promising results on their effects on modulating pathophysiological processes that may also be related to neuropathic pain development. In this context, the main objective of the present thesis was to study the preventive and analgesic effects of two polyphenolic plant extracts in SCI-induced CNP development in mice and to compare it with the effects of epigallocatechin-3-gallate (EGCG), one of the most studied polyphenols in the field of neuropathic pain. Concretely, the two polyphenolic extracts were obtained from grape residual material (GSE) and roasted decaffeinated coffee powder (CE), which were chosen since both grapevine and coffee are known to be rich natural sources of polyphenols. After SCI, the effects of repeated EGCG, GSE and CE treatment on neuropathic pain behaviours were evaluated during the acute, intermediate, and chronic phase of injury. In addition, gliosis and the expression central sensitisation-related biomarkers were

analysed in both spinal cord and pain-processing involved supraspinal structures. On the one hand, the EGCG administration during the first week post SCI resulted in both mechanical allodynia and thermal hyperalgesia development prevention during the acute phase of SCI, accompanied by SCI-induced spinal cord gliosis attenuation. However, all tested doses of EGCG resulted in significant weight loss of mice throughout the experimental period, indicating that systemic toxicity could be associated with such treatment. In contrast, both preventive GSE and CE administration during the first week post SCI, resulted in the attenuation of both mechanical allodynia and thermal hyperalgesia development during the acute phase of SCI without either weight-loss or serum biomarkers increase of hepatotoxicity or nephrotoxicity. Antinociceptive effects of both polyphenolic extracts were associated with the prevention of both gliosis and upregulation of central sensitization-related algogens (pERK, CX3CL1, CX3CR1 and CCR2) in the spinal cord. In addition, while GSE and CE treatments modulated CX3CL1/CX3CR1 signalling and attenuated astrogliosis in both supraspinal structures of the anterior cingulate cortex (ACC) and periaqueductal gray (PAG), as well as microgliosis in the ACC of injured mice, such treatments induced microglial activation in PAG likely characterized by an increase of microglial cells exhibiting an M2 anti-inflammatory phenotype. On the other hand, while repeated administration of EGCG during the third week post SCI modulated neither mechanical allodynia nor thermal hyperalgesia induced by mild SCI during the intermediate phase of injury, the same administration pattern for both GSE and CE modulated these reflexive pain responses with no associated systemic toxicity, hepatotoxic or nephrotoxic effects. In addition, CE administration modulated depressive-like behaviour detected in spinal cord injured mice at intermediate phase of injury. Finally, repeated administration of GSE and CE during the first-, third- and sixth-week post SCI prevented thermal hyperalgesia and mechanical allodynia development up to the chronic phase of injury, with no associated systemic toxicity, hepatotoxic or nephrotoxic effects. In addition, GSE and CE treatments also modulated both affective-motivational disturbances (anhedonia, depression and anxiety) and social interaction impairment developed in chronic SCI-animals. Antinociceptive effects of GSE and CE were associated not only with the modulation of spinal cord microgliosis but also with the modulation of astrogliosis and CCL2/CCR2 and CX3CL1/CX3CR1 signalling in the supraspinal structures ACC, amygdala, dorsal and ventral PAG and rostral ventromedial medulla (RVM) as well as microgliosis in ventral PAG. Overall, the results of the present thesis suggest that a mixture of polyphenols present in natural extracts may be a suitable pharmacological strategy to either prevent or attenuate the development of SCI-induced neuropathic pain by modulating not only the reflexive pain responses (more related to the sensory-discriminative dimension of pain) but also the non-reflexive pain responses (included in the affective-motivational dimension of pain). These compounds not only exert their effects at the site of injury by modulating gliosis and the expression of central sensitisation-related biomarkers but also on supraspinal structures closely related to expression and modulation of central neuropathic pain.

RESUM

El dolor neuropàtic central (DNC) després d'una lesió medul·lar (LME) es desenvolupa en més de la meitat dels pacients, i al voltant d'un terç d'ells defineix el dolor com a intens. El DNC després d'una LME s'ha associat a deficiències en diverses àrees, com el funcionament físic i la mobilitat, el funcionament mental i cognitiu, el funcionament social i la reintegració a la comunitat, el somni, l'ocupació laboral i la qualitat de vida. A més, les persones que experimenten dolor després d'una LME mostren una pitjor salut, una menor satisfacció amb la vida i un risc més gran de patir depressió. Per fer front a aquest problema de salut, s'han utilitzat diversos tractaments farmacològics per alleujar el DNC. Tanmateix, els tractaments actuals solen ser ineficaços perquè només es dirigeixen a un o dos dels diversos mecanismes que componen el DNC. Actualment, amb l'objectiu de dissenyar un enfocament terapèutic personalitzat, el tractament consisteix en un assaig i error de diferents estratègies, que inclouen antidepressius, antiepilèptics, agonistes GABA, anestèsics locals, antagonistes NMDA, cannabinoides i opioides. No obstant això, aquests fàrmacs solen ser inadequats i només un terç dels pacients hi respon en comparació amb el placebo. A més, la millor estratègia farmacològica només redueix la intensitat del dolor en un 20-30% i sol anar acompanyada de greus efectes secundaris. Per tant, atesa la manca de tractaments eficaços, és necessari desenvolupar noves estratègies farmacològiques no només per l'alleujament del dolor neuropàtic sinó també per la prevenció de la seva cronificació. Entre les potencials estratègies farmacològiques dirigides a modular el dolor patològic, es pot destacar l'ús de polifenols, ja que a la literatura científica es poden trobar evidències preclíniques dels seus efectes antinociceptius. D'entre les propietats que poden atribuir-se als polifenols i que poden explicar la modulació del dolor patològic, destaquen les activitats antioxidants o d'eliminació de radicals lliures, immunomoduladores, neuroprotectores, antiapoptòtiques i reguladores de l'autofàgia. Tot i això, encara que diversos estudis s'han dirigit específicament a dilucidar els efectes dels tractaments polifenòlics en el desenvolupament del dolor neuropàtic, la majoria s'han realitzat en models preclínics no relacionats amb la LME, com el dolor neuropàtic perifèric, el dolor neuropàtic diabètic o la neuropatia alcohòlica. A més, encara que es disposa d'estudis sobre el tractament amb polifenols després d'una LME, la majoria s'han centrat en la recuperació motora o en la regeneració de la medul·la espinal, fet que comporta una manca d'informació malgrat els resultats prometedors sobre els seus efectes en la modulació de processos fisiopatològics que també poden estar relacionats amb el desenvolupament del dolor neuropàtic. En aquest context, l'objectiu principal de la present tesi va ser estudiar els efectes preventius i analgèsics de dos extractes vegetals polifenòlics en el desenvolupament de DNC induït per LME en ratolins i comparar-ho amb els efectes de l'epigalocatequina-3-galat (EGCG), un dels polifenols més estudiats en el camp del dolor neuropàtic. Concretament, els dos extractes polifenòlics es van obtenir a partir de material residual de raïm (GSE) i de cafè torrat descafeïnat en pols (CE), elegits per ser fonts naturals riques en polifenols. Després de la lesió, es van avaluar els efectes del tractament repetit amb EGCG, GSE i CE sobre els comportaments de

dolor neuropàtic durant la fase aguda, intermèdia i crònica de la lesió. A més, es va analitzar la gliosi i l'expressió de biomarcadors relacionats amb la sensibilització central tant a la medul·la espinal com a les estructures supraespinals implicades en el processament del dolor. D'una banda, l'administració d'EGCG durant la primera setmana després de la LME va atenuar el desenvolupament d'al·lodínia mecànica i hiperalgèsia tèrmica, acompanyada de l'atenuació de la gliosi a la medul·la espinal durant la fase aguda. Tanmateix, totes les dosis provades d'EGCG van donar lloc a una pèrdua de pes significativa dels ratolins al llarg del període experimental, indicant així que la toxicitat sistèmica podria estar associada a aquest tractament. Per contra, tant l'administració preventiva de GSE com de CE durant la primera setmana posterior a la LME va donar lloc a l'atenuació del desenvolupament de l'al·lodínia mecànica i la hiperalgèsia tèrmica durant la fase aguda sense pèrdua de pes ni augment dels biomarcadors sèrics d'hepatotoxicitat o nefrotoxicitat. Els efectes antinociceptius dels dos extractes polifenòlics es van associar a la prevenció tant de la gliosi com de la regulació a l'alça dels algògens relacionats amb la sensibilització central (pERK, CX3CL1, CX3CR1 i CCR2) a la medul·la espinal. A més, mentre que els tractaments amb GSE i CE van modular la senyalització de CX3CL1/CX3CR1 i van atenuar l'astrogliosi a les estructures supraespinals del còrtex cingulat anterior (CCA) i la substància grisa periaqüeductal (SGPA), així com la microgliosi al CCA dels ratolins lesionats, aquests tractaments van induir l'activació microglial a la SGPA, probablement caracteritzada per un augment de les cèl·lules de la micròglia que presentaven un fenotip antiinflamatori M2. D'altra banda, mentre que l'administració repetida d'EGCG durant la tercera setmana després de la LME no va modular ni l'al·lodínia mecànica ni la hiperalgèsia tèrmica durant la fase intermèdia de la lesió, la mateixa pauta d'administració amb GSE o CE va modular aquestes respostes reflexes del dolor sense efectes associats de toxicitat sistèmica, hepatotòxics o nefrotòxics. A més, l'administració de CE va modular el comportament de tipus depressiu detectat als ratolins lesionats de la medul·la espinal a la fase intermèdia de la lesió. Finalment, l'administració repetida de GSE i CE durant la primera, tercera i sisena setmana després de la LME va prevenir el desenvolupament d'hiperalgèsia tèrmica i al·lodínia mecànica fins a la fase crònica de la lesió, sense efectes associats de toxicitat sistèmica, hepatotòxica o nefrotòxica. A més, els tractaments amb GSE i CE també van modular les alteracions afectivo-motivacionals (anhedonia, depressió i ansietat) i la disminució de la interacció social desenvolupades en els animals amb LME crònica. Els efectes antinociceptius de GSE i CE es van associar no només amb la modulació de la microgliosi a la medul·la espinal, sinó també amb la modulació de l'astrogliosi i la senyalització de CCL2/CCR2 i CX3CL1/CX3CR1 a les estructures supraespinals del CCA, amígdala, SGPA dorsal i ventral, i bulb rostral ventromedial, així com la microgliosi a la SGPA ventral. En conjunt, els resultats d'aquesta tesi suggereixen que una barreja de polifenols presents en extractes naturals pot ser una estratègia farmacològica adequada per prevenir o atenuar el desenvolupament del dolor neuropàtic induït per la LME, modulant no només les respostes reflexives del dolor (més relacionades amb la dimensió sensorial-discriminativa del dolor) sinó també les respostes no reflexives del dolor (incloses en la dimensió afectiva-motivacional del dolor). Aquests

compostos no només exerceixen els seus efectes al lloc de la lesió modulant la gliosi i l'expressió de biomarcadors relacionats amb la sensibilització central, sinó també en estructures supraespinals estretament relacionades amb l'expressió i la modulació del dolor neuropàtic central.

RESUMEN

El dolor neuropático central (DNC) después de una lesión medular (LME) se desarrolla en más de la mitad de los pacientes, y alrededor de un tercio de ellos declara el dolor como intenso. El DNC después de una LME se ha asociado a deficiencias en diversas áreas, como el funcionamiento físico y la movilidad, el funcionamiento mental y cognitivo, el funcionamiento social y la reintegración en la comunidad, el sueño, el empleo y la calidad de vida. Además, las personas que experimentaron dolor después de una LME muestran una peor salud, una menor satisfacción con la vida y un mayor riesgo de sufrir depresión. Para hacer frente a este problema de salud, se han utilizado varios tratamientos farmacológicos para aliviar el DNC. Sin embargo, los tratamientos actuales suelen ser ineficaces porque sólo se dirigen a uno o dos de los diversos mecanismos que componen el DNC. En la actualidad, con el objetivo de diseñar un enfoque terapéutico personalizado, el tratamiento consiste en un ensayo-error de diferentes estrategias, que incluyen antidepresivos, antiepilépticos, agonistas GABA, anestésicos locales, antagonistas NMDA, cannabinoides y opioides. Sin embargo, estos fármacos suelen ser inadecuados y sólo un tercio de los pacientes responde a ellos en comparación con el placebo. Además, la mejor estrategia farmacológica sólo permite reducir la intensidad del dolor en un 20-30%, y suele ir acompañada de graves efectos secundarios. Por lo tanto, dada la falta de tratamientos eficaces, es necesario desarrollar nuevas estrategias farmacológicas no sólo para el alivio del dolor neuropático sino también para la prevención de su cronificación. Entre las potenciales estrategias farmacológicas dirigidas a modular el dolor patológico podría destacarse el uso de polifenoles, ya que en la literatura científica se pueden encontrar evidencias preclínicas de sus efectos antinociceptivos. De entre las propiedades que pueden atribuirse a los polifenoles y que pueden explicar la modulación del dolor patológico destacan las actividades antioxidantes o de eliminación de radicales libres, inmunomoduladoras, neuroprotectoras, antiapoptóticas y reguladoras de la autofagia. Sin embargo, aunque varios estudios se han dirigido específicamente a dilucidar los efectos de los tratamientos polifenólicos en el desarrollo del dolor neuropático, la mayoría de ellos se han realizado en modelos preclínicos no relacionados con la LME, como el dolor neuropático periférico, el dolor neuropático diabético o la neuropatía alcohólica. Además, aunque se dispone de estudios sobre el tratamiento con polifenoles tras una LME, la mayoría de ellos se han centrado en la recuperación motora o en la regeneración de la médula espinal, lo que conlleva a una falta de información a pesar de los prometedores resultados sobre sus efectos en la modulación de procesos fisiopatológicos que también pueden estar relacionados con el desarrollo del dolor neuropático. En este contexto, el objetivo principal de la presente tesis fue estudiar los efectos preventivos y analgésicos de dos extractos vegetales polifenólicos en el desarrollo de DNC inducido por LME en ratones y compararlo con los efectos de la epigalocatequina-3-galato (EGCG), uno de los polifenoles más estudiados en el campo del dolor neuropático. Concretamente, los dos extractos polifenólicos se obtuvieron a partir de material residual de uva (GSE) y de polvo de café

descafeinado tostado (CE), elegidos por ser fuentes naturales ricas en polifenoles. Después de la lesión, se evaluaron los efectos del tratamiento repetido con EGCG, GSE y CE sobre los comportamientos de dolor neuropático durante la fase aguda, intermedia y crónica de la lesión. Además, se analizó la gliosis y la expresión de biomarcadores relacionados con la sensibilización central tanto en la médula espinal como en las estructuras supraespinales implicadas en el procesamiento del dolor. Por un lado, la administración de EGCG durante la primera semana después de la LME atenuó el desarrollo de alodinia mecánica e hiperalgesia térmica, acompañada de la atenuación de la gliosis en la médula espinal durante la fase aguda. Sin embargo, todas las dosis probadas de EGCG dieron lugar a una pérdida de peso significativa de los ratones a lo largo del período experimental, lo que indica que la toxicidad sistémica podría estar asociada a dicho tratamiento. Por el contrario, tanto la administración preventiva de GSE como de CE durante la primera semana posterior a la LME, dio lugar a la atenuación del desarrollo de la alodinia mecánica y la hiperalgesia térmica durante la fase aguda sin pérdida de peso ni aumento de los biomarcadores séricos de hepatotoxicidad o nefrotoxicidad. Los efectos antinociceptivos de ambos extractos polifenólicos se asociaron a la prevención tanto de la gliosis como de la regulación al alza de los algógenos relacionados con la sensibilización central (pERK, CX3CL1, CX3CR1 y CCR2) en la médula espinal. Además, mientras que los tratamientos con GSE y CE modularon la señalización de CX3CL1/CX3CR1 y atenuaron la astrogliosis en las estructuras supraespinales del córtex cíngulo anterior (CCA) y la sustancia gris periacueductal (SGPA), así como la microgliosis en el CCA de los ratones lesionados, dichos tratamientos induceron la activación microglial en la SGPA, probablemente caracterizada por un aumento de las células microgliales que presentaban un fenotipo antiinflamatorio M2. Por otro lado, mientras que la administración repetida de EGCG durante la tercera semana después de la LME no moduló ni la alodinia mecánica ni la hiperalgesia térmica durante la fase intermedia de la lesión, la misma pauta de administración con GSE o CE moduló estas respuestas de dolor reflejo sin efectos asociados de toxicidad sistémica, hepatotóxicos o nefrotóxicos. Además, la administración de CE moduló el comportamiento de tipo depresivo detectado en los ratones lesionados de la médula espinal en la fase intermedia de la lesión. Por último, la administración repetida de GSE y CE durante la primera, tercera y sexta semana después de la LME previno el desarrollo de hiperalgesia térmica y alodinia mecánica hasta la fase crónica de la lesión, sin efectos asociados de toxicidad sistémica, hepatotóxica o nefrotóxica. Además, los tratamientos con GSE y CE también modularon las alteraciones afectivo-motivacionales (anhedonia, depresión y ansiedad) y la disminución de la interacción social desarrolladas en los animales con LME crónica. Los efectos antinociceptivos de GSE y CE se asociaron no sólo con la modulación de la microgliosis en la médula espinal, sino también con la modulación de la astrogliosis y la señalización de CCL2/CCR2 y CX3CL1/CX3CR1 en las estructuras supraespinales del CCA, amígdala, SGPA dorsal y ventral, y bulbo rostral medioventral, así como la microgliosis en la SGPA ventral. En conjunto, los resultados de la presente tesis sugieren que una mezcla de polifenoles presentes en extractos naturales puede ser una estrategia farmacológica adecuada para prevenir o atenuar el desarrollo

del dolor neuropático inducido por la LME, modulando no sólo las respuestas de dolor reflexivo (más relacionadas con la dimensión sensorial-discriminativa del dolor) sino también las respuestas de dolor no reflexivo (incluidas en la dimensión afectiva-motivacional del dolor). Estos compuestos no sólo ejercen sus efectos en el lugar de la lesión modulando la gliosis y la expresión de biomarcadores relacionados con la sensibilización central, sino también en estructuras supraespinales estrechamente relacionadas con la expresión y modulación del dolor neuropático central.

CONTENTS

INTRODUCTION

I. INTRODUCTION

PART I. SPINAL CORD INJURY: DEFINITION, CLASSIFICATION AND PHYSIOPATHOLOGY

1. Spinal Cord Injury definition

Spinal cord injury (SCI) is defined as a damage to the spinal cord resulting from trauma, disease or degeneration, and it occurs when the axons of nerves running through the spinal cord are disrupted, leading a neurologic dysfunction (**Bennett et al., 2020**).

Depending on its aetiology, the SCI can be classified in traumatic and non-traumatic. Traumatic SCI occurs as a result of an external physical impact (for example, motor vehicle injury, fall, sports-related injury or violence), whereas non-traumatic SCI is by cause of an acute or chronic disease process, such as a tumour, infection or degenerative spinal column condition (**Fehlings et al., 2013**).

Epidemiological data on SCI are mostly reported by individual national or provincial databases, which generalize between countries difficult. National net wealth, median age of the specific population, lifestyle, and cultural aspects as well as different rates of violent crime and self-harm are some of the influential factors in both traumatic and non-traumatic SCI incidence. For example, traumatic SCI from land transport is high but relatively stable in developed countries, in contrast to developing countries where its proportion is increasing due to trends in transport mode (transition to motorised transport, regulatory challenges of traffic rules and poor infrastructure). On the other hand, traumatic SCI from low falls in the elderly or non-traumatic SCI derived of diseases are increasing in developed countries with ageing populations (**Lee et al., 2014**).

Nevertheless, the World Health Organisation (WHO) estimates an annual global incidence of SCI between 40 and 80 cases per million population. In other words, between 250 000 and 500 000 people become spinal cord injured every year and this incidence is increasing in both high-income and low middle-income countries (**Kang et al., 2017**). Nearly a half (47.3%) of all spinal cord injuries occur between the ages of 16 and 30, becoming the higher risk of SCI age group. Otherwise, significant sex-specific differences are evident in SCI incidence, whereas females are most at risk in adolescence (15-19 years of age) and older age (60+), males are most at risk in young adulthood (20-29 years of age) and older age (70+). Moreover, reported male-to-female ratios indicate at least 2:1 among adults, sometimes much higher (**WHO**).

About to 90% of the reported SCI cases are due to traumatic causes, though the proportion of non-traumatic SCI appears to be growing (**WHO**). Concretely, the incidence of traumatic SCI in Western Europe is 16 cases per million population (**Lee et al., 2014**).

Based on available evidence on the aetiology of traumatic spinal cord injury (TSCI) across WHO regions, vehicle crashes are the most recent leading cause of injury, closely followed by falls (***Fig.1.B***). Automobile crashes are the TSCI leading cause until age 45, while falls become the leading cause above age 60 (***Fig.1.A***). Furthermore, TSCI arises more commonly in males (80,4%) than in females (19,6%) (***Fig.1.C***).

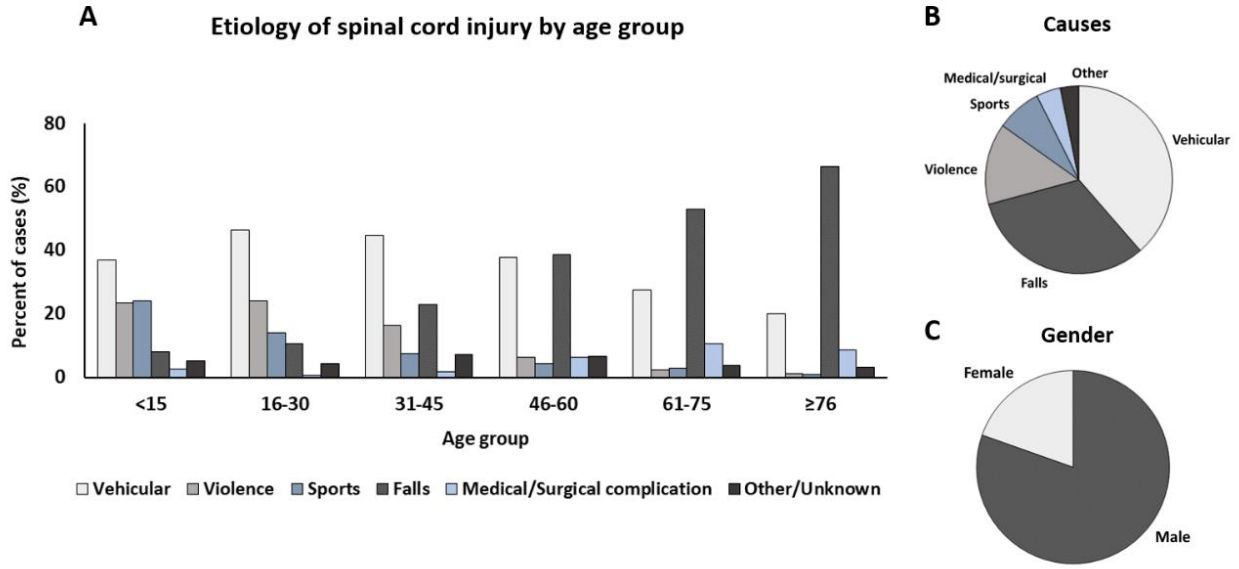


Figure 1. ***Etiology of Traumatic SCI. (A) Etiology by age group (B) TSCI causes and (C) gender distribution.*** Extracted from: Annual Statistical Report for the spinal cord Injury model system. National Spinal Cord Injury Statistical Center (NSCISC) 2019.

SCIs can have a devastating and enduring functional, psychological, and socioeconomic consequence for patients and their families. Although the clinical outcomes of SCI depend on the severity and location of the lesion, as well as availability of timely and quality medical care, loss of independence and persistently increased lifelong mortality rates are the hallmarks of SCI. The highest mortality risk take place during the first year after injury with 2 to 5 times more likely to die prematurely than average population (**WHO**). Concerning the TSCI, the highest mortality risk is placed during the first 24 hours in hospital and the first-year post injury. The most significant predictive factors of survival are patient age and indicators of injury severity, such as neurologic level and degree of injury completeness (**Krause et al., 1997**). Furthermore, SCI is associated with a secondary condition that can be debilitating or even life-threatening including respiratory complications, cardiovascular complications, urinary and bowel complications, spasticity, pain syndromes, pressure ulcers, osteoporosis, and bone fractures (**Sezer et al., 2015**).

Not surprisingly, patients with SCI have been shown a lower quality of life than general population (**Dijkers et al., 1997**). A higher level of education, the ability to walk without assistance, social support (as indicated by marriage or cohabitation) and employment are some of the aspects that were associated with higher quality of life (**Saadat et al., 2010; Jain et al., 2007; Siösteen et al., 1990; DeVivo et al., 1992**). On the other

hand, SCI-related morbidities such neuropathic pain, spasticity and bladder, bowel and sexual dysfunction were all associated with a lower quality of life (**Westgren et al., 1998**).

Both direct and indirect costs of SCI are significant in estimate the economic impact of SCI. Direct costs include rehabilitation and health services, personal assistance, transportation, and special diets. Indirect costs refer to loss of productivity due to premature death or disability, social isolation, and stress. The SCI costs are influenced to a large extent factors such the level and severity of the injury, with higher costs associated with higher injury levels (cervical SCI) and complete SCI compared to incomplete SCI. Direct costs are highest in the first year after injury and then decrease significantly over time whereas indirect costs are maintained throughout the patient's life and may exceed direct costs (**Dryden et al., 2005; Vaikuntam et al., 2019**). It is difficult to compare the cost estimates of SCI between countries due to different categories of direct and indirect costs are used and estimates of costs rely on different statistical techniques (**St. Andre et al., 2011; Sundance et al., 2004**). For this reason, it is difficult to find a global lifetime cost number per SCI patient. In the USA, lifetime cost for a hypothetical individual injured at age 25 was estimated to be between $1 and $3.5 million, $0.85 to $2.1 million if the individual is injured at 50 years of age depending on severity of injury (**Cao et al., 2011**). Actually, total direct costs of caring for individuals with spinal-cord injury exceed $7 billion per year in the USA (**DeVivo et al., 1997**). In Spain, the economic cost in 2007 associated with people injured with TSCI was between €92 million and 212 million according to the injury mechanism (**García-Altés et al., 2012**).

Therefore, SCI not only affects patients' physical, psychological health and social well-being, but also results in a heavy economic burden and multidimensional problem on families, communities, and health care systems.

2. Classification of Spinal Cord Injury

The International Standards for Neurological Classification of Spinal Cord Injury (ISNCSCI) were established by the American Spinal Injury Association (ASIA) Impairment Scale (**Ditunno, 1994**). The ASIA Impairment Scale (AIS) is an internationally recognized clinical tool for SCI classification based on the degree of neurological impairment and involves both sensory and motor examination to determine the sensory and motor levels for the right and left side, the overall neurological level of the injury and completeness of the injury (**Kirshblum, 2011**). It is worth mentioning that AIS has become widely used as an index of recovery after SCI (**Walters 1995a, 1995b, 1996, 1998**) and as an outcome measure for clinical trials (**Bracken 2000; Geisler 1991**).

The sensory examination is based on sensitivity to prototypical touch and soft pinpricks (pinprick) in the different corporal dermatomes, as well as in the preservation of deep anal pressure, while the motor

exploration consists of evaluation of certain muscle groups along with the implementation of specific movements based on the scale MRC (Medical Research Council) and capacity to anal contraction (***Fig.2.A***).

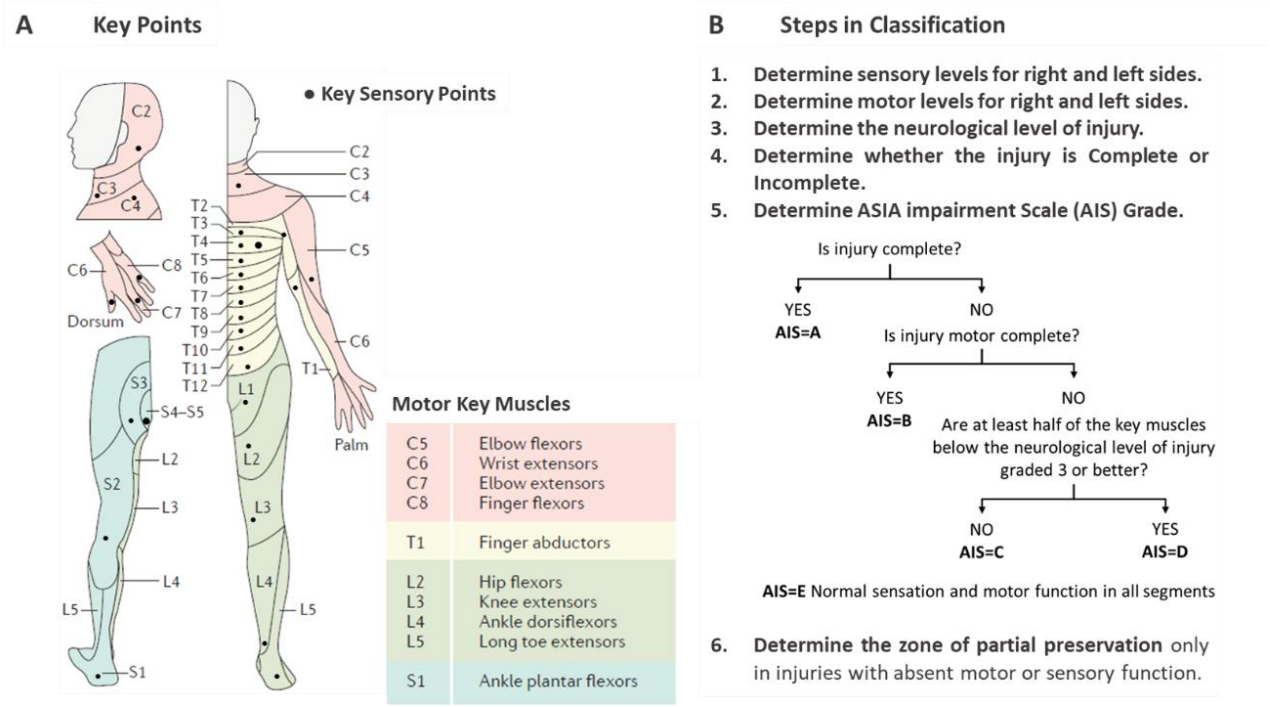


Figure 2. International standards for neurological classification of spinal cord injury. (A) *Key sensory points and motor key muscles described in the International Standards for Neurological Classification of Spinal Cord Injury.* ***(B)*** *Steps to determine the ASIA Impairment Scale (AIS) classification. Part (A) are adapted from* ***Ahuja et al., 2017****; and part (B) is extracted from the American Spinal Injury Association: International Standards for Neurological Classification of Spinal Cord Injury, revised 2019; Atlanta, GA.*

On the other hand, the neurological level refers to the most caudal segment with intact sensation and antigravity muscle function strength, if there is intact sensory and motor function rostrally respectively (**Maynard et al., 1997**). Accordingly, ASIA Impairment Scale classify the SCI from A to E. A complete SCI, AIS=A, is referred as no sensory or motor function preserved in the sacral segments S4-S5, AIS=B is sensory incomplete SCI, AIS=C and AIS=D refer to motor incomplete SCI, and AIS=E occurs when sensation and motor functions are graded as normal in all segments (***Fig.2.B***).

3. Physiopathology of Spinal Cord Injury

In order to understand the spinal and supraspinal mechanisms responsible for SCI pain it is important to acknowledge that associated with spinal injury is a cascade of biochemical, molecular, and anatomical changes that collectively have a devastating impact on the functional integrity of the spinal cord.

The physiopathology of SCI is best described as biphasic, divided into primary and secondary injury phases. The **primary injury phase** includes the initial mechanical injury that compromise the spinal cord integrity. This focal region of damage triggers a pathophysiological cascade comprising the **secondary injury phase**

which can be temporally subdivided into the immediate (≤2 hours), acute (≤48 hours), subacute (48 hours to 14 days), intermediate (14 days to 6 months) and chronic (≥6 months) phases (**Norenberg et al., 2004**) (***Fig.3***).

In murine models of animal experimentation, temporal changes in SCI are shorter than human models. Thus, the immediate and acute phases occupy approximately the first 2-3 weeks post-injury (**Taoka and Okajima, 1998**), the intermediate phase lasts between 3 to 6 weeks post-injury (**Young et al., 2002**) and the chronic phase begins at 6-8 weeks after the injury (**Arnold and Hagg, 2011**) (***Fig.3***).

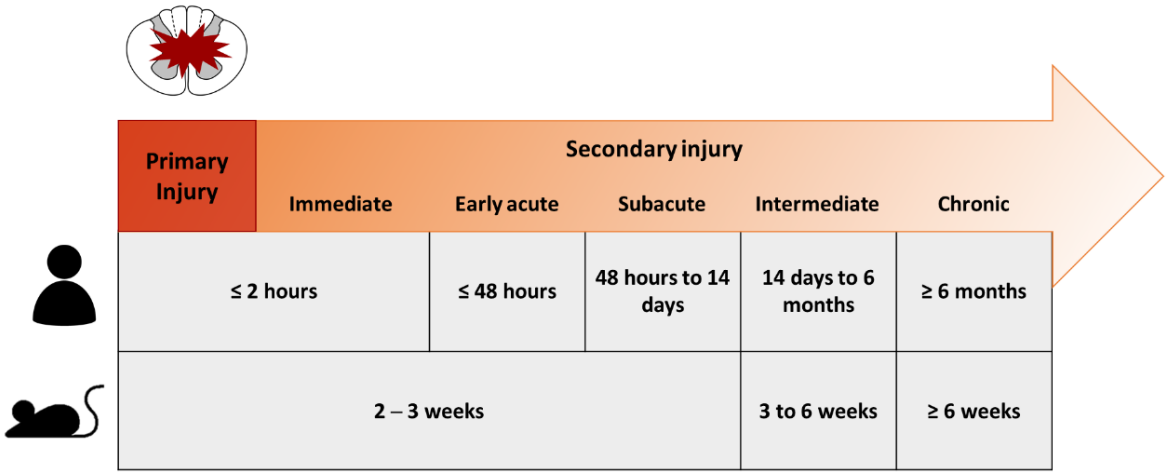


Figure 3. Timeline of SCI phases in humans and murine models. Based on **Norenberg et al., 2004; Arnold and Hagg, 2011**.

Primary injury phase

The primary injury phase involves the mechanical trauma that directly dislocate and/or fracture the vertebral column displacing bone fragments, disc materials, and/or ligaments bruise or tear into the spinal cord tissue. This focal region of damage disrupts axons, blood vessels and cell membranes, and compromises the blood–spinal cord barrier (**Rowland et al., 2008**). Following the initial impact or injury, there is immediate mechanical damage to neural and other soft tissue, including the endothelial cells of the vasculature. Thus, necrosis, or cell death, is the instantaneous result of these mechanical and ischaemic insults. After the insult and for the next few minutes, the injured nerve cells respond with an injury-induced barrage of action potentials. This is accompanied by significant electrolyte shifts, primarily involving the monovalent and divalent cations Na^+ (intracellular concentrations increase), K^+ (extracellular concentrations increase) and Ca^{2+} (intracellular concentrations increase to toxic levels), which contribute to a failure of normal neuronal function and spinal shock, lasting about 24 hours and representing generalised failure of

the circuitry in the spinal neural network. In parallel, haemorrhage occurs, with localised oedema, loss of microcirculation through thrombosis, vasospasm and mechanical damage, and loss of autoregulation of the vasculature, all of which further aggravate the neural injury. The rupture of blood vessels leads to extravasation of plasma and formed elements, the release of plasma proteases in the medullary parenchyma and formed elements, mainly erythrocytes, which are easily haemolysed and release haemoglobin and iron. Disruption of axons leads to interruption of the propagation of action potentials along the ascending and descending medullary pathways, accompanied by destructuring of the white matter by necrosis of the oligodendrocytes. In addition, spinal cord compression occurs as a result of vertebral displacement, followed by oedema and subsequent fibrotic responses, further contributing to neural injury (**Hulsebosch, 2002; Alizadeh et al., 2019**). Together, these events immediately initiate a sustained secondary injury cascade, which involves vascular dysfunction, oedema, ischemia, excitotoxicity, electrolyte shifts, free radical production, inflammation, and restrained apoptotic cell death (**Rowland et al., 2008**), leading to further damage to the spinal cord and neurological dysfunction (**Ahuja et al., 2017**). Cell dysfunction and necrotic/apoptotic cell death are caused by cell permeabilization, pro-apoptotic signalling and ischaemic injury due to the destruction of the microvascular supply of the spinal cord within minutes of injury (**Choo et al., 2007; LaPlaca et al., 2007**).

As a rule of thumb, the kinetic and the duration of the compression are the main determinants of the severity of the traumatic SCI. Impact followed by persistent compression is the most frequently injury form, but other characteristic mechanisms may occur such as impact alone with transient compression, distraction, or laceration/transection (**Alizadeh et al., 2019**). Importantly, primary injury mechanisms rarely transect or fully disrupt the anatomical continuity of the spinal cord. The spared axons crossing the injury site is highly significant therapeutically because represents the neural substrate on which many emerging therapeutic strategies will act (**Rowland et al., 2008**). Hence, the initial mechanical injury activates a serial of pathophysiological processes, that include both biochemical and molecular cascade, resulting in a prolonged secondary injury phase.

Secondary injury phase

The secondary injury phase begins within minutes following the initial primary injury (**Oyinbo et al., 2011**) and is referred to a series of cellular, molecular, and biochemical phenomena that continue to self-destruct spinal cord tissue and impede neurological recovery (**Fehlings et al., 2013**). As mentioned above, the events of this secondary injury process can be divided into multiple contiguous phases: the immediate, acute, intermediate, and chronic stages of spinal cord injury.

- **Immediate phase**

The immediate phase begins at the time of injury, lasts about to 2 hours, and is dominated by the immediate results of the injurious event itself (**Norenberg et al., 2004**). The disruption of axons, the death of neurons and glia, and the accompanying phenomenon of spinal shock results in instantaneous **loss of function** at and below the level of the injury.

One of the first detectable pathological changes following the injury is a generalized **swelling** of the spinal cord often with **haemorrhaging** in the central gray matter. The spinal cord gray matter is more prone to be haemorrhagic than the white matter, because of its dense capillary network that is vulnerable to mechanical damage (**Maikos et al., 2007**). At this point, cells immediately begin undergoing necrotic death due to direct mechanical disruption of its membranes or **ischemia** resulting from vascular disruption (**Rowland et al., 2008**). In addition, within minutes after the injury, the spinal cord swells and occupies the whole spinal canal increasing its pressure beyond arterial blood pressure, causing **secondary ischemia**, one of the major triggers of secondary damages (**Cheriyan et al., 2014; Salegio et al., 2016**).

On the other hand, ischemia caused by vascular damage and swelling results in a hypoperfusion of the spinal tissue at the lesion epicentre. This hypoperfusion slows and eventually blocks the propagation of action potentials along axons, contributing therewith to the spinal shock (**McDonald et al., 2002**). Moreover, ischemia and necrosis trigger the recruitment of immune cells, microglial activation, and the secretion of the pro-inflammatory cytokines, such as tumour necrosis factor alpha (TNFα) and interleukin beta (IL-β) (**Donnelly et al., 2008; Pineau et al., 2007**). Ischemia and necrosis also lead to an accumulation of extracellular glutamate that initiates the processes of excitotoxicity (**Wrathall et al., 1996**).

Another phenomenon responsible of swelling is the formation of **oedemas**, that can be cytotoxic, ionic or vasogenic. Under normal physiological condition, the Na^+/K^+ ATPase and the Ca^{2+} ATPase use ATP-derived energy to maintain the intracellular compartment richer in K^+ and poorer in Na^+ and Cl^-, compared to the extracellular compartment. During ischemia, the maintenance of these ionic gradients will be compromised due to the ATP depletion. Consequently, an influx of Na^+, Cl^- and water in the intracellular compartment will take place, resulting in cell swelling and loss of cytoskeletal integrity, causing cell death (**Kahle et al., 2009**). In addition, during the glutamate excitotoxicity process, N-Methyl-D-aspartate (NMDA)-receptor will be stimulated further promoting the entrance of Na^+, Cl^-, and water in neurons and glia (**Strange et al., 1992**). All these events will lead to **cytotoxic oedema**. The ionic and the vasogenic oedemas result from an increased permeability of the blood–spinal cord barrier. The **ionic oedema** results from an increased trans-endothelial ion transport due to the depletion of ions and water from the interstitial space. The **vasogenic oedema** occurs when further endothelial dysfunction, in part related to cytotoxic swelling of endothelial

cells, leads to the formation of permeability pores in the blood–spinal cord barrier that allows for the passage of large plasma-derived molecules.

- **Early acute phase**

The early acute phase of SCI takes place from approximately 2 to 48 hours following injury. During this phase, haemorrhage is still ongoing, and oedema and inflammation increase (***Fig. 4. A***). Cells cannot maintain their homeostasis which leads to necrosis due to cell swelling. Impairment in ATP production, the disruption of organelles, and the lysis of the plasma membrane cause the release of the intracellular content in the extracellular space and the induction of local inflammatory process (**Rosenblum et al., 1997**). As a result of acute cell death by necrosis, secondary damage processes get induced during this period: glutamate-mediated excitotoxicity, ionic imbalance, free radical production and lipid peroxidation, and immune-associated neurotoxicity. These processes provoke additional axonal injury, cell death, and the propagation of the lesion within the surrounding spared tissue (**Couillard-Despres et al., 2017**). In addition, neutrophils infiltrate the spinal cord from the bloodstream within the first few hours after injury (**Dusart and Schwab, 1994**), which contribute to phagocytosis and clearance of tissue debris but also release inflammatory cytokines, proteases and free radicals that degrade the extracellular matrix, activate astrocytes and microglia, and initiate neuroinflammation (**Taoka et al., 1997**).

- **Sub-acute phase**

The sub-acute phase is considered to last from ≈2 days to 14 days after injury. This phase involves apoptosis, demyelination of surviving axons, Wallerian degeneration, axonal dieback, matrix remodelling, and evolution of a glial scar around the injury site (***Fig. 4. B***).

The increase in extracellular potassium as a consequence of necrotic astrocyte death leads to rapid activation of microglial cells, which synthesise and release cytokines, nitric oxide and prostaglandins, humoral elements that cause further activation of astrocytes and microglial cells far from the injury site. Moreover, lymphocyte and neutrophil infiltrates also contribute to glial reactivation by releasing inflammatory cytokines (TNF-alpha, IL1-beta, IL6) and oxygen free radicals (**Alizadeh et al., 2019**). Thus, activated microglia, in addition to other inflammatory cells such as activated macrophages, polymorphonuclear cells and lymphocytes, infiltrate the injury site and propagate the inflammatory response contributing to apoptosis of neurons and oligodendrocytes (**Ahuja et al., 2017**). Although phagocytic response can be beneficial in removing cell debris from the lesion area and may promote axon growth to some degree through the removal of growth-inhibitory components of myelin debris (**Tator and Fehlings, 1991; Donnelly and Popovich, 2008**) can also induce further damage through the release of cytotoxic products such as free radicals. These reactive oxygen species cause lipid peroxidation, DNA

oxidative damage and protein oxidation, which cause additional necrotic and delayed apoptotic cell death (**Hausmann et al., 2003**).

On the other hand, astrocytes react quickly to SCI by changes in their gene expression, hypertrophy, and process extension. Whereas following SCI astrocytes in the lesion core display cytotoxic oedema and undergo necrotic cell death, in the subacute injury phase, the astrocytes located at the periphery of the lesion start to proliferate and become hypertrophic. These reactive astrocytes initiate the formation of a **glial scar** physically sealing the injury site. This scar not only represents a physical barrier, but also a chemical impediment to axonal regeneration because contains inhibitory molecules that block axon growth.

The fibro-glial scar consists of various cells and extracellular matrix (ECM) developing in and around the lesion. Hypertrophic astrocytes are mostly present in lesion periphery whereas a heterogeneous mix of cells (including NG2+ glia/oligodendrocyte precursor cells, meningeal and/or vascular fibroblast, pericytes, ependymal cells, and phagocytic macrophages) majority occupies the lesion core (**Silver 2004**). These reactive astrocytes contribute to the inhibition of axonal regeneration by secreting chondroitin sulphate proteoglycans (CSPG). Briefly, CSPGs activate the small GTPase ras homolog gene family member A (RhoA) that, in turn, activates associated coiled coil containing protein kinase 2 (ROCK2), a kinase that regulates actin cytoskeletal dynamics. Eventually, the activation of ROCK2 produces the cessation of axonal growth by collapsing of the growth cone (**Huebner 2009**). CSPGs are not only well-known for their contribution to the inhibitory role in axonal regeneration (**Galtrey 2007; Cafferty 2007; Massey 2008**), but also for the inhibition of sprouting (**Barritt 2006**), conduction (**Hunanyan 2010**), and remyelination (**Karimi-Abdolrezaee 2012; Pendleton 2013**). In normal condition, basal levels of CSPGs are expressed in the CNS that play critical roles in neuronal guidance and synapse stabilization (**Dyck 2015**). Nevertheless, following SCI, CSPGs are robustly upregulated reaching their peak of expression at 2 weeks post-SCI and remain upregulated chronically (**Asher 2002; Buss 2009**). In addition, myelin-derived inhibitory molecules (e.g. NOGO, MAG, OmpG; **Yamaguchi, 2001**), semaphorins (**Pasterkamp and Verhaagen, 2001**) and netrins (**Timothy and Bargmann, 2001**) expressed by astrocytes and reactive microglia, also contribute to the inhibition of axonal growth.

Despite the mentioned prejudicial mechanisms, astrocytes and glial scar serve beneficial functions such as stabilization of the injured parenchyma by re-establishing its physical and chemical integrity and promoting the integrity of the blood–brain barrier (BBB), which has an important role in the resolution of the oedema and regulation of the infiltration of immune cells (**Rowland 2008**). Historically, the astrocytic scar has been regarded as the main impediment for axonal regeneration. However, it has been demonstrated that the scar has also a pro-regenerative function (**Rolls 2009**).

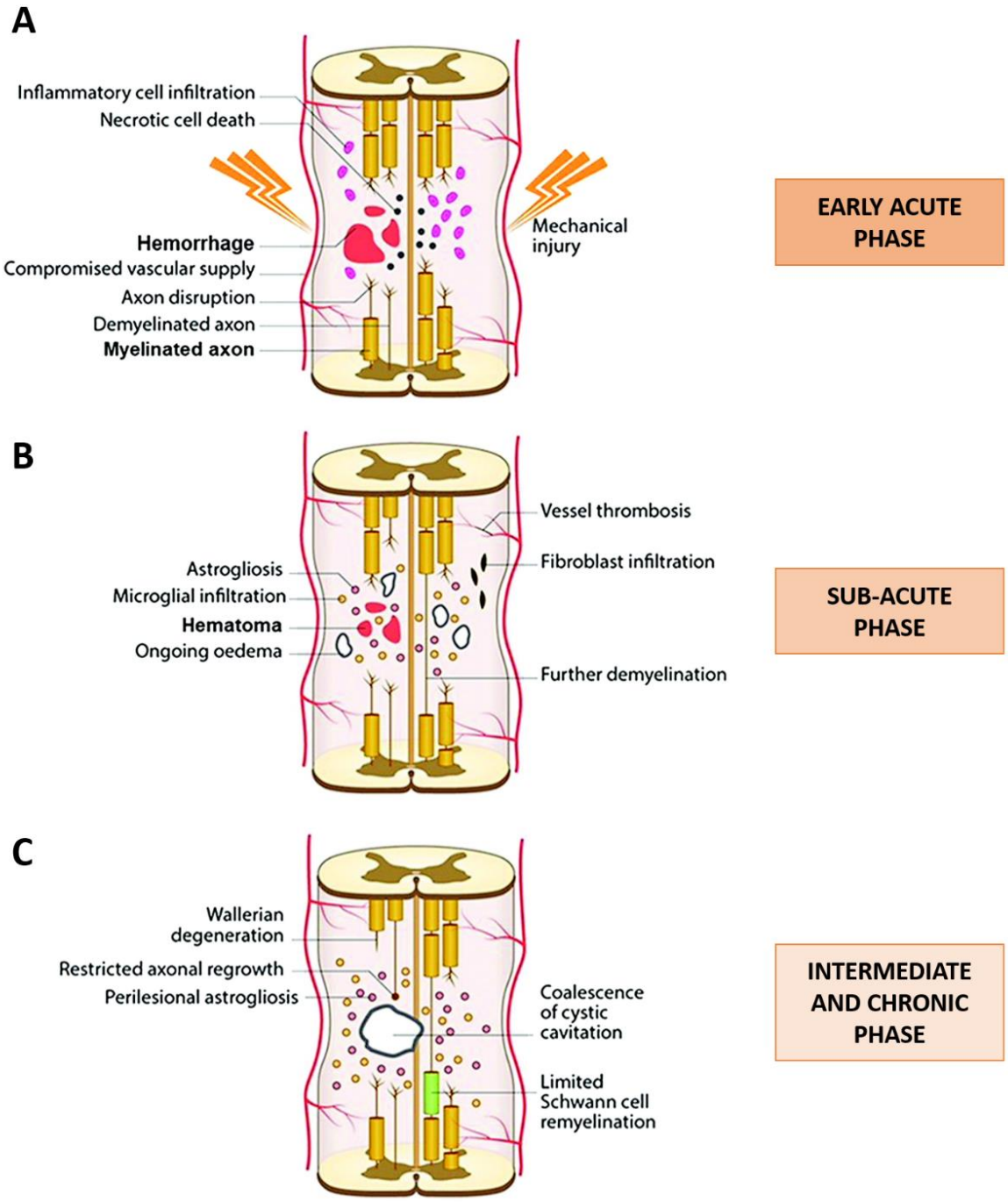


Figure 4. Development of pathophysiological changes following SCI. The **early acute phase** involves hemorrhage, edema, and proapoptotic factors. This leads to further loss of function, more than that resulting from the initial insult occurs due to injury to neurons and oligodendrocytes. Astrocyte infiltration and release of additional proinflammatory factors are seen while demyelinated and injured axons begin to die back **(A)**. In the **subacute phase**, further ischemia and cell death occurs owing to the excitotoxicity produced by the edema and to the persistent inflammatory cell infiltration. In addition, astrocytes proliferate and deposit extracellular matrix molecules into the perilesional area, forming the glial scar **(B)**. In the **intermediate** and **chronic** stages, microcystic cavities follow cell death. These cavities then coalesce forming barriers to regeneration in the chronic stage (>6 months). The final chronic stage scar, which is composed of a network of astrocytic processes and a dense fibrous deposit, acts as a physical and biochemical barrier to neurite outgrowth and cell migration **(C)**. Adapted from **Ashammakhi et al., 2019.**

- **Intermediate phase**

The intermediate phase of SCI takes place from approximately 2 weeks to 6 months following injury and is characterized by the continued maturation of the glial scar and regenerative axonal sprouting (***Fig. 4. C***) (**Hill 2001; Anjum et al., 2020**). While glial scar-reactive astrocytes secrete chondroitin sulphate proteoglycans (CSPG), which limit axonal growth (**Li et al., 2020**) and block axonal conduction (**Petrosyan et al., 2013**), microglial cells surrounding the glial scar secrete inflammatory cytokines and chemokines, ATP, NO, growth factors such as NGF, NT3, BDNF, bFGF, IGF-1 (**Kim and de Vellis, 2005; Gaudet and Fonken, 2018; Bellver-Landete et al., 2019**) and extracellular matrix metalloproteinases (e.g., MMP3 and MMP9; **Yenari et al., 2010**). All these components contribute to the glial cells' activation and/or spinal BBB disruption (**Cregg et al., 2014**). Disruption of the BBB leads to perivascular astrogliosis (**Alvarez et al., 2015**), as well as invasion of hematogenous cells that induce astrocyte reactivity and glial scar formation around the injury site (**Kawano et al., 2012**). In addition, glial scar-fibroblasts contribute to the production of fibronectin, collagen, and laminin in the extracellular matrix of the inured spinal cord (**Cregg et al., 2014**) and are a source of axon-repulsing molecules such as semaphorins that influence axonal regeneration following SCI (**Pasterkamp et al., 1999**).

This dynamic intermediate phase is also marked by attempts at remyelination, vascular reorganization, alterations in the composition of the extracellular matrix (ECM) and remodelling of neural circuits (**Kwon et al., 2004**). Although observable regenerative attempts are obviously insufficient in producing significant functional recovery in severe SCI, it is nonetheless encouraging evidence that regenerative potential does exist in the spinal cord.

- **Chronic phase**

The chronic phase begins approximately 6 months after SCI and extends for the rest of the patients' life. This final phase is characterized by the maturation and stabilization of the lesion consisted of continued scar formation, development of cysts or syrinxes and progressive axonal die-back (***Fig. 4. C***) (**Couillard-Despres et al., 2017**). The process of Wallerian degeneration remains active for many years for severed axons and their cell bodies to be fully removed (**Ehlers et al., 2004**). The lesion itself is marked by cystic cavitation and myelomalacia. The process of cavitation is the result of ongoing apoptotic neuronal and oligodendroglial cell death. Unfortunately, after establishment of the post-traumatic cyst, the lesion may not remain static. In fact, about one third of SCI patients develop syringomyelia (syrinx formation), which can cause **neuropathic pain** and further neurological deficits (**Krebs et al., 2016**). Syringomyelia-associated mechanisms responsible for the development of neuropathic pain include anatomical, neurochemical (excitotoxic) and inflammatory changes (**Rusbridge and Jeffery, 2008**). Syringomyelia typically starts centrally and dissects to the outer spinal cord, resulting in damage to the deeper layers while preserving

the superficial layers and thus causing an imbalance between the various processing pathways, due to death or dysfunction of specific cell types. For instance, neuronal loss in the dorsal horn, without affecting the superficial laminae, results in spontaneous (grooming) and evoked (mechanical and thermal allodynia) pain behaviours in rodents (**Yezierski et al., 1998**). This could be explained as deeper layers contain or are influenced by GABA and glycine inhibition, and therefore selective damage could lead to central disinhibition (**Cronin et al., 2004**). These anatomical syringomyelia-associated changes inevitably lead to changed expression of neurotransmitters or receptors. For instance, Todor et al. found a substantial increase in spinal cord levels of substance P which would likely alter modulation and perception of pain (**Todor et al., 2000**). Inflammatory changes in syringomyelia also play a role in persistent pain, mediated through glial cell production of cytokines and altered expression of nociceptive peptides (**DeLeo and Yezierski, 2001**).

Chronic pain is one of the most frequent secondary complications for individuals with SCI (**Sezer et al., 2015**). Actually, it has been reported that up to 80% of patients with SCI develop or experience clinically significant neuropathic pain (**Rekand et al., 2012**). Furthermore, around 40% of SCI patients develop persistent neuropathic pain and another 30% describes this pain as moderate to severe (**Siddall et al., 2003; Baastrup and Finnerup, 2012**). In order to understand the SCI-related pain and develop new effective treatments, it is vital to acknowledge the anatomy and biological mechanisms of physiological pain and its maladaptive responses after SCI.

PART II. PAIN AFTER SPINAL CORD INJURY

The prevalence of **pain** following SCI ranges from 65 to 80% of patients, depending on the method of data acquisition and type of pain, and around one-third of those affected reporting the pain to be severe (**Yezierski et al., 1996; Siddall et al., 2003; Dijkers et al., 2009**). Although loss of motor and sensory function is regarded as the most significant consequences of SCI, the condition of pain has a direct relationship with the ability of patients to regain an optimal level of activity and quality of life. More than half of SCI patients suffer from chronic pain severe enough to interfere with rehabilitation and activities of daily life, and therefore reduce quality of life (**Siddal et al., 2001**). In a longitudinal study of pain in the 5 years following SCI, patients revealed that dealing with pain associated with SCI is only surpassed by the decreased ability to walk or move, and loss of sexual function (**Siddal et al., 2003**). Therefore, pain is a highly prevalent and disabling condition among people with SCI. Available studies have associated pain after SCI with impairments in a variety of areas, including physical functioning and mobility (**Ballinger et al., 2000; Ravenscroft et al., 2000; Turner et al., 2001; Widerström-Noga et al., 2002; Gironda et al., 2004; Price et al., 2004; Gutierrez et al., 2007; Krause et al., 2007; Murray et al., 2007; Battalio et al., 2018; Rivers et al., 2018**), mental and cognitive functioning (**Jensen et al., 2005; Murray et al., 2007**), social functioning and community reintegration (**Störmer et al., 1997; Ravenscroft et al., 2000; Felipe-Cuervo and Yezierski, 2001; Donnelly and Eng, 2005; Kemp et al., 2011; Battalio et al., 2018**), sleep (**Ravenscroft et al., 2000; Felipe-Cuervo and Yezierski, 2001; Budh et al., 2005**), employment (**Ravenscroft et al., 2000; Valtonen et al., 2006; Middleton et al., 2007**), and quality of life (**Price et al., 2004; Kogos et al., 2005; Gutierrez et al., 2007; Middleton et al., 2007; Murray et al., 2007; Kemp et al., 2011; Rivers et al., 2018**). Moreover, persons who experimented pain after SCI showed poorer health (**Ballinger et al., 2000; Barrett et al., 2003**), lower satisfaction with life (**Putzke et al., 2002a, Putzke et al., 2002b; Budh and Österåker, 2007; Wollaars et al., 2007; Rivers et al., 2018; Jörgensen et al., 2021**) and greater risk of depression (**Haythornthwaite and Benrud-Larson, 2000; Rudy et al., 2003; Hiremath et al., 2017; Lim et al., 2017; Saurí et al., 2017; Graupensperger et al., 2018**).

Pain is a debilitating consequence of SCI based on the nature of the lesion, neurological structures damaged, and secondary pathophysiological changes of surviving tissue (**Yezierski et al., 2005**). The pain following SCI may be nociceptive, neuropathic or a combination of the two types. Nociceptive pain is caused by a damage to non-neural tissue either musculoskeletal or visceral, while neuropathic pain is induced by damage to or dysfunction of the nervous system (either above-level, at-level or below-level) (**IASP**) (***Table 1***).

Nociceptive pain is the most common type of pain following SCI (**Siddall et al., 2003**). However, neuropathic pain related to SCI tends to increase over time, and nociceptive pain decreases slightly, with both being present in about 60% of patients at one year after lesion (**Finnerup et al., 2014**). Concretely, neuropathic

pain is present in 40% to 50% of patients and it usually develops within the first year, tending to become chronic (**Budh et al., 2003; Siddall et al., 2003; Werhagen et al., 2004**).

Table 1. Proposed IASP classification of pain following SCI.

Broad type (Tier 1)	*Broad system (Tier 2)*	*Specific structures/pathology (Tier 3)*
Nociceptive	Musculoskeletal	Bone, joint, muscle trauma or inflammation Mechanical instability Muscle spasm Secondary overuse syndromes
	Visceral	Renal calculus, bowel, sphincter dysfunction, etc. Dysreflexic headache
Neuropathic	Above level	Compressive mononeuropathies Complex regional pain syndromes
	At level	Nerve root compression (including cauda equina) Syringomyelia Spinal cord trauma/ischemia
	Below level	Spinal cord trauma/ischemia

Extracted from **Siddall et al., 2009**

The International Association for the Study of Pain (IASP) defines pain as "An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (**Raja et al., 2020**). The capacity to experience pain has a protective role, warning of imminent or actual tissue damage and eliciting coordinated reflex and behavioural responses to keep such damage to a minimum. If tissue damage is unavoidable, a set of excitability changes in the peripheral and central nervous system establish a profound but reversible pain hypersensitivity in the inflamed and surrounding tissue. This process assists wound repair because any contact with the damaged part is avoided until healing has occurred (**Woolf et al., 1999**).

Pain can be classified following many different criteria such as anatomic, aetiologic, intensity, duration, and pathophysiological (**Orr et al., 2017**). According to the World Health Organization, the most relevant classification criteria in both clinical and research practice are based on its duration and on its pathophysiology. According to its time course and duration, pain can be classified into *acute* and *chronic*. **Acute pain** represents short-term pain that results from the activation of the nociceptive pathways due to an adverse thermal, chemical, or mechanical stimulus. It is considered an adaptive and protective process which motivates the individual to avoid tissue damage, to protect a damaged body part while it heals and to avoid similar experiences in the future. Thus, acute pain is felt immediately following injury with a severe intensity and short-lasting, and generally resolves over days to weeks once the stimulus has disappeared or

the lesion is healed and repaired (**Anwar et al., 2016; Basbaum et al., 2009**). Nevertheless, sometimes this acute pain can persist leading to chronic pain. The transition from acute to chronic pain is known as pain chronification. **Chronic pain** is defined as continuous or intermittent pain that persists despite stimulus absence and apparent healing and therefore it has loosed its warning and protective function. This pathological chronic pain is maladaptive resulting from an abnormal functioning of the nervous system. It represents long-term pain (3 months or longer) and is commonly associated with various diseases, including psychological conditions. Chronic pain must be considered as its own disease rather than as a symptom of another health problem. It may reduce quality of life, well-being, and ability to function over the long-term (**Basbaum et al., 2009**).

On the other hand, the pathophysiological pain classification represents the pathophysiological mechanism of injury to the body resulting in pain. Thus, the two major physiologic pathways are *nociceptive* (also known as *physiologic pain*) and *neuropathic* (usually referred as *pathological pain*) (***Fig. 5***). Nociceptive pain is described as "pain that arises from actual or threatened damage to non-neural tissue and is due to the activation of nociceptors" (IASP). It represents the sensation associated with the detection of potentially tissue-damaging noxious stimuli and therefore has both protective and adaptative role (**Woolf et al., 2010**). In contrast to neuropathic pain, nociceptive pain occurs with a normally functioning of the somatosensory nervous system. *Neuropathic pain* is defined as "pain caused by a lesion or disease of the somatosensory nervous system" (**IASP**). This type of pathological pain is not protective but maladaptive and results from an abnormal functioning of the nervous system (**Woolf et al., 2010**). Neuropathic pain is a clinical description (and not a diagnosis) which requires a demonstrable lesion or a disease that satisfies established neurological diagnostic criteria. Hence, any process that causes demonstrable damage to nervous system, such as metabolic, traumatic, infectious, ischaemic, toxic or immune-mediated pathological conditions, may trigger neuropathic pain development (**Baron et al., 2010; Bouhassira et al., 2019**). Depending on the location of the lesion or disease, neuropathic pain can be divided into peripheral (when damage is located in the peripheral somatosensory nervous system) and central (when injury affects the central somatosensory nervous system) (**IASP**).

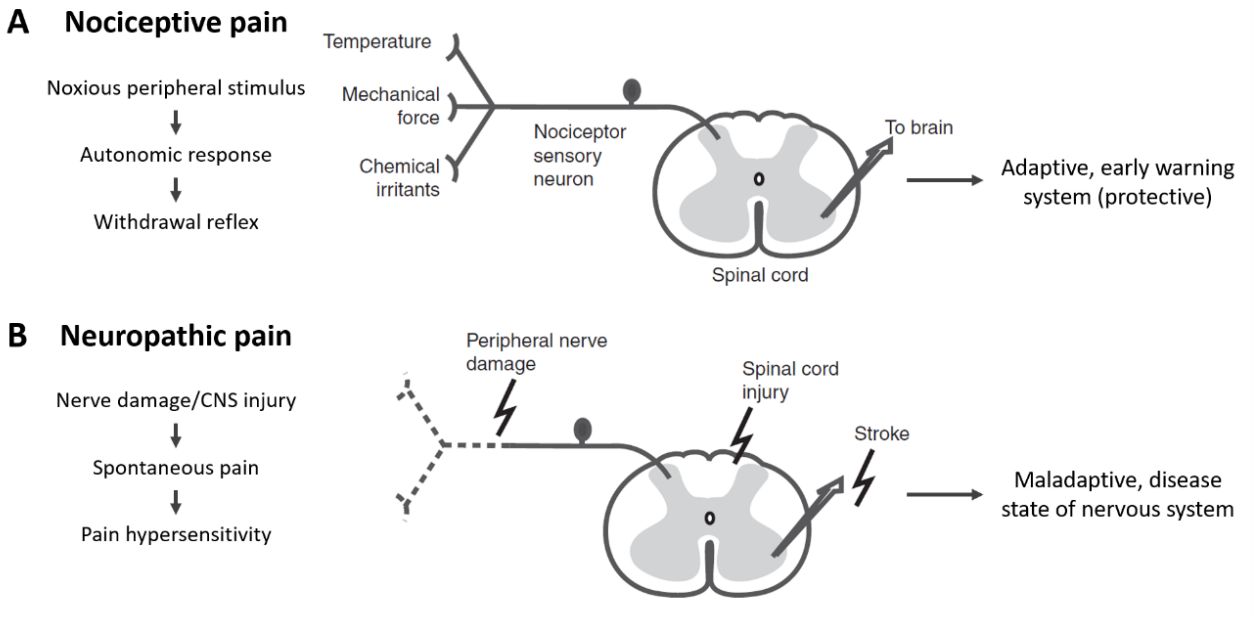


Figure 5. Pain classification depending on its pathophysiology. (A) Nociceptive pain represents the sensation associated with the detection of potentially tissue-damaging noxious stimuli and is protective. (B) Neuropathic pain is a disease state caused by damage to the peripheral or central somatosensory nervous system. Adapted from Vacanti et al. 2011. Essential Clinical Anesthesia, Cambridge University Press, p. 883.

1. Nociceptive pain

Nociceptive pain can be defined as a distressing sensation, as well as an emotional experience, linked to actual or potential tissue damage, with the sole purpose of notifying the body's defence mechanism to react towards a stimulus in order to avoid further tissue damages (**Yam et al., 2018**).

Nociception is the neural process of encoding noxious stimuli, which can be a mechanical, chemical, or thermal stimulus. These noxious stimuli can cause a tissue lesion with the further appearance of algogens in the injury site and surrounding area. These algogens are pronociceptive inflammatory molecules such as ions and small chemicals which can be released by damaged cells (e.g. K+, prostaglandins, ATP, leukotrienes), platelets (e.g. serotonin), plasma (e.g. bradykinin), mast cells (e.g. histamine) and primary nerve afferents (e.g. substance P, CGRP) (**Marchand et al., 2008; Belmonte et al., 2009**) (***Fig.6***).

These noxious stimuli can be detected by *nociceptors* defined as high-threshold sensory receptors of the peripheral somatosensory nervous system that are capable of transducing and encoding noxious stimuli (**IASP**). The capacity of the nociceptors to detect stimuli is based on the presence of specialized signalling molecules in their membranes that convert the stimulus energy into a conformational change, leading to an alteration in ionic permeability and depolarization of the nerve terminal (**Babos and Wisnoff, 2013**).

These molecules can be ion channels that are directly gated by the stimulus or by intracellular messenger systems activated by a variety of chemical substances (**Belmonte 2009**). Relevant examples of ion channels and ionotropic receptors present in nociceptor fibers include members of the TRP-family (TRPV1, TRPV2, TRPV3, TRPM8), acid-sensing ion channels (ASICs), isoforms of the voltage-gated Na^+ channel (Nav1.8 and Nav1.9), and K^+ channels (TREK-1, TRAAK) (**Caterina et al., 1997; Knowlton et al., 2010; Bautista et al., 2006; Yu et al., 2003; Bayliss et al., 2008; Noel et al., 2009**) (***Fig.6***).

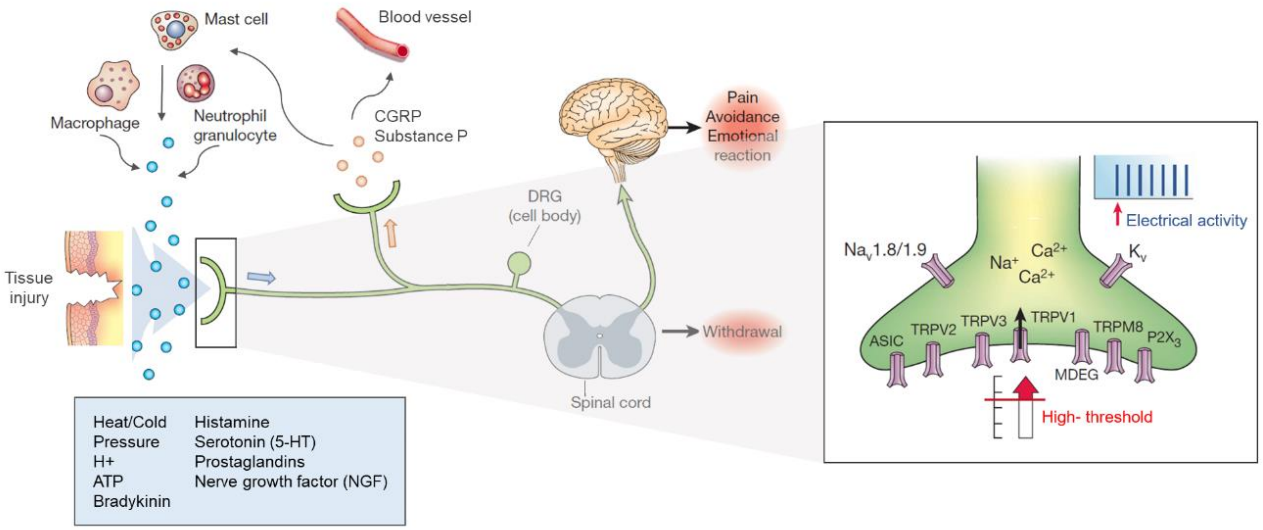


Figure 6. Activation of nociceptors in nociceptive pain. The molecular complexity of the primary afferent nociceptor is illustrated by its response to algogens released at the site of tissue injury. Some of the main pronociceptive inflammatory molecules are indicated in the blue box. Each of these factors excite the terminals of the nociceptor by interacting with cell-surface receptors expressed by these neurons. Nociceptive pain is produced under physiological conditions only by noxious stimuli acting on high-threshold nociceptors. Noxious stimuli are transduced into electrical activity at the peripheral terminals of nociceptors by specific receptors or ion channels sensitive to thermal, chemical, and mechanical stimuli. This activity is conducted to the spinal cord dorsal horn and, after transmission in central pathways, to the cortex, where the sensation of pain is experienced. Activation of the nociceptor also initiates the process of neurogenic inflammation in which nociceptor release neurotransmitters, notably substance P and CGRP, from the peripheral terminal that induce vasodilation, plasma extravasation and the activation of mast cells and neutrophils, contributing to the increase of peripheral pronociceptive mediators. Adapted from **Scholz and Woolf, 2002.**

All these receptors are in the nociceptive fibres, a primary sensory neuron that are highly specialized in respond to pain stimuli and convert them into nerve impulses. Briefly, the activation of voltage-gated sodium channels (e.g., Na_V 1.8/1.9) allows an influx of Na^+ and thus amplifies the transient receptor potentials, reaching depolarization level sufficient to trigger an action potential. Conversely, these transient potentials can also be blocked by potassium channels (K_v), which induce hyperpolarization (**Dubin and Patapoutian, 2010**).

These primary sensory neurons, also known as afferent fibres or first order neurons, are responsible to send the peripheral sensory information to the Dorsal Root Ganglia (DRG) where they have their soma. These neurons are considered pseudo-unipolar meaning that a single axon emanates from the cell body and

bifurcates, with one branch projecting to the periphery and the other projecting to the CNS, where make synapse with the second-order neurons located in the spinal cord dorsal horn (**Yasko et al., 2018**). Most of these fibres are polymodal, meaning that can respond to different modalities, including mechanical, thermal, and chemical stimulation (**Meyer et al., 2006**).

The activation of the nociceptor not only transmits afferent messages to the spinal cord dorsal horn, but also initiates the process of **neurogenic inflammation**. This is an efferent function of the nociceptor whereby can release peptides and neurotransmitters such substance P, calcitonin-gene-related peptide (CGRP) and ATP from their peripheral terminals. These substances induce vasodilation and plasma extravasation (leakage of proteins and fluid from postcapillary venules), as well as activation of many non-neuronal cells, including mast cells and neutrophils (***Fig. 6***). Hence, nociception transduction is a high complex process due to is not limited to the nociceptor but also comprises interactions between nociceptors and neighbouring cells including resident cells (keratinocytes and mast cells) and migrating immune cells (neutrophils and lymphocytes) (**Julius et al., 2001**) (***Fig.6***).

The main groups of primary afferent sensory nerve fibres are classified according to their physical characteristics and conduction velocity: Aβ fibres, Aδ fibres and C fibres. Nociceptive information is mainly transmitted by the Aδ fibres and the C fibres.

Aδ fibres have a medium diameter cell bodies and a thinly myelinated axon (1-6 μm) with a fast conduction velocity (5-40 m/s) (***Fig.7.A***). Threshold for stimulation is high, initiating the reflex response and producing an acute, sharp, well-localized and transient pain, also called first pain (***Fig.7.B***). C fibres have a small diameter cell bodies and unmyelinated axons (0.2-1.5 μm) with a slow conduction (0.5-2 m/s) (***Fig.7.A***). They have a high threshold stimulation and transmit a diffuse, poorly localized, and delayed pain sensation, also called second pain (***Fig.7.B***). These slow-conducting fibres represent three quarters of the sensory afferent input and they recover from fatigue more slowly than those of Aδ nociceptors, producing a longer-lasting pain. Most of C fibres are classified as polymodal nociceptors, that is, they are sensitive to thermal, mechanical, and chemical stimuli. There are a subgroup of C fibres that are called “sleeping” or “silent” fibres due to their lack of response following mechanical or thermal stimuli. However, under inflammation conditions, these fibres respond to these stimuli in the process of sensitization. Aβ fibres have a large diameter cell bodies and a considerable myelinated axon (6-12 μm) which allows a rapid information transmission (35-90 m/s) (***Fig.7.A***). They are mainly involved in detect innocuous stimuli such as vibration, movement, or light touch and therefore they normally do not respond to noxious stimuli. However, under sensitization condition these fibres can promote nociceptive transmission. Besides conducting the non-nociceptive signal in normal condition, the stimulation of Aβ fibres recruits inhibitory interneurons in the

spinal cord dorsal horn, which inhibit nociceptive input in a phenomenon called the Gate Control Theory (**Melzack and Wall, 1965; Price et al., 1999; Julius and Basbaum, 2001; Marchand et al., 2008**).

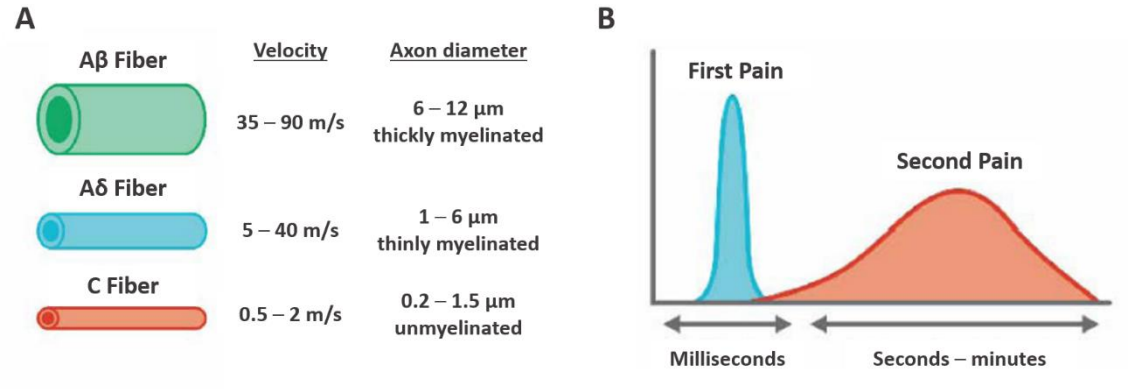


Figure 7. Classification of primary afferents according to their conduction velocity, axon diameter and degree of myelination. **(A)** The first order neurons include large-diameter Aβ myelinated fibers, medium diameter Aδ fibers, and small diameter unmyelinated C-fibers. **(B)** Conduction velocity is related to degree of myelination. Aδ fibers are responsible for the immediate-fast *first pain* that occurs within milliseconds, and C-fibers mediate the *second pain* due to its slow conduction velocity. Adapted from **Yasko et al., 2018**.

Based on their both gene and protein expression, C fibres can be classified into two different categories: peptidergic and non-peptidergic. Peptidergic C fibres releases one or both of the neuropeptides substance P and calcitonin gene related-peptide (CGRP). They also express TrKA neurotrophin in their membrane, the high affinity receptor for nerve growth factor (NFG). The non-peptidergic C fibres do not release any peptides and express the c-Ret neurotrophin receptor which is targeted by glial-derived neurotrophic factor (GDNF). Moreover, a large percentatge of non-peptidergic C fibres also binds to isolectin B4 (IB4) and expresses G protein-coupled receptors of the Mrg family, as well as the purinergic $P2X_3$ receptor on their cell membranes. Both peptidergic and non-peptidergic C fibres are excitatory and have glutamate and ATP in their presynaptic vesicles in the spinal cord branch (**Emery and Ernfors 2018; Dong et al., 2001; Basbaum et al, 2009; Ottestad et al., 2013**).

These primary afferent nerve fibres (Aβ, Aδ and C fibres) project to the spinal cord dorsal horn, which is organized into anatomically and electrophysiological distinct laminae (Rexed laminae) (**Basbaum et al., 2000**) (***Fig. 8***). Thus, each dorsal horn lamina has a distinctive pattern of primary afferent input. Anatomically, Aβ fibres project to deeper lamina III, IV and V; Aδ afferents project to lamina I and V; and C fibres project to the superficial lamina I and II. Concretely, peptidergic C neurons terminates within lamina I and the outer region of lamina II, and the non-peptidergic C fibres terminate in the mid-region of lamina II. Dorsal horn laminae are further organized by electrophysiological properties. Thus, the most ventral portion of lamina II is composed largely of excitatory interneurons that express the gamma isoform of

protein kinase C (PKCγ⁺), which is targeted predominantly by myelinated non-nociceptive afferents (**Neumann et al., 2008**).

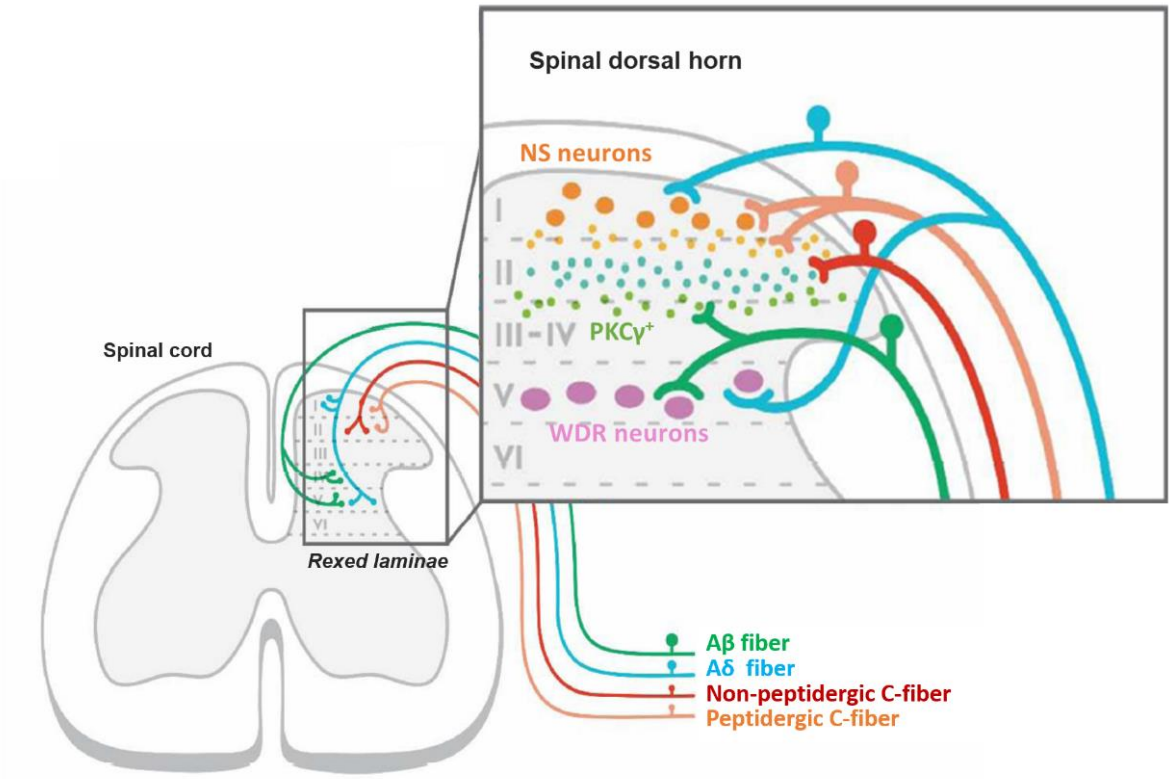


Figure 8. Primary afferent connections in the spinal dorsal horn. Spinal dorsal horn is organized into distinct laminae (Rexed laminae). The superficial laminae (I-II) mainly contain nociceptive-specific (NS) neurons (orange) that receive input from C and Aδ fibres. The deep laminae (III-VI) contain wide dynamic range (WDR) neurons (purple) that receive input from Aδ and Aβ fibres. Aβ fibres also contact with PKCγ⁺ interneurons (green), located in the most ventral part of lamina II. Adapted from **Yasko 2018**.

Hence, afferent fibres terminate in the dorsal horn of the spinal cord where they synapse with interneurons and projection neurons. Actually, afferent information to dorsal horn is processed through a complex neuronal network built by primary afferents, inhibitory and excitatory interneurons, projection neurons, and descending neurons that originate in the brainstem (***Fig. 9***). However, the major neuronal output to the supraspinal structures is carried by the projection neurons (**Ottestad et al., 2013**). These second order neurons can be divided into two categories, depending on the afferent input from the first order neuron: the nociceptive specific (NS) neurons and the wide dynamic range (WDR) neurons (**Meyer et al., 2006**). The NS neurons exclusively transmit noxious signals and are mostly located in laminae I and II. They can be recruited by Aδ fibres or by a combination of Aδ and C fibres. On the other hand, WDR neurons respond to a broad range of stimulus intensities, ranging from innocuous to nociceptive. They are located in the deep dorsal horn (laminae III-V) although the lamina III WDR neurons have long dorsally directed dendrites that penetrate the laminae I and II, and lamina V WDR neurons extend their dendrites to superficial lamina II.

Consequently, WDR neurons can receive inputs from Aβ, Aδ and C fibres. Finally, the non-nociceptive neurons respond to innocuous stimuli and are principally located in laminae I, II, III and IV.

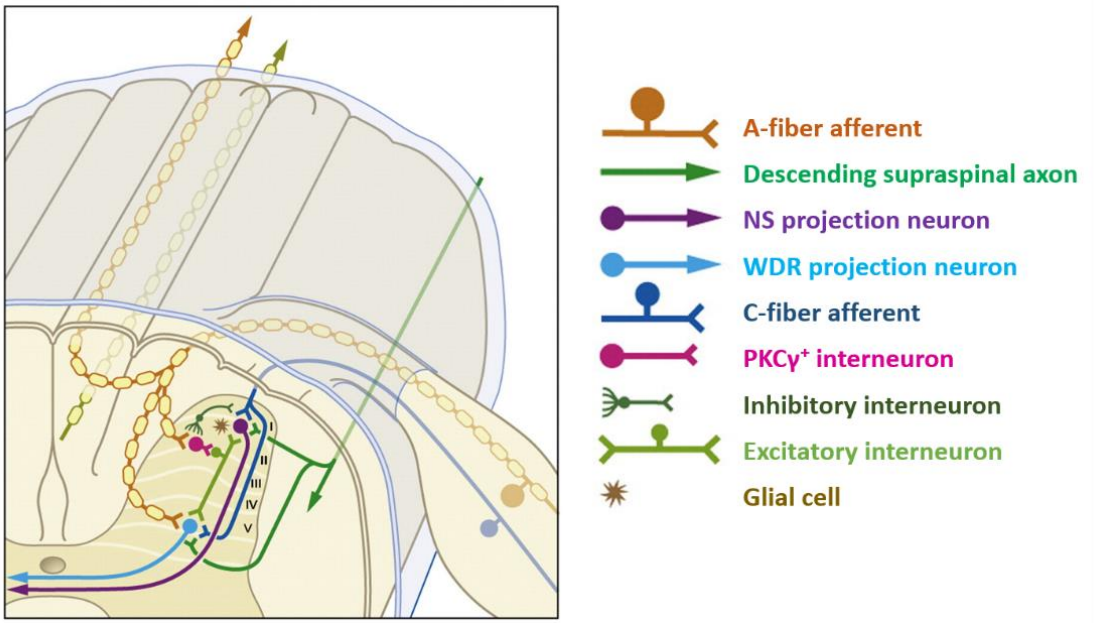


Figure 9. The spinal dorsal horn nociceptive network. Projection neurons (NS and WDR neurons) receive input from primary afferent neurons (Aβ, Aδ and C fibers), descending modulatory pathways (via inhibitory and/or excitatory effects) and spinal interneurons (both of excitatory and inhibitory nature). Finally, glial cells also actively participate in spinal dorsal horn nociceptive network not only expressing receptors for neurotransmitters but also containing synaptic vesicles with neurotransmitters. Adapted from **Berger et al., 2011**.

Dorsal horn interneurons also have a critical role in the modulation of ascending sensory information, specifically in the control of excitability at the segmental level. In all laminae, interneurons make up the great majority of the neuronal population. (**Todd et al., 2010**). There are two types of interneurons depending on the neurotransmitter that they release: excitatory and inhibitory. The main neurotransmitter of excitatory interneurons is glutamate, while γ-aminobutyric acid (GABA) and glycine are the major transmitters of inhibitory interneurons (**Labrakakis et al., 2009**).

Primary afferent neurons are excitatory and use glutamate as their principal fast neurotransmitter. Glutamate can bind to several receptors on post-synaptic neurons in the dorsal horn, including NMDA, AMPA and mGluR. At basal states, NMDA receptor is blocked by a magnesium ion (Mg^{2+}) which can only be removed when the membrane potential is sufficiently depolarised (**Mayer et al., 1984**). Thus, after the depolarization of the first order neurons, continued release of glutamate and neuropeptides such as SP, CGRP and BDNF in the synaptic cleft take place. The binding of these neurotransmitters with their specific receptors (AMPAR and mGluR for glutamate, NK1R for SP, CGRPR for CGRP and TrkB for BDNF) increases the postsynaptic excitability of the second order neuron, a phenomenon known as 'wind-up' (**Mendell and Wall, 1965**). This gradual increase in membrane potential allows a temporal and a spatial summation of the

excitatory inputs onto the nociceptive postsynaptic neuron, leading to sufficient membrane depolarisation to remove the Mg^{2+} from NMDAR. Consequently, this channel is activated, resulting in a rapid increase of Ca^{2+} ion influx (**Davies and Lodge, 1987; Dickenson and Sullivan, 1987**). All this electrical charge passing through these channels changes the second order neuron membrane potential, generating an action potential which allows the information transmission to the third order neurons (***Fig. 10***).

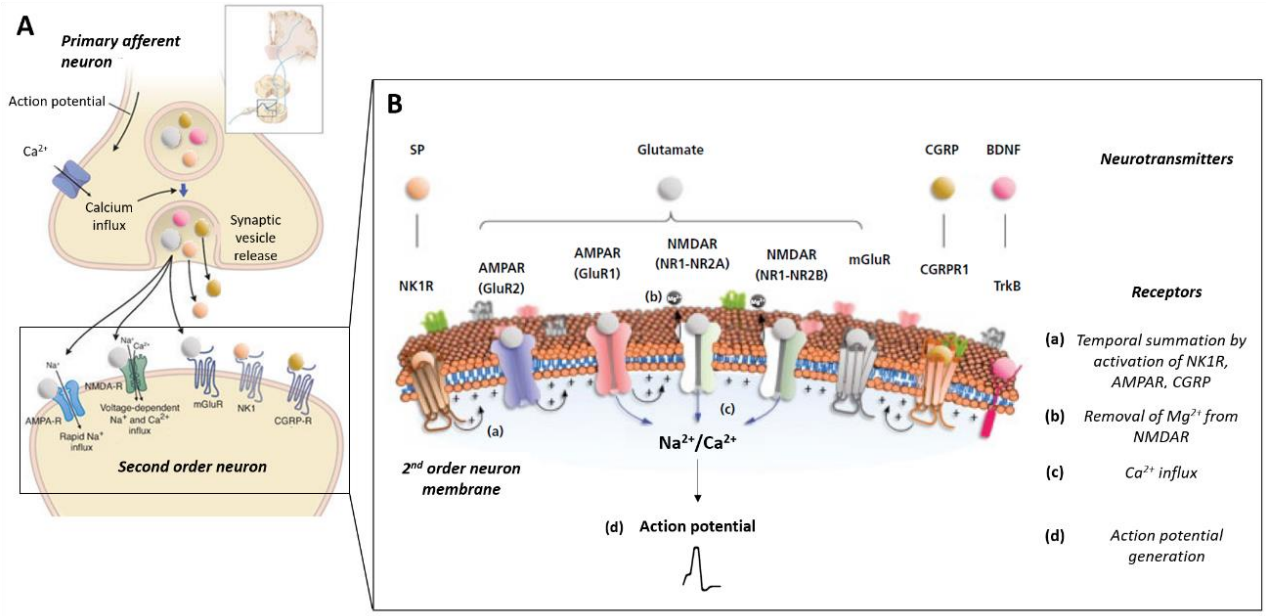


Figure 10. Neurotransmission in the spinal dorsal horn. **(A)** Synapse between primary afferent neuron and second order neuron. After nociceptive input, presynaptic voltage gated calcium channels are activated leading to Ca^{+2} influx and subsequent vesicle release in the synaptic cleft. **(B)** The released neurotransmitters (i.e., glutamate, SP, CGRP and BDNF) act on their respective postsynaptic receptors. All these will depolarize the second order neuron, if sufficient producing action potential, resulting in the nociceptive information transmission. Modified from **Latrémolière et al., 2016**.

These second-order neurons then transmit pain information to different supraspinal structures through three distinct groups of long tracts: spinothalamic, spino-reticular and spino-mesencephalic tracts. Spinothalamic tract is principally made up of neurons from laminae I and V that project to the contralateral nuclei of the thalamus. Depending on the origin and projection of these neurons, three forms of spinothalamic tract afferences have been described. The first one is the neospinothalamic pathway or ventral spinothalamic tract, which directly projects to the nuclei of the lateral complex of the thalamus, involved in the sensory–discriminative component of pain. The second one is the paleo-spinothalamic pathway, or dorsal spinothalamic tract, that projects to nuclei of the posterior medial and intralaminar complex of the thalamus, involved in the motivational–affective aspects of pain. The third one is the monosynaptic spinothalamic pathway, which projects directly to the medial central nucleus of the thalamus, involved in the affective component of pain. Thus, the second order neurons forming these tracts will interact with the thalamic third order neurons, projecting to other regions of the thalamus and regions of the somatosensory cortex (**Willis et al., 1985; Almeida et al., 2004; Marchand et al., 2008**). Spino-reticular

tract arises from the neurons mainly located in laminae V, VII and VIII, and synapses in the reticular formations of the medulla and pons before reaching the thalamus. The afferences of spino-reticular tract are involved in the motivational–affective component of pain as well as in the pain modulation pathways by activating brain structures responsible for descending suppression (**Almeida et al., 2004; Zhang et al., 1990; Bourne et al., 2014**). Spino-mesencephalic tract has its origin in laminae I and IV-VI with some contribution from laminae VII, X, and ventral horn. Neurons in this tract are nociceptive and project to PAG, pretectal nuclei, red nucleus, Edinger-Westphal nucleus and Cajal interstitial nucleus. The spinomesencephalic fibers are involved in aversive behaviour and orientation responses and can activate descending antinociceptive systems (**Almeida et al., 2004; Bourne et al., 2014**).

Thus, the second order neurons from the spinal cord project through these tracts to different nuclei of the thalamus (concretely the ventrobasal and the centromedian nuclei) and other structures such as PAG and parabrachial area (PB) which will project to limbic areas through third order neurons. Briefly, ventrobasal nucleus project their third order neurons to the primary and secondary somatosensory cortices (SI, SII) which are involved in the stimulus localization, intensity, and quality discrimination of pain information. On the other hand, centromedial nucleus also connect through their third order neurons with the structures of the limbic system (insular, anterior cingulate and prefrontal cortex), which process the affective-motivational component of pain (**Marchand et al., 2008; Bushnell et al., 2013; Morton et al., 2016**). Moreover, neurons in the hippocampus are also activated, contributing to the formation of pain-related spatial memory and mood responses. Activation of the amygdala is also present, contributing to pain-related fear memory and pain modulation (**Zhuo et al., 2008**).

Apart to receiving noxious information through the ascending pain pathways described above, the brain modulates pain sensation through the efferent descending pathways, which play an essential role in integrating the nociceptive input by exerting an inhibitory or excitatory/facilitatory modulation (**Millan et al., 2002; Fields et al., 2004; Boadas-Vaello et al., 2016**). Descending pathways arise from a different supraspinal structures, including the cerebral cortex, limbic structures, hypothalamus, parabrachial nucleus, nucleus tractus solitarius, locus coeruleus (LC), dorsal reticular nucleus, periaqueductal gray (PAG) and rostral ventral medulla (RVM) (***Fig. 11***) (**Tracey et al., 2007; Chen et al., 2013**).

The descending pathways starting in supraspinal structures mentioned above, make synapse with primary afferent fibres, inhibitory and excitatory interneurons, and projection neurons in the spinal cord dorsal horn, modulating the pain response (***Fig. 11***) (**Ottestad et al., 2013; Boadas-Vaello et al., 2016**). This modulation is possible due to the release of neurotransmitters by neurons from these descending pathways. Some neurotransmitters have been shown to participate in inhibition and facilitation of pain transmission through binding to their respective receptors located at diverse population of dorsal horn

neurons. Release of excitatory neurotransmitters such as glutamate, substance P and CGRP increases sensitivity to incoming pain signals, which can be mediated by both functional plasticity (e.g., reduction of the required voltage threshold to cause conformational changes in receptors) and by structural plasticity (e.g., up-regulation of excitatory neurotransmitter receptors) (**Kuner et al., 2010**). Contrarily, release of inhibitory neurotransmitters including GABA, endorphins, enkephalins, norepinephrine and serotonin, reduces secondary neurons sensitivity to incoming pain signals by decreasing calcium influx into presynaptic nerve terminals or by evoking hyperpolarization in the postsynaptic neurons (**Babos et al., 2013**).

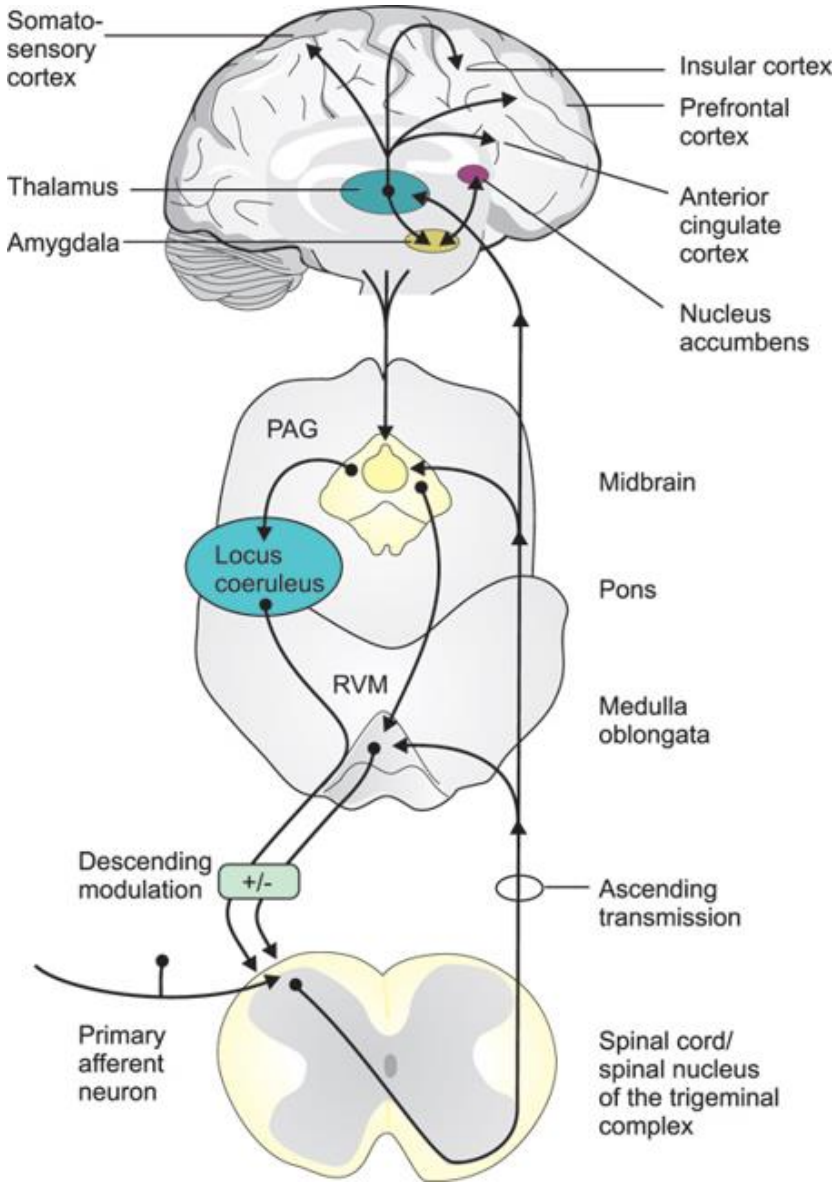


Figure 11. Schematic representation of the pathways and brain regions involved in pain transmission and modulation. First order neurons, which have their nucleus in DRG, transmit nociceptive information from the periphery and make synapse with second order neurons in the spinal DH. Second order neurons project to supraspinal structures and make synapse with third order neurons that in turn project to limbic system structures or to different parts of the cortex. Descending modulatory (inhibitory/excitatory) pathway arising from brainstem nuclei project to spinal cord modulating spinal pain processing by acting in both primary afferent neurons and second order neurons. Extracted from **Brodin et al., 2016**.

Among other structures, the PAG is a well-known target for pain modulation. It plays a key role in receiving inputs from higher brain centres and producing a powerful analgesic effect (**Boadas Vaello et al, 2016**). For instance, the stimulation of opioid receptors located at the ventral PAG produces analgesia through descending mechanisms. Similarly, endogenous cannabinoid receptors in the PAG act to suppress hyperalgesia and allodynia (**Petrosino et al., 2007**). Contrarily, activation of their cholecystokinin (CCK) receptors produces a facilitation of nociception and suppression of opioid driven analgesia (**Fenton et al., 2015**). As the PAG receives ascending pain inputs directly from the dorsal horn as well as from limbic forebrain structures such as the anterior cingulate, insular cortex, and amygdala, it is perfectly situated to integrate multiple environmental and emotional factors into the ultimate perception of pain.

The connection between the PAG, RVM and dorsal horn of SC is one of the best characterized nociceptive systems. This is mainly based on the high expression of μ-opioid receptors (MOR) in PAG neurons which make synapses to the RVM neurons. Axons from the RVM conform descending long tracts that project to the dorsal horn and directly inhibit laminae I, II, and V neurons (**Lau et al., 2014**).

The RVM is also an important structure in the descending modulation of pain perception. It can either facilitate or inhibit nociceptive inputs using an ON-and OFF-cells network. OFF-cells inhibit the pain perception due to their role in decreasing activation just before nociception. On the other hand, ON-cells facilitates the pain perception by expressing MOR and CCK receptors in their membranes. In addition, RVM is considered the main region of origin of serotonergic descending pain modulation pathways (**Mason et al., 2001**).

2. Neuropathic pain

Neuropathic pain is a maladaptive or pathological pain which, in contrast to nociceptive pain, is not protective. This type of pain is the result of abnormal functioning of the nervous system and is characterised by sensory abnormalities due to the hypersensitivity state. This hypersensitivity state may occur peripherally or centrally and can arise from a functional alteration of molecules involved in pain perception, transduction, and transmission (e.g., reduction of the threshold necessary to cause receptor activation due to its phosphorylation) or from structural plasticity (e.g., increased number of synaptic spines, neurotransmitter receptors overexpression) (**Babos et al., 2013**). These pathophysiological mechanisms involved in neuropathic pain lead to sensitization, defined as increased responsiveness of nociceptive neurons to their normal input, and/or recruitment of a response to normally subthreshold inputs (**IASP**). The clinical manifestation of this sensitization includes three sensory symptoms: allodynia, hyperalgesia (***Fig. 12***), and spontaneous pain (**Jensen & Finnerup, 2014**).

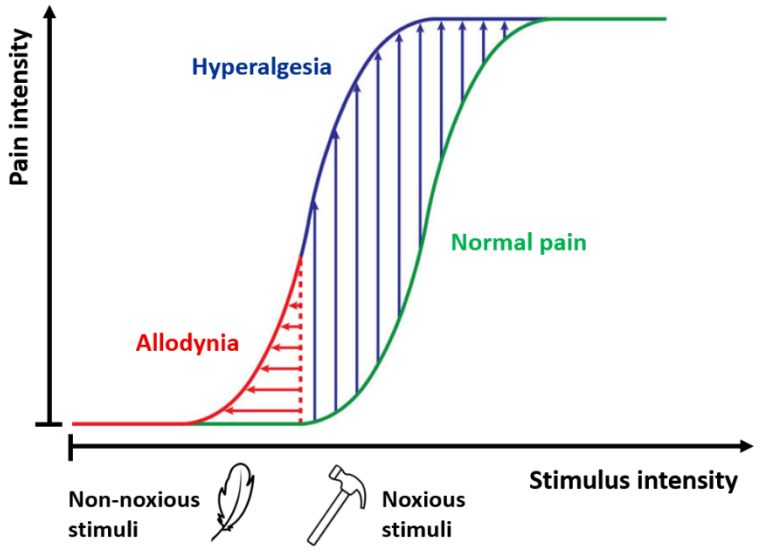


Figure 12. Sensitization to pain. Representation of the shift in pain thresholds during a pain state showing both enhanced response to noxious stimuli (hyperalgesia) and pain triggered by non-noxious stimuli (allodynia). These two painful states do not always coexist because of they are driven by distinct mechanisms in different sets of sensory neurons. Allodynia is mediated by low-threshold mechano- or thermosensitive afferent nerve fibres and hyperalgesia is mediated presumably by C fibres. Modified from **Lolignier et al., 2015**.

Both allodynia and hyperalgesia are forms of evoked, or stimulus-dependent, pain (**Cohen and Mao, 2014**) in contrast to spontaneous pain symptom which is stimulus-independent. On the one hand, allodynia is defined as pain due to a stimulus that does not normally provoke pain (IASP) and reflects a decreased threshold for response. On the other hand, hyperalgesia is defined as increased pain from a stimulus that normally provokes pain (IASP) and reflects increased pain on suprathreshold stimulation (***Fig 12***). Finally, spontaneous pain is caused by ectopic spontaneous impulse generation within the nociceptive pathways, so it does not originate in response to a stimulus (**Costigan et al., 2009**).

The clinical symptoms of neuropathic pain resulting from SCI include sensations of pins and needles, shooting, hot/burning, tingling, pricking, stabbing, shooting, squeezing, painful cold and paroxysmal (electric shock-like) pain, often associated with dysesthesia (an unpleasant abnormal sensation) and/or paresthesia (an abnormal sensation that is not painful or unpleasant) (**Defrin et al., 2001; Attal and Bouhassira, 2004**). Moreover, allodynia and hyperalgesia are usually present at- and below- injury level in patient with incomplete lesions (**Finnerup et al., 2007**).

When neuropathic pain is caused by a lesion or disease in the central nervous system, such as SCI, it is called **central neuropathic pain**. Central neuropathic pain resulting from SCI can also be classified based on its location: at- and below-level of the injury (***Fig. 13***).

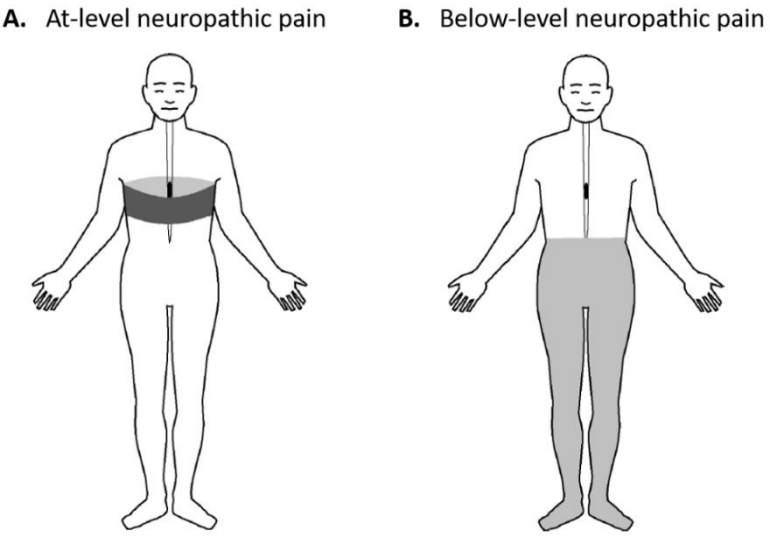


Figure 13. Typical distribution of neuropathic pain following spinal cord injury. Typical patterns of at-level (**A**) and below-level (**B**) neuropathic pain following mid-thoracic spinal cord injury are illustrated. The shading represents the distribution of pain. Extracted from **Siddall and Finnerup, 2006.**

Although neuropathic pain above the level of injury can also occur, this pain is not directly related to SCI. For this reason, the term ‘‘above-level pain’’ has been omitted in the most recent classification proposed by the International Spinal Cord Injury Pain (ISCIP). Thus, patients with SCI do not experience neuropathic pain from their injury above the neurological level, and above-level pain has been used to describe pain which is not specific to SCI such as syndromes of complex regional pain (sympathetic-reflex dystrophy, causalgia or shoulder-hand syndrome) and peripheral nerve compression (compressive mononeuropathies, carpal tunnel syndrome due to wheelchair use, post-thoracotomy pain after surgery or even painful diabetic polyneuropathy due to the immobility in some cases). (**Davidoff et al., 1991; Baastrup and Finnerup, 2012**). As these types of pain may also occur at- and below- the level of injury, “above-level pain” is now termed ‘‘other neuropathic pain’’ to differentiate from neuropathic pain as a direct consequence of the SCI.

At-level neuropathic pain has a characteristic segmental or dermatomal pattern within two to three segments above or below the level of injury. Due to its distinctive distribution, this pain is also referred to as segmental, radicular, transitional zone, border zone, end zone and girdle pain. It is reported in 12-42% of SCI patients (**Siddall et al., 2003**) and is classically described as electric, shooting, or burning, often associated with allodynia or hyperesthesia of the affected dermatomes. It may be induced by lesion of either nerve roots or to the spinal cord itself. Neuropathic pain arising from nerve root damage is generally unilateral whereas pain resulting from spinal cord damage is usually bilateral (**Riddoch, 1938; Burke and Woodward, 1976**). Syringomyelia and cauda equina injuries are syndromes included in this type of pain (**Siddall and Finnerup 2006**).

Below-level neuropathic pain is located diffusely below the level of injury, concretely more than three dermatomes below the neurological level of injury. It is reported in 23-34% of patients (**Siddall et al., 2003**) and is characterized by sensations of burning, aching, stabbing or electric shocks. It is a central pain occurring as a result of the spinal cord lesion, affecting the spino-thalamocortical pathways. This type of pain is considered the most severe and excruciating, and although is often classified as a stimulus independent pain, it can be spontaneous and/or evoked, often associated with allodynia and hyperalgesia (**Siddall and Finnerup, 2006**).

3. Pathophysiology of central neuropathic pain after spinal cord injury

After SCI, a cascade of biochemical, cellular, and molecular changes take place. These pathological and functional changes contribute to the mechanism responsible for the development of neuropathic pain, including neuropathic pain at- and below-level of the injury. Therefore, neuropathic pain resulting from SCI is related to changes that can occur in the following three levels: periphery, spinal cord and supraspinal structures (**Vierck et al., 2000; Siddall and Middleton 2015**). As a result, peripheral, spinal and supraspinal generators have been proposed to trigger neuropathic pain after SCI (***Fig.14***).

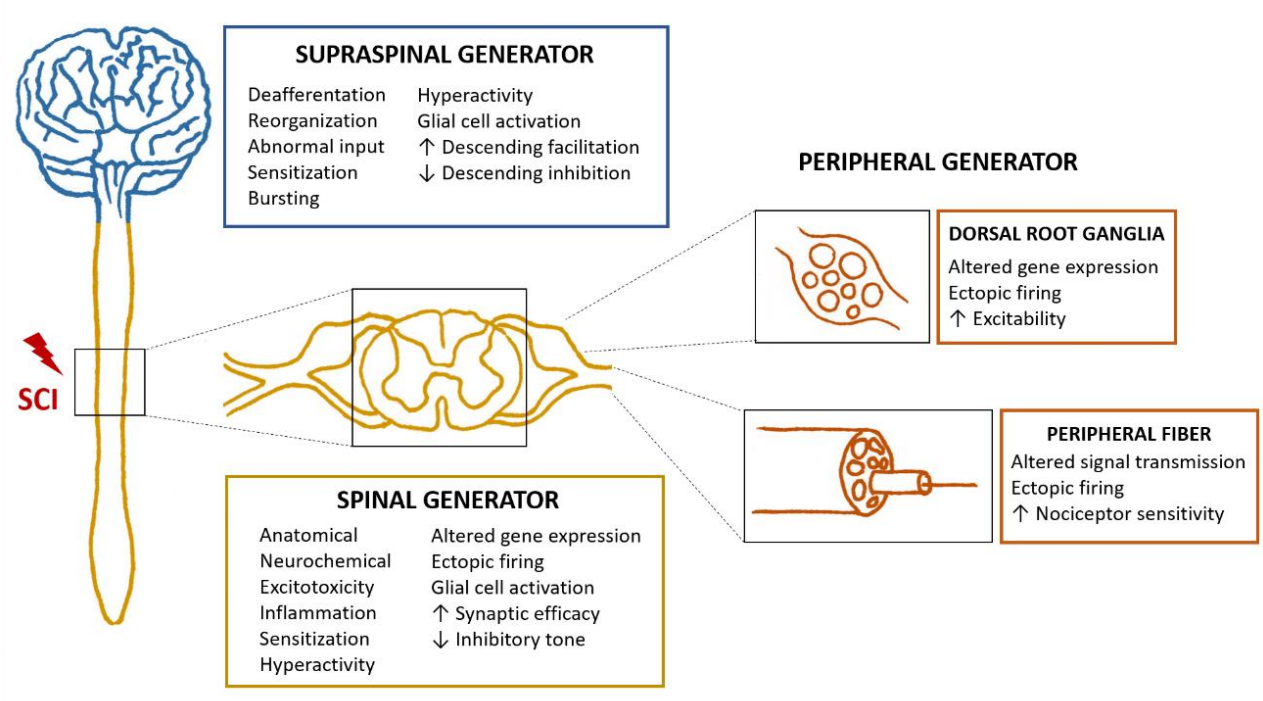


Figure 14. Overview of peripheral and central changes contributing to the development and maintenance of neuropathic pain associated with SCI. A peripheral generator emerges from anatomical and functional changes, leading to the increased nociceptor sensitivity and excitability. A spinal generator evolves due to the collective impact of anatomical, neurochemical and excitotoxic changes, leading to the increased excitability of spinal neurons. A supraspinal generator results from the loss of afferent input from spinal segments below the level of injury (deafferentation) and other injury-induced changes at supraspinal level (e.g., sprouting, unmasking connections), leading to the activation of specific supraspinal regions involved in pain perception and modulation. Based on (**Yezierski et al., 2006**) and (**Meacham et al., 2017**).

Peripheral mechanisms

Damage resulting from SCI not only compromises spinal cord integrity, but also can result in impingement of nerve roots entering in the spinal cord. This may lead to functional and structural changes in the primary afferents and in dorsal root ganglia (DRG) neurons, resulting in **peripheral sensitization** (**Carlton et al., 2009**). Peripheral sensitization is defined as increased responsiveness and reduced threshold of nociceptive neurons in the periphery to the stimulation of their receptive fields (**IASP**). Peripheral sensitization after SCI was demonstrated by Carlton and colleagues (2009) when improved responses to evoked stimuli and greater spontaneous activity in the absence of stimulus were obtained from recording peripheral nociceptive activity of spinal cord injured rats (**Carlton et al., 2009**). In addition, it has also been reported that SCI produces CNP and enhanced spontaneous activity of primary afferent neurons via hyperexcitable nociceptors (**Bedi et al., 2010**).

When nociceptor is sensitized, it reduces its threshold and increase in the magnitude of a response to noxious stimulus (**Bessou et al., 1969**). As a result, previously ineffective stimuli may become effective and spontaneous activity may also develop (**Kuner et al., 2010**). Post-translational and/or transcriptional changes in primary sensory neurons are necessary to generate peripheral sensitization. Some of the changes require retrograde transport of signals to the cell body, activation of signal transduction cascades, changes in transcription and orthograde transport of proteins to the peripheral terminals (**Svensson and Yaksh, 2002**). However, other changes are faster and simpler, for example the reduction of activation threshold following the receptors phosphorylation. When bradykinin or prostaglandins bind to G-protein-coupled receptors (GPCR), induce activation of protein kinases A and C which in turn phosphorylate ion channels and receptors in nociceptor peripheral terminals. As a result, the activation threshold of transducer receptors is reduced, and the excitability of the peripheral terminal membrane increases (**Scholz and Woolf, 2002**).

Additionally, some immunohistochemistry studies have shown that SCI can also physically alter primary sensory neurons. Thus, after SCI, CGRP immunoreactive nociceptors exhibit sprouting of new branches within the dorsal horn, contributing to the development of new abnormal connections (**Polistina et al., 1990; Hou et al., 2009**). On the other hand, it has been demonstrated that SCI also implies functional changes of axonal properties, such as increase of nodal Na+ current, nerve impulses amplitude, and conduction velocity, and a decrease of threshold for firing action potentials (**Ahmed et al., 2010; Isose et al., 2010**). These functional changes and the expression of voltage-gated sodium channels (VGSCs) predominantly distributed in DRG neurons that mediate nociceptive transmission via primary Aδ- and C-fibres contribute to enhance neuronal excitability and to maintain peripheral sensitization of primary afferent neurons after neurotrauma and inflammation (**Weston et al., 2009; Ahmed et al., 2010**). Long-

lasting spontaneous activity of nociceptors and persistent pain seem to be maintained by upregulation of Nav1.8 channels in primary afferents (**Yang et al., 2014**), continuing cAMP-PKA signaling (**Bavencoffe et al., 2016**) and increased expression of TRPV1 in dorsal root ganglia (**Wu et al., 2013**).

Taken together, the generation of spontaneous and ectopic activity and increase of the excitability of the primary afferent neurons produce an enhanced release of excitatory neurotransmitters and proinflammatory cytokines, which activate and sensitize postsynaptic neurons and glial cells in the dorsal horn. Hence, providing one peripheral mechanism of dorsal horn neuronal hyperexcitability contributing to central neuropathic pain following SCI (**Gwak and Hulsebosch, 2011**). To sum up, central neuroinflammatory processes after SCI trigger nociceptors to become hyperactive and, in turn, the hyperactive state of primary sensory neurons promotes central sensitization and hyperexcitability of dorsal horn neurons.

Spinal mechanisms

Both at- and below- level neuropathic pain has been demonstrated to be dependent on the presence of a spinal generator (**Finnerup et al., 2003**). However, it has been suggested that below-level neuropathic pain is not only dependent of a spinal generator but is also dependent of further pathophysiological changes at supraspinal levels, which contribute to the development and maintenance of such pain (**Lenz et al., 2004; Finnerup and Jensen, 2004**).

Regarding at-level neuropathic pain, different studies registered neuronal hyperexcitability, high discharge frequency, and abnormal pattern of background activity in the dorsal horn at or near the level of spinal injury (**Loeser and Ward, 1967; Loeser et al., 1968; Hao et al., 1992; Yezierski and Park, 1993; Scheifer et al.,2002**). On the other hand, animal models of SCI demonstrate that this increased excitability may be due to the increased activation of NMDA and metabotropic glutamate receptors (**Mills et al., 2002**) or/and up-regulation of sodium channels (**Hains et al., 2003**), as well as a reduced GABAergic (**Zhang et al., 1994; Drew et al.,2004**), opioid (**Xu et al., 1994**), serotonergic (**Hains et al., 2002**) and noradrenergic (**Hao et al., 1996**) inhibition. Taken together, all these changes may contribute to the development of allodynia and hyperalgesia, characteristic responses of at-level neuropathic SCI pain.

As to below-level neuropathic pain, the implication of spinal generator was demonstrated in a study in which patients with below-level pain had an increased hypersensitivity at-level compared to SCI patients without pain (**Finnerup et al., 2003**). It is widely accepted that spinal white matter damage, and more concretely the interruption of spinothalamic axons, contribute to below-level SCI pain (**Burchiel et al., 2002**). However, the importance of grey matter damage in such pain has also been demonstrated by different studies. For example, it has been reported that pain associated with syringomyelia is more

prevalent when a central cavity compromises dorsal pathways (**Milhorat et al., 1996**). On the other hand, it was demonstrated that ischemic involvement of spinal grey matter was a critical determinant of hyperalgesia after an interruption of the spinothalamic tract (**Vierck et al., 2000**). Similarly, quisqualic acid injections into the spinal grey matter produced below-level hyperalgesia without white matter damage (**Yezierski et al., 1998**). Another study showed that a common pathological feature of patients with below-level pain was the involvement of lesions to the spinal grey matter when compared with patients without pain (**Finnerup et al., 2003**). In conclusion, below-level neuropathic pain not only depends to the interruption of spinothalamic projections to rostral targets but is also potentiated by interruption and/or activation of other pathways, including propriospinal systems.

SCI induces direct primary and indirect secondary damages to spinal dorsal horn sensory neurons (described in 'Physiopathology of Spinal Cord Injury' section). A flow chart of biochemical, cellular, and molecular cascades following SCI that play a significant role in producing pathological and functional changes contributing to the SCI associated neuropathic pain is shown in ***Figure 15***.

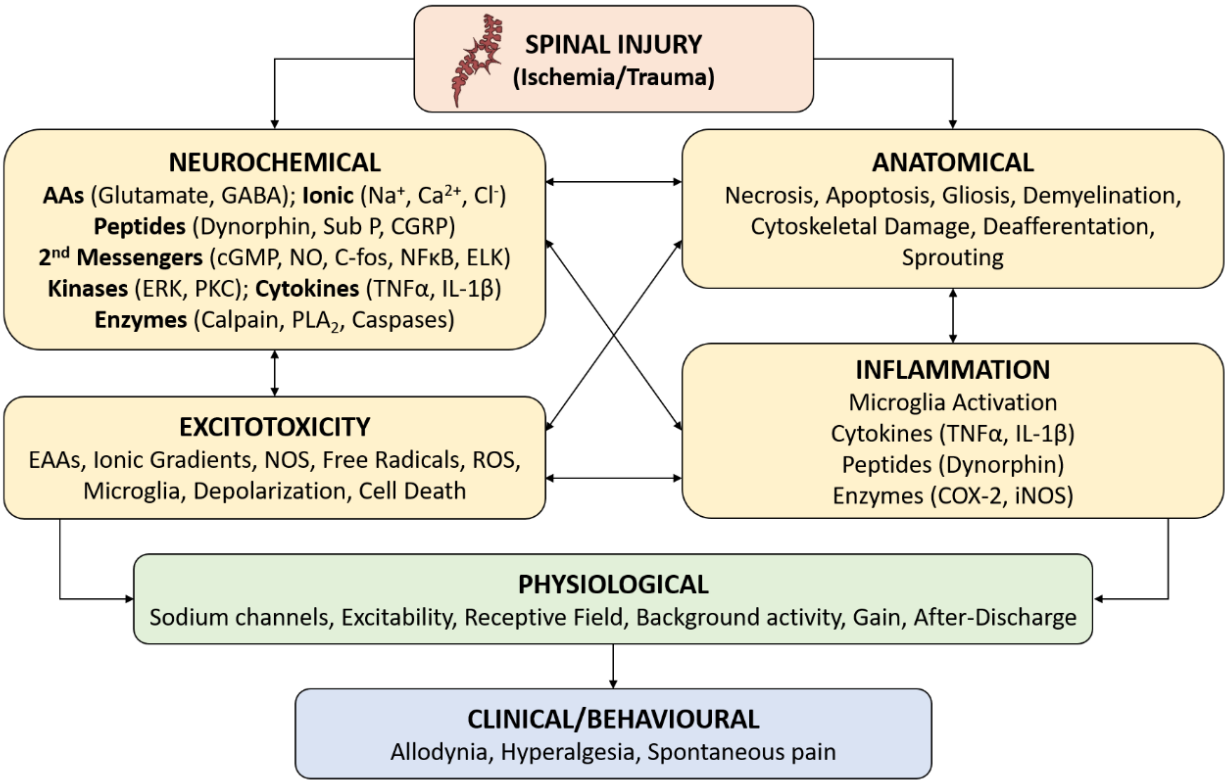


Figure 15. Biochemical, cellular and molecular components of the spinal injury cascade that contribute to the neuropathic pain development after SCI. The cascade has four major components (neurochemical, excitotoxic, anatomical and inflammation) that collectively interact to produce functional changes in spinal and supraspinal neurons. The end point of this cascade is the onset of clinical symptoms such as allodynia, hyperalgesia, and spontaneous pain. Abbreviations: AAs, amino acids; Sub P, substance P; cGMP, cyclic guanidine monophosphate; CGRP, calcium gene-related peptide; NO, nitric oxide; NF-kB, nuclear factor kappa B; PKC, protein kinase C; ERK, extracellular signal-regulated kinase; TNFα, tumour necrosis factor; IL-1β, interleukin-1β; PLA2, phospholipase A2; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2. Modified from **Yezierski et al., 2006**.

These changes contribute to the development of neuropathic pain and involve two major events: central sensitization and synaptic plasticity (**Woolf and Costigan, 1999; Ji and Woolf, 2001; Ji et al., 2003**). **Central sensitization** consists of an increased responsiveness of nociceptive neurons in the central nervous system to their normal or subthreshold afferent input (IASP) and is a consequence of early-translational changes as well as later transcription-dependent changes in effector genes. On the other hand, **neuroplasticity** consists of changes in the activation pattern either of structure or function that involves alteration of the strength of existing connections and sprouting of new neural connections (**Ding et al., 2005**) and includes activity-dependent changes in neurons and specific signalling molecules in signal transduction pathways. All these changes, including the development of maladaptive plasticity in the nervous system, aberrant sensory neuron activation, increased synaptic transmission, alterations in synaptic connectivity, as well as neuro-immune interactions (**Eide et al., 1998; Baron et al., 2006**), lead to alterations in the basal state of excitability that ultimately contributes to the development of spinal generators in both at- and below-level neuropathic pain.

In fact, maladaptive synaptic plasticity following SCI represents a potential mechanism for persistent pain (**Ji, 2003**) and the strengthening of synaptic connections between CNS neurons require **(a) initiating events** such as glutamate-induced intracellular calcium increase, **(b) maintaining forces** such as protein kinases activation, **(c) perpetuating forces** such as activation of cAMP, CREB, NFkB and **(d) terminal results** ending with an altered synaptic efficacy and increased excitability (***Fig.16***).

(a) Initiating events

The immediate results following SCI are due to direct physical trauma to the spinal cord and include the disruption of the blood-spinal cord barrier (**Maikos et al., 2007**), and the local disruption of blood vessels as well as neuronal and glial cells membranes (**Rowland et al., 2008**). On the one hand, the disruption of the blood-spinal cord barrier facilities the extravasation of thrombin and trypsin in the injured spinal cord parenchyma space, and the infiltration of phagocytic inflammatory cells (largely macrophages and neutrophils). On the other hand, the breakdown of cellular membranes activates phospholipases, which are responsible to lipid peroxidation and membrane hydrolysis. Consequently, polyunsaturated fatty acids will be released, causing cell damage through arachidonic acid and eicosanoid pathways (**Anderson and Hall, 1993; Tator et al., 1995**) and affecting physiologic processes by altering membrane structure, fluidity, permeability, and function. These membrane lipid perturbations contribute to Na^+/K^+-ATPase inactivation and loss of ionic homeostasis, causing and K^+ efflux and Na^+ and Ca^{2+} influx into the cells. The high levels of intracellular Ca^{2+} cause continued or additional activation of phospholipases, mitochondrial dysfunction with further compromise of cellular energy metabolism, and activation of Ca^{2+}-dependent proteases. As a result, the membrane potential cannot be maintained, and impulse transmission is lost. The ultimate

outcome of this series of chemical and cellular events is necrotic death with the consequent loss of function (**Anderson et al., 1993**). Finally, vascular disruption causes haemorrhage and swelling, which combine to produce ischemia. Ischemic environment is another factor that lead to necrotic death of neurons and glial cells, which will release hydrons (H+), potassium ions (K+) and excitatory amino acids (EAAs) triggering an excitotoxic state. The elevation of EAAs, especially glutamate, activates several receptors on post-synaptic neurons in the dorsal horn (including NMDA, AMPA and mGluR) culminating in a rapid increase of Na^{2+} and Ca^{2+} ion influx. As described previously, NMDA receptors, which mainly allow Ca^{2+} inflow, are voltage-dependent blockage by Mg^{2+} under resting potentials (**Mayer et al., 1984**). However, the continued release of excitatory substances after SCI (e.g., glutamate, substance P, CGRP and BDNF) and the further activation of their receptors (AMPA, NK1, CGRPR, TkrB) induce a persistent depolarization leading to the loss of Mg^{2+} blocker of the NMDA and allowing the entry of Ca^{2+} into the second order neurons. Importantly, this Ca^{2+} influx is also promoted by AMPA receptors, which under physiological conditions are impermeable to Ca^{2+}. However, under pathophysiological conditions such as SCI, there is an expression shift from its GluR2 to GluR1 subunit, allowing the pass of Ca^{2+} (**Latremoliere and Woolf, 2009; Luo et al., 2014**). In addition, mGluR receptors also contribute to the intracellular Ca^{2+} increase mediating the release of Ca^{2+} from the endoplasmic reticulum to intracellular space (**Latremoliere and Woolf, 2009**).

As a result, there are an activation of both calcium-dependent enzymes, promoting the formation of prostaglandins (PGE2), reactive oxygen, ROS (O_2, OH) and nitrogen species, NOS (NO, ONOO-, NO_2) (**Lewén et al., 2000; Vanegas and Schaible, 2001**), and intracellular proteases (e.g., calpains) which induce cytoskeleton disruption and axonal degeneration (**Ray and Banik, 2003**).

The molecular factors and neurotransmitters released in the primary injury contribute to the more protracted secondary injury phase. Pathophysiological processes include microglia and astrocyte reactivation, and high stimulation of primary afferent neurons (e.g., Aδ/C) conducting to hyper-excitability and sensitization of nociceptive neurons at dorsal horn (e.g., NS and WDR neurons). Concretely, trypsin and thrombin coming from the extravasation of plasma in the injury site stimulate the primary afferent neurons by activating the protease-activated receptors (PAR) which induce the release of SP, prostaglandins, glutamate and BDNF to the second order neurons (**Cenac and Vergnolle, 2005**). Diffusible mediators such as NO can also act on first order neurons by activating soluble guanylate cyclase (GC) which in turn generates cGMP. Intracellular elevation of the cGMP levels mediates neuronal hyperexcitability via the NO-cGMP-PKG signalling axis (**Ding et al., 2010, Wang et al., 2015**).

Activated glia in both superficial and deep dorsal horn laminae are key cellular contributors to persistent activation of dorsal horn neurons following SCI. The main factors that contribute to glial activation are ionic imbalances, neuroinflammation and alterations of cell cycle proteins after SCI. Activated glial cells increase

the expression or/and release of neurotransmitters, proinflammatory cytokines (TNFα, IL-1β, IL-6), chemokines (MCP1/CCL2, FKN/CX3CL1) and its receptors (CX3CR1), ROS, RNS and ATP (**Cooney et al., 2014; Gundersen et al., 2015; Hamby et al., 2008; Riazi et al., 2015; Pineau et al., 2010; Cekanaviciute et al., 2016**).

Hence, the released neurotransmitters, cytokines and chemokines act on their respective receptors located at dorsal horn second order neurons (e.g., SN and WDR neurons), ultimately producing a dramatic increase in intracellular Ca^{2+}, via both Ca^{2+} influx from membrane channels as well as Ca^{2+} release from intracellular stores (**Ladeby et al., 2005**; **Hulsebosch et al., 2009; Waxman and Hains, 2006**). Significantly, some of these soluble factors also will enhance the activation of primary afferent neurons and glial cells, that in turn will release more excitatory substances (positive feedback) (**Gwak et al., 2012**).

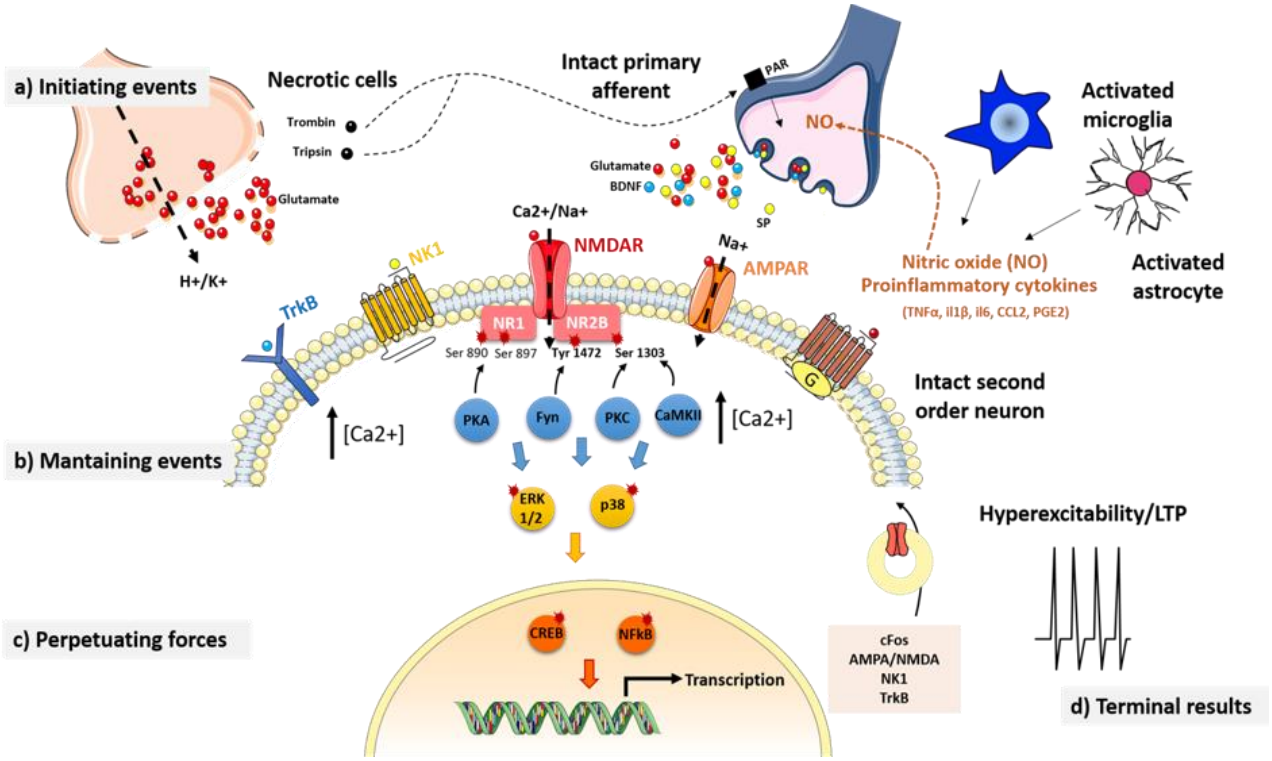


Figure 16. Simplified scheme of central sensitization mechanisms at spinal cord after SCI. **a) Initiating events.** Necrotic cells release high concentrations of glutamate, hydrons and potassium ions, as well as thrombin and trypsin. On the other hand, activated glia release nitric oxide (NO) and proinflammatory cytokines. In parallel, non-damaged primary afferents are activated by released thrombin, trypsin, and diffusible NO, producing the release of excitatory neurotransmitters such as BDNF, SP and glutamate. All these extracellular mediators interact with their receptors in the second order neuron causing a dramatic rise in intracellular calcium. **b) Maintaining events.** Intracellular calcium increase is the initiator of maintaining events that consist in the activation of calcium-dependent kinases (PKA, PKC, CAMKII, Fyn) which in turn will sensitize NDMAR and AMPAR via their phosphorylation. **c) Perpetuating forces.** These intracellular effectors will activate/phosphorylate mitogen map kinases (ERK, p38 MAPK) which in turn will activate some transcription factors (CREB, NFkB). As a result, several genes that play an important role for the perpetuation of the synapsis connection (c-fos, AMPA/NMDAR, NK1,TrkB) will be transcripted. **d) Terminal results.** Consequently, a strengthening of the synapsis (also called LTP) will activate persistently the spinothalamic tract leading to central sensitization. Extracted from **Castany 2017**.

(b) Maintaining forces

Intracellular Ca^{2+} increase trigger the activation of calcium-dependent kinases, such as protein kinase C (PKC), protein kinase G (PKG) and calcium/calmodulin-dependent kinase (CaMK). Calcium/calmodulin can also activates adenylate cyclase (AC), resulting in a cAMP increase and PKA activation (**Woolf et al., 2001**). These intracellular effectors will be responsible for direct or indirect phosphorylation of NMDAR subunits (NR1, NR2B) on specific residues located on their C-terminus. The phosphorylation of different residues on NMDAR subunits has several consequences such as an increasing in channel open time and kinetics (**Yu et al., 1997; Woolf et al., 1998; Woolf and Salter, 2000**), an increased responsiveness to glutamate (**Tingley et al., 1997**) and facilitation of calcium permeability (**Chen and Roche, 2007**), as well as a reduction on its endocytosis (**Wu and Zhuo, 2009**). Moreover, the activation of these intracellular pathways also leads to phosphorylation of AMPA receptors, reducing their response threshold (**Soderling and Derkach, 2000**). All these changes will contribute to NDMAR and AMPAR sensitization, suggesting a possible involvement of these receptors in long term plastic changes, through enhancing the sensitization of secondary nociceptive neurons (**Qu et al., 2009; Mao et al., 2014**).

The disinhibition of the spinal cord interneurons also plays an important role. Under normal circumstances, inhibitory interneurons exert a tonic inhibitory activity on secondary afferent neurons in the dorsal horn by releasing inhibitory neurotransmitters (e.g gamma-amino-butyric-acid (GABA) and glycine) (**Taylor et al., 2009**). However, this inhibition can be lost as a result of both direct neuronal death due to cytotoxic agents such as glutamate, cytokines and ROS released in injury site, and/or attenuation of inhibitory function in the setting of increased flux of BDNF and TNFα from activated microglia. Concretely, BDNF can cause a positive shift in the chloride equilibrium potential in dorsal horn nociceptive neurons by down-regulation of KCC2 (K^+–Cl^- cotransporter), and hence reduce inhibition that is normally exerted by GABA receptors (**Coull et al. 2005; Lu et al., 2008**). This hypofunction of GABAergic and glycinergic tone allows the persistent excitability of secondary nociceptive neurons contributing to their sensitization (**Rafati et al., 2008; Meisner et al., 2010**).

(c) Perpetuating forces

The activation of intracellular pathways mentioned above (PKA, PKC, CaMKII) not only are responsible for AMPA and NDMA receptors sensitization via its phosphorylation, but also will activate/phosphorylate mitogen map kinases (MAPKs) including ERK, p38 MAPK and JNK. It has been demonstrated that these MAPKs have a major role in regulating neuronal plasticity in pathological pain (**Yang et al., 2004; Liu and Zhou, 2015**) with the phosphorylation and the consequent activation of some transcription factors such as cAMP-response-element binding protein (CREB), NF-kβ, Elk-1 and ATF-2 among others (**Woolf et al., 2001; Ji et al., 2003; Liu and Zhou, 2015**). These transcription factors will induce the transcription of different

nociceptor genes, including cell membrane receptors (NK1, TrkB) (**Ji et al., 1997; Dubner et al., 1992; McCarson et al., 1994**), ion channels (AMPAR, NMDAR), enzymes (COX-2), cytokines (TNF-α, IL-1β) and other proteins and peptides (c-fos, BDNF, dynorphin, CGRP) (**Samad et al., 2001; Hunt et al., 19987**). The activation of these intracellular pathways take place in the second order neurons but also in glial cells, contributing to the hypersensitivity state of the secondary nociceptive neurons (**Gwak et al., 2017**).

(d) Terminal results

The molecular events described above lead to hypersensitivity state of the dorsal horn second order neurons through the establishment of spinal cord long term potentiation (LTP)-like phenomena (**Sandkühler et al., 2007**). This central amplification take place following decreased inhibition within the central system (disinhibition), increased spontaneous and post-discharge activity, as well as, enhanced synaptic facilitation due to increased response in amplitude, duration, and spatial extent. Consequently, low threshold sensory inputs now activate the WDR neurons, that under these circumstances respond to both innocuous and noxious stimuli (**Costigan et al., 2009; Wolf et al., 2011**).

In conclusion, SCI provokes maladaptive responses in nociceptive pathways that drive spontaneous pain and sensory amplification. Neuronal death and maladaptive plasticity provide the structural basis for persistently altered processing of both nociceptive and innocuous afferent input. Typical clinical phenotype of this central sensitization and LTP will be allodynia, hyperalgesia and/or spontaneous pain (**Jensen and Finnerup, 2014**).

Supraspinal mechanisms

After SCI there are also significant changes at supraspinal structures, that potentially contribute to the central mechanisms responsible for the onset and progression of neuropathic pain. Following SCI, the injured cord sends an abnormal input to supraspinal structures that together with effects of deafferentation and sprouting of undamaged fibres contribute to the development of supraspinal generators and/or amplifiers (**Vierck et al., 1991; Lenz et al.,2000**). The thalamus has a key role in modulating nociceptive information and in mediating different pain components: sensory discriminative (lateral pain pathway) and affective-motivational (medial pain pathway) (**Ab Aziz et al., 2006**). However, due to central sensitization and hyperexcitability of spinothalamic neurons after SCI, there is a greater stimulation of thalamic neurons via glutamate neurotransmission and sensitization via calcium influx (**Boadas-Vaello et al., 2016**). In fact, changes in thalamic biochemistry, gene expression, blood flow and neurons response properties have been shown in animal models and patients with neuropathic pain after SCI (**Pattany et al., 2002; Gustin et al.,**

2014; Hubscher et al., 2006; Hains et al., 2005; Whitt et al., 2013). Thus, after SCI, thalamus suffers a reorganization and molecular neuroplasticity changes that together contribute to the central sensitization.

The descending modulation (both facilitatory and inhibitory) by supraspinal structures has also a key role in central sensitization. Indeed, PAG and RVM are two supraspinal structures that play an important role in the descending modulation of nociception (explained in "Nociceptive pain" section). The main descending projection of PAG is the RVM, which project to the spinal dorsal horn neurons, in both superficial and deep layers (**Heinricher et al., 2009; Ossipov et al., 2010**). RVM can either facilitate or inhibit its nociceptive inputs using an ON- and OFF-cells network: OFF-cells are tonically active and produce an anti-nociceptive effect, whereas ON-cells promote nociception when are activated by a noxious stimulus sufficient to elicit a withdrawal response (**Boadas-Vaello et al., 2016**).

One of the features that differentiate these two types of cells is the different expression of receptors in their membranes. Whereas OFF-cells mainly express NMDA, AMPA and TRPV1 receptors (**Palazzo et al., 2008; Heinricher et al., 2009**), ON-cells express MOR, CCKB, NMDA, AMPA, NK1, TrK-B and TRPV1 receptors (**Guo et al., 2006; Budai et al., 2007; Zhang et al., 2009; Da Silva et al., 2010; Price et al., 2013**). In physiological conditions, PAG neurons release glutamate over OFF-cells causing their depolarization via NMDA/AMPA receptors and therefore producing an antinociceptive effect in the spinal dorsal horn neurons (**Heinricher and Tortorici, 1994**). On the other hand, PAG neurons release β-endorphin over ON-cells causing their hyperpolarization via MO receptor and provoking the inhibition of the descending facilitation (**Ossipov et al., 2009**). Moreover, PAG neurons stimulate GABAergic interneurons located at RVM, causing the inhibition of ON-cells. In addition, ON-cells can also be inhibited by the stimulation of their TRPV1 receptors (**Palazzo et al., 2010**) (***Fig. 17. A***).

However, under neuropathic pain condition, the ON- and OFF-neurons suffer several molecular changes due to the hyperexcitation of ascending nociceptive pathways. On the one hand, OFF-cells will overexpress GABA-A and kappa opioid receptors, thus becoming more sensitive to GABA and β-endorphins released by hyperactivated RVM interneurons and PAG neurons, respectively (**Gutstein et al., 1998; Heinriche et al., 2009**). Consequently, the hyperpolarization of these OFF-neurons will reduce their anti-nociceptive effect on dorsal horn spinal cord neurons (**Satoh et al., 1995**) (***Fig. 17. B***). On the other hand, ON-neurons will overexpress NMDA/AMPA, Trk-B and NK1 receptors whereas will reduce their expression of MO receptor. As a result, the inhibitory signal from PAG over ON-neurons will be less effective and the release of glutamate, SP and dynorphin from ascending inputs will stimulate ON-neurons, causing their hyperexcitability (**Boadas-Vaello et al., 2016**) (***Fig. 17. B***).

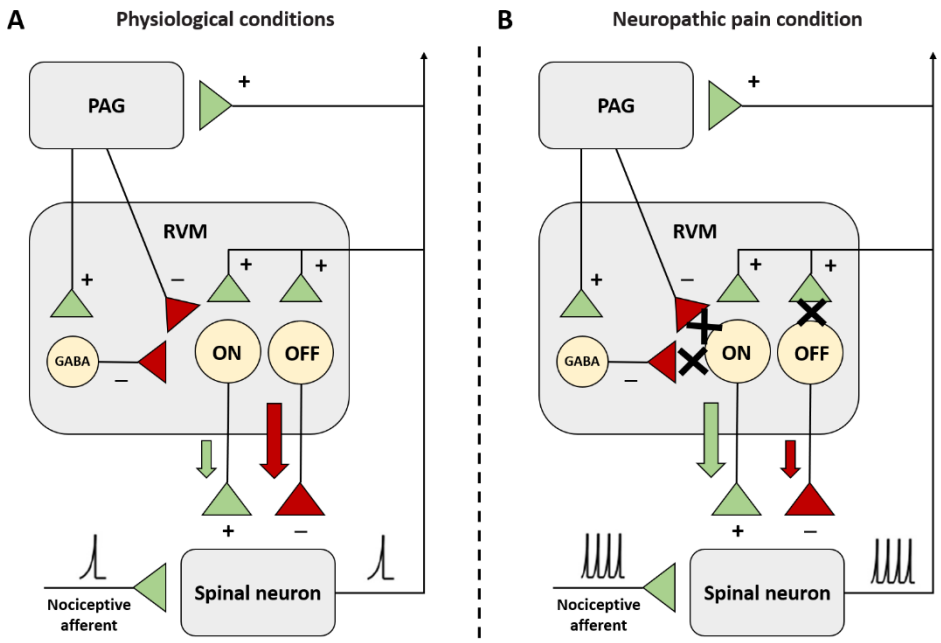


Figure 17. Schematic representation of ON- and OFF-neurons from RVM under physiological and neuropathic pain condition. (**A**) Under physiological conditions, spinal neurons send nociceptive inputs to PAG, which in turn stimulate the ON- and OFF-neurons from RVM. Excited PAG neurons also excite GABAergic interneurons at RVM and inhibit ON-neurons. As a result, ON-neurons are inhibited and OFF-neurons are excited, causing a decrease in the firing response over spinal nociceptive neuron. (**B**) The hyperexcitability of dorsal horn neurons after SCI change the expression of ion channels and receptors in ON- and OFF cells from RVM. On the one hand, ON-neurons become insensitive to inhibitory inputs from GABAergic interneurons and PAG neurons, and more sensitive to excitatory inputs from spinothalamic pathway. On the other hand, OFF-neurons become insensitive to excitatory inputs from the spinothalamic pathway. As a result, there is a potentiation of ON-neurons inputs over spinal cord neurons, causing descent facilitation from RVM. Based on **Boadas-Vaello et al., 2016**.

Furthermore, neuropathic pain also induces sensitization of PAG neurons via overexpression of NMDA, AMPA and NK1 receptors, and an increased release of glutamate, SP and BDNF. These BDNF-positive PAG neurons project their axons to ON-RVM neurons, enhancing their depolarization via TrK-B, NMDA and AMPA receptors (**Guo et al., 2006**). Different models of neuropathic pain also have shown an increase of microglial and astroglial reactivity in RVM. The mediators released by activated glia facilitate the excitation of ON-RVM neurons, enhancing their excitatory effect on dorsal horn neurons (**Guo et al., 2007; Roberts et al., 2009; Wei et al., 2008**).

In summary, all this evidence demonstrates that neuropathic pain after SCI triggers plastic changes in descending pain modulatory pathway, resulting in pain facilitation in the spinal cord. Further suggesting a significant role of supraspinal structures in development and maintenance of neuropathic pain after SCI.

In conclusion, the previously described pathophysiological changes occurring after SCI at peripheral, spinal and supraspinal level contribute to the generation and maintenance of neuropathic pain at- and below-level of injury.

4. The affective-motivational dimension of central neuropathic pain

Pain is a multifactorial and highly personal experience that contains both sensory and affective dimensions (**Price et al., 2000**). The sensory-discriminative dimension includes stimulus localization, intensity, and quality discrimination whereas the affective-motivational dimension involves the affective component of pain that comprises emotional reactions and stimulus related selective attention (**Melzack et al., 1968; Treede, et al., 1999; Auvray, et al., 2010**). Therefore, pain is a multidimensional composition created by complex interactions between afferent sensory inputs and their processing throughout the nervous system from the periphery to the brain and affective brain circuitry, with additional complexity given by memory, expectations, attention, and mood (**Kuner and Kuner, 2021**). Is well known that the negative affection of pain, like anxiety or depression, accompany the pain perception during the chronic phase. Moreover, it has been demonstrated that pain-related negative affect is more disabling than pain itself and has a severe effect on the daily activities in chronic pain patients (**Crombez et al., 1999**).

The persistent pain associated with SCI has been reported to interfere with cognitive, emotional, and physical health, affecting daily life activities with mood changes, depressive symptoms, and stress (**Avluk et al., 2014**). Specifically, nearly half of spinal cord injured patients with chronic pain suffer from depression (**Shin et al., 2012; Ataoğlu et al., 2013**), anxiety (**Post and van Leeuwen, 2012; Budh & Österåker, 2007**), decreased quality of life (**Boakye et al., 2012; Ataoğlu et al., 2013**) and cognitive impairments (**Berryman et al., 2013; Craig et al., 2017; Sachdeva et al., 2018**). Moreover, suicide attempts and suicide ideation are approximately three times greater in SCI patients than in general population (**DeVivo et al., 1991; Soden et al., 2000**). Importantly, epidemiological data suggest that females have a higher prevalence of chronic pain and a higher vulnerability in the development of comorbid pain and emotional disorders (**Miller and Cano, 2009; Goesling et al., 2013**).

In conclusion, cognitive and emotional factors have an important influence on pain perception (***Fig. 18***). In fact, different brain imaging studies have revealed that the activity of afferent pain pathways is altered by the attention, positive and negative emotions, empathy, and the administration of a placebo. Moreover, the descending modulatory pain pathways are involved in pain relief, with attentional states and emotions that activates different supraspinal structures. Furthermore, it has been demonstrated that chronic pain lead to anatomical and functional alterations in supraspinal structures implicated in pain modulation, resulting not only in persistent pain but also in altered cognition and affect (**Bushnell et al., 2013**).

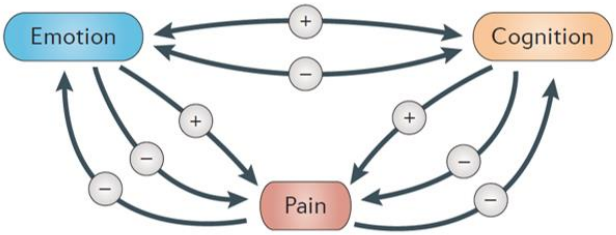


Figure 18. Feedback loops between pain, emotion, and cognition. The negative feedback loop between impaired pain modulatory and pain processing lead not only to increased chronic pain but also to cognitive and emotional deficits that are comorbid with the pain. Thus, pain can have a negative effect on emotions and on cognitive function. Inversely, negative emotional state can increase the pain sensation whereas positive emotional state can reduce it. Likewise, cognitive states such as attention or memory can either increase or decrease pain. Simultaneously, emotion and cognition can also reciprocally interact. Extracted from **Bushnell, 2013**.

Multiple brain pathways (including sensory, limbic, and associative regions) are involved in pain processing. Some of the supraspinal structures involved in cognitive and emotional modulation of pain which suffer anatomical and functional alterations in chronic pain state are prefrontal cortex (PFC), anterior cingulate cortex (ACC), insula, nucleus accumbens (NAc) and amygdala (AMG) (**Schweinhardt and Bushnell, 2012; Apkarian et al., 2005; Tracey and Johns, 2010**).

Among these brain areas, the **ACC** plays an important role in the affective component of pain through its expression, modulation, and emotion processing. The neurons of the ACC receive nociceptive projections from the thalamus and somatosensory cortices, and emotional fear-anxiety-depression information from the amygdala. These connections suggest that ACC may act as a substrate to link both sensory and affective dimensions of pain (**Johansen et al., 2001; Shackman et al., 2011; Bushnell et al., 2013; Bentley et al., 2016**). Neuroimaging studies have shown increased activity in the ACC during chronic and neuropathic pain (**Hsieh et al., 1995; Peyron et al., 2000; Tseng et al., 2013**). In fact, neuronal hyperexcitability and synaptic plasticity in ACC during the chronification of neuropathic pain is considered one of the most important pathological events for this process (**May et al., 2008; Seifert and Maihöfner, 2011**). Moreover, neurophysiological and molecular changes (**Wrigley et al., 2008; Xu et al., 2008; Yamashita et al., 2014**) as well as increased cerebral blood flow (**Watanabe et al. 2018**) in the ACC have been associated with development of chronic and neuropathic pain. Additionally, different studies have demonstrated the necessary implication of ACC in aversive component of neuropathic pain (**Qu et al., 2011**) and in pain expectation (**Porro et al., 2002**). Finally, different studies have shown astrocyte and microglia activation in the ACC in models of chronic, and neuropathic (**Narita et al., 2006; Kuzumaki et al., 2007; Lu et al., 2011; Chen et al., 2012; Yamashita et al., 2014; Miyamoto et al., 2017**). The actual data suggest that the activation of glial cells in the ACC is probably time-dependent and chronic pain model type-dependent. In the SCI model, lower glutamatergic metabolism and greater glial activation in ACC are important contributors to

the development and maintenance of severe neuropathic pain (**Chang et al., 2013; Widerstrom-Noga et al., 2013**).

There are two **amygdala** nuclei particularly relevant to pain: the central amygdaloid nucleus (CeA) and the basolateral amygdala (BLA) (**Kuner and Kuner, 2021**). On the one hand, CeA is essential for the negative emotional processing of pain and is also called "nociceptive amygdala" (**Neugebauer et al., 2015**). On the other hand, BLA plays a critical role in generation the fear response after exposure to a potentially noxious stimuli and is essential for memory consolidation of cued fear. Moreover, BLA sends glutamatergic projections to the NAc, PFC, and hippocampus, all of which are important in the regulation of motivated behavior. It has been observed that in neuropathic pain condition, the neuronal proliferation and excitability in both CeA and BLA is increased (**Gonçalves et al., 2008**). The hyperactivity of BLA neurons in chronic pain states leads to deactivation of the mPFC, which is accompanied by the induction of chronic pain-associated cognitive decline (***Fig. 19***) (**Ji et al., 2010**). Indeed, several studies have demonstrated that role of amygdala is essential in maintaining anxio-depressive and nocifensive behaviours in persistent neuropathic pain (**Pedersen et al., 2007; Ansah et al., 2010; Tanimoto et al., 2003**).

NAc plays a critical role in reward and aversion in chronic pain. NAc receives excitatory glutamatergic inputs from the amygdala, PFC and hippocampus, and dopaminergic input from the ventrotegmental area (VTA), which in turn projects dopaminergic axons to the cortex, amygdala and NAc, comprising the center of the reward pathway (**Willner et al., 1991; Volkow et al., 2019**). Alterations in NAc dopamine content govern the balance between aversion and reward-seeking behavior. However, in chronic pain state this equilibrium is altered through different mechanisms, thus promoting aversion outputs (**Eimer et al., 2018; Lammel et al., 2012; Schwartz et al., 2014; Soares-Cunha et al., 2020; Volkow et al., 2019**). Moreover, alterations in strength of mPFC-NAc connectivity are proposed to predisposing factor for pain chronicity (**Baliki et al., 2012**). On this line, optogenetic activation of mPFC-NAc modulates sensory hypersensitivity as well as behaviours related to neuropathic pain (**Lee et al., 2015**) and induces resilience to chronic social defeat stress (**Bagot et al., 2015**).

PFC executes an important role in chronic pain through its connectivity with the NAc (**Baliki et al., 2012**). In fact, several studies show the implication of PFC-NAc connectivity in alterations of reward system in neuropathic pain states (***Fig. 19***) (**Kai et al., 2018; Lee et al., 2015; Zhou et al., 2018; Schwartz et al., 2017**). Moreover, different animal models of chronic/neuropathic pain present morphological and functional reorganization of mPFC with altered dendritic spine density, long-term alterations in gene expression, altered responses to excitatory glutamatergic inputs and grey matter loss (**Metz et al., 2009; Alvarado et al., 2013; Kelly et al., 2016; Kang et al., 2019; Apkarian et al., 2004**). On the other hand, a major subpopulation of mPFC neurons project to the PAG, which is involved in both descending inhibition and

descending facilitation of nociceptive pathways. Altered excitability and local connectivity of mPFC-PAG neurons have been reported in a mouse model of neuropathic pain, suggesting an alteration on endogenous analgesic network which may contribute to persistence of chronic pain (***Fig. 19***) (**Cheriyan et al., 2018**). In summary, alterations in both PFC activity and connectivity with PAG and NAc in chronic/neuropathic pain states trigger cognitive comorbidities as well as decreased reward and increased sensory hypersensitivity.

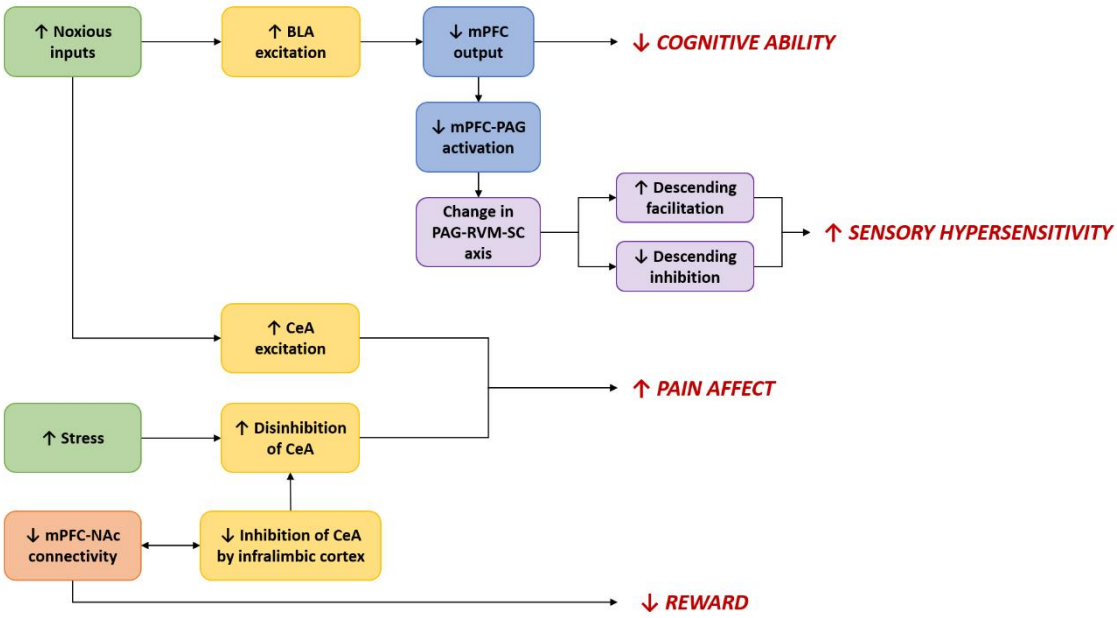


Figure 19. Flowchart of relationships between the activity status of the central (CeA) and basolateral (BLA) amygdaloid nuclei, medial prefrontal cortex (mPFC) and nucleus accumbens (NAc) in chronic pain. Changes in cognitive function, sensory hypersensitivity, pain affect, and reward are shown. Based on **Kuner and Kuner, 2021.**

5. Animal models of pain following spinal cord injury

Pain resulting from SCI is notoriously difficult to treat, therefore improve the management of pain symptoms is a priority area of research (**Anderson et al., 2004**). Resolving this issue requires animal models that mimic the clinical situation of human SCI in both terms of injury mechanism and pain phenotype. Over the last few decades, animal models were widely used in order to study the development and maintenance of central neuropathic pain-like behaviour after SCI as well as successful treatment strategies (***Table 2***). Among them, SCI rodent models are arguably the most prominent (**Ahmed et al., 2019**) because of their rapid adaptation to the experimental environment and to the given tasks for sensorimotor evaluation. Moreover, their facile manipulation, high availability, low infection rate after surgery, ease of maintenance and their certainly anatomical, physiological and genetic similarity to humans make the rodents a prime

candidate for SCI translational research. In ***Table 2*** are indicated the main models of neuropathic pain after SCI which have been simulated in rats and then adapted to mice (**Nakae et al., 2011**).

Table 2. Rodent spinal cord injury models for pain research.

TYPE OF SPINAL CORD INJURY		DESCRIPTION	REFERENCES
Mechanical	**Contusion/Hemi-contusion**	Contusion injury performed by weight-drop or impactor method	Kuhn and Wrathall, 1998
	Clip Compression	Compression injury induced with clips calibrated to exert a specific force	Steward et al., 1999
	Canal stenosis	Injury induced by placing square-shaped pieces of silicon into the epidural space	Sekiguchi et al., 2004
	Complete transection	Spinal cord transection performed by spring scissor	Weber and Stelzner, 1977
	Hemi-section	Spinal cord hemi-section performed by spring scissor	Christensen et al., 1996
Chemical	**Electrolytic**	Spinothalamic tract is selective lesioned using a microelectrode	Mathers and Falconer, 1991
	Photochemical	Intravenous injection or spinal cord bath of photosensitive dyes rose Bengal or erythrosine B accompanied with selective irradiation	Hao et al., 1991 Verdú et al., 2003
	Excitotoxic	Intraspinal or intrathecal injection of an excitotoxic agent	Yezierski et al., 1993

Considering compression injury is the most common type of SCI in humans mainly caused by falls or by physical impacts, **contusion or hemi-contusion** is probably the most suitable method to get the same type of injury in rodents (**Young et al., 2002**). In fact, it is the oldest and most widely used SCI model which elicits motor and sensory dysfunction including neuropathic pain, mechanical allodynia, and thermal hyperalgesia (**Tanabe et al., 2009; Hoschouer et al., 2010**). Following injury, motor analysis is required to exclude unilateral paralysis and possible unilateral contusion. When motor dysfunction is recovered, pain behaviour can be analysed (**Baastrup et al., 2010; Kang et al., 2015**). In 1911, Allen (**Allen, 1911**) first introduced the drop-weight technique (revised by **Anderson, 1982**) to create reproducible spinal cord injuries in the dog. Several investigators have subsequently employed this technique for use in a variety of mammalian species, including monkeys (**Albin et al., 1968**), sheep (**Yeo et al., 1975**) cats (**Faden and Jacobs, 1984**), rats (**Wrathall**

et al., 1985), mice (**Kuhn and Wrathall, 1998**) and pigs (**Bernards and Akers, 2006**). Nowadays, spinal cord contusion or hemi-contusion injury can be obtained using the weight-drop method (**Maybhate et al., 2012; Wang et al., 2012**) or the impactor method (**Anderson et al., 2009; Gaudet et al., 2017**). In the weight-drop method, spinal cord is exposed via laminectomy and then injured by dropping a specific weight from determined height by means of a weight-drop apparatus. In the impactor method, injury is produced via an impactor by applying a specific force to the exposed spinal cord area. While the weight-drop method is more rudimentary but has more scope for variation, the impactor method is more specific, accurate and precise (**Geremia et al., 2017; Verma et al., 2019**). In these methods, different degree and severity of SCI can be obtained depending on the weight and height selected, that will determine the applied force to the cord. However, the impact of the injury tends to differ due to variability of the method. In an attempt to gain more control over the injury, electronic and pneumatic compression devices are developed in order to monitoring biochemical parameters such as impact velocity and tissue displacement (**Verma et al., 2019; Jakeman et al., 2000**).

Clip compression provokes an ischemic state of the spinal cord simulating common clinical injuries and outcomes after compressive SCI in humans. The clip compression model was first described in rats by Rivlin and Tator in 1978 (**Rivlin and Tator, 1978**). Briefly, follow laminectomy, a calibrated clip is dorsoventrally closed over the spinal cord for a specific duration of time (usually 1 minute) and then removed to induce a compressive injury (***Fig. 20***) (**Nashmi and Fehlings, 2001; Bruce et al., 2002; Densmore et al., 2010**). Clips are available with a wide range of closing forces that can be applied for different periods of time, resulting in injuries of varying severity. For example, clip calibrated to exert a force of 50g and 35g produces a severe and moderate injury, respectively. This model has been adapted for use in the mouse (**Steward et al., 1999**) with a smaller aneurysm clip producing an injury that varies in grade with respect to both behavioural and histological outcomes (**Joshi and Fehlings, 2002**). Due to the similarity to human traumatic SCI, the compression injury is a suitable model for translational research (**Sun et al., 2017**). In addition, it has recently been shown that SCI by clip compression induces anxiety and depression-like behaviours associated with an imbalance in the production/release of pro- and anti-inflammatory cytokines in both spinal cord and plasma of female rats (**do Espírito Santo et al., 2019**).

Canal stenosis model consists in place square-shaped pieces of silicon into the epidural space (**Sekiguchi et al., 2004; Ito et al., 2007**). This model permits simulate the lumbar canal stenosis induced by entrapment of the cauda equine and/or lumber nerve roots by hypertrophy of osseous and soft tissue structures surrounding the lumbar spinal canal. The typical pathology is an ischemic injury due to reduced blood flow to the peripheral nerve, resulting in demyelination and axonal degeneration. Canal stenosis animal models can present hypersensitivity and mechanical hypoalgesia (**Sekiguchi et al., 2004**). Based on the above, canal stenosis is a suitable SCI model to study the pathophysiology of chronic, light pressure to the spinal cord.

Complete transection and **hemi-section** models consist in total or partial transection of spinal cord by spring scissors after previous laminectomy (**Weber and Stelzner, 1977**) (***Fig. 20***). On the one hand, complete spinal transection model reflects symptoms of complete SCI in humans, manifesting at-level and below-level neuropathic pain (**Scheifer et al., 2002; Densmore et al., 2010**). However, this model is more appropriate to study degeneration as well as tissue engineering strategies, such as scaffolds or biomaterials, for regeneration and functional recovery (**Krishna et al., 2013**). Moreover, it is also a suitable model to study musculoskeletal pain pathology highly associated with muscular spasticity that occurs in spinal cord complete transection (**Densmore et al., 2010**). On the other hand, hemi-section injury is a useful model to neuropathic pain studies. Mechanical allodynia and thermal hyperalgesia are bilaterally observed in above-level and below-level (**Hains et al., 2001; Hains et al., 2000; Bennett et al., 2000; Gwak et al., 2004**) and motor dysfunction only appears in the ipsilateral injured side, perduring from 5 days to 4 weeks (**Hains et al., 2003; Coronel et al., 2011**).

Electrolytic model is designed to unilaterally injury the area of spinothalamic tract and is often used to study CNS neuronal pathways. The technique involves the insertion of a thin metal electrode into the spinal cord. Restricted damage to the cells in this region is achieved by heating the electrode tip by passage of a high electric current (**Mathers and Falconer, 1991**). This model resembles central pain syndromes following SCI in humans, such as bilateral hypersensitivity above- and below-level hyperalgesia and allodynia. Considering above mentioned, spinothalamic tract lesion model provides a useful method to study the underlying mechanisms of neuropathic pain associated to SCI (**Wang and Thompson, 2008**).

Photochemical model consists in the intravenous injection of the photosensitive dyes rose Bengal (**Watson, 1986**) or erythrosine B (**Cameron et al., 1990; Hao et al., 1992**), or alternatively dura-covered spinal cord bath with rose Bengal (**García-Alías et al., 2003; Verdú et al., 2003**). After injection or application, dye is activated by an argon ion laser to produce single oxygen molecules at the endothelial surface of spinal cord vessels, resulting in platelet response and subsequent vessel occlusion and parenchymal tissue infarction (**Ishikawa et al., 2002**). Moreover, this photochemical reaction also causes an alteration of the grey and white matter of the spinal cord (**Verdú et al., 2003**). This experimental model, developed by Watson and collaborators in 1986 (**Watson et al., 1986**), has proven to be one of the most reliable and reproducible experimental models of SCI and is widely used to study neurotrauma in rodents (**Ishikawa et al., 2002; García-Alías et al., 2003; Piao et al., 2009**). The major advantage of this model is that it does not induce mechanical trauma to the spinal cord, instead the injury is purely ischaemic in origin. Animal models of photochemical SCI present hypersensitivity at- and below- level of irradiation (**Hao et al., 1992; Hao et al., 1999; Erichsen et al., 2005**). Since the extent of injury is difficult to control, it is important to evaluate motor score deficits (**Xu et al., 1992**).

Excitotoxic model consists in intraspinal or intrathecal injection of an excitotoxic agent such as quisqualic acid or excitatory amino acids (e.g. glutamate, N-methyl-D-asparate, kainate) which produces neuronal loss, cavity formation, astroglial scaring, and prominent inflammation (**Yezierski et al., 1993**) (***Fig.20***). Nearly 100% of excitotoxic models develop varying degrees of hypersensitivity including long-lasting spontaneous pain, mechanical allodynia and thermal hyperalgesia (**Yezierski et al., 1998; Fairbanks et al., 2000**). The major advantage of this model is that it reproduces selected components of the secondary injury cascade and specific areas of tissue damage can be correlated with behavioural changes (**Yezierski et al., 1998**).

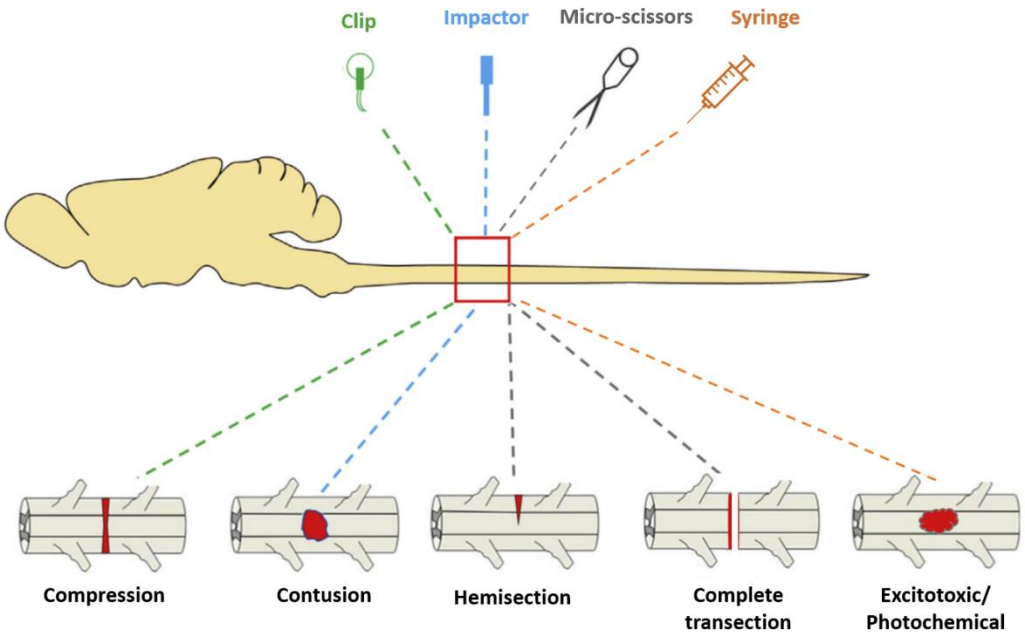


Figure 20. Diagram showing some of injury models in rodents' spinal cord. Compression model is performed using a clip to create the injury. In the contusion model an impactor is dropped from a predefined height. For transection model, a surgical blade is used to carry out different types of transection injury. In the excitotoxic/photochemical models is injected or directly applied an excitotoxic/photosensitive agent that initiates the injury. Modified from **Ahmed et al., 2019**.

Like most pain studies (**Mogil et al., 2009**), pain following SCI has been mainly studied in young male rodents (**Kramer et al., 2017**). Although this choice can be more readily justified due to upwards of 70% of SCI patients are young adulthood males (**Thompson et al., 2015**), the prevalence and intensity of neuropathic pain following SCI are reported to be equivalent between male and female patients (**Budh et al., 2003; Cardenas et al., 2004**). Moreover, female SCI patients have a higher prevalence of nociceptive pain with an increased overall pain prevalence (**Budh et al., 2003**). On the other hand, several studies have suggested that male and females neuropathic pain models may process painful stimuli in different ways, therefore requiring different treatment approaches (**Bartley et al., 2013; Gaudet et al., 2017; Acosta-Rua et al., 2011**). For this reason, nowadays urge the necessity to develop female models of SCI neuropathic pain in order to study specific female SCI physiopathology and further effective treatments.

PART III. CURRENT TREATMENT OF NEUROPATHIC PAIN FOLLOWING SPINAL CORD INJURY AND POLYPHENOLIC COMPOUNDS AS POTENTIAL PHARMACOLOGICAL STRATEGY

1. Current treatment of neuropathic pain following spinal cord injury

A thorough clinical examination of pain condition should precede any treatment to make sure of the presence of neuropathic pain and to identify any possible somatic cause of pain other than SCI. The classification of pain as well as its localization, duration, intensity, and characteristics are crucial to optimize the therapy (**Siddall and Finnerup, 2006**). The clinical examination must include a neurological status with a mapping of sensory phenomena in the painful area, indicating the presence of neuropathic pain (**Hagen and Rekand, 2015**). Moreover, data collection of previous surgical and medical treatment is also important to choose appropriate pain management. The International Spinal Cord Injury Pain Basic Data Set represents an international consensus on clinical data and relevant assessments scales required for pain assessment in SCI patients (**Widerström-Noga et al., 2014**).

Pharmacological treatment

It is well known that neuroinflammatory and plastic changes which take place immediately after SCI produce higher chances to develop pain sensitization states that will eventually chronify, thus making the pain management even more difficult. For this reason, the primary intervention immediately after SCI is to stabilize the spinal cord by preventing neuroinflammatory and excitotoxic processes. For purpose of neuroprotection, methylprednisolone sodium succinate (MPSS) is the only systematically prescribed treatment after SCI (**Bracken et al., 1984**). This steroid is commonly used to limit death of spinal parenchyma in an acute stage after SCI. Despite their extensive use over the past several decades, the appropriateness of MPSS for SCI approach remains a contentious topic, suggesting that the evidence of harm is more consistent than the evidence of potential benefit (**Hurlbert et al., 2014; Fehlings et al., 2014**). A recent systematic review (**Fehlings et al., 2017**) indicates that 24-hour NASCIS II (National Acute Spinal Cord Injury Studies) MPSS regimen has no impact on long-term neurological recovery when all postinjury time points are considered. However, there is also moderate evidence that subjects receiving the same MPSS regimen within 8 hours of injury achieve a small positive benefit on long-term motor recovery compared with patients receiving placebo or no treatment. Therefore, administration of MPSS in the first 8 hours post SCI is the only pharmacological therapy provides real efficacy in terms of motor improvement.

Due to the wide range of symptoms and poor understanding of the underlying mechanisms of SCI neuropathic pain, it is not surprising that its treatment is far from being achieved effectively (**Meacham et al., 2017**). Moreover, most of the current analgesics are based on data collected from peripheral models of

neuropathic pain (**Hagen and Rekand, 2015**), although pain derived from lesions to the central nervous system, such as SCI, is more difficult to treat. Nowadays, treatment of neuropathic pain consists in a "trial-and-error" of different strategies, including pharmacological and non-pharmacological, in order to design a personalized therapeutic approach that alleviate not only pain but also the other symptoms (**Martínez-Salio et al., 2012; Attal et al., 2019**). Since SCI neuropathic pain comprise several pathological mechanisms, current treatments are often ineffective because they target only one or two of these mechanisms (**Nijs et al., 2019**). In fact, the selected pharmacological approach to treat pain after SCI is usually inadequate and only one third of patients with NP respond to pharmacological treatments when compared with placebo (**Attal et al., 2011; Attal, 2019**). Moreover, in the best case only 20-30% of pain intensity is reduced, frequently accompanied by severe side effects (**Dworkin et al., 2007; Finnerup et al., 2010**).

The Neuropathic Pain Special Interest Group (NeuPSIG) of the IASP published a guideline for pharmacological treatment of NP, fully applicable to NP after SCI. This guideline was based on a systematic review and meta-analysis of published and unpublished trials, and used the Grading of Recommendations Assessment, Development, and Evaluation (GRADE) to rate the quality of evidence and strength of recommendations (**Finnerup et al., 2015**). Therefore, pharmacological treatment of neuropathic pain following SCI is based on this guideline that comprises first-line, second-line and third-line treatment approaches depending on the evidence for their use based on GRADE (**Finnerup et al., 2015; Attal, 2019**). First-line drugs are the ones given first because they showed strong evidence for their use based on GRADE and include antidepressants as tricyclic antidepressants (TCAs) –particularly amitriptyline–, serotonin-norepinephrine reuptake inhibitors (SNRI) –particularly duloxetine –, and antiepileptics as calcium channel α2δ ligands –particularly gabapentin and pregabalin–. Second-line treatment is reserved for patients in whom the first-line therapy has failed, stopped working or has side effects that are not tolerated; and include weak opioids such as tramadol, and lidocaine and capsaicin topical agents. Finally, third-line treatment has weak evidence for their use based on GRADE and is reserved for patients who have not responded to both initial (first-line therapy) and subsequent (second-line therapy) treatment. Third-line drugs include strong opioids such as morphine and oxycodone and botulinum toxin type A. The most currently used pharmacological drugs against neuropathic pain with their mechanisms of action, effects and side effects are summarised in ***Table 3***.

Table 3. NeuPSIG recommended drugs for neuropathic pain treatment.

DRUG	MECHANISM OF ACTION	EFFECTS	SIDE EFFECTS
FIRST LINE TREATMENT			
Antidepressants - TCA			
Amitriptyline (and others such as clomipramine)	Blocking the monoamine reuptake; blockade of neuronal membrane ion channels and anticholinergic effects	Increase monoamine concentrations in the synapsis; diminution of the ion influx	Somnolence, anticholinergic effects (e.g. dry mouth, blurred vision, urinary retention), cardiac conduction abnormalities and weight gain
Antidepressants – SNRIs			
Duloxetine and venlafaxine	Serotonin and norepinephrine reuptake inhibition	Increase amine presence in the synaptic cleft potentiating their inhibitory effect	Nausea, somnolence, dizziness, increased sweating, increased blood pressure, anorexia, sexual dysfunction, abdominal pain and constipation
Antiepileptics			
Gabapentin and pregabalin	Blockage of Na_+ and Ca_{2+} channels and enhance of GABA effects	Reduction of central sensitization and subsequent neuronal hyperexcitability	Somnolence, sedation, dizziness, ataxia, weight gain and edema
SECOND LINE TREATMENT			
Topical agents			
Lidocaine	Blockade of Na_+ ion channels	Decrease Na_+ influx resulting in a reduction of neuronal excitability	Local erythema, rash and itching
Capsaicin	Agonist of TRPV1	Leads to TRPV1 desensitization and defunctionalisation of epidermal nerve fibers	Local erythema, rash, itching, pain and sometimes high blood pressure
Weak opioids			
Tramadol	μ-receptor agonist and inhibits monoamine reuptake	Activation of μ-receptor which eventually produce a depletion in neurotransmitter release; increase monoamine presence in the synapsis	Nausea, vomiting, constipation, dizziness, somnolence, urinary retention, cognitive deprivation, hyperhidrosis and a high risk of drug abuse and addiction
THIRD LINE TREATMENT			
Strong opioids			
Morphine, oxycodone and methadone	μ-receptor agonist	Activation of μ - receptor which eventually produce a depletion in neurotransmitter release	Nausea, vomiting, constipation, dizziness, somnolence, urinary retention, cognitive deprivation, hyperhidrosis and a high risk of drug abuse and addiction
Neurotoxins			
Botulinum toxin type A	Inhibition of acetylcholine release and act as a neuromuscular blocking agent		Pain at injection site with hypersensitivity and risk of infection in the painful area.

Extracted from **Deulofeu 2019**. Based on **Dworkin et al., 2007; Baron et al., 2010; Baastrup and Finnerup, 2012; Finnerup et al., 2015; Colloca et al., 2017; Zilliox et al., 2017; Attal, 2019.**

Some studies that demonstrate the specific efficacy of these drugs in relief of SCI-derived neuropathic pain are commented below:

Regarding analgesic effect of **amitriptyline**, conflicting evidence is seen between studies, including human and animal models studies. In a randomized study performed in 2002, amitriptyline did not show a significant reduction of neuropathic pain after SCI compared with placebo (**Cardenas et al., 2002**). However, few years later, another randomized controlled trial found a significant analgesic effect of this tricyclic antidepressant in SCI neuropathic pain (**Rintala et al., 2007**). In addition, a recent randomized longitudinal comparative study (**Agarwal and Joshi, 2017**) demonstrated the effectiveness of amitriptyline in traumatic SCI-induced NP, thus supporting the use of this tricyclic antidepressant in the management of NP after SCI. Regarding animal studies, a model of NP following SCI evidenced the absence of efficacy of amitriptyline in pain relieve (**Hama and Sagen, 2007**).

Among the various antidepressants, amitriptyline is the most commonly administered in a SCI rehabilitation setting (**Janzen et al., 2012**). However, it may not be an optimal treatment for SCI individuals due to the increased adverse effects and the low rates of adherence to amitriptyline treatment (**Moore et al., 2012; Mehta et al., 2015**). Other tricyclic antidepressants such as secondary amine tricyclics (e.g., nortriptyline and desipramine) are considered to be first-line choice in management of neuropathic pain in general (**Vranken et al., 2012; O'Connor et al., 2009**) because they cause less sedation and fewer anticholinergic effects compared to first-generation TCAs (e.g., amitriptyline, clomipramine, and doxepin) (**Shaw and Saulino, 2020**). However, they have not been studied in SCI patients and/or in animal models of SCI.

Analgesic effect of the non-tricyclic antidepressants such as duloxetine and trazodone have been evaluated in the randomized, placebo controlled double-blinded studies without showing any effect in patients with SCI (**Davidoff et al., 1987; Vranken et al., 2011**). On the other hand, other study that evaluated the efficacy of venlafaxine in SCI-induced pain showed significant benefits in nociceptive pain intensity, but no effects were observed on neuropathic pain sites (**Richards et al., 2015**). Therefore, although SNRIs such as duloxetine or venlafaxine are widely used in several peripheral neuropathic pain conditions (**Hoshino et al., 2015; Tawfik et al., 2018; Yamashita et al., 2016; Sindrup et al., 2003; Yucel et al., 2005**), there is no evidence in the efficacy of these antidepressants in SCI-induced neuropathic pain.

Lithium is another antidepressant drug that has shown promise in treating NP in chronic SCI. Yang and colleagues demonstrated that oral administration of lithium reduced SCI-induced NP after 6 weeks of treatment with subsequently 6-month clinical follow-up, including some patients even had complete elimination of their pain (**Yang et al., 2012**). On the other hand, another study of 27 SCI patients treated with oral lithium supported the effectiveness of this antidepressant. Characteristics of the patients included paraplegia, tetraplegia as well as complete and incomplete lesions. Treatment duration was 12 weeks and

sensory evaluation was assessed every 2 to 3 weeks. Almost half of patients had 50% or greater reduction in pain, and patients with incomplete injuries and tetraplegics showed a better improvement in pain (**Rozak and Shaw, 2019**). Although several animal studies showed the effectiveness of lithium in peripheral NP (**Banafshe et al., 2012; Shimizu et al., 2000; Gao et al., 2013**), this drug has not yet been tested in SCI animal models of neuropathic pain. Nevertheless, it has been demonstrated that lithium chloride (LiCl) contributes to blood–spinal cord barrier integrity and functional recovery from SCI by stimulating autophagic flux, indicating that LiCl may be a useful therapeutic intervention for patients with SCI, preventing BSCB disruption and facilitating locomotor recovery (**Tong et al., 2018**). Considering beforementioned, further studies on neuropathic pain following SCI are needed to confirm the effectiveness of lithium.

Pregabalin and **gabapentin** are the most studied drugs against neuropathic pain following SCI. Two mechanisms of action for gabapentinoids drugs in the spinal dorsal horn have been proposed: (1) decrease of presynaptic neurotransmitter release due to an inhibition of endosomal VGCCs recycling which results in a reduced channel expression (**Tran-Van-Minh and Dolphin, 2010**) and (2) decrease of postsynaptic excitability by inhibition of excitatory synaptogenesis mediated by interaction between α2δ-1 subunit of VGCCs and extracellular matrix proteins such as thrombospondins. However, it remains to be determined whether is sufficient to account for the broad clinical spectrum of pregabalin and gabapentin (**Eroglu et al., 2009**).

Although there are several randomized controlled trials which have reported gabapentin and pregabalin efficacy in relieving neuropathic pain following SCI (**Levendoglu et al., 2004; Siddall et al., 2006; Cardenas et al., 2013; Rintala et al., 2007**), other studies questioned their efficacy with controversial results. Thus, it has been suggested that gabapentin lost its efficacy in patients reporting neuropathic pain symptoms for more than 6 months (**Ahn et al., 2003**). Moreover, it presents some limitations, as for instance, only those patients able to tolerate short and long terms side effects had a significant pain relieve (**Putzke et al.,2002**). Related to this, literature data suggest that pregabalin is more efficacious than gabapentin in relieve neuropathic pain after SCI. However, pregabalin is followed by more side effects than gabapentin, hindering the treatment adherence (**Tzellos et al., 2008; Yu et al., 2019**). In parallel with clinical evidence, gabapentin and pregabalin sometimes fail to provide analgesia in animal models of neuropathic pain fallowing SCI, despite being the first-choice analgesics for chronic neuropathic pain (**M'Dahoma et al., 2014; Kimura et al., 2016**).

Tramadol is a weak opioid that has been shown to decrease pain intensity and severity in a randomized placebo-controlled study of SCI-related NP. However, adverse events were substantial and caused 43% of treated patients to withdraw from the study. (**Norrbrink et al., 2009**). Moreover, evidence quality of the study has been questioned because of wide confidence intervals (**Guy et al., 2016**).

Strong opioid **morphine** was assessed in a double-blind placebo-controlled study including 9 SCI patients showing no effect in NP relieve (**Attal et al., 2002**). Moreover, other animal study showed the ineffectiveness of morphine in NP following SCI (**Hook et al., 2017**). However, morphine and clonidine combination treatment showed favourable effects on SCI-induced NP in two studies including a total of 23 patients (**Siddall et al., 2000; Uhle et al., 2000**). Oral administration of another strong opioid, oxycodone, has demonstrated additional improvement of pain in NP-derived SCI patients pre-treated with antiepileptic drugs (**Barrera-Chacon et al., 2011**). In summary, use of opioids alone or with other medications may be an option for patients who have not responded to first- and second-line medications. However, they have a lot of side effects along with the risk of drug abuse may complicate a long-term use. Moreover, chronic opiates administration may result in an increased sensitivity to painful stimuli, a phenomenon called "opioid induced hyperalgesia (OIH)" (**Roeckel et al., 2016**).

Several case reports have suggested that use of topical analgesics such as **lidocaine** and **capsaicin** can be beneficial in treating SCI-related NP (**Hans et al., 2008; Wasner et al., 2007; Trbovich et al., 2015; Freo et al., 2016; Crul et al., 2020**). However, the evidence is scarce and controversial. For instance, one report indicated that 0.6% capsaicin patch increased thermal pain (**Wasner et al., 2007**) whereas other case report indicated capsaicin 8% plaster alleviated pain (**Trbovich et al., 2015**).

Another new approach for treatment of well-localized neuropathic pain is intradermal use of **botulinum toxin A** (BTX-A) injections. In a randomized, double-blind, placebo-controlled study on 40 patients with SCI-associated NP, the effect of BTX-A was assessed. Between 45% and 55% of patients treated with BTX-A reported pain relief of 20% or greater (**Han et al., 2016**). In addition, other case report (**Han et al., 2014**) and case series (**Jabbari et al., 2003**) supported the effectiveness of BTX-A on SCI-associated NP. In conclusion, BTX-A treatment should be studied further on SCI-associated NP patients for stablish its effectiveness.

Cannabis-based medications have been suggested to have a beneficial effect on SCI-derived NP. In fact, several studies in preclinical animal models showed reduced hypersensitivity in SCI-NP (**Hama and Sagen, 2010; Ahmed et al., 2010**). On the other hand, a pilot study comparing **dronabinol** against an active placebo diphenhydramine showed no significant difference in relief of below-level SCI pain (**Rintala et al., 2010**). Yet another small study (n=3) testing a cannabinoid analogue demonstrated significant improvements in SCI-NP patients (**Karst et al., 2003**). For now, these studies are so far too limited, and the efficacy of cannabinoids continues to remain unproven as a reliable analgesic for NP following SCI.

It is worth mentioning that one of the biggest problems of pharmacological SCI-NP treatment is that drug efficacy is generally dose-dependent. Consequently, when pain becomes unbearable, higher doses of drug treatment are usually prescribed. In parallel, it has been demonstrated that higher drugs doses imply major

and more serious side effects (**Table 3**) becoming a serious problem for treatment adherence and the daily well-being of SCI patients (**Hagen and Rekand, 2015**).

Non-pharmacological treatment

Interventional and surgical treatment

Commonly, patients with SCI and chronic neuropathic pain conditions need complementary non-pharmacological treatments which can help to alleviate their pain. In general, invasive treatments for SCI-NP have not been sufficiently studied to permit an adequate evaluation of their efficacy. In concordance with actual literature, **spinal cord stimulation**, **deep brain stimulation** and **dorsal root entry zone (DREZ)** operations below level are not recommended for SCI pain due to treatments do not reduce NP or when reduced it, the effectiveness of the treatment decrease over time (**Cioni et al., 1995; Richardson et al., 1980; Falci et al., 2002; Lagauche et al., 2009**). In contrast, there are some evidences of efficacy for **motor cortex stimulation** and for the ablation of spinal cord neuronal hyperactivity in several segments above lesion level (**Prévinaire et al., 2009; Chun et al., 2011**).

Non-invasive treatment

Survey studies (**Widerström-Noga and Turk, 2003; Dalyan et al., 1999; Cardenas et al., 2006; Budh et al., 2004**) reported that most people with SCI-associated persistent NP have tried at least one type of alternative therapy to manage their pain. Non-invasive treatments include both **physical interventions** – such as exercise, massage, acupuncture, and transcutaneous electrical nerve stimulation –, and **psychological treatments** – such as cognitive restructuring, relaxation, and self-hypnosis techniques –.

Several human studies showed that exercise in the form of strength training, cardiovascular training, or informal physical activities is beneficial for reduce overall pain in SCI patients (**Hicks et al., 2003; Curtis et al., 1999; Nawoczenski et al., 2006**). Despite the specific effect of exercise in SCI-NP has not been systematically tested in humans, several animal studies showed antinociceptive behaviours after various weeks of exercise training (**Hutchinson et al., 2004; Kuphal et al., 2007**). Currently, no clinical trials of massage therapy in persons with SCI-chronic pain have been reported in the literature, but survey studies have shown that massage therapy is one of the most frequently used and effective non-pharmacological pain therapies for SCI patients (**Widerström-Noga and Turk, 2003; Cardenas et al., 2006**). Similarly, between 15% and 35% of SCI patients have tried acupuncture for relief of pain (**Budh et al., 2004; Dalyan et al., 1999; Cardenas et al., 2006**). Concretely, a retrospective chart-review study showed that 67% of patients treated with a standardized scalp acupuncture protocol reported reduced ratings of their below-level NP (**Rapson et al., 2003**). In a study of transcutaneous electrical nerve stimulation, between 29% and 38% of SCI patients reported favourable effect of this treatment in NP relief (**Norrbrink et al., 2009**).

Finally, psychological-based treatments may be effective for reducing SCI-associated NP, providing a more cost-effective intervention with no side effects (**Ehde and Jensen, 2007**). One technique is cognitive behavioural treatment in which the patient is trained to use adaptive coping thoughts ("cognitive restructuring") and behaviours (e.g., distraction and relaxation techniques) to manage pain. Several randomized controlled trials supported the effectiveness of this therapy for reducing NP and related conditions such as depression or anxiety (**Ehde et al., 2004; Budh et al., 2006**).

In summary, there is scarce conclusive clinical trials of both pharmacological and non- pharmacological treatment conducted in SCI-NP community. The main reason is the poorly size sample of studies due to logistic issues related to transportation and patient burden as well as participants concern about addiction and adverse effects of pharmacological medication (**Widerström-Noga, 2017**). On the other hand, the current existing pain drugs reveal various but typically low levels of analgesic efficacy and are generally coupled with deleterious effects. Furthermore, under the lack of effective alternatives, the major abuse liabilities of gabapentinoids and opiods become an ongoing critical crisis. In this context, it is imperative to develop novel analgesic agents with superior efficacy, lower side effects and reduced abuse liability. To address this concern, preclinical experiments are essential to develop potential treatments against NP following SCI. Among them, the use of polyphenolic compounds may be a promising option since scientific literature provides pre-clinical experimental evidence on their antinociceptive effects in animal models of neuropathic pain.

2. Polyphenolic compounds as potential pharmacological strategy to treat neuropathic pain after spinal cord injury

Polyphenolic compounds constitute one of the most important natural products within the plant kingdom, with more than 8000 phenolic structures currently known. Some rich natural sources of polyphenols include fruits, vegetables, whole grains and several other foods and beverages such as coffee, wine, and chocolate (**Bravo 1998; Waterman and Lockwood, 2007**). Polyphenols present at least one hydroxyl group (–HO) in their aromatic ring(s) and they can be conjugated with sugars, amines, organic acids, carboxylic, lipids, as well as other phenols (**Tsao et al., 2010**). According to the number of phenol rings and the structural elements that bind these rings to one another, polyphenols can be classified as phenolic acids, phenolic alcohols, flavonoids, stilbenes and lignans (***Fig.21***) (**D'Archivio et al., 2007**).

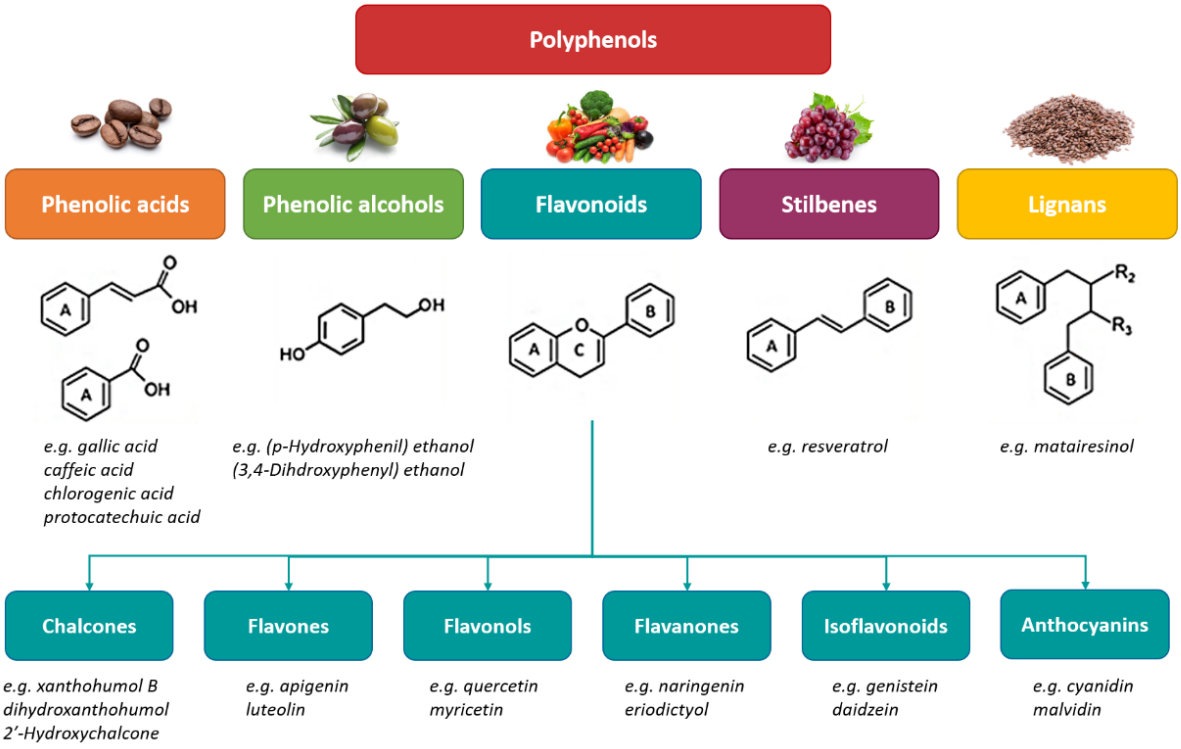


Figure 21. Classification of the polyphenolic compounds according to the number of phenol rings and the structural that bind these rings to one another. Based on **Boadas-Vaello et al., 2017** and **Hano and Tungmunnithum, 2020**.

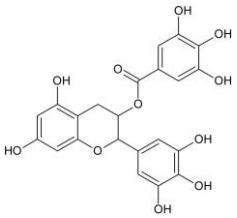
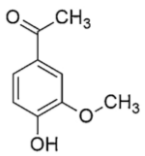
The scientific literature provides not only wide pre-clinical experimental evidence on the antinociceptive effect of natural polyphenols in both peripheral and central neuropathic pain, but also in the nociceptive and inflammatory pain (**Boadas-Vaello et al., 2017**). These polyphenolic compounds include flavonoids (**Meotti et al., 2006; Choi et al., 2012; Kuang et al., 2012; An et al., 2014; Uddin et al., 2020; Basu et al., 2020**), lignans (**Kassuya et al., 2003; Kassuya et al., 2006; Hu et al., 2015**), phenolic acids (**Bagdas et al., 2013; Xu et al., 2016; El Gabbas et al., 2019**) and nonflavonoid polyphenols such as resveratrol (**Sharma et al., 2007; Yin et al., 2013; Tao et al., 2016; Xie et al., 2017; Xu et al., 2018; Li et al., 2019**), egallic acid (**Jain et al., 2013; Mansouri et al., 2015; Naghizadeh et al., 2016**), curcumin (**Zhao et al., 2012; Jeon et al., 2013; Ji et al., 2013; Li et al., 2013; Di et al., 2014; Zanjani et al., 2014; Zhu et al., 2014; Liu et al., 2016**) and rosmarinic acid (**Lucarini et al., 2013; Rahbardar et al., 2018**) among others (***Table 4***).

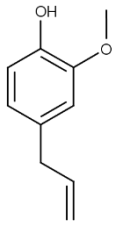
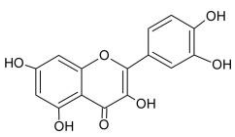
According to literature, these polyphenols alleviate neuropathic pain through numerous and diverse mechanisms, including modulation of serotonergic, noradrenergic, GABAergic, glutamatergic (NMDAR-dependent) and peptidergic neurotransmission; regulation of several cell membrane receptors and ion channels such as P2X3, CX3CR1, TRL4, TRPV1, K^+ and Na^+ channels; and inhibition of intracellular MAPK pathways (ERK1/2, JNK, p38MAPK), protein-kinases (PKA, PKC), phosphodiesterases (PDE2), phospholipases (PLC), apoptotic pathways (Bax/Bcl2, caspases), enzymes (iNOS, COX-2, FASN), and transcription factors (NF-κB, STAT). Furthermore, the inhibition of these signalling pathways is accompanied by downregulation of

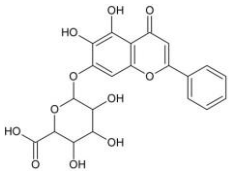
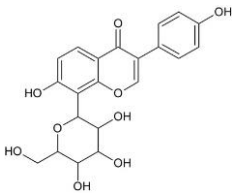
pro-inflammatory agents, such as cytokines (TNF-α, IL-1β, IL-6) and prostaglandins (PGE2), and free radicals (ROS, NO). In contrast, upregulation of anti-inflammatory cytokines (IL-10) is also stated. In summary, free radical scavenging/antioxidant, immunomodulatory, neuroprotective, antiapoptotic and autophagy-regulating activities are attributed to polyphenols and may account for pain modulation; among their cellular targets there are not only neurons but also spinal astrocytes and microglia (that are activated following persistent nociceptive inputs, contributing to neuropathic pain maintenance) (**Boadas-Vaello et al., 2017**).

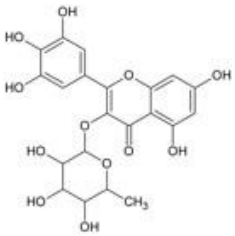
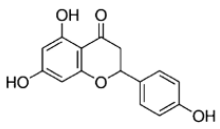
In ***Table 4*** are indicated some of the representative polyphenols that exert anti-nociceptive effects in animal models of pain with the cellular and/or molecular mechanisms underlying these effects.

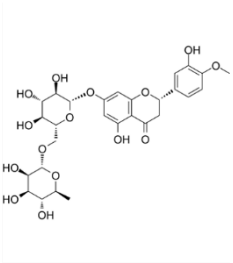
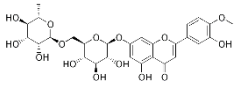
Table 4. Polyphenols which exert anti-nociceptive effects, proposed mechanism of action and pharmacological effects in animal models.

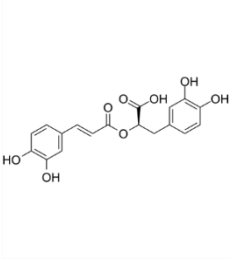
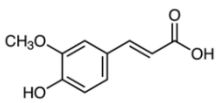
POLYPHENOL	CHEMICAL STRUCTURE	PROPOSED MECHANISM OF ACTION	ANTINOCICEPTIVE EFFECTS IN ANIMAL MODELS
Epigallocatechin -3-gallate		↓RhoA, ↓FASN, ↓MPO, ↓iNOS ↓COX-2, ↑BDNF, ↓PARP ↓MDA (lipid peroxidation) ↓pro-inflammatory cytokines (TNF-α, IL-1β). Modulation of p38MAPPK, JNK, NF-kappaB pathways ↓Bax, ↑Bcl-2, ↓capase-3	**↓ Hyperalgesia and/or allodynia after:** Spinal cord injury (SCI) (**Álvarez-Pérez et al., 2016; Renno et al., 2014**) Chronic constriction injury (CCI) (**Xifró et al., 2015; Kuang et al., 2012; Bosch-Mola et al., 2017**) Sciatic nerve crush injury (SNCI) (**Renno et al., 2017**) Spinal nerve ligation (SNL) (**Choi et al., 2012; Krupkova et al., 2014, An et al., 2014**) STZ induced diabetic neuropathic pain (**Morgado et al., 2012; Raposo et al., 2015**)
Apocynin		↓NADPH-oxidase, ↓MPO, ↓ROS, ↑SOD ↓MDA Modulation of p38MAPPK, JNK, NF-kappaB pathways	**↓ Hyperalgesia and/or allodynia after:** Spinal cord injury (SCI) (**Hassler et al., 2012; 2014**) Chronic constriction injury (CCI) (**Choi et al., 2013**)

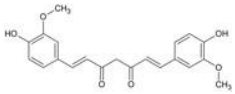
POLYPHENOL	CHEMICAL STRUCTURE	PROPOSED MECHANISM OF ACTION	ANTINOCICEPTIVE EFFECTS IN ANIMAL MODELS
		↓pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-18) ↓Bax, ↑Bcl-2, ↓capase-3, ↓caspase-1	STZ-induced diabetic neuropathic pain (**Olukman et al., 2018**)
Eugenol		↓pro-inflammatory cytokines and mediators (TNF-α, IL-1β, IL-6, NF-κB subunit p65, p38MAPK, iNOS) ↓oxidative stress (↑SOD, ↓MDA, ↑CAT and ↑GSH-PX) Modulation of NF-κB pathway ↓apoptotic factors (caspase 3) Inhibition of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels ↓substance P, ↓CGRP ↑dynorphin	**↓ Hyperalgesia and/or allodynia after:** Spinal cord injury (SCI) (**Ma et al., 2018**) Chronic constriction injury (CCI) (**Yeon et al., 2011**) Sciatic nerve ligation (SNL) (**Lionnet et al., 2010**) Sciatic nerve cuff-implanted induced neuropathic pain (**Guénette et al., 2007**) Acrylamide-Induced neuropathy (**Prasad et al., 2013**) Monoiodoacetate-induced model of osteoarthritis (**Ferland et al., 2012**) Formalin-induced pain (inflammatory pain) (**Lugo-Lugo et al., 2019**)
Quercetin		Interaction with L-arginine-NO, serotonin and GABAergic systems Scavenges reactive oxygen and nitrogen species. Inhibits phospodiesterases Exerts inhibitory effects on prominent pro-inflammatory signalling pathwyas ($STAT_1$, NF-kappaB, MAPK) Inhibits mTOR/p70S6K pathway-mediated changes of synaptic morphology and synaptic protein levels in spinal DH	**↓ Hyperalgesia and/or allodynia after:** Chronic constriction injury (CCI) (**Çivi et al., 2016**) Spinal nerve ligation (SNL) (**Ji et al., 2017**) Spared nerve injury (SNI) (**Muto et al., 2018**) Diabetic neuropathic pain (**Wang et al., 2020; Yang et al., 2019; Anjaneyulu and Chopra, 2003**)

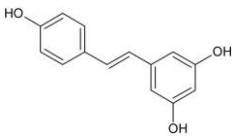
POLYPHENOL	CHEMICAL STRUCTURE	PROPOSED MECHANISM OF ACTION	ANTINOCICEPTIVE EFFECTS IN ANIMAL MODELS
			Paclitaxel-induced neuropathy (chemotherapy-induced neuropathic pain) (**Gao et al., 2016**) Oxaliplatin-induced neuropathy (chemotherapy-induced neuropathic pain) (**Azevedo et al., 2013; Schwingel et al., 2014**) Alcoholic neuropathy (**Raygude et al., 2012**) Formalin/carrageenan-induced pain (inflammatory pain) (**Valério et al., 2009**)
Baicalin		↓ROS, ↓NO, ↓Nrf2 ↓ PGE2 ↓ lipid peroxidation ↓pro-inflammatory cytokines (TNF-α, IL-6, IL-17 and IL-1β) ↓ TRPV1 mRNA and protein overexpression ↑ α2-adrenoceptor expression ↓pERK ↑pAkt-mediated adult hippocampal neurogenesis ↓Histone Deacetylase 1 (HDAC1) expression Prevention of histone-H3 acetylation	**↓ Hyperalgesia and/or allodynia after:** Chronic constriction injury (CCI) (**Wang et al., 2020**) Spinal nerve ligation (SNL) (**Tsai et al., 2013; Cherng et al., 2014; Huang et al., 2020**) Partial sciatic nerve transection (PST) (**Lai et al., 2018**) STZ-induced diabetic neuropathic pain (**Li et al., 2018**) Bone cancer pain induced by tumour cell implantation (**Li et al., 2020**) Inflammatory pain induced by CFA (**Fang et al., 2020**) Join pain/osteoarthritis induced by meniscectomy (**Chen et al., 2018**)
Puerarin		↓pro-inflammatory cytokines (IL-6, IL-1β, TNF-α) Modulation of ERK, CREB, NFκB, TGF-β/Smad and BDNF pathways	**↓ Hyperalgesia and/or allodynia after:** Chronic constriction injury (CCI) (**Liu et al., 2014; Xu et al., 2012**) Spared nerve injury (SNI) (**Zhao et al., 2017**)

POLYPHENOL	CHEMICAL STRUCTURE	PROPOSED MECHANISM OF ACTION	ANTINOCICEPTIVE EFFECTS IN ANIMAL MODELS
		↓purinergic receptors ($P2X_3$, $P2X_{2/3}$ and $P2X_7$), ↓TRPV1, ↓TRPA1, ↓excitability and block Nav1.8 ↓CGRP, ↓substance P, ↓VEGF	Partial sciatic nerve transection (PST) (**Xie et al., 2018**) STZ-induced diabetic neuropathic pain (**Liu et al., 2014**) Paclitaxel-induced neuropathy (chemotherapy-induced neuropathic pain) (**Zhang et al., 2019; Wu et al., 2019**) Vincristine-induced neuropathy (chemotherapy-induced neuropathic pain) (**Xie et al., 2020**) Inflammatory pain induced by CFA (**Ullah et al., 2018**)
Myricitrin		↓MPO activity Inhibition of the nociceptors activation via inhibition of PKC pathways ↓pro-inflammatory mediators (TNF-α, NO) Modulation (↓ phosphorylation) of ion channels and p38MAPK	**↓ Hyperalgesia and/or allodynia after:** Spinal nerve ligation (SNL) (**Hagenacker et al., 2010**) Partial sciatic nerve ligation (PSNL) (**Meotti et al., 2006**) Inflammatory pain induced by CFA (**Meotti et al., 2006**) Intraplantar injection of a variety of chemical algogens (**Córdova et al., 2011**) Acetic acid-induced visceral pain (**Meotti et al., 2006, 2007; Ferreira et al., 2013**) Formalin/carrageenan-induced pain (**Ferreira et al., 2013**)
Naringenin		↓pro-inflammatory cytokines and mediators (TNF-α, IL-6, IL-1β, IL-12, TGF- β, MCP-1, NO, MMP-9) Antioxidant protection (↑SOD activity, ↓MPO activity, ↓Nrf2 levels)	**↓ Hyperalgesia and/or allodynia after:** Chronic constriction injury (CCI) (**Kaulaskar et al., 2012**)

POLYPHENOL	CHEMICAL STRUCTURE	PROPOSED MECHANISM OF ACTION	ANTINOCICEPTIVE EFFECTS IN ANIMAL MODELS
		↓lipid peroxidation ↓ glial cell activation (↓GFAP, ↓Mac-1) Modulation of NFκB and NO-cGMP-PKG pathways Inhibition of NaV1.8 Voltage-Gated Sodium Channels	Partial sciatic nerve ligation (PSNL) (**Dallazen et al., 2017**) Spinal nerve ligation (SNL) (**Hu and Zhao, 2014**) Paw incision model of postsurgical pain (**Zhou et al., 2019**) STZ-induced diabetic neuropathic pain (**Singh et al., 2020; Al-Rejaie et al., 2015; Hasanein et al., 2014**) Inflammatory pain induced by acetic acid, PBQ, formalin, capsaicin, carrageenan, PGE2, CFA, KO_2 and LPS (**Pinho-Ribeiro et al., 2016a; Pinho-Ribeiro et al., 2016b; Manchope et al., 2016; Dallazen et al., 2017; Xue et al., 2019**) Chronic Sleep Deprivation-Induced Pain (**Arora et al., 2021**)
Hesperidin		↓pro-inflammatory cytokines (TNF-α, IL-6, IL-1β) ↓P2X3 mRNA and protein expression Inhibition of P2X3 and suppression of P2X3 receptors upregulation Antioxidant protection (↑SOD activity, ↓NO) ↓lipid peroxidation	**↓ Hyperalgesia and/or allodynia after:** Chronic constriction injury (CCI) (**Carballo-Villalobos et al., 2016; 2017; Tao et al., 2019**) Partial sciatic nerve ligation (PSNL) (**Aswar et al., 2014**) STZ-induced diabetic neuropathic pain (**Visnagri et al., 2014**)
Diosmin		↓pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-33/St2) ↓glial cells activation (↓GFAP -astrocytes-, ↓Iba-1 -microglia-, ↓Olig2 – oligodendrocytes, mRNA expression) Activation of NO/cGMP/PKG/ATP-sensitive potassium channels signalling pathway	**↓ Hyperalgesia and/or allodynia after:** Chronic constriction injury (CCI) (**Bertozzi et al., 2017; Carballo-Villalobos et al., 2018**) STZ-induced diabetic neuropathic pain (**Jain et al., 2014**)

POLYPHENOL	CHEMICAL STRUCTURE	PROPOSED MECHANISM OF ACTION	ANTINOCICEPTIVE EFFECTS IN ANIMAL MODELS
		Modulation of NF-κB pathway Modulation of opioid and D_2 dopaminergic pathways Antioxidant protection (↑SOD activity, ↓NO, ↓ROS, ↑GSH) ↓MDA (lipid peroxidation)	Lipopolysaccharide-Induced Inflammatory Pain (**Fattori et al., 2020**)
Rosmarinic acid		↓pro-inflammatory cytokines and mediators (TNF-α, COX2, PGE-2, IL-1β, MMP2 and NO) ↓glial cells activation (↓GFAP -astrocytes-, ↓Iba-1 -microglia-) ↓apoptotic factors (Bax, caspases 3, 9) ↓MDA (lipid peroxidation)	**↓Hyperalgesia and/or allodynia after:** Chronic constriction injury (CCI) (**Rahbardar et al., 2017; 2018; El Gabbas et al., 2019**) STZ-induced diabetic neuropathic pain (**Hasanein et al., 2014**) Oxaliplatin-induced neuropathy (chemotherapy-induced neuropathic pain) (**Areti et al., 2018**) Formalin/carrageenan-induced pain (inflammatory pain) (**Boonyarikpunchai et al., 2014**)
Ferulic acid		↓pro-inflammatory cytokines and mediators (TNF-α, IL-1β, IL-6, COX-2 and NO) ↑anti-inflammatory cytokines (IL-10) ↓lipid peroxidation (↓LPO, ↓MDA) Antioxidant protection (↑SOD activity, ↑GSH, ↓NOS, ↓ROS) Antiapoptotic effects (↓Bax, ↑Bcl-2, ↓capase-3) ↓substance P, NF-κβ p65 and total calcium levels ↑ spinal NA and 5-HT	**↓Hyperalgesia and/or allodynia after:** Chronic constriction injury (CCI) (**Aswar and Patil, 2016; Xu et al., 2016**) STZ-induced diabetic neuropathic pain (**Dhaliwal et al., 2020**) Reserpine-induced pain (fibromyalgia pain model) (**Xu et al., 2013**) Vincristine-induced neuropathy (chemotherapy-induced neuropathic pain) (**Vashistha et al., 2017**)

POLYPHENOL	CHEMICAL STRUCTURE	PROPOSED MECHANISM OF ACTION	ANTINOCICEPTIVE EFFECTS IN ANIMAL MODELS
Curcumin		↓pro-inflammatory cytokines and mediators (TNF-α, IL-1β, IL-6, MCP-1, MIP-1α and NO) ↓phosphorylation of NR1 subunit of NMDA ↓P2Y12 and $P2X_3$ receptor ↓BDNF, COX2, 11-β-HSD1, p-Akt Cx43, CX3CR1 and astroglial NALP1 inflammasome Modulation of ERK, STAT3, JNK, NF-kappaB pathways Modulation of monoamine system and opioid receptors ↓astrocytes activation (↓GFAP) ↓lipid peroxidation (↓MDA, ↓LPO) Antioxidant protection (↑SOD, ↑GSH, ↑catalase) Activation of NO/cGMP/PKG/ATP-sensitive potassium channels signalling pathway ↓ TTX-R sodium currents	**↓ Hyperalgesia and/or allodynia after:** Chronic constriction injury (CCI) (**Li et al., 2009; Zhao et al., 2012; Ji et al., 2013; Jeon et al., 2013; Cao et al., 2014; Zhu et al., 2014; Zanjani et al., 2014; Di et al., 2014; Limcharoen et al., 2021**) Spared nerve injury (SNI) (**Liu et al., 2016**) Spinal nerve ligation (SNL) (**Lee et al., 2014; Pastrana-Quintos et al., 2020**) STZ-induced diabetic neuropathic pain (**Sharma et al., 2006; 2007; Li et al., 2013; Zhao et al., 2014; Banafshe et al., 2014; Meng et al., 2015; Jia et al., 2018**) HIV-gp120-associated neuropathic pain (**Zhao et al., 2017**) Alcoholic neuropathy (**Kandhare et al., 2012; Kaur et al., 2017**) Formalin-induced pain (**Han et al., 2012**) Inflammatory pain induced by CFA or capsaicin (**Yeon et al., 2010; Singh and Vinayak, 2015; Chen et al., 2015**) Incisional surgery induced-postoperative pain (**Zhu et al., 2014**) Brachial plexus avulsion (BPA)-induced neuropathic pain (**Xie et al., 2019**) Vincristine-induced neuropathy (chemotherapy-induced neuropathic pain) (**Babu et al., 2015**)

POLYPHENOL	CHEMICAL STRUCTURE	PROPOSED MECHANISM OF ACTION	ANTINOCICEPTIVE EFFECTS IN ANIMAL MODELS
			Oxaliplatin-induced neuropathy (chemotherapy-induced neuropathic pain) (**Zhang et al., 2020**)
Resveratrol		↓pro-inflammatory cytokines and mediators (TNF-α, IL-1β, IL-6, COX-2, iNOS, NO and $ONOO^-$) ↑anti-inflammatory cytokines (IL-10) and ↑anti-inflammatory cytokine receptors (IL-1RA, IL-1R2 and IL-4Rα) ↓phosphorylation of p38 and ERK1/2 ↓p65 and IκB-α expression ↓$P2X_7$ and $P2X_3$ receptors ↓CX3CR1 and Nav1.7 expression ↑activation of potassium channels ↑Sirt1 expression and ↓acetyl-histone H3 ↑IGF-1 ↓NOS and COX2 activity ↑ SOD activity ↓lipid peroxidation (↓MDA) Antioxidant protection (↑catalase) Modulation of NF-κB pathway Inhibition of AMPK Downregulation of NMDA (NR_1, NR_2B) expression ↓glia activation (↓GFAP, ↓Iba-1) Modulation of spinal and supraspinal serotonergic systems	**↓ Hyperalgesia and/or allodynia after:** Chronic constriction injury (CCI) (**Yin et al., 2013; Zhao et al., 2014; Bagriyanik et al., 2014; Tao et al., 2016; Xie et al., 2017; Xu et al., 2018; Jia et al., 2020**) Partial sciatic nerve ligation (pSNL) (**Guo et al., 2019**) Spinal nerve ligation (SNL) (**Pérez-Severiano et al., 2008**) STZ-induced diabetic neuropathic pain (**Sharma et al., 2007; Kumar et al., 2007; Kumar and Sharma, 2010; Cui et al., 2020**) Paclitaxel-induced neuropathy (chemotherapy-induced neuropathic pain) (**Li et al., 2019**) Vasculitic peripheral neuropathy (VPN) (**Pan et al., 2019**) Bone cancer pain model (**Cheng et al., 2014**) Formalin/carrageenan-induced pain (inflammatory pain) (**Gentilli et al., 2001; Torres-López et al., 2002; Granados-Soto et al., 2002**) Inflammatory pain induced by CFA (**Singh et al., 2016**)

More related to the present thesis, several studies have demonstrated both anti-nociceptive and neuroprotective effects of certain polyphenols in animal models of SCI. These natural phenolic compounds include flavonoids such as epigallocatechin-3-gallate (EGCG) and other simpler phenols such as apocynin and eugenol.

Epigallocatechin-3-gallate (EGCG) is the most abundant catechin in green tea (*Camellia sinensis*) and it has shown neuroprotective (**Tiraihi et al., 2010**), anti-nociceptive (**Renno et al., 2014**), anti-inflammatory (**Lambert et al., 2010**), antioxidant (**Elbling et al., 2005**), and anti-apoptotic properties (**Schroeder et al., 2009**) in SCI animal models. Regarding antinociceptive effects of this polyphenol in SCI-derived neuropathic pain, EGCG (30 mg/kg i.p.) treatment reduced thermal hyperalgesia after SCI by down-regulating RhoA, FASN and TNF-α proteins expression in mice (**Álvarez-Pérez et al., 2016**). In addition, another study showed that EGCG (20 mg/kg, i.v.) administration inhibited chronic SCI-induced mechanical and thermal hypersensitivity (**Renno et al., 2014**). On the other hand, treatment with EGCG (50 mg/kg, i.p.) was shown to attenuate malondialdehyde (MDA) levels as an indicator of lipid peroxidation, neuronal apoptosis, spinal tissue loss and motor dysfunction after SCI in rats (**Tiraihi et al., 2010**). Reduced activity of myeloperoxidase (MPO) and attenuated myelin degradation, IL-1β, TNF-α, nitrotyrosine, iNOS, cyclooxygenase 2 (COX-2) and poly-(ADP-ribose) polymerase (PARP) could also be detected after SCI in rats treated with EGCG (50 mg/kg, i.p.) (**Khalatbary and Ahmadvand, 2011**). Moreover, EGCG treatment (10 and 20 mg/kg, i.t.) upregulated the glial cell line-derived neurotrophic factor, BDNF, and Bcl-2 inhibited pro-apoptotic Bax after SCI (**Tian et al., 2013**). In this fashion, Renno and collegues also displayed that EGCG modulated spinal cord neuronal degeneration by reducing Bax and increasing Bcl-2 (**Renno et al., 2015**). Finally, the inhibitory effects of EGCG on caspase-3 expression was also reported, thus evidencing its anti-apoptotic role after SCI (**Ahadi et al., 2019**).

In this way, **apocynin**, also called acetovanillone, is an organic polyphenolic compound isolated from a variety of plant sources such as Canadian hemp root (*Apocynum cannabinum*) or small plant that grows at high altitudes in the western Himalayas (*Picrorhiza kurroa*), with numerous biological activities (**Simonyi et al., 2012**). Intrathecal lumbar injections with apocynin (0.1 mg/kg) exhibited analgesic effect on chronic SCI rats reducing allodynia to thermal and mechanical stimuli as well as reactive oxygen species (ROS) and the downstream lipid peroxidation products (**Hassler et al., 2014**). Another study showed that apocynin (50 mg/kg, i.p.) decreased oxidative damage, inhibited the SCI-induced neuronal death, diminished the inflammatory response by reducing the secretion of inflammatory cytokines and resulted in the promotion of locomotor function in SCI rats (**Sun et al., 2017**). In another study with clip compression SCI mice, apocynin administration (5 mg/kg, i.p.) also caused a significant reduction in expression levels of inflammatory cytokines (IL-1β, TNF-α), p-JNK, p38, and NF-κB, all of them related to neuropathic pain

development and maintenance after SCI (**Impellizzeri et al., 2011**). In a more recent study, Liu and collaborators (**Liu et al., 2020**) demonstrated the inhibitory effects of apocynin (100 mg/kg, i.p.) on NADPH oxidase, p-JNK, caspase-1, pp38, p-NF-κB p65, TNF-α, and IL-1β to suppress the inflammatory, oxidative and apoptotic pathways in a rat model of SCI hemi-contusion. Besides, Zhang and co-workers exhibited the potential neuroprotective effects of apocynin (5 mg/kg, i.p.) by suppressing neuroinflammation through inhibiting the production of NADPH oxidase-mediated ROS in spinal cord injured mice (**Zhang et al., 2019**).

Eugenol (4-allyl-2-methoxyphenol) is a methoxyphenol with a short hydrocarbon chain that belongs to the phenylpropanoids class of chemical compounds and it can be isolated from certain essential oils especially from clove oil, nutmeg, cinnamon, basil, and bay leaf. Eugenol has shown its anti-nociceptive, anti-inflammatory, antioxidant and anti-apoptotic properties in SCI animal models. Concretely, in a rat model of SCI (**Ma et al., 2018**) eugenol markedly improved locomotor function and relieved neuropathic pain accompanied by decreased inflammation (decreased TNF-α, IL-1β, IL-6 and NF-κB subunit p65), oxidative stress (increased superoxide dismutase, catalase and glutathione peroxidase activities and decreased malondialdehyde activity) and neural apoptosis (decreased caspase 3) in both spinal cord and serum. Moreover, downregulated pathway molecules NF-κB and p38 MAPK were also found in the spinal cord (**Ma et al., 2018**).

On the other hand, flavonoid **quercetin** showed neuroprotective effects inhibiting the activation on p38MAPK/iNOS signaling pathway and thus regulating secondary oxidative stress following acute SCI in rats (**Song et al., 2013**). Further, Fan and colleagues found that quercetin significantly reduced necroptosis of oligodendrocytes after SCI without influencing their apoptosis and regeneration by supressing macrophages/microglia polarization to M1 phenotype through inhibition of STAT1 and NF-κB pathway in SCI rats (**Fan et al., 2019**). Other study revealed that quercetin promoted locomotor and electrophysiological functional recovery, astrocyte activation and axonal regeneration after acute SCI in rats, possibly through BDNF and JAK2/STAT3 signalling pathways (**Wang et al., 2018**). Although quercetin executes antioxidant effects, neurological functional recovery, increased axonal preservation, decreased the size of the cystic cavity, reduced the proinflammatory cytokines, and increased anti-inflammatory cytokines in SCI animal models (**Çiftçi et al., 2016; Wang et al., 2018**), their efficacy in neuropathic pain treatment after SCI has not been yet proved. However, promising results in other experimental models of neuropathic pain (**Valério et al., 2009; Anjaneyulu and Chopra 2003; Raygude et al., 2012; Azevedo et al., 2013; Ji et al., 2017; Yang et al., 2019**) make hypothesize that quercetin may be a good treatment for neuropathic pain following SCI.

Similarly, other flavonoids such as **baicalin**, **puerarin**, **myricitrin**, **naringenin** and **hesperidin** have also been shown to have neuroprotective and anti-inflammatory properties after SCI (**Cao et al., 2010; Kang et al., 2018; Sang et al., 2004; Tian et al., 2013, 2015, Zhang et al., 2016**). Thus, the effect of **baicalin** treatment (100 mg/kg, i.p.) on spinal cord injured rats dramatically decreased oxidant stress (decreased malondialdehyde and increased glutathione levels), inflammation (decreased TNF-α and NF-κB) and apoptosis (decreased Bax and Bcl-2 levels and increased caspase-3 expression) (**Cao et al., 2010**). Moreover, a more recent study showed that baicalin has protective effects in improving SCI and lower limb motor function, has a significant anti-inflammatory effect and regulates serum metabolic disorder caused by SCI in rats (**Kang et al., 2018**). On the other hand, the neuroprotective effect of **puerarin** in SCI has also been demonstrated in a study in which puerarin treatment significantly increased BBB score in SCI rats and decreased neuron loss, inhibited glial cells activation, alleviated inflammation, and inhibited cell apoptosis in spinal cord. Moreover, these effects were associated with the activation of PI3K/Akt signalling pathway (**Zhang et al., 2016**). Another study showed that the neuroprotective mechanism of puerarin was related to a decrease in glutamate release and mGluRs-1 mRNA expression (**Tian et al., 2013**). Another flavonoid which exerts antioxidant and anti-inflammatory activities in SCI is **myricitrin**. Specifically, this polyphenol showed these effects through inhibition of COX-2, TGF-β1, p53 and upregulation of Bcl-2/Bax signaling pathway in a rat model of SCI (**Lei et al., 2017**). In the same vein, **naringenin** also has a protective effect on SCI, possibly through inhibiting neuroinflammation. Concretely, naringenin inhibited SCI-induced neutrophil activation via the repression of miR-223 (**Shi et al., 2016**). In addition, **hesperidin** ameliorated SCI-induced hindlimb paralysis, down-regulated pro-inflammatory mediators and oxidative stress, increased serum bilirubin and Fe^{2+} and activated Nrf-2/HO-1 pathway in SCI rats (**Heo et al., 2020**).

On the other hand, different studies have introduced phenolic acids in combating post-SCI complications through modulating inflammation, oxidate stress and apoptotic pathways. For instance, in a recent study by Ma and colleagues (**Ma et al., 2020**) **rosmarinic acid** showed a neuroprotective effect by improving function recovery and suppressing cell apoptosis, oxidative stress and inflammation through modulating the Nrf2/HO-1, TLR4/NF-κB and caspase-3 pathways after SCI. These effects were also demonstrated in a study in which rosmarinic acid enhanced antioxidant status, decreased oxidative stress and ameliorated inflammatory mechanisms by down-regulating NF-κB and pro-inflammatory cytokines in rats after SCI (**Shang et al., 2017**). **Ferulic acid** also showed its neuroprotective effects in SCI through enhancing functional recovery of rats after SCI via inhibiting microRNA-590 to upregulate vascular endothelial growth factor (VEGF) expression (**Li et al., 2017**). Moreover, another study exposed the ameliorating role of ferulic acid in apoptosis and autophagy through mTOR, PI3K/Akt pathway, PARP-1, AIF, Beclin-1, and LC3 in spinal cord injured rats (**Zhang et al., 2017**). Lastly, the beneficial effects of **curcumin** on SCI have been extensively demonstrated. On the one hand, the anti-inflammatory effect of curcumin post-SCI was associated with

inhibition of NF-κB, IL-1β, IL-6, and TNF-α, upregulation of Nrf2 (**Jin et al., 2014**) and modulation of the TLR4/NF-κB signalling pathway (**Ni et al., 2015**). Curcumin also induced an antioxidative protection through increasing SOD activity and decreasing MDA levels (**Kavakli et al., 2011**). In addition, curcumin inhibited the Akt/mTOR signalling pathway which in turn inhibited neuronal apoptosis and modulated autophagy after SCI (**Li et al., 2021**). On the other hand, curcumin suppressed inflammatory cytokines with pro-apoptotic roles, including ILs and TNF-α (**Ruzicka et al., 2018**), transforming growth factor-beta (TGF-β), as an upstream apoptotic receptor, and NF-κB which is involved in the apoptotic and inflammatory pathways (**Yuan et al., 2019**). Other study showed that treatment with curcumin after traumatic SCI noticeably downregulated the levels of proinflammatory cytokines and labile Zinc accumulation, thereby ameliorated the locomotor activity, spinal cord edema, and apoptosis (**Ni et al., 2014**). Another neuroprotective effect of curcumin in the treatment of SCI is its role in CDGSH iron sulphur domain 2 (CISD2) upregulation. CISD2 increases Bcl-2/Beclin-1 binding to modulate autophagy/apoptosis pathways, prevents mitochondrial dysfunction and cell death, inhibits the increase of excitotoxic Ca^{2+} in the endoplasmic reticulum in response to stress, suppresses NO signalling and prevents apoptosis in neuronal cells (**Lin et al., 2016**).

Furthermore, the stilbene **resveratrol** is considered a potent neuroprotectant on SCI. In terms of pharmacological mechanism, resveratrol is an activator of SIRT1 that can protect neurons against oxidative stress, inflammation, and apoptosis (**Borra et al., 2005; Bastianetto et al., 2015**). After SCI, resveratrol enhanced the expression of SIRT1, p-AMPK, Bcl-2, LC3, and Beclin-1 while decreasing the expression of p62, caspase-3, caspase-9, and Bax. Moreover, different studies showed that resveratrol exerts its neuroprotective effect by regulating apoptosis and autophagy through SIRT1/AMPK and AMPK/mTOR signalling pathways (**Zhao et al., 2017; Yan et al., 2017; Meng et al., 2018**). In addition, other studies revealed anti-inflammatory, anti-apoptotic and antioxidant effects of resveratrol after SCI (**Liu et al., 2011; Hu et al., 2017; Wang et al., 2018**).

Although most of the polyphenols mentioned above have not yet been tested against neuropathic pain resulting from SCI, it is worth mentioning that they have shown antinociceptive effects in animal models of peripheral neuropathic pain, nociceptive pain, and inflammatory pain (see ***Table 4*** for more details). Moreover, their anti-inflammatory, antioxidant, anti-apoptotic and neuroprotective effects in SCI models suggest that these polyphenols could probably have antinociceptive effects against SCI-derived neuropathic pain by acting on the cellular, molecular, and biochemical phenomena that occur in the secondary SCI lesion. Thus, these polyphenols could exert their effects on the pathological and functional changes that contribute to the mechanism responsible for the development of neuropathic pain after SCI.

Despite their efficacy in attenuating post-SCI pathophysiological complications, polyphenols present several problems such as their poor absorption, rapid metabolism and, therefore, their low bioavailability influenced not only by their transmembrane capacity but also by their structure (**Rasouli et al., 2017; Chen et al., 2018**). In this way, polyphenols are extremely degradable, leading to very low bioavailability and thus to a significant decrease in their biological activities (**Chen et al., 2018**). Bioavailability is defined as the proportion of the nutrient that is digested, absorbed and metabolised through normal pathways. Therefore, it is not only important to define concentration of polyphenol in specific treatment, but even more important is to know how much of that is bioavailable (**Srinivasan et al., 2001**).

In this fashion, the chemical structure of polyphenols, rather than the concentration, determines the rate and degree of absorption and the nature of the metabolites circulating in the plasma (**D'archivio et al., 2007**). Generally, most dietary polyphenols metabolized in the small intestine and are further methylated and modified into its glucuronide and sulphated metabolites by liver or other organs (**Cassidy and Minihane, 2017**). A way to obtain direct evidence on the bioavailability of phenolic compounds is to measure their concentration in plasma or urine after ingestion of either pure compounds or foodstuffs with known content of the compounds of interest (**Marrugat, et al. 2004; Tian et al., 2006; Fitó et al., 2007**). For instance, ingestion of the pure compound epigallocatechin gallate (50-1600 mg) resulted in a maximum concentration of 0.28-7.4μM in plasma (**Ullmann et al., 2003**), in contrast to green tea infusion (containing 105 mg of epigallocatechin gallate) which resulted in 0.13-0.31 μM/plasma (**Unno et al., 1996**), or green tea extract (110-328 mg epigallocatechin gallate) which resulted in 0.26-0.7 μM/plasma (**Kimura et al., 2002**). In general, plasma concentrations of polyphenols rarely exceed 1μM and maintenance of a high plasma concentration requires repeated ingestion over time (**Van Het Hof et al., 1999**); indeed, peak concentrations are most often reached 1-2 h after ingestion (**Unno et al., 1996; Aziz et al., 1998**), except for those polyphenols that require degradation prior to absorption (**Hollman et al., 1997**).

On the other hand, it is also important to determine the bioavailabilty of polyphenols metabolites in the tissues of interest. The data collected reveal that some phenolic metabolites may accumulate in certain gut organ or tissue and may present differently in target tissue or plasma. Therefore, plasma concentrations of polyphenols are not directly correlated with concentrations in target tissues, and distribution between blood and tissues also differs between the various polyphenols. In conclusion, polyphenols bioavailability integrates several variables, such as intestinal absorption, metabolism by the microflora, intestinal and hepatic metabolism, nature of circulating metabolites, binding to albumin, cellular uptake, accumulation in tissues, and biliary and urinary excretion (**D'archivio et al., 2007; Chen et al., 2018**). In general, the bioavailability of polyphenols is low and may be improved by some structural modifications or by targeted nano-delivery. The main advantages of nanoparticles in the treatment of SCI include facilitating passage

across the blood-spinal cord barrier, improving bioavailability by increasing water solubility, and long-term delivery of the drug at the spinal cord lesion (**Hu et al., 2009; Zhao et al., 2009; Shin et al., 2014; Peng et al., 2020**). In fact, several studies showed the efficacy of nanoparticles in SCI and other neurodegenerative diseases. For instance, a lipid-based discoidal nano-formulation of curcumin improved the locomotor function more efficiently than free curcumin in SCI rats. Moreover, nano-formulated curcumin more significantly decreased the glial scar, preserved the white matter, and downregulated NF-κB, IL-1β, TNF-α, and IL-6 (**Krupa et al., 2019**). Other study showed the antinociceptive effects of curcumin-loaded Poly (d, l-lactide-co-glycolide) (PLGA) nanovesicles in different pain models (formalin test, zymosan-induced hyperalgesia, and sciatic nerve ligation), thus demonstrating the efficacy of nanovesicles enhancing curcumin bioavailability (**Pieretti et al., 2017**). On the other hand, EGCG-loaded polyethylen glycol (PEG)ylated-PLGA nanoparticles were more effective than the same dose of naïve drug in reducing neuroinflammation and neuronal death in a mouse model of temporal lobe epilepsy (**Cano et al., 2018**). In summary, these results suggest that employing novel drug delivery systems could improve the efficacy of polyphenols by overcoming their pharmacokinetic limitations.

The current knowledge about both the development and treatment of spinal cord injury-induced neuropathic pain, with special mention of polyphenolic compounds as potential pharmacological strategies, have been reviewed in the introduction section. In particular, the key points to be considered are:

- SCI-induced CNP is developed in more than half of patients. CNP implies also a high probability of severe disabilities in daily life and is commonly associated with an increased vulnerability to develop emotional disorders, especially when this pain becomes chronic.
- Several pharmacological treatments have been used to alleviate CNP, but they are mostly inefficient. Actually, the best pharmacological strategy results in a reduction of only 20–30% in pain intensity, frequently accompanied by severe side effects.
- The lack of suitable treatments leads to CNP chronification. Hence, it is necessary to design new pharmacological strategies to modulate CNP development and prevent its chronification.
- Among the current strategies aimed at modulating pathological pain, the use of polyphenols may be highlighted since preclinical results evidence their potential antinociceptive effects. However, most studies have been conducted in experimental models unrelated to SCI-induced CNP.
- Polyphenolic compounds constitute one of the most important natural products within the plant kingdom. Some rich natural sources of polyphenols include fruits, vegetables, whole grains and several beverages such as coffee and wine. They have shown antinociceptive effects in animal models of peripheral neuropathic pain, nociceptive pain and inflammatory pain and their anti-

inflammatory, antioxidant, anti-apoptotic and neuroprotective effects in SCI models suggest that polyphenols could exert antinociceptive effects on SCI-induced neuropathic pain.

- Among the few polyphenols that have been shown to have an antinociceptive effect in SCI model, EGCG is noteworthy, which has a short-term antihyperalgesic effect when administered during the first week after SCI. However, the preventive effects of EGCG during the whole SCI acute phase is still unknown and the pharmacological safety of this polyphenol remains controversial because of its suggested systemic toxicity.

In this context, the present work aimed to study the antinociceptive effects of two vegetal polyphenolic extracts on SCI-induced CNP in mice. Extracts were obtained from grape stalk residual material (GSE) and from roasted decaffeinated coffee powder (CE) since both grapevine and coffee are known to be rich natural sources of several polyphenols. The current knowledge about antinociceptive effects of this molecular family make both extracts suitable candidates to be evaluated as potential pharmacological strategies towards SCI-induced CNP modulation.

HYPOTHESIS AND OBJECTIVES

II. HYPOTHESIS AND OBJECTIVES

HYPOTHESIS

In light of all the above, the present work is aimed to explore the **hypothesis** that: *"Natural polyphenolic extracts can be obtained from both grape stalk and coffee using physiological serum as solvent, and the repeated administration of such extracts may safety exert spinal cord injury-induced neuropathic pain development attenuation in mice by modulating SCI-related gliosis*"

OBJECTIVES

The **global objective** of the present thesis is to evaluate the preventive effects of both Grape Stalk and Coffee polyphenolic extracts on mild SCI induced-central neuropathic pain development in mice, considering both as better alternative treatments to Epigallocatechin-3-gallate (EGCG). The following specific objectives were set to accomplish this global aiming:

1. **To assess the preventive effects of Epigallocatechin-3-gallate (EGCG) repeated administration on central neuropathic pain development following SCI in female CD1 mice, in the acute phase of injury.**

- Evaluation of reflexive pain responses development in the spinal cord injury acute phase by repeated administration of 10, 15 and 20 mg/kg EGCG (i.p. diluted in 10% dimethyl sulfoxide (DMSO)-saline solution) during the first week post injury.
- Evaluation of mice weight throughout the spinal cord injury acute phase as indicator of potential systemic toxicity effects related to treatments.
- Assessment of the preventive EGCG effects on central neuropathic pain-related microgliosis and astrogliosis in the dorsal horn of spinal cord injured mice.

2. **To assess the preventive effects of Grape Stalk polyphenolic extract (GSE) repeated administration on central neuropathic pain development following SCI in female CD1 mice, in the acute phase of injury.**

- Obtaining and characterization of Grape Stalk polyphenolic extract (GSE), obtained using physiological serum as solvent.

- Evaluation of systemic effects of preventive GSE administration by means of hepatotoxicity and nephrotoxicity biomarkers as well as weight assessment.
- Evaluation of reflexive pain responses development in the spinal cord injury acute phase by repeated administration of 10, 15 and 20 mg/kg GSE during the first week post injury.
- Assessment of the preventive GSE effects on central neuropathic pain-related microgliosis and astrogliosis in the dorsal horn of spinal cord, as well as in supraspinal structures associated with either sensory-discriminative or affective-motivational behaviours.
- Assessment of the preventive GSE effects on the expression of molecular markers related to central sensitisation in spinal cord and selected supraspinal structures.

3. To assess the preventive effects of repeated administration of Coffee polyphenolic extract (CE) on central neuropathic pain development following SCI in female CD1 mice, in the acute phase of injury.

- Obtaining of Coffee polyphenolic extract (CE) using physiological serum as solvent and its characterisation.
- Evaluation of systemic effects of preventive CE administration by means of hepatotoxicity and nephrotoxicity biomarkers as well as weight assessment.
- Evaluation of reflexive pain responses development in the acute phase of spinal cord injury after repeated administration of 10 and 15mg/kg CE during the first week post injury.
- Assessment of the preventive CE effects on central neuropathic pain-related microgliosis and astrogliosis in the spinal cord dorsal horn, as well as in supraspinal structures associated with either sensory-discriminative and affective-motivational behaviours.
- Assessment of the preventive CE effects on the expression of molecular markers of central sensitisation in spinal cord and selected supraspinal structures.

4. To assess the antinociceptive effects of EGCG, GSE and CE repeated administration on SCI-induced central neuropathic pain in female CD1 mice, until the intermediate injury phase.

- Evaluation of reflexive pain responses until the intermediate phase of spinal cord injury after repeated administration of most suitable doses of EGCG, GSE and CE, according to previous experiments, during the third week after injury.
- Evaluation of systemic effects of GSE and CE administration during the third week after injury by means of hepatotoxicity and nephrotoxicity biomarkers as well as weight assessment.
- Evaluation of non-reflexive pain responses at the intermediate phase of spinal cord injury after repeated administration of most suitable doses of GSE and CE, according to previous experiments, during the third week after injury.

5. **To assess the preventive effects of repeated administration of GSE and CE on central neuropathic pain development following SCI in female CD1 mice, until the chronic phase of injury.**

- Evaluation of reflexive pain responses until the chronic phase of spinal cord injury after repeated administration of most suitable doses of GSE and CE, according to previous experiments, during the first, third and sixth week after injury.
- Evaluation of non-reflexive pain responses at the chronic phase of spinal cord injury after repeated administration of most suitable doses of GSE and CE, according to previous experiments, during the first, third and sixth week after injury.
- Assessment of the preventive GSE and CE effects on central neuropathic pain-related microgliosis and astrogliosis in spinal cord and supraspinal structures associated with either sensory-discriminative and affective-motivational behaviours in spinal cord-injured mice.
- Assessment of the preventive GSE and CE effects on central neuropathic pain-related chemokines and chemokines receptors expression in supraspinal structures associated with either sensory-discriminative and affective-motivational behaviours.

MATERIALS AND METHODS

III. MATERIALS AND METHODS

1. Experimental design

The main objective of the present thesis is to study the preventive effects of two polyphenolic plant extracts (GSE and CE) on the central neuropathic pain development induced by spinal cord injury in mice and to compare it with the effects of a widely studied reference polyphenol such as EGCG. In addition, a characterisation of the phenolic compounds of both extracts was carried out. For this purpose, a set of different experiments was designed, and the results obtained were divided into five chapters.

Chapter I. Preventive effects on Spinal Cord Injury-induced neuropathic pain development by repeated administration of EGCG in CD1 mice.

This experiment was designed to evaluate the preventive effects of repeated EGCG treatment on spinal cord injury-induced neuropathic pain development in mice during the acute phase. To this end, CD1 female mice were subjected to mild spinal cord injury and daily treated with EGCG during the first week post-surgery (10, 15 or 20 mg/kg; i.p.). The locomotor recovery and nociceptive behaviour was assessed once per week – at 7, 14, 21 days post injury (dpi) – until the end of the experimental period (**Fig.22**). The evaluation consisted in the performance of Basso mouse scale (BMS), Hargreaves test and Von Frey test to assess locomotor functional recovery, thermal hyperalgesia and mechanical allodynia, respectively.

At the end, immunohistochemical analysis of spinal cord samples were performed to study astrogliosis (GFAP) and microgliosis (Iba1).

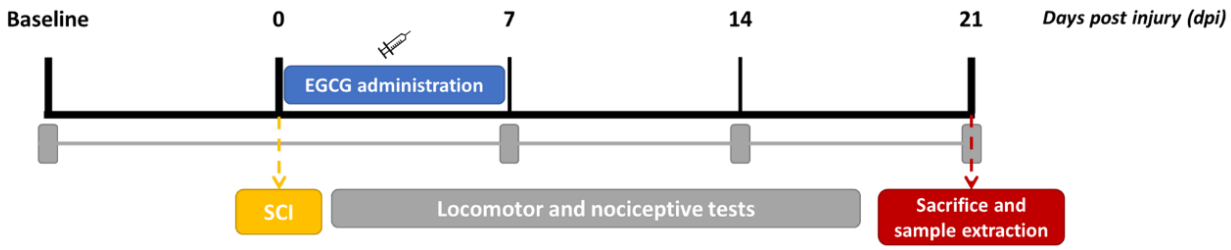


Figure 22. Schematic protocol design of nociceptive test and sample extraction of EGCG treatment during the acute phase of the spinal cord injury.

Chapter II. Preventive effects on Spinal Cord Injury-induced neuropathic pain development by repeated administration of GSE in CD1 mice.

This experiment was conducted to evaluate the preventive effects of repeated GSE treatment on spinal cord injury-induced neuropathic pain development in mice during the acute phase. First, GSE was obtained from plant material (see "Grape Stalk Extract (GSE) and Coffee Extract (CE) obtaining" section for more details). The total amount of polyphenols in GSE was determined by the Folin-Ciocalteu assay, and the identification and quantification of phenolic compounds was accomplished by different high-performance liquid chromatography high resolution mass spectrometry (HPLC-HRMS) techniques. Once GSE was obtained and characterized, female CD1 mice were subjected to mild spinal cord contusion and treated daily with GSE during the first postoperative week (10, 15 or 20 mg/kg; i.p.). Thermal hyperalgesia (Hargreaves test), mechanical allodynia (Von Frey filaments) and locomotor functional recovery (Basso mouse scale; BMS) were weekly evaluated – at 7, 14, 21 days post injury (dpi) – until the end of the experimental period (**Fig.23**).

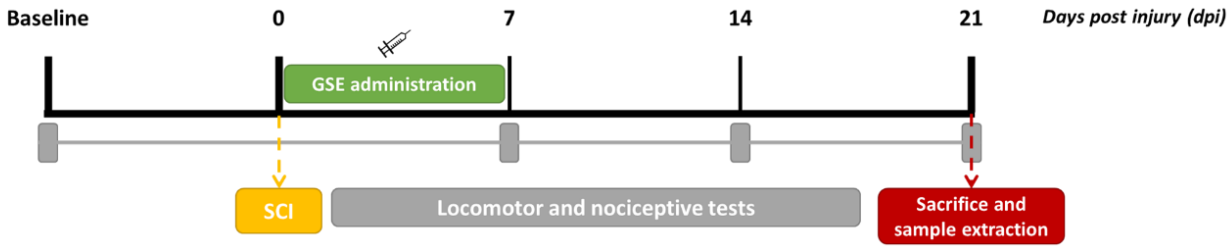


Figure 23. Schematic protocol design of nociceptive test and sample extraction of GSE treatment during the acute phase of the spinal cord injury.

At the end of the experiment period, serum levels of hepatotoxicity and nephrotoxicity biomarkers were analysed to assess the pharmacological safety of GSE treatment. Furthermore, gliosis, ERK phosphorylation as well as the expression of CCL2 and CX3CL1 chemokines and their receptors, CCR2 and CX3CR1, were analysed in the spinal cord. Gliosis and CX3CL1/CX3CR1 expression were also analysed in anterior cingulate cortex (ACC) and periaqueductal gray matter (PAG) since are supraspinal structures involved in pain perception and modulation.

Chapter III. Preventive effects on Spinal Cord Injury-induced neuropathic pain development by repeated administration of CE in CD1 mice.

This experiment was performed to evaluate the preventive effects of repeated CE treatment on spinal cord injury-induced neuropathic pain development in mice during the acute phase. Thus, the first step was to obtain the CE (see section "Grape Stalk Extract (GSE) and Coffee Extract (CE) obtaining" for details) and characterise its phenolic compounds by means of high-performance liquid chromatography high resolution mass spectrometry (HPLC-HRMS). Next, female CD1 mice were subjected to mild spinal cord injury and daily treated with CE during the first week post-surgery (10 or 15mg/kg; i.p.). As in the previous Chapter, thermal hyperalgesia (Hargreaves test), mechanical allodynia (Von Frey filaments) and locomotor functional recovery (Basso mouse scale; BMS) were weekly evaluated (**Fig.24**).

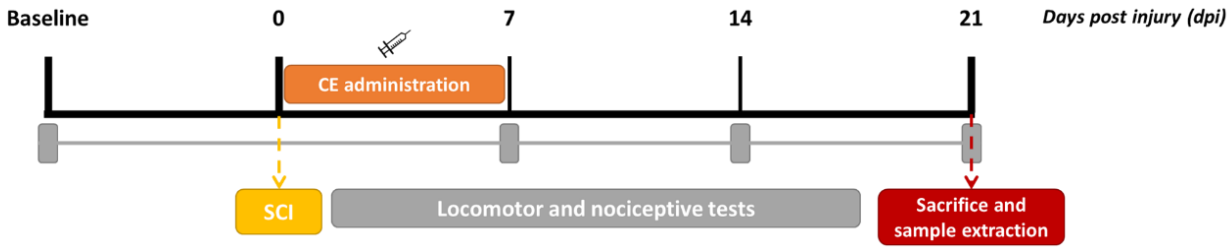


Figure 24. Schematic protocol design of nociceptive test and sample extraction of CE treatment during the acute phase of the spinal cord injury.

At the end of the experiment, the previous analysis done for GSE treatment (Chapter II) were carried out in the same conditions: immunohistochemical and western blot analysis to assess the astrogliosis (GFAP) and microgliosis (Iba1) in the spinal cord, periaqueductal gray matter (PAG) and anterior cingulate cortex (ACC); protein expression of ERK phosphorylation, MCP1/CCR2 and CX3CL1/CX3CR1 in spinal cord; CX3CL1/CX3CR1 expression in ACC and PAG; and analysis of hepatotoxicity and nephrotoxicity biomarkers to assess the pharmacological safety of CE treatment.

Chapter IV. Antinociceptive effects on Spinal Cord Injury-induced neuropathic pain by repeated administration of EGCG and Polyphenolic Extracts in CD1 mice during the intermediate phase of injury.

This fourth study included two experiments: (i) the first aimed to elucidate the analgesic effects on neuropathic pain of EGCG if administered during the third postoperative week, i.e., after the acute phase of SCI was over and neuropathic pain was established in the animals; (ii) the second experiment aimed to assess the same aspects but using GSE and CE treatments.

IV.I. Antinociceptive effects on SCI-induced neuropathic pain by repeated administration of EGCG in CD1 mice during the intermediate phase of injury.

This experiment was performed to evaluate the possible antinociceptive effect of repeated EGCG treatment on spinal cord injury-induced neuropathic pain in mice during the intermediate phase of the lesion. To this end, the doses of EGCG that exerted the best preventive effects in the previous experiments were selected to be tested in this study. In this way, female CD1 mice were subjected to mild spinal cord contusion and treated daily with EGCG during the third week post-surgery (15 or 20 mg/kg; i.p.). Thermal hyperalgesia (Hargreaves test), mechanical allodynia (Von Frey filaments) and locomotor functional recovery (Basso mouse scale; BMS) were weekly evaluated until the end of the experimental period (**Fig.25**).

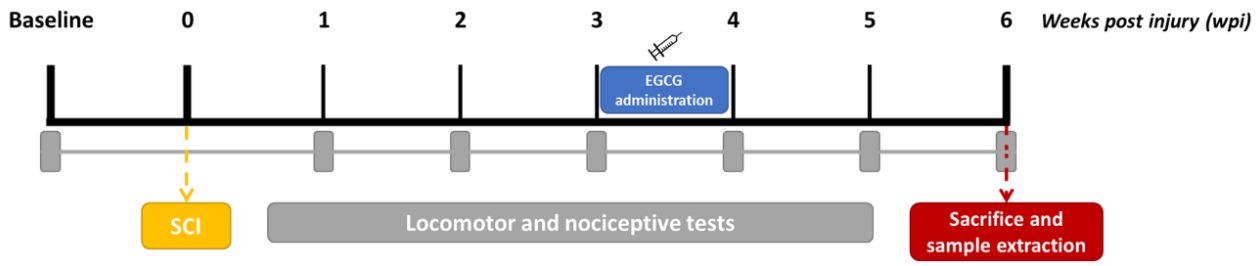


Figure 25. Schematic protocol design of EGCG treatment during the intermediate phase of the spinal cord injury.

IV.II. Antinociceptive effects on SCI-induced neuropathic pain by repeated administration of GSE and CE in CD1 mice during the intermediate phase of injury.

This experiment was performed to evaluate the possible antinociceptive effect of repeated GSE or CE administration on spinal cord injury-induced neuropathic pain in mice during the intermediate phase of the lesion. To this end, the doses of GSE and CE that exerted the best preventive effects in the acute phase of injury were selected to be tested in this study. In this way, CD1 female mice were subjected to mild spinal cord contusion and treated daily with 15 mg/kg of GSE or 10 mg/kg of CE during the third week post-surgery. Thermal hyperalgesia (Hargreaves test), mechanical allodynia (Von Frey filaments) and locomotor functional recovery (Basso mouse scale; BMS) were weekly evaluated. In order to elucidate possible affective disturbances, the open field, dark and light box, forced swim and reward-seeking behaviour (RSB) test were evaluated during the last week of the experimental period (**Fig.26**). Finally, to assess the pharmacological safety of the GSE and CE treatments, analysis of hepatotoxicity and nephrotoxicity biomarkers were performed.

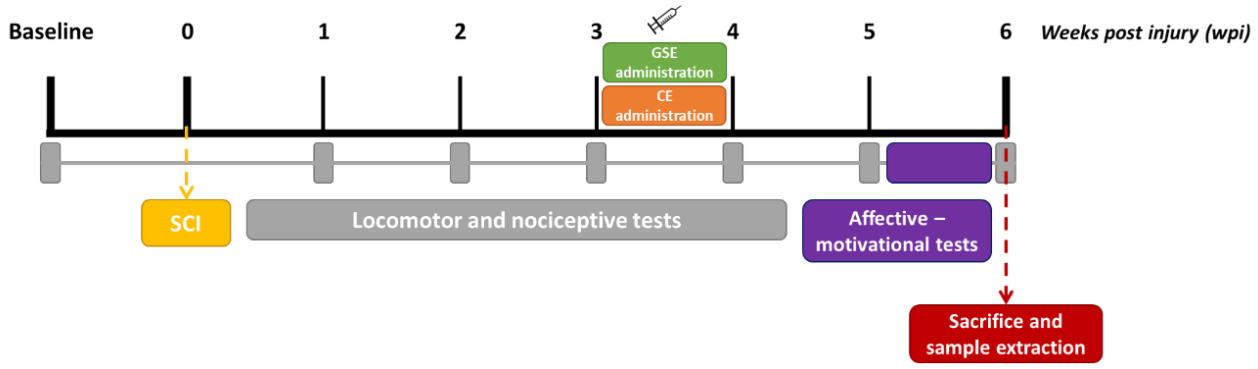


Figure 26. Schematic protocol design of GSE and CE treatment during the intermediate phase of the spinal cord injury.

V. Preventive effects on SCI-induced neuropathic pain development by repeated administration of Polyphenolic Extracts in CD1 mice during the acute, intermediate and chronic phase of injury.

This final experiment was designed to evaluate the possible preventive effect of repeated GSE or CE treatment on SCI-induced neuropathic pain development in mice during the acute, intermediate, and chronic phase of the lesion. Therefore, CD1 female mice were subjected to mild spinal cord contusion and daily treated with 15 mg/kg of GSE or 10 mg/kg of CE during the first-, third- and sixth-week post-surgery. Reflexive pain responses of thermal hyperalgesia (Hargreaves test) and mechanical allodynia (Von Frey filaments), as well as locomotor functional recovery (Basso mouse scale; BMS) were weekly evaluated. To prove possible affective disturbances, non-reflexive pain responses were evaluated by means of the open field, dark and light box, forced swim, reward-seeking behaviour (RSB) and social interaction tests during the last week of the experimental period (**Fig.27**).

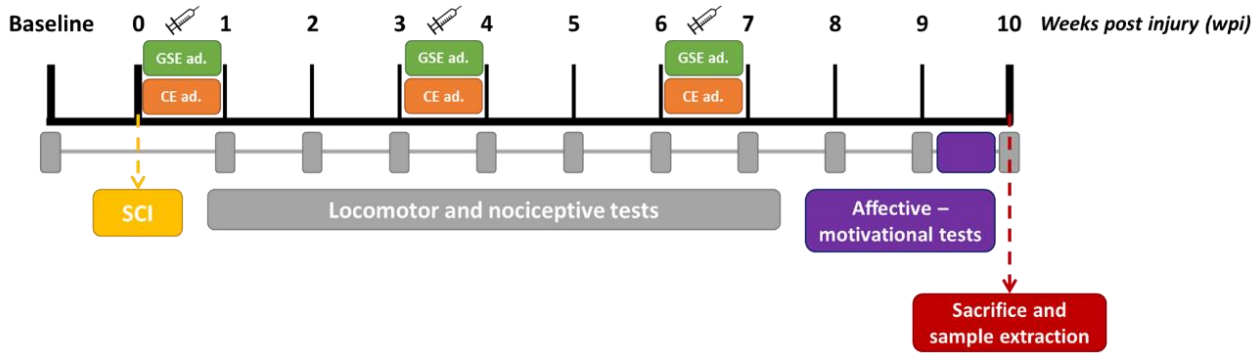


Figure 27. Schematic protocol design of GSE and CE treatment until the chronic phase of the spinal cord injury.

At the end of experimental period, serum levels of hepatotoxicity and nephrotoxicity biomarkers were analysed to assess the pharmacological safety of the GSE and CE treatments in this dosing regimen. In addition, molecular studies of gliosis in the spinal cord, as well as gliosis and expression of CCL2/CCR2 and CX3CL1/CX3CR1 in the ACC, amygdala, dorsal and ventral PAG and RVM were performed, as these are all

supraspinal structures involved in either sensory-discriminative and affective-motivational dimensions of pain.

2. Animals

Adult female ICR CD1 mice (20-30 g) were purchased from Janvier Laboratories (Le-Genest-SaintIsle, France). Animal sample size was calculated using GRANMO (Version 7.12 April 2012) and the spreadsheet of the University of Boston (Sample Size Calculations (IACUC); Boston University). However, it was reduced as much as possible without losing statistical significance. Finally, a total of 220 mice were used to perform all the experiments of the present thesis. For chapters I, III and IV, a total of 138 mice were used to evaluate the effects of EGCG, GSE and CE treatments on locomotor recovery and evoked pain responses during the acute phase of the spinal cord injury, including several biochemical assessments. For the fifth chapter, a total of 82 mice (20-25g) were used to evaluate the effects of the best tested doses of EGCG, GSE and CE treatments on locomotor recovery, evoked pain responses, and affective-motivational behaviours during the intermediate and chronic phases of the spinal cord injury.

All animals were housed in groups of 4-5 in standard Marcolon cages (28x28x15 cm) with a wood shaving bedding, at 21±1ºC of temperature, 40-60% of humidity, under a 12:12 hours light-dark cycle and fed ad libitum with a standard diet of mouse pellets (TEKLAD 2014, Harlan Interfauna Ibérica, Sant Feliu de Codines).

All experimental procedures and animal husbandry were performed according to the ethical principles of the I.A.S.P. for the evaluation of pain in conscious animals (**Zimmerman, 1983**) and the European Parliament and the Council Directive of 22 September 2010 (2010/63/EU), along with the approval Ethical Committee on Animal Experimentation (CEEA) of the University of Barcelona and the Department of Agriculture, Livestock, Fisheries, Food and Natural Environment of the Generalitat de Catalunya (DAAM number 9918-P3). All the experiments were carried out at the Campus Bellvitge (Universitat de Barcelona) animal house.

3. Drugs and polyphenolic extracts

The epigallocatechin-3-gallate (EGCG) was purchased from Sigma-Aldrich (Ref: 4143) and was dissolved in dimethyl sulfoxide (DMSO) (Ref: 4540, Sigma-Aldrich, USA)/saline solution (1:9; v/v) (**Xifró et al 2015**).

3.1 Grape Stalk Extract (GSE) and Coffee Extract (CE) obtaining

The two polyphenolic extracts used in the present thesis were obtained from different plant sources. The grape stalk extract (GSE) was obtained from the grape residual material coming from several wine cooperatives in *"L'Empordà"* (Catalunya) and the coffee extract (CE) was obtained from the decaffeinated, ground, and roasted coffee bean.

To collect the GSE particles, grape stalk residual material was cut with a chopper grinder (WCG75 Pro Prep) and was then sifted with a digital electromagnetic sieve (CISA BA 200N) to achieve a particle size between 0.5 and 1 mm. For CE, processed coffee powder was used.

In order to obtain the extracts, 3 grams of either grape stalk or coffee particles were mixed with 50mL of saline solution (0.9% Vitulia Physiological Serum) as a solubilizer which was refluxed and left stirring at 100 °C for 2 hours. The resulting solutions were first filtered with chromatographic filters (Scharlau Nylon Syringe filter, ø 13mm, 0.45 µm, NY13045200) and were then filtered and sterilized with 0.22 µm vacuum filter bottles (FPE-204-250, Biofil).

3.2 Total polyphenol content in extracts

The total amount of polyphenols in both extracts was determined by the Folin-Ciocalteu assay. This method, also called the gallic acid equivalence method (GAE), consists of using the Folin-Ciocaiteu reagent, which is a mixture of phosphomolybdic acid ($H_3PMo_{12}O_{40}$) and phosphotungstic acid ($H_3PW_{12}O_{40}$) that reduces to molybdenum and tungsten oxide in presence of phenolic and polyphenolic compounds (**Singleton 1999**). This reduction reaction gives a blue-green colour to the solution: the higher polyphenolic quantity, the more intense the blue solution is.

The Folin-Ciocalteu reagent does not just react with phenolic compounds, but it reacts with any reducing substance. It therefore measures the total reducing capacity of the sample. The reagent has also been shown to be reactive towards thiols, many vitamins, the trioses glyceraldehyde and dihydroxyacetone, the nucleotide base guanine, and some inorganic ions (**Everette 2010**). For this reason, the Folin-Ciocalteu method gives a rough approximation of total phenolic content and other methods like High-performance liquid chromatography (HPLC) are necessary to determine the exact concentration of polyphenols in both extracts.

The Folin Ciocalteu method was performed using gallic acid as standard. The calibration curve was obtained by preparing different standard concentrations of gallic acid within the range 100-1000 mg/L. Briefly, a 100 µL aliquots of diluted extracts (1:2, 1:3, 1:4), gallic acid standard solutions (100-1000 mg/L) and a blank (saline solution) were put in different tubes. Then, 3.9 mL of Mili-Q water, 900 µL of 20% sodium carbonate

and 600 µL of Folin-Ciocalteu reagent were added. The tubes were shacked and were then allowed to incubate for 2 hours at room temperature. After incubation, the absorbance against a blank was measured spectrophotometrically at 760 nm (Hitachi U-2000 VIS/UV spectrophotometer). Total polyphenolic content was expressed as milligrams of gallic acid equivalents (GAE) per litre of extracts.

3.3 Characterization of phenolic compounds in extracts

3.3.1 Qualitative analysis

Qualitative determination of polyphenols in GSE was accomplished by high-performance liquid chromatography high resolution mass spectrometry (HPLC-HRMS) carried out by the scientific technical services of the University of Barcelona.

The GSE analysis sample was prepared by filtering through 0.45 µm syringe filter and further diluted (1/5) with 0.1% formic acid.

A high-performance liquid chromatography with UV detection coupled with electrospray ionization Fourier transformation mass spectrometry (HPLC-UV-ESI-FTMS) was performed to identify the polyphenols presents on GSE. The HPLC system consisted of an Accela chromatograph equipment. A Luna HST 2.5 µm (10 cm × 2.0 mm) column (Phenomenex) was used. The mobile phase consisted of 0.1% formic acid (A) and acetonitrile (B) with the following gradient (t (min), % B): (0, 0), (0.5, 0), (10, 50), (12, 50), (25, 95), (28, 95), (28.5, 0), (35, 0). The flow rate was 400 µL/min, with no split before the MS. The oven temperature was 50ªC and automatic injection system temperature was 10ºC. Injection volume was 10 µL. This HPLC system was coupled to an LTQ-Orbitrap Velos (Thermo) mass spectrometer fitted with Electrospray source working in negative ionization mode and under MS conditions indicated in **Table 5**.

Table 5. MS (HPLC-UV-ESI-FTMS) analysis conditions.

Equipment	LTQ Orbitrap Velos (Thermo Instruments)
Capillary Temperature	375 ºC
Gas	Sheath 50, Aux 20, Sweep 2
Source voltage	2.8 kV (ESI -)
Slens RF Level	60%
Acquisition	FTMS
Full scan	m/z 100-1000
MS^2 experiments	Isolation width m/z 1.5, CE 35%

Chromatograms and spectra acquired in negative ion mode in both FTMS and MS^2 of the identified ions were used to tentatively identify some compounds looking up in Phenol-Explorer database (http://phenol-explorer.eu/).

3.3.2 Quantitative analysis

Quantitative determination of polyphenols in both GSE and CE was accomplished by high-performance liquid chromatography high resolution mass spectrometry (HPLC-HRMS) carried out by the scientific technical services of the University of Barcelona.

Both GSE and CE analysis samples were prepared by filtering through 0.45 µm syringe filter. GSE was diluted (1/5 and 1/50) with 0.1% formic acid and CE sample was diluted (1/10, 1/100 and 1/250) with 0.1% formic acid.

HPLC-TOFMS analysis for GSE

A HPLC-UV-ESI-TOFMS was performed to identify and quantify the polyphenols presents on GSE. The HPLC system consisted of an Agilent 1200RR chromatograph equipment. A Luna HST 2.5 µm (10 cm × 2.0 mm) column (Phenomenex) was used. The mobile phase consisted of 0.1% formic acid (A) and acetonitrile (B) with the following gradient (t (min), % B): (0, 0), (0.5, 0), (10, 50), (12, 50), (25, 95), (28, 95), (28.5, 0), (35, 0). The flow rate was 400 µL/min, with no split before the MS. The oven temperature was 50ªC and automatic injection system temperature was 10ºC. Injection volume was 10 µL. This HPLC system was coupled to a QSTAR Elite (ABSciex) mass spectrometer fitted with a TurboIon spray source working in negative ionization mode with the following TOFMS conditions: full scan analysis from m/z 100 to 600 and product ion scan m/z 449.

HPLC-FTMS analysis for CE

A HPLC-UV-ESI-FTMS was performed to identify and quantify the polyphenols presents on CE. The HPLC system consisted of an Ulltimate 3000 (Dionex) chromatograph equipment. A Kinetex EVO C18 1.7µm (10 cm × 2.0 mm) column (Phenomenex) was used. The mobile phase consisted of 0.1% formic acid (A) and acetonitrile 0.1% formic acid (B) with the following gradient (t (min), % B): (0, 0), (0.5, 0), (5, 10), (8, 90), (15, 20), (17, 50), (17.5, 50), (18, 95), (19, 95), (19.5, 0), (22,0). The flow rate was 500 µL/min, with no split before the MS. The oven temperature was 50ªC and automatic injection system temperature was 4ºC. Injection volume was 2 µL. This HPLC system was coupled to a LTQ-Orbitrap Velos (Thermo) mass spectrometer fitted with Electrospray source working in negative ionization mode with the following MS conditions: full scan analysis from m/z 100 to 2000 at 30000 resolution using the FTMS.

4. Surgical procedure

In order to obtain a central neuropathic pain mouse model without locomotor paralysis, mild spinal cord contusion was performed following procedures explained elsewhere (**Álvarez-Pérez et al 2016, Castany et al 2018, 2019**). Briefly, animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and placed prone on a heating pad to maintain constant the levels of body temperature. After back shave and disinfection with povidone iodide, T8–T9 thoracic spinal cord segments were exposed via dorsal laminectomy and two grams of weight was dropped from 25 mm high onto metallic stage located over the exposed spinal cord (**Fig. 28**). Then, the wound was closed and disinfected with povidone. After this, animals received 0.5 mL of saline solution to restore an eventual blood volume deficit and were then allowed to recover in warmed cages with accessible food and water. In sham animals, used as controls, T8–T9 thoracic spinal cord segments were exposed via dorsal laminectomy but not contusioned.

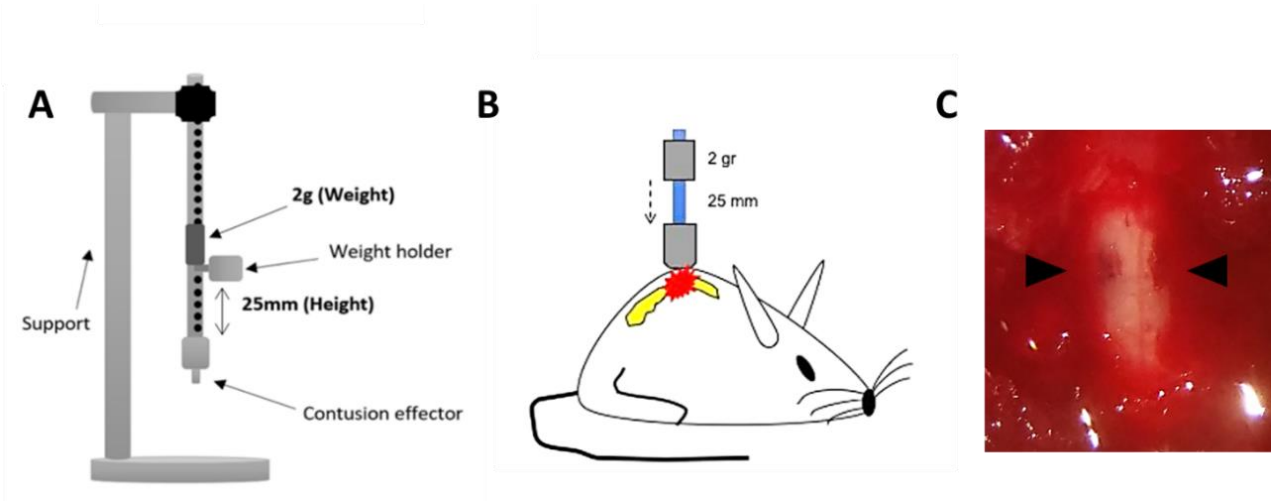


Figure 28. Spinal cord contusion procedure. **(A)** Scheme of the weight drop apparatus. **(B)** Schematic representation of the SCI-injury. (C) Image of the lesion produced after the contusion in the spinal cord of the mice.

5. Functional evaluation

5.1 Locomotor activity

Locomotor activity of the animals was evaluated by means of the Basso Mouse Scale (BMS) test (**Basso et al., 2006**). Concisely, animals were placed separately into a circular plastic open field (70 cm diameter x 24 cm wall height) and allowed them to move freely for 5 minutes. Meanwhile, the hindlimb movements were scored according to the Basso Mouse Scale, considering their stepping, paw position, coordination, and trunk stability. The BMS score ranges from 0 (no hindlimb movement) to 9 (normal movement-coordinated gait).

5.2 Nociceptive tests

5.2.1 Mechanical allodynia

Mechanical allodynia was detected by assessing 50% withdrawal thresholds using a set of von Frey monofilaments (bending force range 0.04–2 g) following the up-down paradigm, as previously described (**Chaplan et al., 1994**). Animals were placed in plastic tube test chambers with a metal mesh floor which allowed full access to the plantar surface of the hind paws (**Fig. 29. A**). Behavioural accommodation was allowed for approximately 1 hour, until cage exploration and major grooming activities ceased. Then, von Frey monofilaments were perpendicular applied to the plantar surface with sufficient force to cause slight buckling against the paw (**Fig. 29. B**). First, the 0.4 g filament was applied and then, the strength of the next filament was decreased when the mouse responded or increased when it was no responded. Clear paw withdrawal, shaking or licking were considered to be a response. This up-down procedure was limited to four assessments after the first response. Each filament was applied for 2 seconds with inter-stimulus intervals of 5-10 seconds. Both paws were evaluated since SCI model results in a bilateral injury and it is not possible to use contralateral paw as a natural intraindividual control. The mechanical threshold that produced 50% of responses was calculated using the Dixon formula:

$$50\%\ \text{paw withdrawal threshold (g)} = [(10(Xf + \kappa\delta)/10000)]$$

where Xf is the value (in logarithmic units) of the final von Frey filament used, κ is a fixed tabular value for the pattern of positive/negative responses and δ is the mean difference (in logarithmic units) between stimuli.

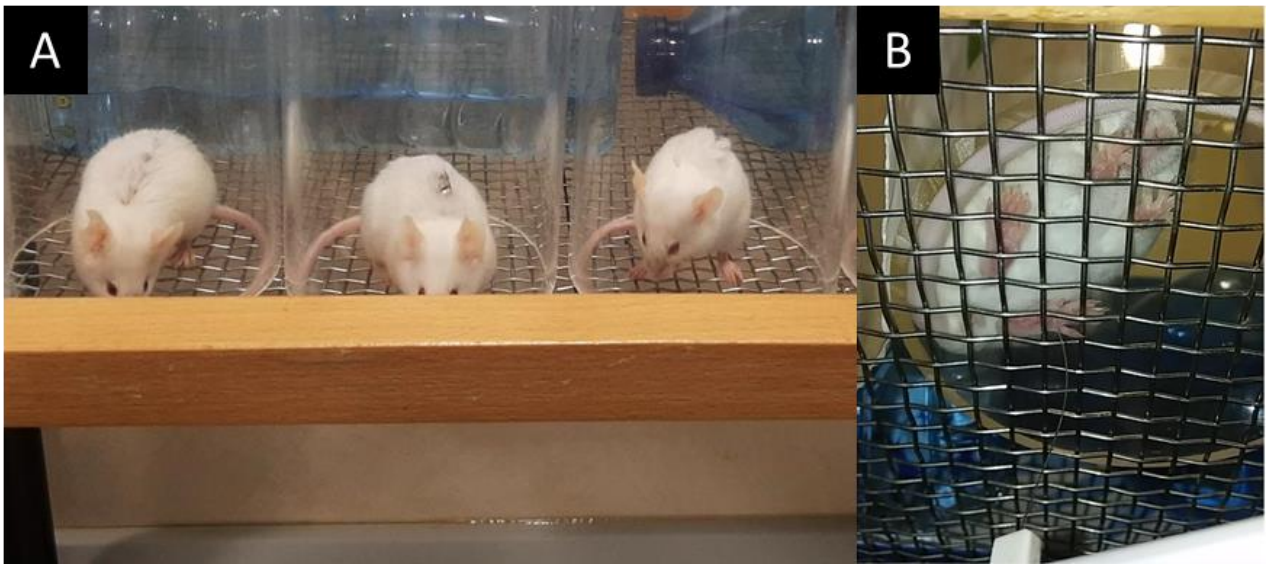


Figure 29. Set up of von Frey test. **(A)** Illustration of arenas with individual subjects placed atop the wire floor grid. **(B)** Close up illustration of the grid floor and a representative filament on approach to the plantar surface of the hind-paw.

5.2.2 Thermal hyperalgesia

Thermal hyperalgesia was assessed by measuring the hind paw withdrawal latency in response to a thermal stimulus. Plantar test was performed according to the Hargreaves method (**Hargreaves et al., 1988**) using a plantar test analgesimeter (#37370; Ugo Basile, Comerio, Italy).

Mice were placed into plastic test enclosures with an elevated glass floor and allowed to acclimate for approximately 1 hour, until cage exploration and major grooming activities ceased (**Fig. 30. A**). Then, the light of a projection lamp was focused onto the plantar surface of the hind paw (**Fig. 30. B**) with a time-limit of 30 seconds to avoid skin damage. Withdrawal latency was automatically recorded by a time-meter coupled to infrared detectors directed to the plantar surface of the paw. The sum of the mean withdrawal latencies for both hind paws were determined from the average of three separate trials, conducted at 5 minutes' intervals.

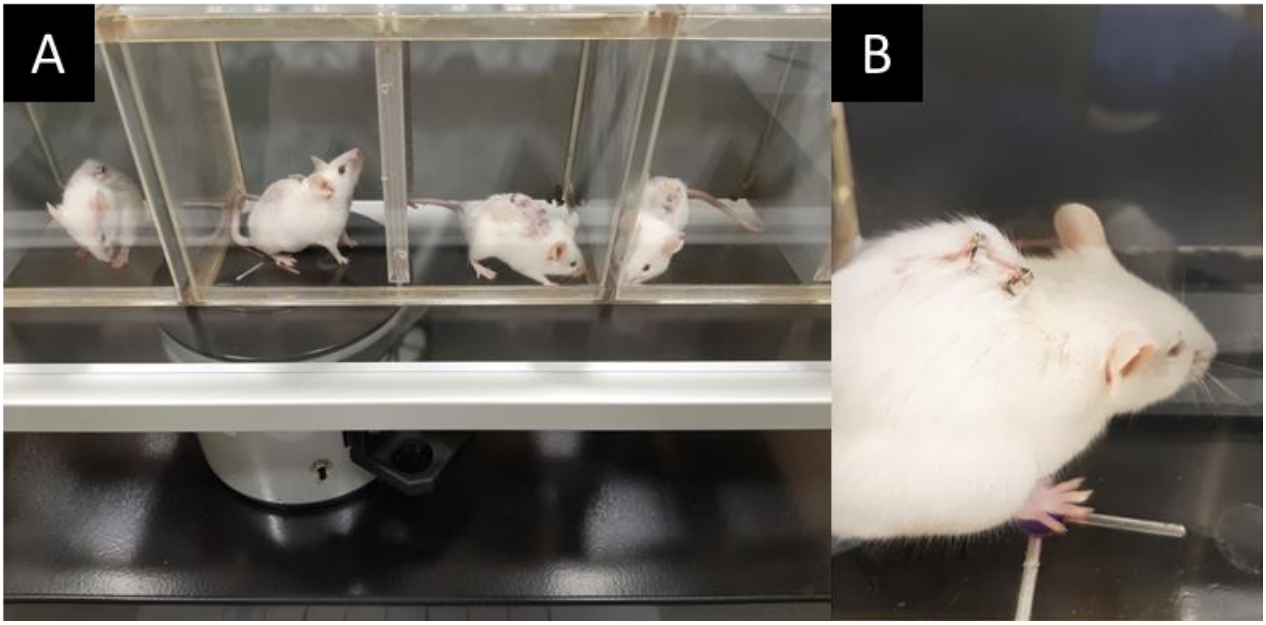


Figure 30. Set up of Plantar test. **(A)** Illustration of animal enclosure. **(B)** Close up illustration of the infrared emitter/detector positioned directly underneath the centre of mouse paw.

6. Affective-motivational evaluation

6.1 Open field

The Open Field Test is a classic test based on natural behaviours of rodents when they enter an open field to which they have not previously been exposed. Thus, each mouse was placed in the periphery of a white Plexiglass enclosure (50x50x45cm) in dim lighting (15-20 lux) and videotaped from above for 10 minutes as it freely explored the arena (**Fig. 31**). Movement patterns were quantified based on the global activity,

percentage of time spent in each zone (peripheral, middle and central), entries in zone, latency to entry in zone and zone transition, using a Panlab Smart video tracking software (SMART V3.0.06; Harvard apparatus, Spain).

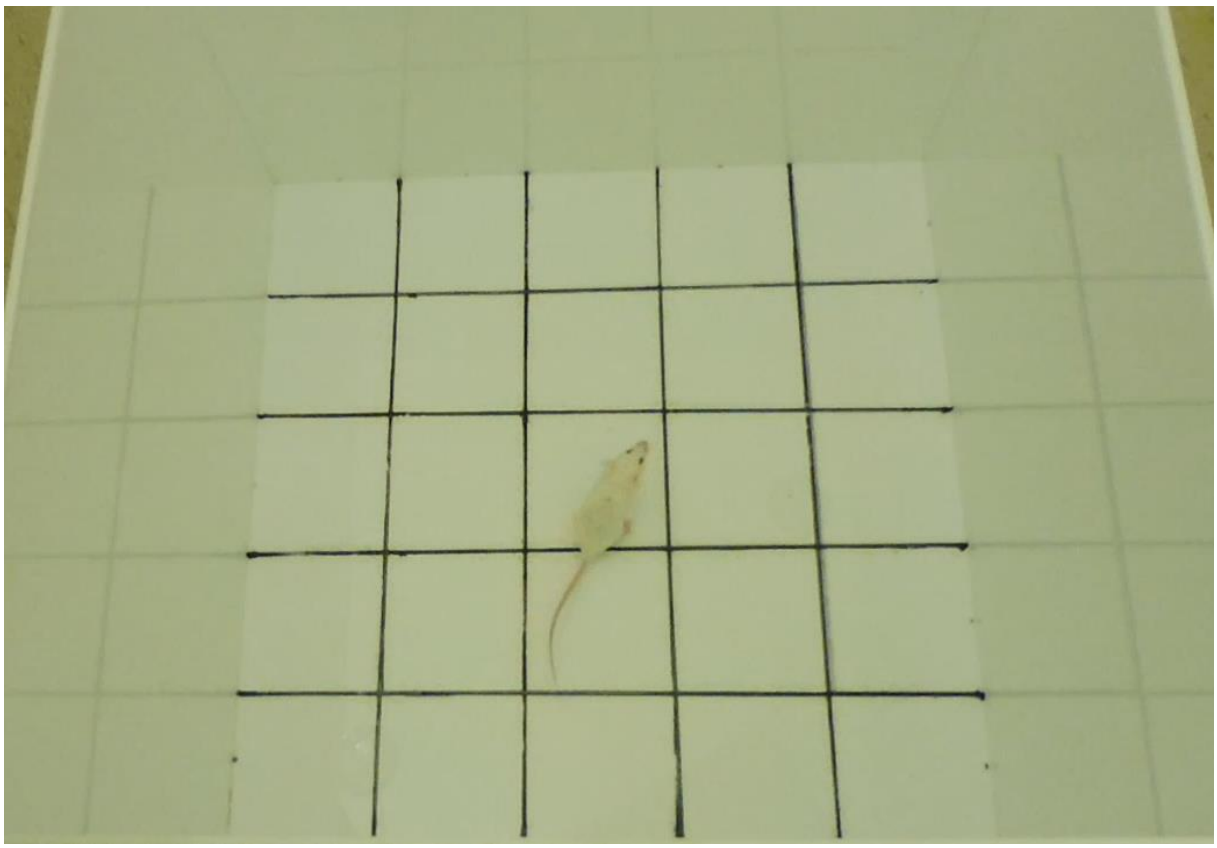


Figure 31. Mouse performing the Open Field test.

6.2 Light and dark box

Light and dark box test is commonly used for the assessment of the anxiolytic-like behaviour in mice (**J. Crawley & Goodwin, 1980**) and it is based on the innate aversion of mice to brightly illuminated areas and on the spontaneous exploratory behaviour in response to mild stressors, which are, novel environment and light (**Bourin & Hascoët, 2003**).

The test apparatus consisted of a rectangular box divided into two compartments, one-third dark (16x25x24 cm) and two-thirds light (25x25x24 cm), connected by a door (7x10 cm) located at floor level. The dark compartment was a black Plexiglas chamber covered with a roof (0-5 lux), while the light compartment was made of white Plexiglass and illuminated with dim light (20-25 lux) (**Fig. 32**). An intermediate zone between the two chambers was defined surround the door. Animals were placed individually in the middle of the dark chamber facing away from the door and allowed to freely explore the chambers for 10 minutes while video camera recorded from above.

The time spent in each chamber, global activity, the number of entries in the light chamber and the number of crossings between chambers was measured using the Panlab Smart video tracking software (SMART V3.0.06; Harvard apparatus, Spain). Increased number of entries and time spent in the light chamber is associated with decreased anxiety-like behaviour (**Bourin & Hascoët, 2003**).

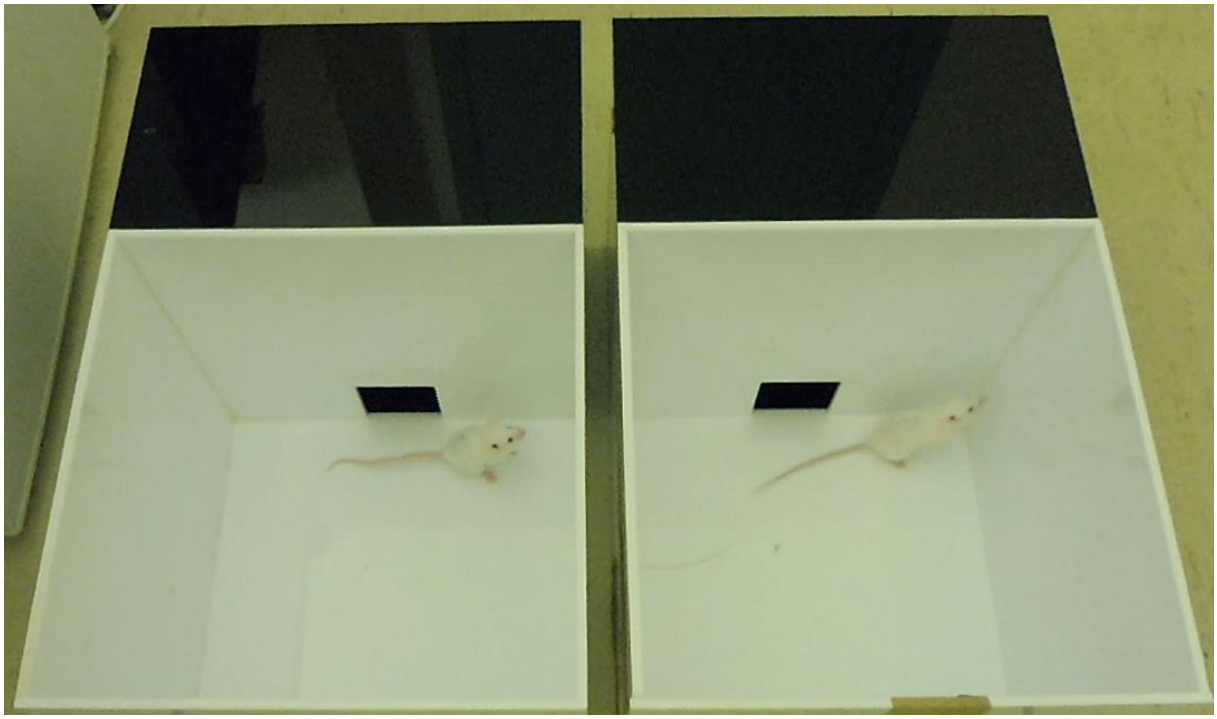


Figure 32. Mice performing the Light and dark box test.

6.3 Forced Swim

The forced swim or Porsolt test is widely used for testing depression in mice (**Porsolt et al., 1977**) and it is based on their innate escape-related mobility behaviour when they are forced to swim in an inescapable transparent cylinder filled with water. The purpose of agitation is searching, and it is highly energy consuming, while the reason of immobility is energy conservation. After antidepressant treatment, animals struggle more even in a desperate situation, and spend less time immobile.

Mice were individually placed in glass cylinders (height 40 cm, diameter 15 cm) filled with water (25±1ºC) to a depth of 30 cm (**Fig. 33**). Test duration was 6 minutes. The global activity, activity level (percentage of immobility, low and high activity) and immobility number were analysed by Panlab Smart video tracking software (SMART V3.0.06; Harvard apparatus, Spain).

Figure 33. Mice performing the Forced Swim test.

6.4 Social Interaction

The social interaction test was performed based on Crawley's sociability and preference for social novelty test (**J. N. Crawley, 2003**) which consists in free choice by a subject mouse to spend time in any of three box's compartments during two experimental sessions, including indirect contact with one or two unfamiliar mice. The apparatus was consisted of a rectangular Plexiglas box with three theoretical areas (20 x 42 cm). Two identical, grid enclosures with removable lids large enough to hold a single mouse were used. These were placed vertically inside the apparatus, one in each side area, and contained or not a female CD1 naïve mouse.

The experimental test consisted in three trials: habituation (adaptation), social affiliation aspect of the test (session I) and social novelty/preference session of the test (session II). For the habituation, subject mouse was placed at the middle of apparatus and allowed to acclimate for 5 minutes. For session I, one of the control mice ("Stranger mouse 1") was placed inside a grid enclosure located in one of the side areas. Then, subject mouse was placed at the middle of apparatus and allowed to freely explore the areas for 10 minutes while video camera recorded from above. The placement of Stranger 1 in the left or right side of the chamber was systematically altered between trials. For session II, a second control mouse ("Stranger mouse

2") was placed inside an identical grid enclosure located in the opposite side area (that had been empty during the session I). Then, subject mouse was placed at the middle of apparatus and allowed to freely explore the areas for 10 minutes while video camera recorded from above (***Fig. 34***).

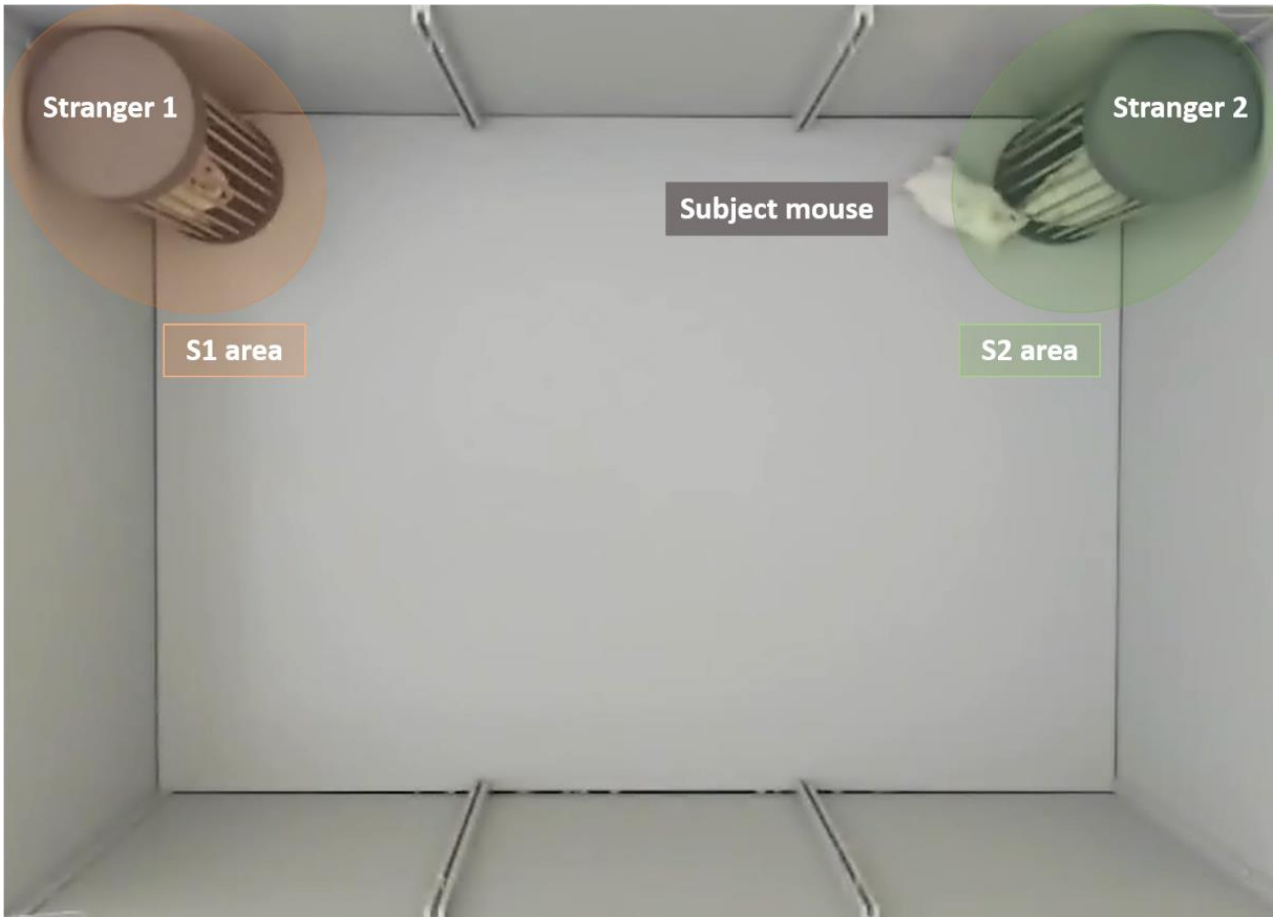


Figure 34. Mouse performing the Session II of Social interaction test. The grid enclosures containing Stranger mouse 1 and 2 are placed in each side area. The stranger mouse 1 area (orange) and stranger mouse 2 area (green) are also indicated. Subject mouse, who can freely explore the arena, is direct contacting (nose-to-nose) with Stranger 2 mouse.

Four outcomes were scored in this test:

- **Direct contact** is considered the most important and reliable parameter to assess social interaction and it is the result of the total number of direct contacts (nose-to-nose and nose-to-tail) between the tested mouse and the enclosure ones.
- The **latency to first direct contact** refers to the time until the tested mouse did the first direct interaction to the stranger mouse 1 or 2.
- **Entries in stranger mouse area** refers to the number of times that tested mouse entries to the stranger mouse 1 or 2 side area.

- **Preference time in stranger mouse area** is the net time that tested mouse spend in the stranger mouse 1 or 2 side area.

Entries and preference time in stranger mouse area were measured using the Panlab Smart video tracking software (SMART V3.0.06; Harvard apparatus, Spain) and direct contact and latency to first direct contact were manually measured to acquire more precision.

This test allows the assessment of social affiliation/motivation, as well as social memory and novelty. "Sociability" is defined as propensity to spend time with another unfamiliar mouse, as compared to time spent alone in an identical but empty chamber. "Preference for social novelty" is defined as propensity to spend time with a previously unencountered mouse rather than with a familiar mouse (**Moy et al., 2004**).

6.5 Approach and consumption of white chocolate as a measure of reward-seeking behaviour (RSB)

The basic test procedure was adapted from Merali (**Merali et al., 2003**) and it was performed as explained elsewhere (**de la Puente et al., 2015**). Approach and consumption of white chocolate as a measure of reward-seeking behaviour (RSB) consisted on evaluating four measures: latency and number of approaches to eat ("motivational-related behaviour") and the amount and duration of consumption ("consummatory-related behaviour"). Latency to eat is the time it takes the mouse to lick or bit chocolate for the first time. Number of approaches refers to the total of mouse approaches to lick or bit the chocolate. Duration of consumption is the time that mouse spends licking or biting the chocolate and consumption is the amount of chocolate that mouse ingests, which is assessed in grams by weighting the piece of chocolate before and after testing (**Fig. 35. A**).

Mice were habituated to chocolate 3 days before starting test to avoid food neophobia responses. For each animal, ~4g of white chocolate fragments (Milkybar®, Nestle, S.A.) were placed on a glass dish located in one corner of the home cage (**Fig. 35. B**). On test day, each animal had its own piece of chocolate (~2g) and the test started as soon as each animal was placed in the opposite side where the chocolate was located. The placement of chocolate in the left or right corner of the home cage was systematically altered between trials. Test duration was 10 minutes, and it was performed during 3 consecutive days. Increased latency to eat chocolate as well as decreased number of approaches, amount and duration of consumption are associated with anhedonia-like behaviour.

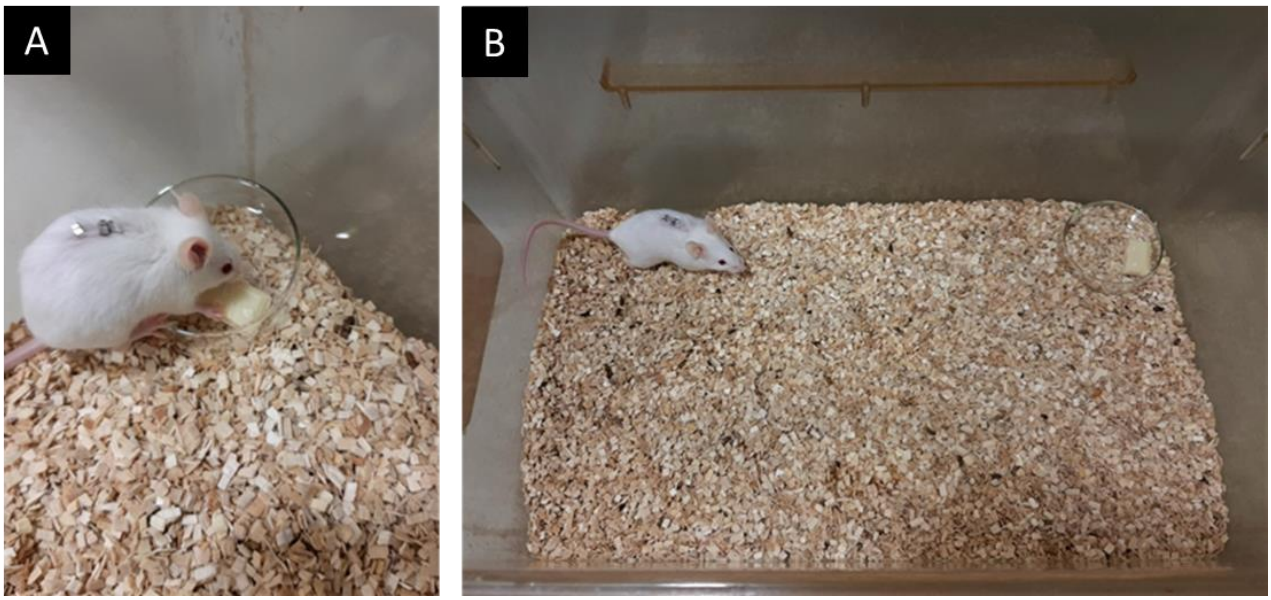


Figure 35. Mouse performing the RBS test. **(A)** Mouse consuming chocolate during the RBS test. **(B)** Home cage with a piece of chocolate placed on a glass dish located in one corner.

7. Animal sample collection

At the end of each experimental protocol, the animals were thoroughly anaesthetized with sodium pentobarbital (90 mg/kg; i.p.) and serum, spinal cord and supraspinal structures samples were carefully collected.

7.1 Serum samples

When the animal entered the anaesthetic plane (absence of foot reflex and breathing located in the abdominal region) was placed in decubitus supine position and blood was extracted through the insertion of an intracardiac needle. Subsequently, the obtained blood was centrifuged during 15 minutes at 4000 rpm to obtain the serum, which was frozen immediately in dry ice and stored at -80ºC until its analysis.

7.2 Central nervous system samples for immunohistochemical analysis

Animals selected for the immunohistochemical analysis were perfused intraventricularly with 500mL of 4% paraformaldehyde solution in phosphate-buffered saline (PBS, 10 mM sodium phosphate buffer, pH 7.4). Afterwards, spinal cord, brain and brainstem tissue were carefully removed.

- **Spinal cord samples**

After animal's perfusion, spinal cord was exposed by a dorsal laminectomy and was then meticulously extracted and immersed in a Zamboni fixing solution (4% paraformaldehyde and 0.3% of picric acid in PBS; **Zamboni and De Martino 1967**). The spinals cords samples were conserved in Zamboni solution at least 14

days after its extraction and then were preserved in 30% sucrose solution in PBS. The samples were stored at 4ºC until histological analysis.

- **Supraspinal cord samples**

The entire brain was removed from each mouse and post-fixed in 4% paraformaldehyde at 4°C.

7.3 Central nervous system samples for western blotting analysis

The central nervous system tissue samples for the western blotting analysis were rapidly extracted from the animal after the blood collection.

- **Spinal cord samples**

Spinal cord was exposed by a dorsal laminectomy and was then meticulously extracted and stored at -80ºC.

- **Supraspinal cord samples**

The entire brain was removed from the cranium of each mouse and stored at -80ºC.

8. Biochemical analysis

8.1 Immunohistochemical analysis

- **Spinal Cord**

For the immunohistochemical analysis, T7-T10 spinal cord segments were embedded in Tissue Freezing Medium (0201-08926, Leica, Barcelona) and cut transversely with a cryostat (CM1520, Leica, Barcelona) into 60 µm thick sections that were collected in six-wells porcelain plates. First, sections were washed 2 times for 10 minutes with saline phosphate buffer (PBS, 0.1M, pH = 7'4) and 2 times for 10 minutes with 0.1M PBS/0.3% Triton (PBS-Triton). Next, tissue sections were blocked with 1% bovine foetal serum in PBS-Triton (PBS-Triton-FCS) for 1 h and were then incubated with Rabbit Anti-GFAP (1:200, ab7260, Abcam) or Rabbit Anti-IBA1 (1:200, 019-19741, Fujifilm Wako) during 48 hours at 4ºC in a humidified chamber to avoid tissue drying out. The sections were washed three times for 10 minutes with PBS-Triton and then incubated overnight at 4ºC in a humidified chamber with AffiniPure Goat anti-Rabbit IgG conjugated with cyanine 3 (Cy3) (1:200, Ref # 111-165-144, Jackson ImmunoResearch, USA). Finally, two 10 minutes-washes were again performed with PBS-Triton and one 10 minute-wash with PBS, and the samples were mounted on previously gelatinized slides. Ounce mounted, slides were dehydrated through its immersion in increasing concentrations of ethanol baths (70%, 96% and 100%) and were covered with cover glass fixed with DPX mounting media (1.01979.0500, Merck, Germany).

Histological sections were observed with an epifluorescence microscope (Leica DMR-XA; Leica Microsystems) attached to a digital camera (FMVU-13S2C-CS; Point Gray Research, Canada) used to capture the images (x200). The result images were analysed with the free software Image-J (Image Processing and Analysis in Java, National Institute of Health, NIH, USA). For each animal in the present study, a minimum of eight immunolabeled histological sections were analysed.

Imaging of the sections immunolabeled for IBA1 was counted for reactive and non-reactive microglia cells and was expressed as a percentage of two phenotypes. The percentage of reactive microglial cells was considered as an index of the degree of microgliosis. Regarding to GFAP immunolabeling, the immunopositive area for GFAP was measured as a percentage of immunoreactivity.

- **Supraspinal Structures**

Serial PAG coronal sections (12 µm) from the central part of the superior colliculi to the upper edge of the inferior colliculi corresponding to PAG between 6.72–8.04 mm from the bregma (**Paxinos and Watson, 1997**) were cut (Leica 1800 cryostat; Leica Microsystems, Wetzlar, Germany). For ACC, serial coronal sections (12 µm) through the prefrontal cortex ACC between 2.2 mm and 4.2 mm from the Bregma (**Paxinos and Watson, 1997**) were also prepared.

The sections were collected on gelatin-coated microscopic slides, air-dried and processed for immunohistochemical staining. First, the sections were washed with PBS containing 0.05% Tween 20 (PBS-TW20) and 1% bovine serum albumin for 10 min, and then treated with 3% normal donkey serum in PBS-TW20 for 30 min. Then, the sections were incubated with the following primary antibodies: Rabbit Anti-GFAP (1:250, DAKO), Rabbit Anti-IBA (1:100, Wako), Mouse Anti-NEUN (1:100, Chamicon), Mouse Anti-OX42 (1:100, Santa Cruz) and Rabbit Anti-CD206 (1:100, Abcam) in a humid chamber at room temperature for 12h. The binding of primary antibodies was visualized by secondary antibodies (TRITC-, FITC- or Cy5 conjugated, affinity purified goat anti-rabbit or anti-mouse; 1:100; Jackson) for 90 min at room temperature. Immunostained sections were rinsed, stained with Hoechst 33342 to detect positions of the cell nuclei, and mounted in a Vectashield aqueous mounting medium (Vector Laboratories Inc., Burlingame, CA). The control sections were incubated with omission of the primary antibody and displayed no immunostaining.

Supraspinal structures sections were analysed using a Nikon Eclipse NI-E epifluorescence microscope equipped with a Nikon DS-Ri1 camera driven by NIS-Elements software (Nikon, Prague, Czech Republic). For each animal in the present study, a minimum of three immunolabelled histological sections were analysed. In addition, for image analysis on each section, 4 non-overlapping regions of interest (ROIs) were defined

to calculate the proportion of immunostaining. The term “immunostaining proportion” for each antibody refers to the ratio of the area of positive pixels to the area of ROI multiplied by 100.

8.2 Western Blotting analysis

- **Spinal cord**

For the western blotting analysis, T7-T10 spinal cord segments were homogenized in modified RIPA buffer (50 mM Tris–HCl pH 7.5, 1% Triton X-100, 0.5% sodium deoxycholate, 0.2% SDS, 100 mM NaCl, 1 mM EDTA, 2 mM PMSF, 1 µg/µL aprotinin, 1 µg/µL leupeptin, and 2 mM sodium orthovanadate) and were then centrifuged at 18,000g at 4°C for 30 min. The protein concentration from the obtained supernatant was determined by Protein Assay DC™ (Bio-Rad). Samples were then stored at −80°C until use.

Samples containing equal amounts of protein (10 – 20 micrograms) were mixed with 2× Laemmli sample buffer (S3401, Sigma-Aldrich) and boiled at 95ºC for 10 min. Samples were fractioned by 10–15% (w/v) SDS-PAGE gels and transferred onto a nitrocellulose membrane, then blocked, with either 5% non-fat dry milk or bovine serum albumin (BSA), in Tris-0.1%Tween 20-buffered saline (T-TBS) for 2 h at room temperature.

Membranes were incubated with the following primary antibodies overnight at 4ºC: Rabbit Anti-IBA1 (1:700, 0019-19741, FUJIFILM Wako Chemicals), Rabbit Anti-GFAP (1:800, SAB4300647, Sigma-Aldrich), Rabbit Anti-MCP1 (1:1000, ab25124, Abcam), Rabbit Anti-CCR2 (1:1000, ab203128, Abcam), Rabbit Anti-CX3CL1 (1:1000, ab25088, Abcam), Rabbit Anti-CX3CR1 (1:1000, ab8021, Abcam), Rabbit Anti-Extracellular signal-regulated kinases (total ERK ½) (1:1000, 9102, Cell Signaling), and diphosphorylated ERKs (pERK ½) (1:1000, 9101, Cell Signaling). Rabbit Anti-GAPDH antibody (1:10,000, G9545, Sigma-Aldrich) was used as a loading control.

The immunoblots were washed three times for 10 minutes with T-TBS and then incubated for 1:30h at room temperature with horseradish peroxidase-conjugated goat antirabbit IgG (1/50000, AP132P, Sigma-Aldrich) and revealed by chemiluminescence Clarity Western ECL Substrate (170-5061, Bio-Rad). Band pixel intensities were quantified by Gel-Pro Analyzer software (Media Cybernetics, USA) and normalized to the corresponding GAPDH intensity. pERK ½ was normalized to total ERK, and subsequently, normalized with GAPDH immunoreactivity intensity.

- **Supraspinal Structures**

In order to obtain the PAG area, a 2 mm thick coronal slice of the mesencephalon was carefully removed at the position from the central part of the superior colliculi to the upper edge of the inferior colliculi corresponding to PAG between 6.72 mm and 8.04 mm from the Bregma (**Paxinos and Watson, 1997**). Radial segments corresponding approximately with ipsilateral and contralateral dorsal PAG and ventral PAG were

dissected under a stereomicroscope according to the boundaries defined using anatomical criteria (**Keay and Bandler, 2001**). For ACC, a 2 mm thick coronal slice of the prefrontal cortex was carefully removed at the position between 2.2 mm and 4.2 mm from the Bregma (**Paxinos and Watson, 1997**). To obtain the rostral ventromedial medulla (RVM), a 2 mm coronal slice was cut though the medulla, 1 mm from posterior edge of the inferior colliculi corresponding approximately to 1 mm from the interaural line. This section corresponds to the RVM between –10.8 mm and –11.4 mm from the bregma (**Paxinos and Watson, 1997**). A tissue triangle was then dissected under a stereomicroscope to isolate the RVM area, including the nucleus raphe magnus, gigantocellularis, and gigantocelularis pars alpha. Finally, to obtain the amygdala, a 1.5 mm thick coronal section was carefully prepared at the position between –1.5 and –3.0 mm from Bregma (**Paxinos and Watson, 1997**) and a triangular segment containing Amygdalar nuclei was dissected.

The ACC, amygdala, vPAG, dPAG and RVM tissue samples of individual animals were homogenized in RIPA buffer (Abcam) containing protease inhibitors (LaRoche, Switzerland), and were then centrifuged at 15,000g at 4ºC for 20 min. Protein concentration from the tissue supernatant was measured by Nanodrop ND-1000 (Thermo Scientific) and normalized to the same levels.

Each sample, containing 50 µg of protein, was separated by SDS-polyacrylamide gel electrophoresis, and transferred onto nitrocellulose membranes. The membranes were then blocked with 1% BSA in PBST (3.2 mM Na2HPO4, 0.5 mM KH2PO4, 1.3 mM KCl, 135 mM NaCl, 0.05% Tween 20, pH 7.4) for 1 h at room temperature and incubated with the following primary antibodies overnight at 4ºC: Rabbit Anti-IBA1 (1:100, FUJIFILM Wako Chemicals), Rabbit Anti-GFAP (1:10000, Abcam), Rabbit Anti-MCP1 (1:1000, ab25124, Abcam), Rabbit Anti-CCR2 (1:1000, ab203128, Abcam), Rabbit Anti-CX3CL1 (1:2000, Abcam), Rabbit Anti-CX3CR1 (1:2000, Abcam) and Rabbit Anti-CatS (1:1000, Abcam). Mouse Anti-α-tubulin antibody (1:1000, Cell Signaling) was used as a loading control.

Blots was washed in PBST and incubated with peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Sigma, 1:1000) at room temperature for 1 h. Protein bands were visualized using the ECL detection kit (Amersham) on an LAS-3000 chemiluminometer reader (Bouchet Biotech) and analyzed using densitometry image software.

8.3 Hepatotoxicity and nephrotoxicity analysis

Serum samples were analysed to study the possible hepatotoxicity and nephrotoxicity produced by the different polyphenols present in the extracts. To this end, the ALANINE AMINOTRANSFERASE (ALT/GPT) kit (COD 11533, BioSystems), ASPARTATE AMINOTRANSFERASE (AST/GOT) kit (COD 11531, BioSystems) and UREA-BUN COLOR kit (COD 11536, BioSystems) have been used. A thermostatic water bath at 37ºC and a

spectrophotometer (ZUZI (model 4211/20)) able to read at 600nm (UREA) or 340 nm (AST/ALT) were also used in the present analysis.

- **ALT and AST**

Alanine aminotransferase (ALT or GPT) catalyzes the transfer of the amino group from alanine to 2-oxoglutarate, forming pyruvate and glutamate. The catalytic concentration is determined from the rate of decreasee of NADH, measured at 340 nm, using the lactate dehydrogenase (LDH) coupled reaction.

$$Alanine + 2 - Oxoglutarate \xrightarrow{ALT} Pyruvate + Glutamate$$

$$Pyruvate + NADH + H^+ \xrightarrow{LDH} Lactate + NAD^+$$

Two different reagents were used in the ALT analysing procedure:

- Reagent A: Tris 150 mmol/L, L-alanine 750 mmol/L, lactate dehydrogenase > 1350 U/L, pH 7.3.
- Reagent B: NADH 1.9 mmol/L, 2-oxoglutarate 75 mmol/L, Sodium hydroxide 148 mmol/L, sodium azide 9.5 g/L.

Aspartate aminotransferase (AST or GOT) catalyzes the transfer of the amino group from aspartate to 2-oxoglutarate, forming oxalacetate and glutamate. The catalytic concentration is determined from the rate of decrease of NADH, measured at 340 nm, employing the malate dehydrogenase (MDH) coupled reaction.

$$Aspartate + 2 - Oxoglutarate \xrightarrow{AST} Oxalacetate + Glutamate$$

$$Oxalacetate + NADH + H^+ \xrightarrow{MDH} Malate + NAD^+$$

Two different reagents were necessary for the AST analysing protocol:

- Reagent A: Tris 121 mmol/L, L-aspartate 362 mmol/L, malate dehydrogenase >460 U/L, lactate dehydrogenase > 660 U/L, pH 7.8.
- Reagent B: NADH 1.9 mmol/L, 2-oxoglutarate 75 mmol/L, Sodium hydroxide 148 mmol/L, sodium azide 9.5 g/L.

Procedure for measure both AST and ALT was the same. Initially, working reagent was prepared mixing the reagent A with B in a proportion of 4:1 and was then set up to the reaction temperature (37ºC) using the thermostatic water bath. Afterward, 50 µL of the serum sample were mixed with 1 mL of working reagent

into 1cm light path cuvette. After 1 minute, the initial absorbance was recorded and at 1-minute intervals thereafter for 4 minutes. The difference of consecutive absorbances was determined to calculate the average absorbance difference per minute (ΔA/min). The AST/GOT and ALT/GPT concentrations in the sample were calculated using the following general formula:

$$\frac{Vt\ x\ 10^{-}}{\Delta A/\min x\ \varepsilon\ x\ l\ x\ VS} = U/L$$

The molar attenuation coefficient (ε) of NADH at 340 nm is 6300, the lightpath (l) is 1 cm, the total reaction volume (Vt) is 1.05, the sample volume (VS) is 0.05, and 1 U/L equals to 0.0166 µkat/L.

- **UREA**

Urea in the sample originates, by means of the coupled reactions described below, a coloured complex that can be measured by spectrophotometry (2,3,4).

$$Urea + H_2O \xrightarrow{urease} 2NH_4^+ + CO_2$$

$$NH_4^+ + Salicylate + NaCIO \xrightarrow{nitroprusside} Indophenol$$

The necessary reagents to measure the urea concentration in serum samples are the following:

- Reagent A1: Sodium salicylate 62 mmol/L, sodium nitroprusside 3.4 mmol/L, phosphate buffer 20 mmol/L, pH 6.9.
- Reagent A2: Urease > 500 U/mL.
- Reagent B: Sodium hypochlorite 7 mmol/L, sodium hydroxide 150 mmol/L.
- Reagent S: Glucose/Urea/Creatinine Standard. Glucose 100 mg/dL, urea 50 mg/dL (8.3 mmol/L, BUN 23.3 mg/dL), creatinine 2 mg/dl.

To start the procedure reagent A was prepared mixing the reagent A1 and A2 in a proportion of 1:24. Next, all the reagents were set to the temperature room and were then pipetted into designed test tubes:

- Blank: 1mL Reagent A
- Standard: 10µL Reagent S with 1mL Reagent A
- Sample: 10µL of serum sample with 1mL Reagent A

The test tubes were mixed thoroughly and were then incubated for 10 minutes. Afterwards, 1mL of Reagent B was added into each test tube, which was mixed and incubated for 10 more minutes. Finally, the standard and the samples absorbance (A) was registered at 600 nm against the blank absorbance. The urea concentration in the sample was determined using the following generic formula:

$$\frac{A_{Sample}}{A_{Standard}} \; x \; C_{Standard} \; x \; Sample \; dilution \; factor = C_{Sample}$$

9. Statistical analysis

All functional, histological and biochemical analysis were performed in a blinded manner using a code for each mouse. Results are shown as mean ± standard deviation of the mean (SEM). The normal distribution of the data was analysed by Kolmogorov Smirnov (when sample > 30) or Shapiro Wilk (when sample < 30) test before further applying parametric or non-parametric statistical analyses. Data that followed a normal distribution were analysed using repeated measures MANOVA (Wilks' criterion) and analysis of variance (ANOVA) followed by Duncan's test, when applicable. Data that did not follow a normal distribution were analysed using Friedman statistic test for non-parametric repeated measures and Kruskal Wallis followed by U de Mann-Whitney test. In all statistical analysis, the α level was set at 0.05 using the statistical package SPSS 25.0 for Windows.

RESULTS

CHAPTER I

Preventive effects on Spinal Cord Injury-induced neuropathic pain development by repeated administration of EGCG in CD1 mice.

IV. RESULTS

CHAPTER I. Preventive effects on Spinal Cord Injury-induced neuropathic pain development by repeated administration of EGCG in CD1 mice.

A previous study performed by Álvarez-Pérez (**Álvarez-Pérez, 2016**) showed that repeated administration of EGCG during the first week post-SCI significantly reduced thermal hyperalgesia in Balb/c mice up to 14 dpi by modulating both spinal cord astrogliosis and microgliosis. However, the preventive effects of EGCG during the whole spinal cord injury acute phase remain unknown. To this end, a dose-response (10, 15 and 20 mg/kg; i.p.) study was performed to elucidate the preventive effects of EGCG on SCI-induced neuropathic pain development throughout the acute phase. To be consistent, female CD1 mice were used since such strain is wildly used for pharmacological assessments. After spinal cord injury, the effects of repeated EGCG treatment on weight, thermal hyperalgesia, mechanical allodynia, locomotor activity and spinal cord gliosis were assessed up to 21 dpi.

Preventive EGCG treatment results in weight loss in spinal cord injured mice.

Following a protocol animal welfare supervision based on Morton D.B and Griffiths P.H. guidelines (**Morton and Griffiths, 1985**), changes in coat and skin, vibrissae of nose, nasal secretions, signs of autotomy or aggressiveness were not detected in any experimental group within the experimental period.

As for systemic effects evaluation, different statistical tests were used to evaluate the role of both time and treatment on animal's weight. After Shapiro-Wilk normality test confirmed a normal distribution of the data (all time-points p's>0.188), the MANOVA analysis indicated significant effects on day ($F_{(3,15)}$= 19.667, p<0.001) and treatment ($F_{(4,17)}$= 5.682, p=0.04) factors as well as a significant day x treatment interaction ($F_{(12,39.978)}$= 3.504, p=0.01). On further ANOVA analysis, significant differences between experimental groups were observed during all the post-injury days, from 7 to 21 (all p's<0.05). Concretely, the subsequent post hoc analysis revealed a significant (all p's<0.005) weight loss of all treated animals at all time-points when compared with Sham group (***Fig. 36***). That is, none of treated groups reached the same Sham group weight suggesting systemic adverse effects associated with either EGCG or DMSO treatment.

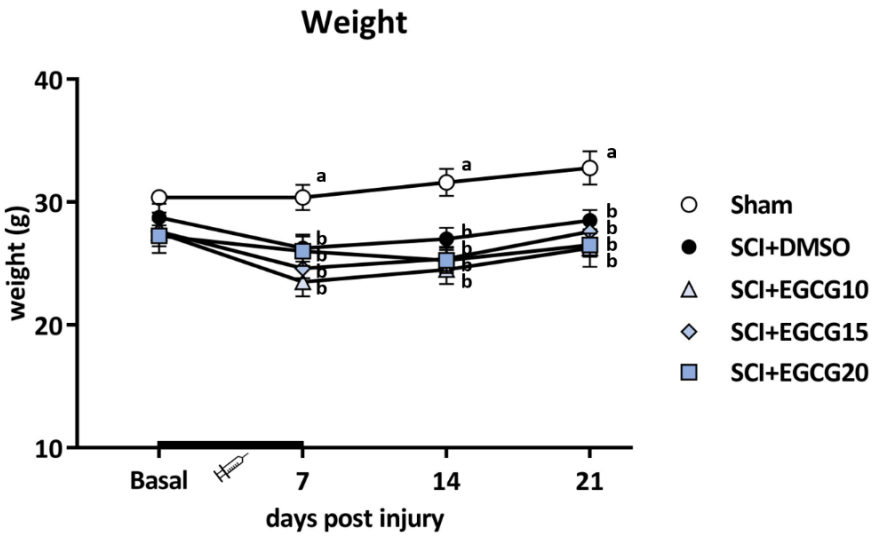


Figure 36. Time-course assessment of mice weight after preventive EGCG treatment, during the spinal cord injury acute phase. Each point and vertical line represent the mean ± SEM. a-b: groups not sharing a letter are significantly different, $p < 0.05$, by Duncan's test. Treatment administration week (basal to 7 dpi) is highlighted with a thick black line. Experimental groups: Sham (n=5), SCI+DMSO (n=4), SCI+EGCG10 (n=4), SCI+EGCG15 (n=5), SCI+EGCG20 (n=4).

Preventive EGCG treatment modulates both mild spinal cord injury-induced mechanical allodynia and thermal hyperalgesia development.

Before reflexive-pain responses evaluation, the BMS test was performed to rule out locomotor disturbances that may override the functional evaluation. Shapiro-Wilk normality test showed that locomotor activity data did not follow a normal distribution (all p's<0.01) and, although Friedman's test showed significant differences throughout the experimental period (p<0.001), Kruskal-Wallis test indicated no significant group differences at any post-injury day (all p's>0.144) (***Fig. 37. A***). It is worth mentioning that only paw position alterations were observed in some of the SCI animals, but no horizontal locomotion alterations were detected, indicating that all mice were able to move freely without neither paralysis nor major impairment in the coordination and locomotor functions. These results allow performing properly the evoked-pain responses evaluation (thermal hyperalgesia and mechanical allodynia) since no significant locomotor deficits were observed during the experimental period.

Referring to mechanical allodynia, Shapiro-Wilk normality test showed that data did not follow a normal distribution (all p's<0.001). Significant differences in the distribution of the data were found by Friedman's test (p=0.017) and further Kruskal-Wallis test indicated significant differences between groups at all post-injury days (7, 14 and 21 dpi) (all p's<0.012). Concretely, subsequent Mann Whitney U test revealed significant decrease of paw withdrawal mechanical thresholds of SCI+DMSO mice when compared with all other experimental groups at 7, 14 and 21 dpi (***Fig. 37. B***). Furthermore, at 7 and 14 dpi, no significant differences were detected between Sham and SCI EGCG-treated animals. Thus, all tested EGCG doses

prevented mechanical allodynia development after SCI up to 14 dpi. However, at 21 dpi, the SCI groups treated with EGCG15 and EGCG20 only showed a significant attenuation of mechanical allodynia compared to Sham, while the EGCG10 dose exerted preventive effects since it no showed significant differences when compared with the Sham group (***Fig. 37. B***).

In contrast to mechanical allodynia, thermal hyperalgesia data followed a normal distribution ($p>0.05$) according to the Shapiro-Wilk test. MANOVA revealed significant effects on day ($F_{(3,12)} = 47.908$, $p<0.001$), treatment ($F_{(4,14)} = 38.744$, $p<0.001$), and interaction day x treatment ($F_{(12,32.041)} = 7.066$, $p<0.001$) factors. On further ANOVA, significant group differences were found at all assessment time-points after the lesion (all p's<0.001). Specifically, the subsequent post hoc analysis revealed that at 7 dpi, SCI+DMSO group showed a significant decrease in thermal paw withdrawal thresholds in comparison to all other experimental groups, indicating preventive effects on thermal hyperalgesia development by all EGCG tested doses. These preventive effects were maintained for the EGCG15 and EGCG20 groups up to 14 and 21 dpi, showing no significant differences with the Sham group. However, EGCG10 dose lost its anti-hyperalgesic effect at 14 and 21 dpi, showing no significant differences with the spinal cord injured mice without treatment (SCI+DMSO) (***Fig. 37. C***).

Preventive EGCG treatment modulates astrogliosis and microgliosis in spinal cord injured mice.

Immunohistochemical analysis of spinal cord samples were performed in order to evaluate the preventive effects of EGCG treatment in spinal astrogliosis and microgliosis. To analyse spinal astrogliosis, anti-GFAP antibody was incubated on histological spinal cord sections and the percentage of GFAP immunoreactivity area was quantified. Statistical analysis showed that data did not follow a normal distribution ($p<0.001$) according to the Shapiro-Wilk test. On further Kruskal-Wallis test, significant differences were found between groups ($p<0.001$) and subsequent Mann Whitney U test revealed that such differences were shown between all doses (all p's<0.01) except between EGCG15 and EGCG20 groups ($p=0.916$). Concretely, a significant increase of the GFAP immunoreactivity area was found in the SCI+DMSO group when compared with Sham group, and while EGCG groups do not reach the same levels of astrogliosis observed in the Sham group, all EGCG treatments clearly significant attenuated spinal cord astrogliosis since all of them showed significant decreased GFAP immunoreactivity area when compared with SCI+DMSO. It is worth mentioning that EGCG15 and EGCG20 doses exerted better effect when compared with EGCG10 dose (***Fig. 38***).

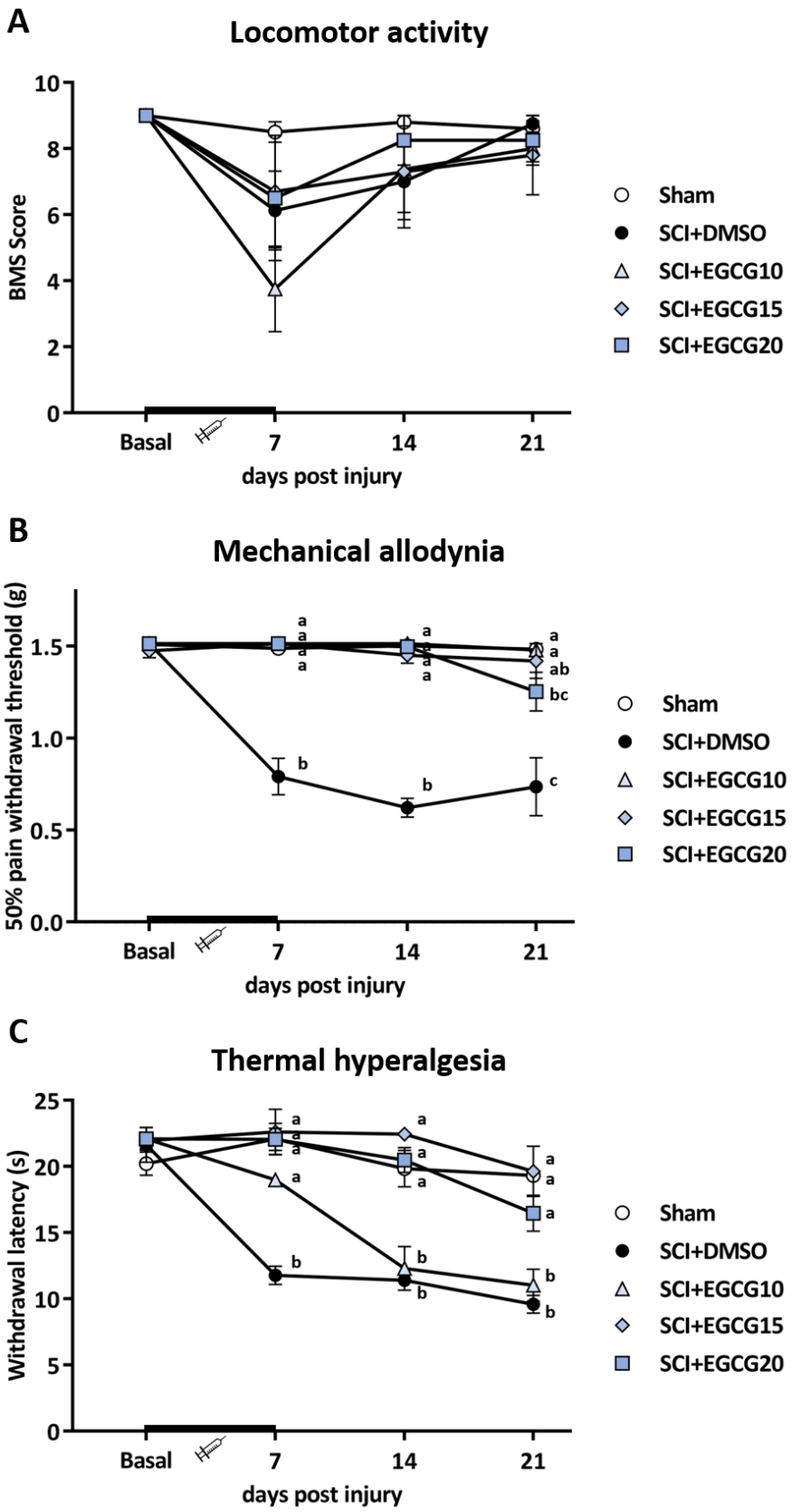


Figure 37. Time-course assessment of locomotor activity, mechanical allodynia and thermal hyperalgesia after preventive EGCG treatment, during the spinal cord injury acute phase. Each point and vertical line represent the mean ± SEM. a-c: groups not sharing a letter are significantly different, $p < 0.05$, by posthocs' test. Treatment administration week (basal to 7 dpi) is highlighted with a thick black line. Experimental groups: Sham (n=5), SCI+DMSO (n=4), SCI+EGCG10 (n=4), SCI+EGCG15 (n=5), SCI+EGCG20 (n=4).

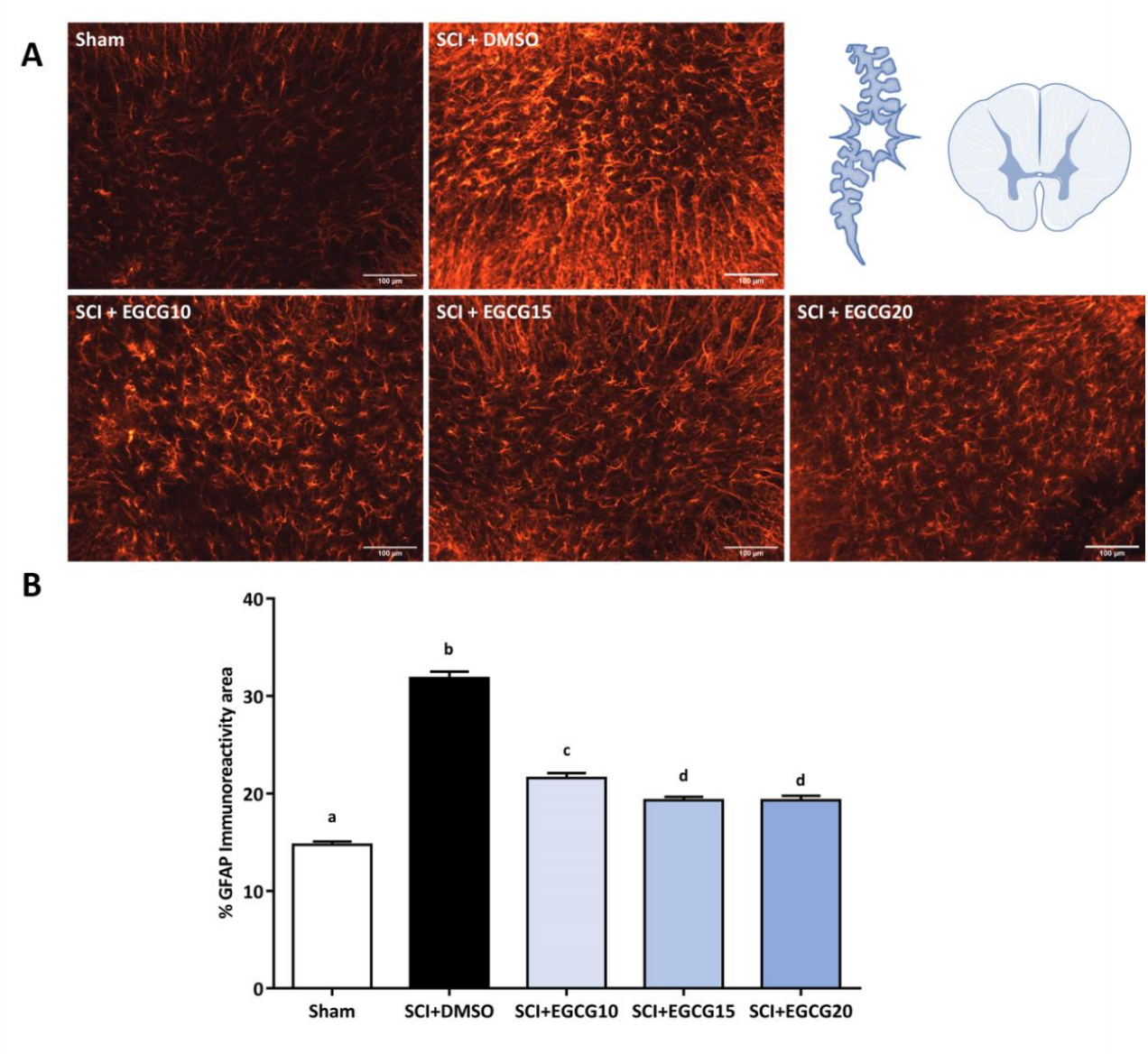


Figure 38. Effects of EGCG treatment (10, 15 and 20 mg/kg) on spinal GFAP immunoreactivity area in SCI mice at the end of experimental period. (**A**) Representative histological images of the spinal cord immunostained against GFAP of each group (Scale bar 100 µm). Experimental groups: Sham (n=3) (slices=66), SCI+DMSO (n=4) (slices=62), SCI+EGCG10 (n=4) (slices=63), SCI+EGCG15 (n=5) (slices=75), SCI+EGCG20 (n=4) (slices=29). (**B**) Histogram representing the percentage of dorsal horn GFAP-immunoreactivity. Data is expressed as mean ± SEM. a–d: Groups not sharing a letter showed significant differences, $p<0.05$.

Referring to spinal microgliosis, anti-IBA1 antibody was incubated on histological spinal cord sections and reactive and non-reactive microglia cells were counted and expressed as a percentage of two phenotypes. According to the Shapiro-Wilk test, data did not follow a normal distribution ($p<0.001$). And Kruskal-Wallis test indicated significant differences between groups ($p<0.001$) in both percentage of reactive and non-reactive microglia cells. Subsequent Mann Whitney U test showed a significant ($p<0.001$) increase of percentage of reactive microglia cells in SCI+DMSO mice when compared with Sham. In parallel, EGCG15 and EGCG20 treatment groups showed a significant decrease in the percentage of reactive cells in comparison to SCI+DMSO (all p's<0.001), but none of them reached the percentage level of the Sham group

since they all significant differed from this control group (all p's<0.001). In contrast, EGCG10 treatment group showed no significant difference of reactive cells percentage compared to SCI+DMSO group (p=0.324) (***Fig. 39. B***). As for the intra-group analysis of the cell types' percentages, Kruskal-Wallis test indicated statistical differences between reactive and non-reactive microglia cells for all experimental groups (all p's<0.01,) (***Fig. 39. B***).

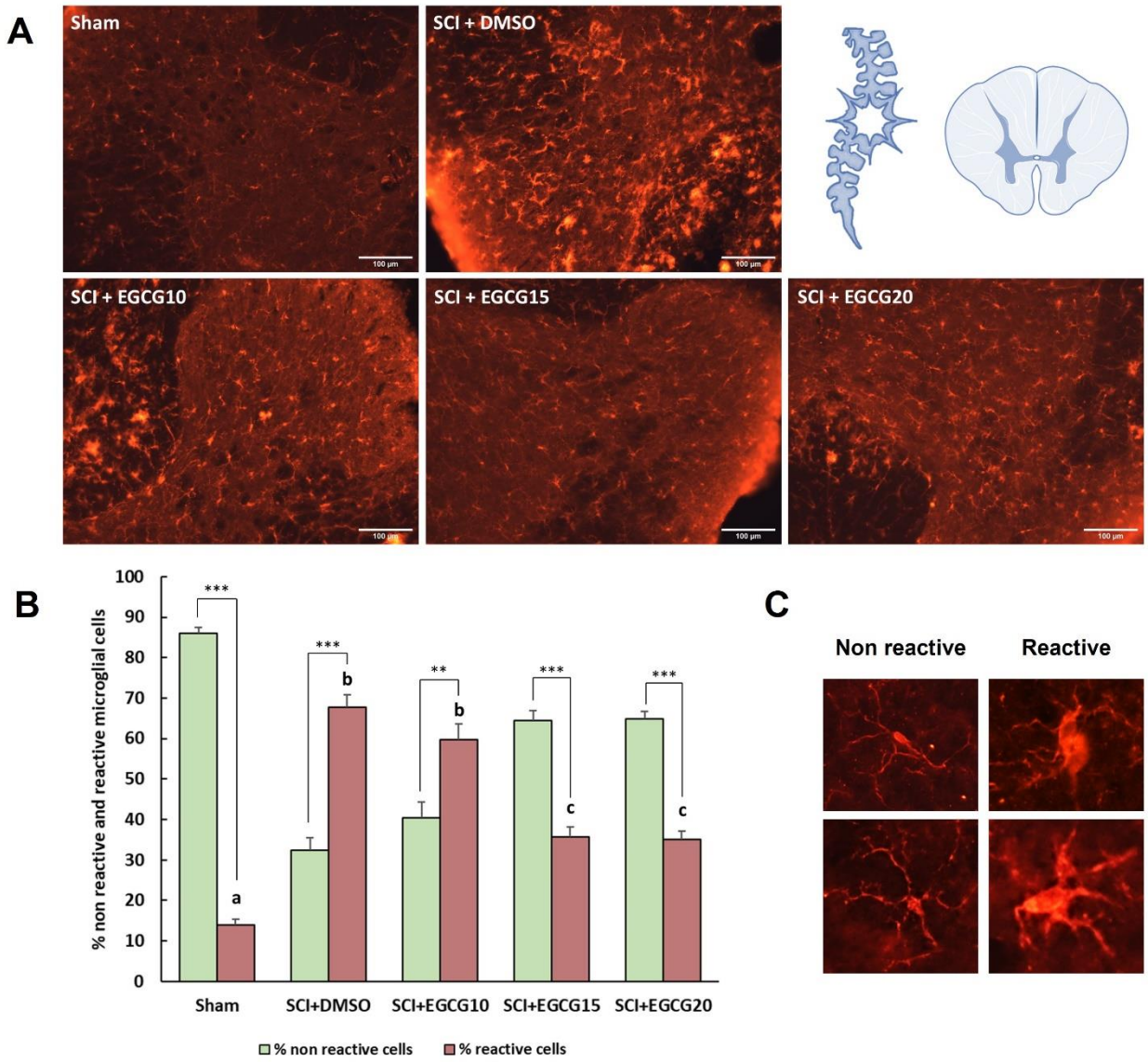


Figure 39. Effects of EGCG treatment (10, 15 and 20 mg/kg) on spinal IBA1 immunoreactivity in SCI mice at the end of experimental period. (**A**) Representative histological images of the spinal cord immunostained against IBA1 of each group (Scale bar 100 µm). (**B**)Histogram representing the percentage of reactive and non-reactive microglia cells in the spinal dorsal horn. Data is expressed as mean ± SEM. a–c: Groups not sharing a letter showed significant differences in %reactive cells, p<0.05. Intra-groups significant differences: *** p<0.001 %, ** p<0.01 %non-reactive vs %reactive. Experimental groups: Sham (n=3) (slices=28), SCI+DMSO (n=4) (slices=23), SCI+EGCG10 (n=4) (slices=20), SCI+EGCG15 (n=5) (slices=33), SCI+EGCG20 (n=4) (slices=30)**.** (**C**) Examples of non-reactive and reactive microglial cells. Note that reactive cells have an amoeboid form, and a larger nucleus and shorter branching processes compared to non-reactive cells.

Summary results Chapter I

In the present chapter, we have demonstrated that preventive EGCG administration during the first week post SCI, resulted in mechanical allodynia development prevention (10 and 15 mg/kg; i.p.) or an attenuation (20mg/kg; i.p.) up to 21 dpi, and thermal hyperalgesia development prevention up to 7dpi (10mg/kg; i.p.) or 21 dpi (15 and 20 mg/kg; i.p.).

In parallel, immunohistochemical studies revealed that all EGCG tested doses significantly attenuated SCI-related astrogliosis, being EGCG15 and EGCG20 the doses showing better attenuation when compared with EGCG10. Similarly, SCI-related spinal cord microgliosis was also attenuated by both EGCG15 and EGCG20 treatments reducing the percentage of reactive cells, whereas EGCG10 did not reduce it.

Altogether, these results suggest potential role of EGCG treatment in modulating the SCI-induced central sensitization during the acute phase of injury by modulating the spinal cord gliosis. However, it is worth mentioning that all tested doses resulted in significant weight loss during the whole experimental period which may indicate that systemic toxicity would be associated with such treatment. Considering that EGCG would exert antinociceptive effects but the use of DMSO as a vehicle (**Galvao et al., 2014**) may be associated with adverse side effects, new polyphenolic treatments using a physiological solvent as serum may be more suitable to modulate SCI-induced central neuropathic pain. In addition, considering that several mechanisms of action have been reported for different polyphenols, the use of a mixture of them in serum could be a good option. Hence, it was decided to develop new plant based polyphenolic extracts to be tested in the SCI-induced neuropathic pain model. Concretely, the two polyphenolic extracts used in the present thesis were obtained from different plant sources, grape residual material and roasted coffee bean, using physiological serum as solvent.

CHAPTER II

Preventive effects on Spinal Cord Injury-induced neuropathic pain development by repeated administration of Grape Stalk Extract (GSE) in CD1 mice.

CHAPTER II. Preventive effects on Spinal Cord Injury-induced neuropathic pain development by repeated administration of Grape Stalk Extract (GSE) in CD1 mice.

Once elucidated the necessity to develop novel polyphenolic extracts with both higher efficacy and lower side effects using saline solution as a vehicle, it was firstly decided to investigate the preventive effects of polyphenolic grape stalk extract (GSE) obtained from residual grape material collected from wine cooperatives of *L'Empordà region* (Girona, Catalonia).

To this end, the first step was the GSE obtaining, the process of which is described in the "Materials and Methods" section. Subsequently and before its administration, the GSE was characterized to determine its suitability to be used as potential treatment to modulate central pathological pain development.

Characterization of phenolic compounds in GSE

Total polyphenol content in GSE

Throughout the thesis several batches of GSE were obtained to perform the scheduled experimental procedures. According to the Folin-Ciocalteu assay, the total polyphenolic content of GSE batches used in the present thesis are indicated in the ***Figure 40***. It can be observed that the total GSE polyphenolic content obtained after extraction procedure was approximately 1100 mg GAE/L on average. This polyphenols concentration allows preparing suitable volume of administration of experimental doses.

Polyphenol content in GSE (mg GAE/L)
First Batch: 960 ± 33
Second Batch: 1014 ± 39
Third Batch: 1104 ± 60
Fourth Batch: 1285 ± 82

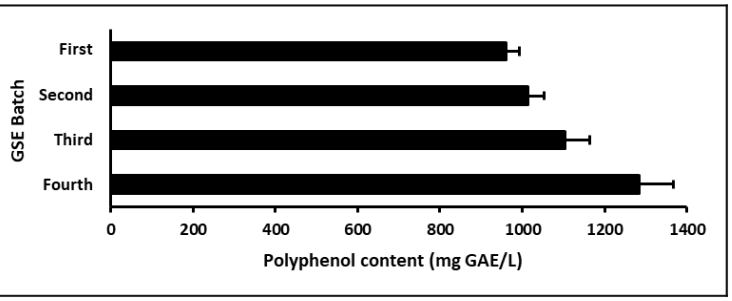


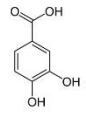
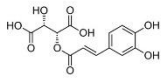
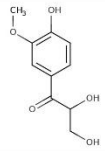
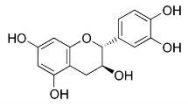
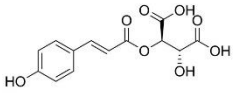
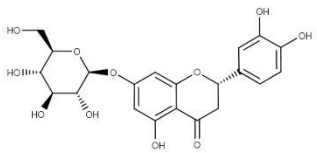
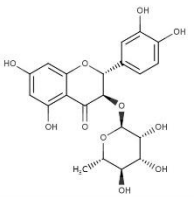
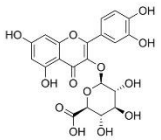
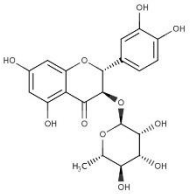
Figure 40. Total polyphenolic content in GSE calculated according to the Folin-Ciocalteu method. Polyphenol content is expressed as milligrams of gallic acid equivalents (GAE) per litre of extract. All values are presented as mean ± SD (n=6).

Qualitative analysis of polyphenols in GSE by HPLC-UV-ESI-FTMS

Considering that GSE specific polyphenolic compounds were not identified before, a qualitative analysis was firstly performed in order to determine potential polyphenolic molecules that would be subsequently analysed to properly be identified. Hence, after HPLC-UV-ESI-FTMS, different ions obtained on FTMS were identified based on the strongest peaks present in UV chromatograms at 310 and 280 nm. Chromatograms and spectra of the obtained ions were used to tentatively identify some compounds looking up in Phenol-Explorer database (http://phenol-explorer.eu/). Moreover, other peaks with weak intensity in UV

chromatograms were also identified because they showed very clear signals in FTMS. The tentative identification of polyphenols in the extract and HPLC-FTMS data are indicated in **Table 6.**

Table 6. Tentative identification of phenolic compounds in GSE by HPLC-UV-ESI-FTMS data.

Peak	Rt (min)	m/z	MS^2 ions	Formula	Tentative identification	Structure
1	3.93	153. 01892	109.02934	$C_7H_6O_4$ $C_6H_6O_2$	Protocatechuic acid	
2	5.23	311.03990	179.03470 149.00887	$C_{13}H_{12}O_9$ $C_9H_8O_4$ $C_4H_6O_6$	Caffeoyltartaric acid/ caftaric acid	
3	5.25	211.06059	181.05044 163.03982	$C_{10}H_{12}O_5$ $C_9H_{10}O_4$ $C_9H_8O_3$	2,3-Dihydroxy-1-guaiacylpropanone	
4	5.7	359.09728	197.04522	$C_{15}H_{10}O_{10}$ $C_9H_{10}O_5$	Syringic acid glucoside	
5	6.49	289.07086	245.08168	$C_{15}H_{14}O_6$ $C_{14}H_{14}O_4$	Catechin or Epicatechin	
6	6.53	295.04523	163.03990	$C_{13}H_{12}O_8$ $C_9H_8O_3$	p-Coumaroyl tartaric acid	
7	7.47	447.14984	401.14481	$C_{19}H_{28}O_{12}$ $C_{18}H_{26}O_{10}$	Unknown	
8	7.9	449.10741	287.05545	$C_{21}H_{22}O_{11}$ $C_{15}H_{12}O_6$	Eriodictyol-7-O-glucoside	
9	8.37	281.13863	237.14926	$C_{15}H_{22}O_5$ $C_{14}H_{22}O_3$	Unknown	
10	9.96	449.107771	303.05045 285.0399	$C_{21}H_{22}O_{11}$ $C_{15}H_{12}O_7$ $C_{15}H_{10}O_6$	Dihydroquercetin-O-rhamnoside	
11	10.03	477.06638	301.03499	$C_{21}H_{18}O_3$ $C_{15}H_{10}O_8$	Quercetin-3-O-glucuronide/ Miquelianin	
12	10.19	449.10784	303.05047 285.03998	$C_{21}H_{22}O_{11}$ $C_{15}H_{12}O_7$ $C_{15}H_{10}O_6$	Dihydroquercetin-O-rhamnoside	

Quantitative identification analysis of polyphenols in GSE by HPLC-UV-ESI-TOFMS

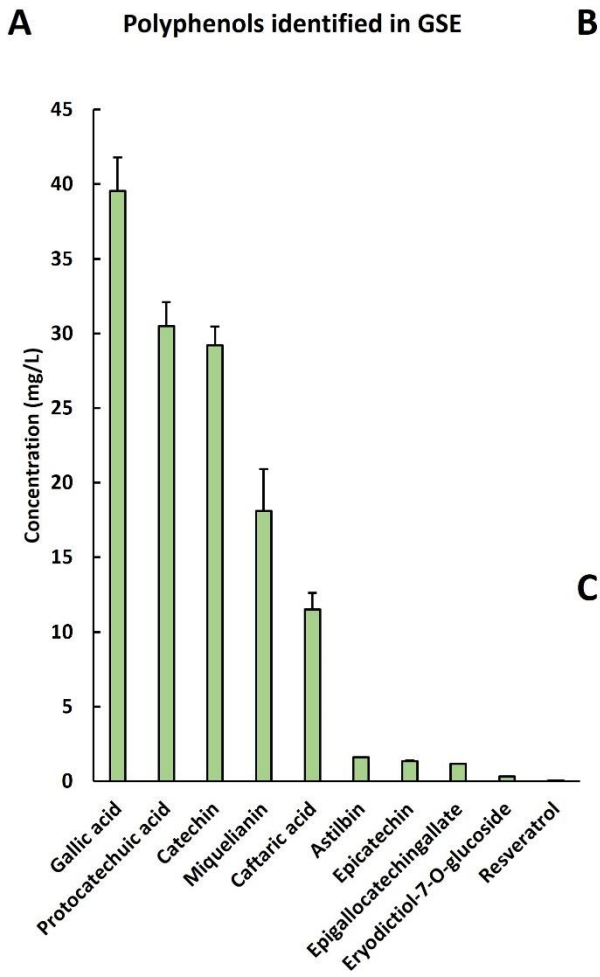
Once qualitative analysis was performed, the tentative identification of polyphenols in GSE was corroborated by HPLC-UV-ESI-TOFMS using standards solutions. First, the standard solutions of proposed polyphenols were analysed by HPLC-TOFMS and were then compared with the corresponding selected peaks obtained from GSE analysis. It was concluded that all the polyphenols tentatively identified were present in the GSE extract excepting the octyl gallate which presented a peak at the same retention time, but the mass was different. Moreover, the presence of trans-resveratrol, gallic acid, epigallocatechin gallate and epicatechin was also confirmed using standards solutions of these polyphenols.

After their identification, the polyphenolic compounds of GSE were quantified with an external standard calibration curve in TOFMS mode integrating the area of the peak present to the 30 mDa trace chromatogram centred on the exact mass of the compound.

The calibration curves were obtained by preparing different concentrations of standard compounds within the range 10-1000 ng/mL, except for epicatechin whose range was from 5 to 500 ng/mL. All the calibration curves were presented a good correlation (r^2>0.99) and precision (80-120%).

Eriodictyol-7-O-glucoside was not quantified in TOFMS mode because in its trace m/z 449 chromatogram more peaks were eluted closely. For this reason, its quantification was performed in trace m/z 287 chromatogram. It is worth mentioning that the eriodictyol-7-O-glucoside quantification was difficult to elucidate due to the presence of other compounds with same m/z ratio and M^2 spectra overlapping. Thus, other quantification methods like Ultra Performance Liquid Chromatography (UPLC) would be useful to a more accurate quantification.

As a result, three polyphenols resulted as major compounds: Gallic acid, Protocatechuic acid and Catechin (***Fig. 41***). Also, Miquelianin and Caftaric Acid were identified with a considerable concentration. Finally, other minor polyphenols were identified, which were: Astilbin, Epicatechin, Epigallocatechingallate, Eriodictyol-7-O-glucoside and Resveratrol (***Fig. 41***)**.**



B

Polyphenol	Concentration (mg/L)	SEM
Gallic acid	39,55	1,3
Protocatechuic acid	30,5	0,9
Catechin	29,2	0,7
Miquelianin	18,12	1,6
Caftaric acid	11,5	0,7
Astilbin	1,61	0,0
Epicatechin	1,33	0,0
Epigallocatechingallate	1,16	0,0
Eryodictiol-7-O-glucoside	0,31	0,0
Resveratrol	0,05	0,0

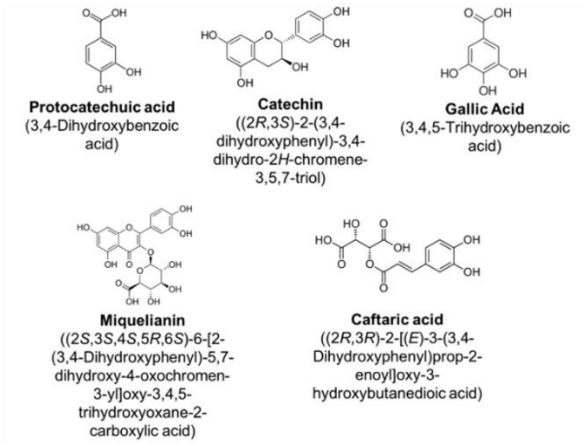


Figure 41. Quantification of phenolic compounds in GSE by HPLC-UV-ESI-TOFMS. Results are presented by (A) Histogram and (B) values data table. Quantification of each polyphenol identified in the GSE was performed in triplicate and all values are presented as mean ± SD (n=3). In (C) the molecules structures of major compounds are presented.

Once it was observed that GSE is characterized by a rich polyphenolic content at concentrations high enough to be administered by a reasonable injection volume, a dose-response study was scheduled. That is, SCI mice were treated with the whole extract (10, 15 and 20 mg/kg; i.p.) during the first week post-injury. Afterwards, mechanical allodynia and thermal hyperalgesia were weekly assessed up to the end of SCI acute phase. At the end of the experimental period, serum levels of hepatotoxicity and nephrotoxicity biomarkers were analysed to assess the pharmacological safety of the GSE treatment. Finally, to explain the potential effects exerted by GSE on neuropathic pain behaviours, astrogliosis and microgliosis were analysed in both the spinal cord and supraspinal structures (periaqueductal gray matter, PAG; and anterior cingulate cortex, ACC). Moreover, in order to get some mechanistic insights, the protein expression of ERK phosphorylation as well as the expression of MCP1/CCL2 and CX3CL1 chemokines and their receptors CCR2 and CX3CR1 were analysed. The obtained results are explained below.

Preventive GSE treatment does not trigger weight loss neither hepatotoxic nor nephrotoxic effects.

Throughout the study and following a protocol animal welfare supervision based on Morton D.B and Griffiths P.H. guidelines (**Morton and Griffiths, 1985**), the general aspect of the animals was normal and changes in coat and skin, vibrissae of nose, nasal secretions, signs of autotomy or aggressiveness were not detected in any experimental group of mice.

Regarding the weight control, Kolmogorov-Smirnov normality test revealed that data did not follow a normal distribution at basal and 7 dpi (p's<0.01). Then, while the Friedman test revealed that the distribution of weight data significantly varied from the beginning of the experiment until 7 dpi (p<0.001), the Kruskal-Wallis test indicated no significant differences between groups (p's>0.05) (***Fig. 42***). Kolmogorov-Smirnov normality test confirmed a normal distribution of the data corresponding to weight at 14 and 21 dpi (p's>0.05). The MANOVA analysis indicated that there are significant effects on day factor ($F_{(3,64)}$= 74.379, p<0.001) and on the day x treatment interaction ($F_{(12,169)}$= 3.211, p<0.001) but not on treatment factor ($F_{(4,66)}$= 0.21, p>0.05) in the weight of the animals at 14 and 21 dpi. Moreover, no significant group differences were identified by further ANOVA analysis (all p's>0.05) (***Fig. 42***). These results indicate that neither surgeries nor treatments have significant effects on the animals' weight and weight changes throughout the experimental period would be related to other factors effecting similarly to all animals.

Biomarkers of hepatotoxicity and nephrotoxicity in animals' serum were quantified at the end of experimental period in order to analyse the pharmacological safety of the polyphenolic GSE treatment. In addition, the values obtained from the GSE treatment were compared with the values of animals treated with DMSO and the highest tested dose of EGCG (EGCG20). Shapiro-Wilk normality test showed that data corresponding to both ALT/GPT and AST/GOT did not follow a normal distribution (p's<0.05) and the distribution of the UREA data was normal (p>0.05). While ANOVA analysis of the nephrotoxicity biomarker revealed non-significant group differences ($F_{(6,38)}$=0.707, p=0.646) (***Fig. 43. C***), Kruskal-Wallis analysis of the hepatotoxicity biomarkers revealed significant differences between groups (p's<0.05) (***Fig. 43. A, B***). Specifically, SCI animals administered with DMSO and EGCG20 showed significantly higher ALT values compared to the rest of the experimental groups (***Fig. 43. A***). Referring to AST biomarker, only SCI animals treated with EGCG20 had higher AST values compared to SCI+Saline, SCI+GSE10 and SCI+GSE15 groups, although did not differ significantly from Sham, SCI+EGCG20 and SCI+DMSO groups (***Fig. 43. B***). Taken together, these results suggest no systemic toxicity associated with GSE administration without hepatotoxic or nephrotoxic effects.

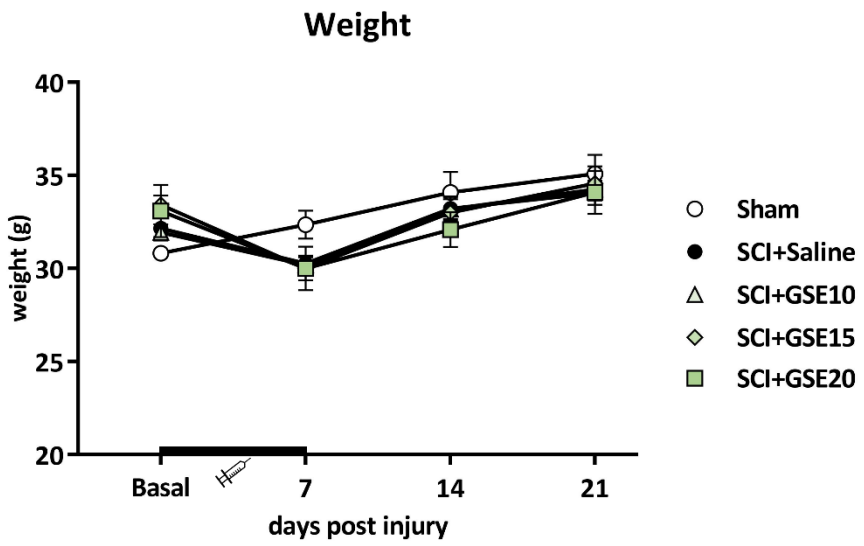


Figure 42. Mice weight control during the injury acute phase of SCI after preventive GSE treatment. Each point and vertical line represent the mean ± SEM. Treatment administration week (basal to 7 dpi) is highlighted with a thick black line. Experimental groups: Sham (n=11), SCI+Saline (n=18), SCI+GSE10 (n=16), SCI+GSE15 (n=14), SCI+GSE20 (n=12).

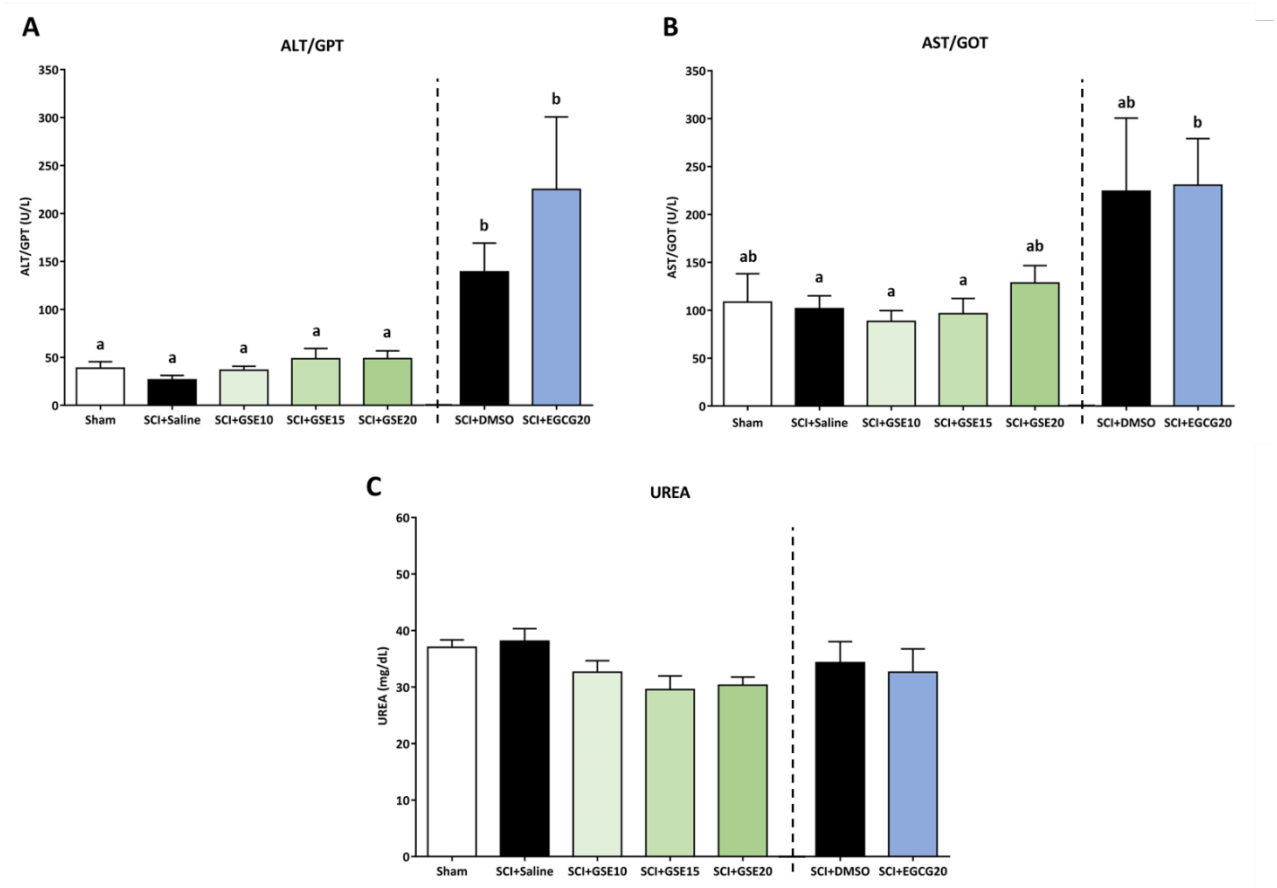


Figure 43. Biomarkers quantification of (A-B) hepatotoxicity and (C) nephrotoxicity, in the serum of each experimental group at the end of experimental period. The results are represented as the mean ± SEM. a-b: groups not sharing a letter are significantly different, $p < 0.05$, by posthoc test. Experimental groups: Sham (ALT/GTP n=6; AST/GOT n=6; UREA n=6), SCI+Saline (ALT/GTP n=6; AST/GOT n=6; UREA n=6), SCI+GSE10 (ALT/GTP n=6; AST/GOT n=5; UREA n=6), SCI+GSE15 (ALT/GTP n=5; AST/GOT n=6; UREA n=7), SCI+GSE20 (ALT/GTP n=5; AST/GOT n=5; UREA n=5), SCI+DMSO (ALT/GTP n=5; AST/GOT n=4; UREA n=9), SCI+EGCG20 (ALT/GTP n=5; AST/GOT n=4; UREA n=6).

Preventive GSE treatment modulates both mild spinal cord injury-induced mechanical allodynia and thermal hyperalgesia development.

Before reflexive pain responses evaluation, the locomotor activity of the mice was assessed to ensure the lack of motor disturbances that may adversely affect the reliability of both plantar and Von Frey tests outcomes. Non-parametric tests Friedman and Kruskal-Wallis were applied to analyse BMS data since it did not follow a normal distribution during the whole experimental period (Kolmogorov Smirnov, all p's<0.001). Although Friedman's test detected differences over experimental time (p's<0.001), no significant group differences were found on any of the post injury days (p>0.05) by the application of Kruskal-Wallis test (***Fig. 44. A***). It is worth mentioning that observed mild BMS alterations only were referred to altered paw position but not to altered horizontal locomotion. Thus, all mice were able to move freely without neither paralysis nor major impairment in the coordination and locomotor functions. These results justify that evoked pain responses, such as thermal hyperalgesia and mechanical allodynia, cannot be associated with locomotor impairment, as no significant locomotor deficits were observed during the study.

Referring to reflexive pain responses, mechanical allodynia data did not follow a normal distribution at any of the experimental time points (Kolmogorov-Smirnov test, all p's<0.01). Thus, Friedman test was applied showing that distribution of the data was not the same (p<0.001) throughout the experimental period and further Kruskal-Wallis test revealed that these significant differences were between groups at all post-injury days (7, 14 and 21 dpi) (all p's<0.001). Specifically, subsequent Mann Whitney U test revealed significant mechanical sensitivity increase of SCI+Saline group when compared with the rest of experimental groups (all p's<0.05). Moreover, no differences were recorded between Sham group and GSE10 and GSE15 treatment groups at all post-injury days (all p's>0.05), indicating that these doses of GSE completely prevented the mechanical allodynia development after SCI during the whole acute phase of injury. On the other hand, the dose of 20GSE completely prevented the mechanical allodynia at 7 and 14 dpi (no significant differences between Sham, p's>0.05) and significant attenuated it at 21 dpi (showing significant differences between Sham, p<0.05) (***Fig. 44. B***).

As for thermal hyperalgesia, data did not follow a normal distribution for all time-points of functional assessment (p's<0.05) according to Kolmogorov-Smirnov test. Significant differences in the distribution of the data were found by Friedman's test (p<0.001) and further Kruskal-Wallis test indicated significant differences between groups at all post-injury days (7, 14 and 21 dpi) (all p's<0.001). During all time-points of functional assessment, spinal cord injured mice without treatment (SCI+Saline) showed significant decrease of thermal paw withdrawal thresholds compared to Sham and all GSE treated groups (Mann Whitney U, all p's<0.05). That indicates preventive effects on thermal hyperalgesia for all tested doses of GSE. More specifically, GSE15 and GSE20 doses completely prevented thermal hyperalgesia development

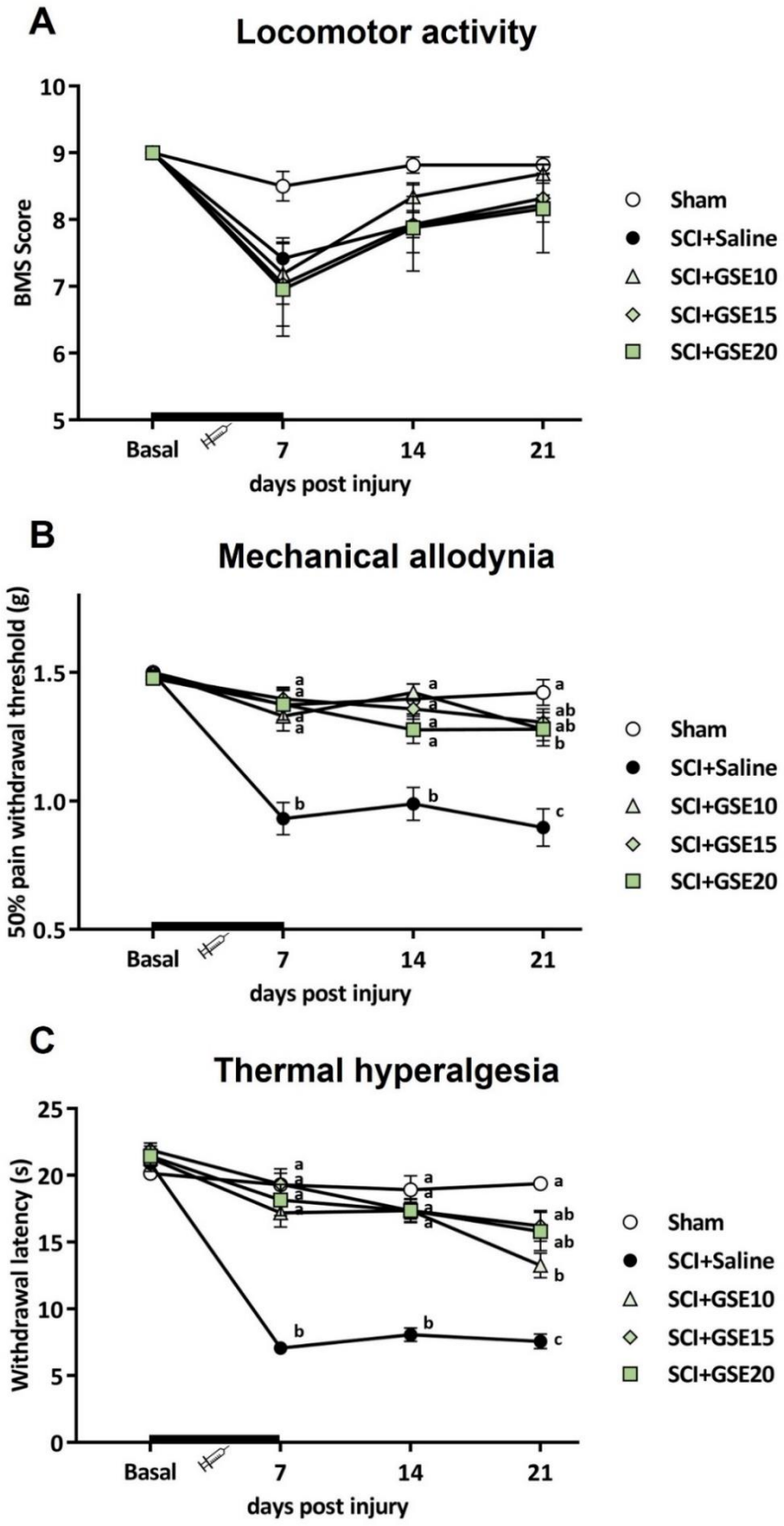


Figure 44. Time-course assessment of locomotor activity (A), mechanical allodynia (B) and thermal hyperalgesia (C) during the injury acute phase of SCI after preventive GSE treatment. Each point and vertical line represent the mean ± SEM. a-b: groups not sharing a letter are significantly different, $p < 0.05$, by posthoc test. Treatment administration week (basal to 7 dpi) is highlighted with a thick black line. Experimental groups: Sham (n=11), SCI+Saline (n=18), SCI+GSE10 (n=16), SCI+GSE15 (n=14), SCI+GSE20 (n=12).

up to 21 dpi, showing no significant differences with Sham group (p's>0.05). The GSE10 dose significantly prevented thermal hyperalgesia development up to 14 dpi (p>0.05 vs. Sham) and significantly attenuated at 21 dpi (p<0.01 vs. Sham) (***Fig. 44. C***).

Preventive GSE treatment modulates both astrogliosis and microgliosis in spinal cord of SCI mice.

It is well known that, after SCI, astrocytes undergo significant cellular, molecular, and functional changes along with profound alterations in their gene expression. These reactions include hypertrophy of astrocytic processes and soma, increased proliferation, and upregulation of intermediate filaments such as GFAP in the astrocytes that are located close to the site of SCI. These modifications are some of the hallmarks of a phenomenon known as reactive astrogliosis (**Pekny and Nilsson, 2005; Pekny, 2007; Sofroniew, 2009**). Thus, in order to evaluate the preventive effects of GSE treatment in spinal astrogliosis, immunohistochemical and western blot analysis in spinal cord samples were performed.

On the one hand, to evaluate the hypertrophy and the proliferation of astrocytes, anti-GFAP antibody was incubated on histological spinal cord sections. Then, the percentage of GFAP immunoreactivity area was quantified as an indicator of these parameters. Statistical analysis showed that data did not follow a normal distribution (p<0.05) according to the Shapiro-Wilk test. For this reason, non-parametric test of Kruskal-Wallis was applied showing significant differences on %GFAP immunoreactivity area in all groups (all p's<0.01) except between GSE10 and GSE15 treatment groups (p>0.05). An increase of the GFAP immunoreactivity area was found in the SCI+Saline group when compared with the rest of experimental groups (all p's<0.001). Although GSE groups do not maintain the same levels of astrogliosis as in the Sham group (p's<0.001), all GSE treatments clearly attenuated spinal astrogliosis compared to SCI+Saline. It is worth mentioning that GSE10 and GSE15 doses exerted better effect when compared to GSE20 dose (***Fig. 45. B***). Representative histological sections illustrating spinal GFAP immunostaining (***Fig. 45. A***.) clearly show qualitative differences on both astrocyte morphology and number of astrocytic cells between experimental groups. Concretely, SCI+Saline group showed hypertrophy of astrocytic processes and soma and an increased astrocytic proliferation compared to Sham. The GSE treatment groups were more similar to the Sham than SCI+Saline group in terms of morphology and number of astrocytes.

On the other hand, western blot analysis of the total spinal GFAP protein was also performed to complement the immunohistochemical results previously obtained. According to Shapiro-Wilk normality test GFAP western blot data did not follow a normal distribution (p<0.001) and further Kruskal-Wallis test indicated significant differences on GFAP protein expression between groups (p<0.01). Specifically, SCI+Saline group showed significant increase of GFAP expression when compared to Sham and GSE20 treatment group (p<0.01). As for GSE10 and GSE15 treatment groups did not showed significant differences with either Sham or SCI+Saline groups (p>0.05) (***Fig. 46***) suggesting a slight modulation without reaching

the same Sham's GFAP expression. Instead, the dose of GSE20 showed a lack of significance when compared with Sham indicating that this dose would exert preventive effects on astrogliosis development.

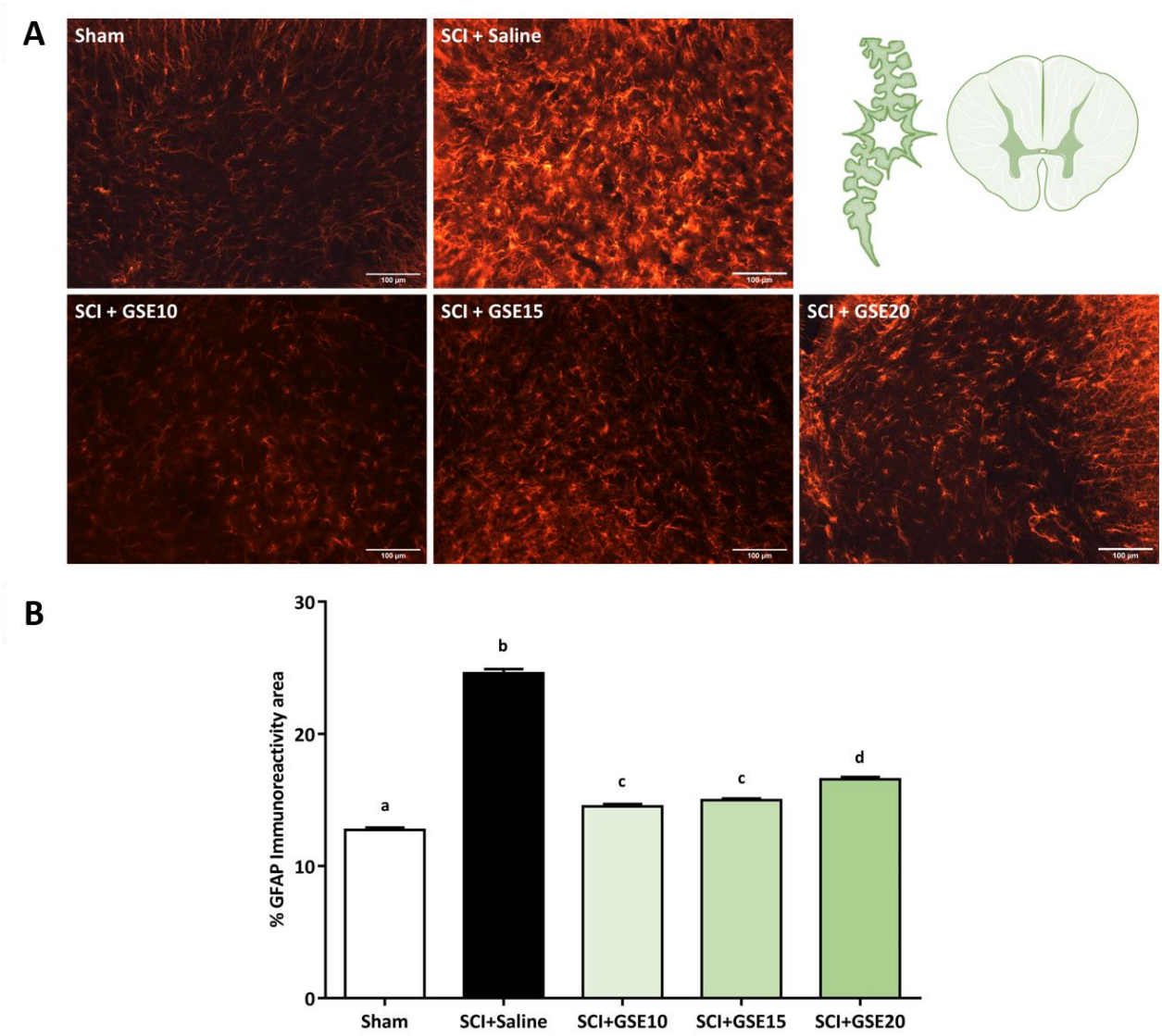


Figure 45. Effects of GSE treatment (10, 15 and 20 mg/kg) on spinal GFAP immunoreactivity area in SCI mice at the end of experimental period. (**A**) Representative histological images of the spinal cord immunostained against GFAP of each group (Scale bar 100 µm). (**B**) Histogram representing the percentage of dorsal horn GFAP-immunoreactivity. Data is expressed as mean ± SEM. a–d: Groups not sharing a letter showed significant differences, $p<0.05$. Experimental groups: Sham (n=4) (slices=60), SCI+Saline (n=4) (slices=62), SCI+GSE10 (n=4) (slices=68), SCI+GSE15 (n=5) (slices=84), SCI+GSE20 (n=5) (slices=54).

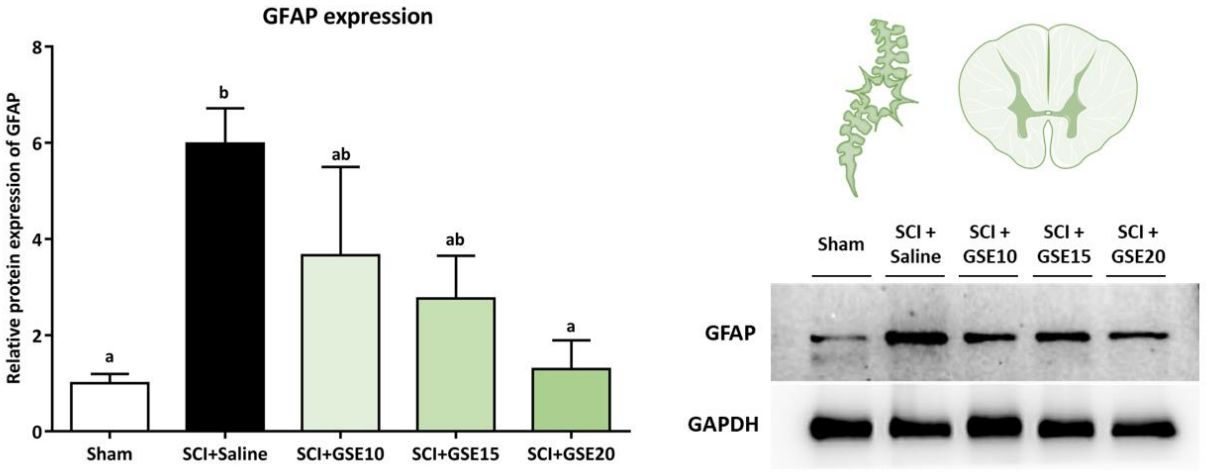


Figure 46. Spinal GFAP expression after preventive GSE treatment (10, 15, 20 mg/kg) on spinal cord injured mice at the end of experimental period. Protein expression was normalized to GAPDH. Data is expressed as a relative percentage respect to Sham group (mean ± SEM). a–b: Groups not sharing a letter showed significant differences, p<0.05. Experimental groups: Sham (n=10), SCI+Saline (n=9), SCI+GSE10 (n=5), SCI+GSE15 (n=5), SCI+GSE20 (n=5).

In addition to astrogliosis, microglial activation has been well documented in the spinal cord after contusive SCI (**Popovich et al., 1997; Sroga et al., 2003; Nesic et al., 2005; Zai and Wrathall, 2005**). Specifically, in response to SCI, spinal quiescent ramified microglia proliferate and transform into reactive microglia, which have a rod-like/amoeboid form, lack branching processes and contain numerous lysosomes and phagosomes (**Kreutzberg, 1996; Stence et al., 2001**). In order to evaluate the effects of GSE treatment on spinal microgliosis after SCI, anti-IBA1 antibody was incubated on histological spinal cord sections. Then, reactive and non-reactive microglia cells were counted and expressed as a percentage of two phenotypes. Shapiro-Wilk test confirmed that immunohistochemical data did not follow a normal distribution ($p<0.001$). Hence, non-parametric tests have been applied. Kruskal-Wallis test indicated significant differences between groups ($p<0.001$) in both, percentage of reactive and non-reactive microglia cells. Subsequent Mann Whitney U test showed a significant ($p<0.001$) increase of percentage of reactive microglia cells in SCI+Saline group when compared with Sham. In parallel, all GSE treatment groups showed a significant decrease in the percentage of reactive cells compared to SCI+Saline (all p's<0.001), but none of them reached the percentage level of the Sham group since they all significant differed from this control group (all p's<0.001). It is worth noting that GSE treatment groups (GSE10, GSE15 and GSE20) did not showed significant difference between them (p's>0.05) (***Fig. 47. B***). Finally, Kruskal-Wallis test indicated statistical intra-group differences between reactive and non-reactive microglia cells for all experimental groups (all p's<0.001) (***Fig. 47. B***).

Representative histological sections illustrating spinal IBA1 immunostaining (***Fig. 47. A***) exhibit differences on both microglia morphology and number of microglial cells between experimental groups. Concretely,

SCI+Saline group showed a more numerous amoeboid microglial cells with less and shorter branching processes compared to Sham. The GSE treatment groups were more similar to the Sham than SCI+Saline group in terms of morphology and number of microglial cells.

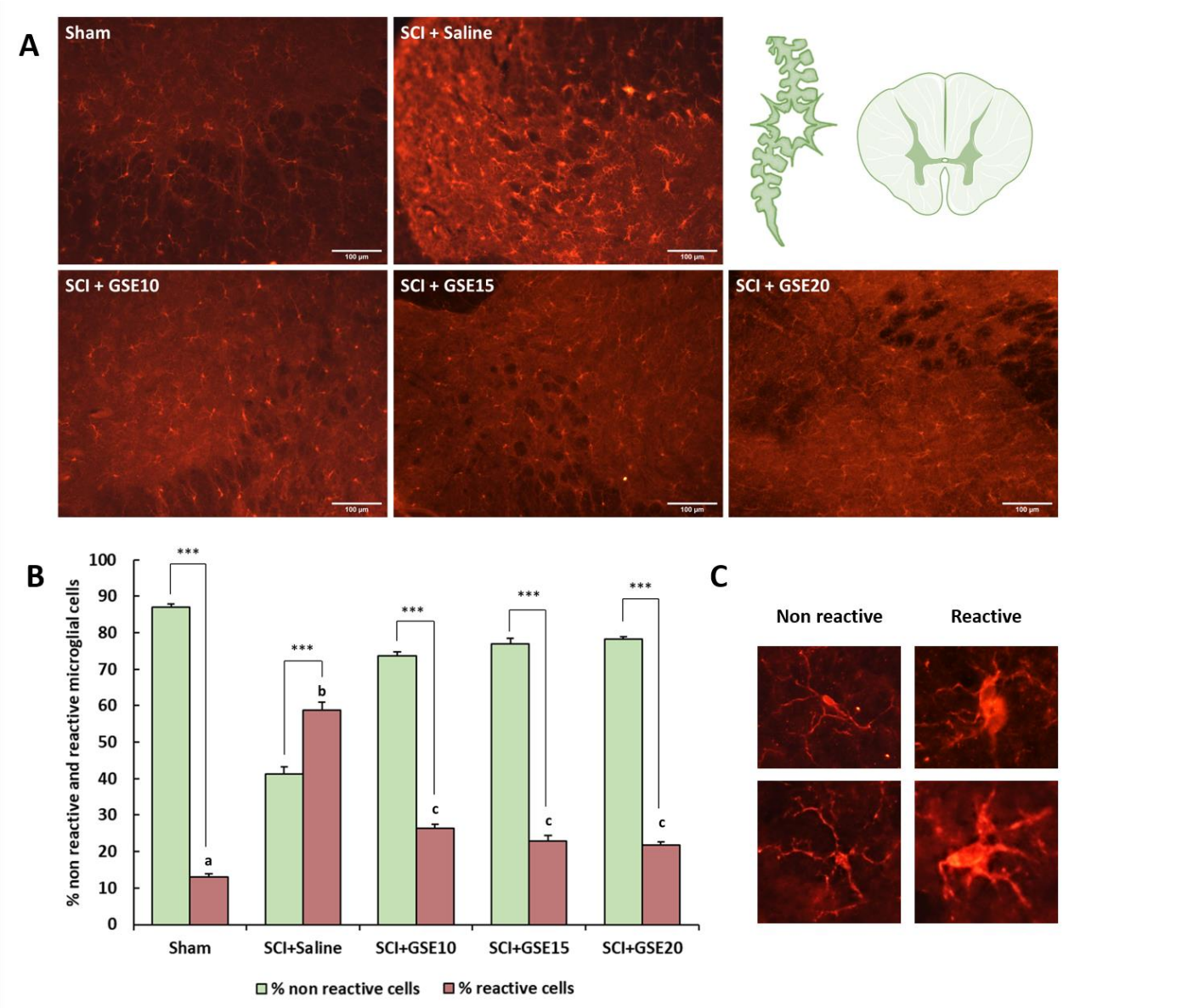


Figure 47. Effects of GSE treatment (10, 15 and 20 mg/kg) on spinal IBA1 immunoreactivity in SCI mice at the end of experimental period. (**A**) Representative histological images of the spinal cord immunostained against IBA1 of each group (Scale bar 100 µm). (**B**) Histogram representing the percentage of reactive and non-reactive microglia cells in the spinal dorsal horn. Data is expressed as mean ± SEM. a–c: Groups not sharing a letter showed significant differences in %reactive cells, $p<0.05$. Intra-groups significant differences: *** $p<0.001$ %non-reactive vs %reactive. Experimental groups: Sham (n=6) (slices=53), SCI+Saline (n=5) (slices=34), SCI+GSE10 (n=6) (slices=65), SCI+GSE15 (n=4) (slices=47), SCI+GSE20 (n=6) (slices=78). (**C**) Examples of non-reactive and reactive microglial cells. Note that reactive cells have an amoeboid form, and a larger nucleus and shorter branching processes compared to non-reactive cells.

Moreover, western blot analysis of the total spinal IBA1 protein was also performed to complement the immunohistochemical results above mentioned. According to Shapiro-Wilk normality test IBA1 western blot data did not follow a normal distribution ($p<0.001$). Subsequent Kruskal-Wallis test indicated significant

differences on IBA1 protein expression between groups (p<0.01). Specifically, SCI+Saline group showed significant increase of IBA1 expression when compared to the rest of experimental groups (p<0.001). While GSE10 and GSE20 prevented IBA1 upregulation at Sham levels (p's>0.05), GSE15 attenuated its expression since it significant differed from Sham levels (p<0.05) (***Fig. 48***).

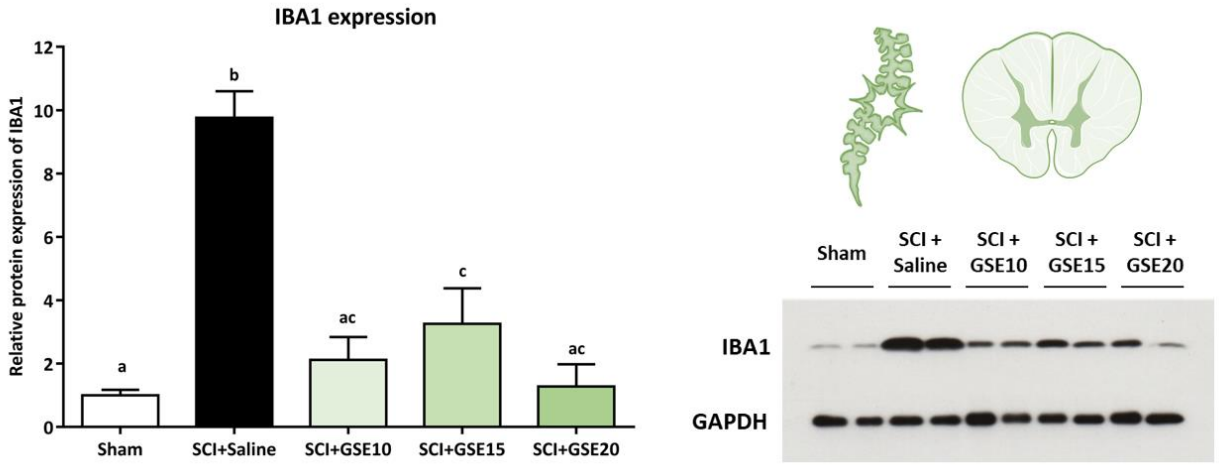


Figure 48. Spinal IBA1 expression after preventive GSE treatment (10, 15, 20 mg/kg) on spinal cord injured mice at the end of experimental period. Protein expression was normalized to GAPDH. Data is expressed as a relative percentage respect to Sham group (mean ± SEM). a–b: Groups not sharing a letter showed significant differences, p<0.05. Experimental groups: Sham (n=8), SCI+Saline (n=7), SCI+GSE10 (n=4), SCI+GSE15 (n=4), SCI+GSE20 (n=4).

Preventive GSE treatment modulates the expression of chemokines-related neuron glia crosstalk in the spinal cord of SCI-mice.

Several chemokines such as MCP1/CCL2 and CX3CL1 have been proposed as potential candidates for integrating inflammation and central neuropathic pain after SCI (**Knerlich-Lukoschus et al., 2008; Gao et al., 2009; Sandhir et al., 2011; Verge et al., 2004**). The above results showing SCI associated astrogliosis and microgliosis attenuation by GSE administration would lead to chemokines release modulation in the spinal cord. In this fashion, molecular experiments to elucidate the expression of chemokines (MCP1/CCL2 and CX3CL1) and their receptors (CCR2 and C3XCR1) in the spinal cord at 21 dpi were performed and the results are explained below.

According to Shapiro-Wilk normality test both MCP1/CCL2 and CCR2 western blot data did not follow a normal distribution (p<0.05). Subsequent Kruskal-Wallis test showed that MCP1/CCL2 was not overexpressed in SCI+saline group according to Sham and none of the GSE treated groups showed significant differences with either the Sham or the SCI+Saline group (all p's>0.05) (***Fig. 49. A***). In contrast, its receptor CCR2 was significant overexpressed in SCI+Saline group when compared to Sham (p<0.01). Although all tested doses of GSE decreased CCR2 expression when compared to SCI+Saline group, only

GSE10 and GSE20 doses reached Sham expression levels (p's>0.05) whereas GSE15 dose did not differ from either Sham or SCI+Saline CCR2 expression levels (p's>0.05) (***Fig. 49. B***).

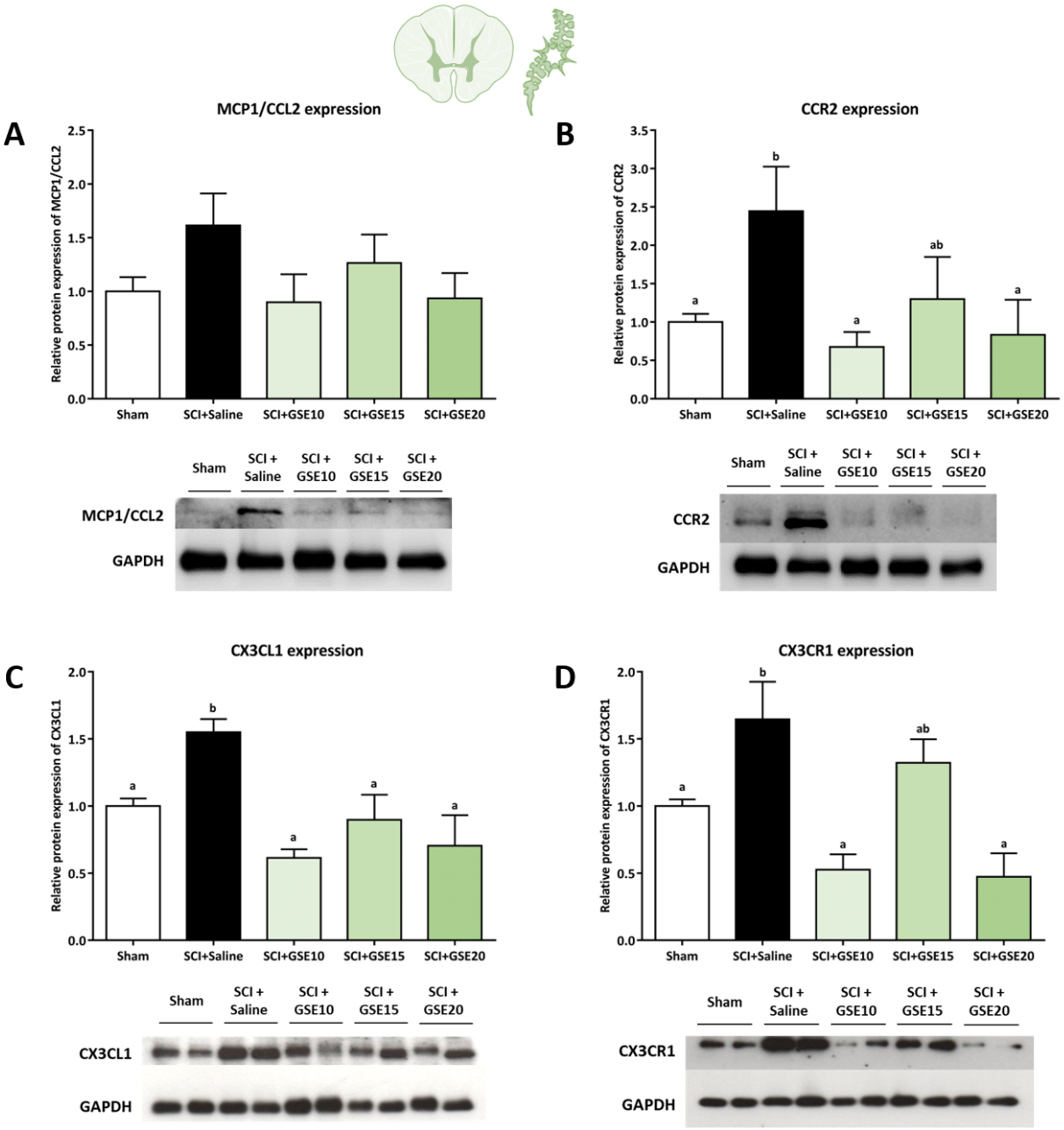


Figure 49. Spinal expression of chemokines (MCP1/CCL2 and CX3CL1) and their receptors (CCR2 and CX3CR1) after preventive GSE treatment (10, 15, 20 mg/kg) on spinal cord injured mice at the end of experimental period. Protein expression was normalized to GAPDH. Data is expressed as a relative percentage respect to Sham group (mean ± SEM). a–b: Groups not sharing a letter showed significant differences, p<0.05. Experimental groups: Sham (MCP1/CCL2 n=9; CCR2 n=10; CX3CL1 n=9; CX3CR1 n=8), SCI+Saline (MCP1/CCL2 n=9; CCR2 n=9; CX3CL1 n=8; CX3CR1 n=7), SCI+GSE10 (MCP1/CCL2 n=5; CCR2 n=5; CX3CL1 n=4; CX3CR1 n=4), SCI+GSE15 (MCP1/CCL2 n=5; CCR2 n=5; CX3CL1 n=4; CX3CR1 n=4), SCI+GSE20 (MCP1/CCL2 n=5; CCR2 n=5; CX3CL1 n=4; CX3CR1 n=4).

Regarding spinal fractalkine CX3CL1 expression, Shapiro-Wilk normality test confirmed a normal distribution of the data (p>0.05). On further ANOVA analysis, significant group differences were found (p<0.001).

Specifically, post-hoc analysis indicated a significant increase of CX3CL1 in SCI+Saline group when compared with the rest of experimental groups ($p<0.05$). Indeed, all tested doses of GSE treatment significantly prevented CX3CL1 overexpression at Sham levels ($p>0.05$) (***Fig. 49. C***). On the other hand, according to Shapiro-Wilk normality test, CX3CR1 western blot data did not follow a normal distribution ($p<0.05$) and subsequent Kruskal-Wallis test indicated significant group differences ($p<0.01$). Concretely, CX3CR1 was significant overexpressed in SCI+Saline group when compared with Sham ($p<0.05$) and GSE10 and GSE20 treatment groups (p's<0.05). In contrast, CX3CR1 expression levels of GSE15 group did not significantly differ from either Sham or SCI+Saline group (p's>0.05) (***Fig. 49. D***).

Preventive GSE treatment downregulates ERK phosphorylation in spinal cord of SCI mice.

As mentioned in the introduction of the present thesis, pERK is one of the pivotal central sensitization-related biomarkers, which in turn is related with gliosis reactivation (**Yang et al., 2004; Liu and Zhou, 2015; Gwak et al., 2017**). In order to elucidate whether GSE treatment after SCI can modulate spinal ERK phosphorylation, western blot analysis against ERK1/2 and pERK1/2 were performed.

Shapiro-Wilk normality test confirmed a normal distribution of the data ($p>0.05$) and significant group differences were detected by ANOVA analysis in ERK1/2 phosphorylation (pERK1/2) ($p<0.001$). As expected, injured mice (SCI+Saline) showed a significant increase of pERK1/2 in comparison to Sham group ($p<0.05$). On the other hand, all tested doses of GSE treatment in SCI mice prevented the pERK1/2 upregulation detected in SCI+Saline mice (all p's<0.05). While GSE15 and GSE20 doses prevented ERK phosphorylation at Sham levels, GSE10 decreased its phosphorylation even at lower Sham levels (***Fig. 50***).

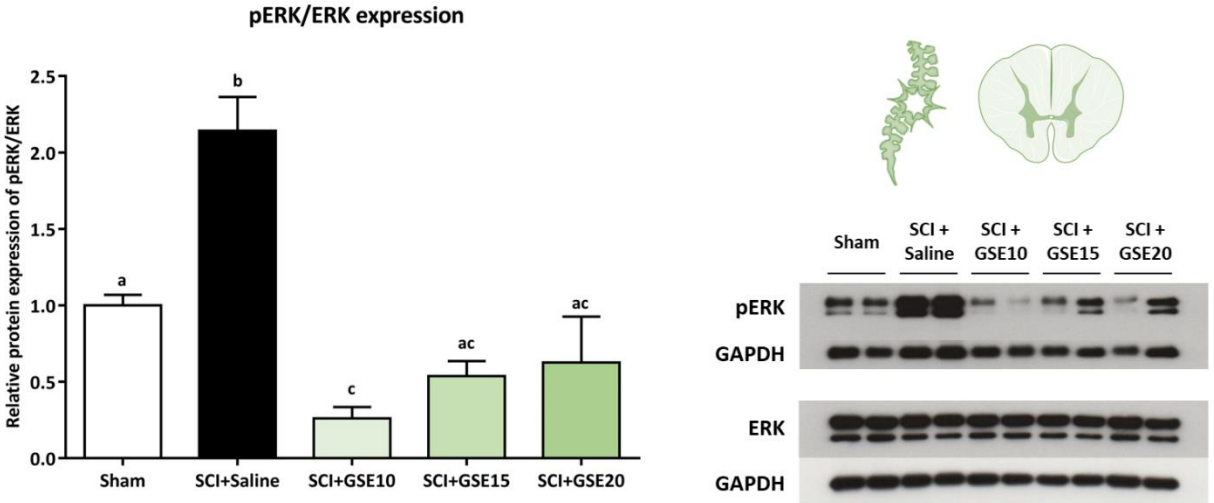


Figure 50. Spinal levels of ERK phosphorylation after preventive GSE treatment (10, 15, 20 mg/kg) on spinal cord injured mice at the end of experimental period. Protein expression was normalized to GAPDH. Data is expressed as a relative percentage respect to Sham group (mean ± SEM). a–c: Groups not sharing a letter showed significant differences, $p<0.05$. Experimental groups: Sham (n=9), SCI+Saline (n=8), SCI+GSE10 (n=4), SCI+GSE15 (n=4), SCI+GSE20 (n=4).

Preventive GSE treatment modulates astrogliosis and microgliosis in ACC of SCI mice.

The anterior cingulate cortex (ACC) is associated not only with perception but also the affective component of pain. It has been reported that glial activation in ACC is an important contributor to the development and maintenance of severe neuropathic pain in SCI model (**Chang, 2013; Widerstrom-Noga et al., 2013**). Moreover, according to its psychobiological function, ACC disturbances may be related also to non-reflexive pain responses (**Tsuda et al., 2017**). In order to elucidate whether SCI-related condition triggers ACC-gliosis and if GSE treatment may modulate it, both immunohistochemical and western blot analysis were performed.

On the one hand, to evaluate the astrogliosis in ACC, anti-GFAP antibody was incubated on histological sections. Then, the proportion of GFAP immunoreactivity area was quantified. Shapiro-Wilk test showed that data did not follow a normal distribution ($p<0.05$). For this reason, non-parametric test of Kruskal-Wallis was applied showing significant differences on %GFAP immunoreactivity area between groups ($p<0.001$). As a result, Mann Whitney U test showed a significant increase in GFAP immunoreactivity area in the SCI+Saline group compared to Sham ($p<0.001$). The only dose of GSE treatment that was successful in reducing astrogliosis in ACC was GSE20 compared to SCI+Saline ($p<0.01$) although it did not reach at sham levels ($p<0.001$). In contrast, GSE15 treatment group showed no significant differences with the SCI+Saline group ($p>0.05$). Finally, the GSE10 dose not only did not reduce the GFAP immunoreactive area but showed even more astrogliosis than the SCI+Saline group ($p<0.01$) (***Fig. 51***).

On the other hand, western blot analysis of the total GFAP protein in ACC was also performed to complement the immunohistochemical results previously obtained. Shapiro-Wilk normality test confirmed a normal distribution for GFAP western blot data ($p>0.05$). On further ANOVA, significant group differences were found ($p<0.001$). In particular, the post-hoc analysis indicated that GFAP was significant overexpressed in SCI+Saline animals when compared with Sham animals ($p<0.05$). Referring to GSE treatments, both GSE10 and GSE15 doses attenuated astrogliosis showing significant differences with the SCI+Saline group ($p's<0.05$). However, none of both reached at Sham GFAP expression levels ($p's<0.05$). On the other hand, GSE20 group showed even less astrogliosis compared to the Sham group ($p<0.05$) (***Fig. 52***). In summary, while GSE10 and GSE15 treatments attenuated SCI-induced astrogliosis in ACC, GSE20 completely prevented it.

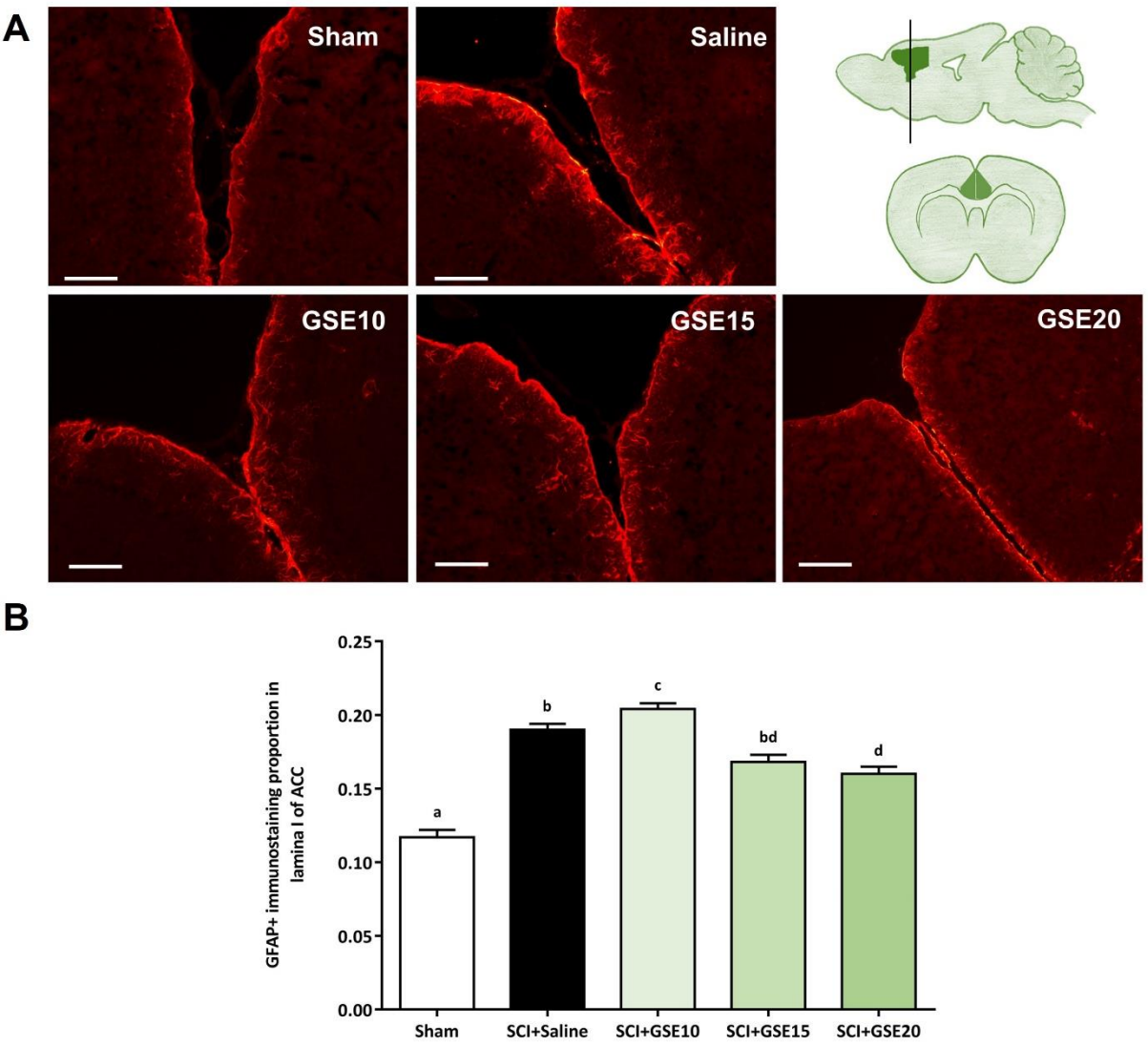


Figure 51. Effects of GSE treatment (10, 15 and 20 mg/kg) on GFAP immunoreactivity proportion in ACC of SCI mice at the end of experimental period. (**A**) Representative histological images of the ACC immunostained against GFAP of each group (Scale bar 200 µm). (**B**) Histogram representing the proportion of GFAP-immunoreactivity in ACC. Data is expressed as mean ± SEM. a–d: Groups not sharing a letter showed significant differences, $p<0.05$. Experimental groups: Sham (n=5), SCI+Saline (n=5), SCI+GSE10 (n=5), SCI+GSE15 (n=5), SCI+GSE20 (n=5).

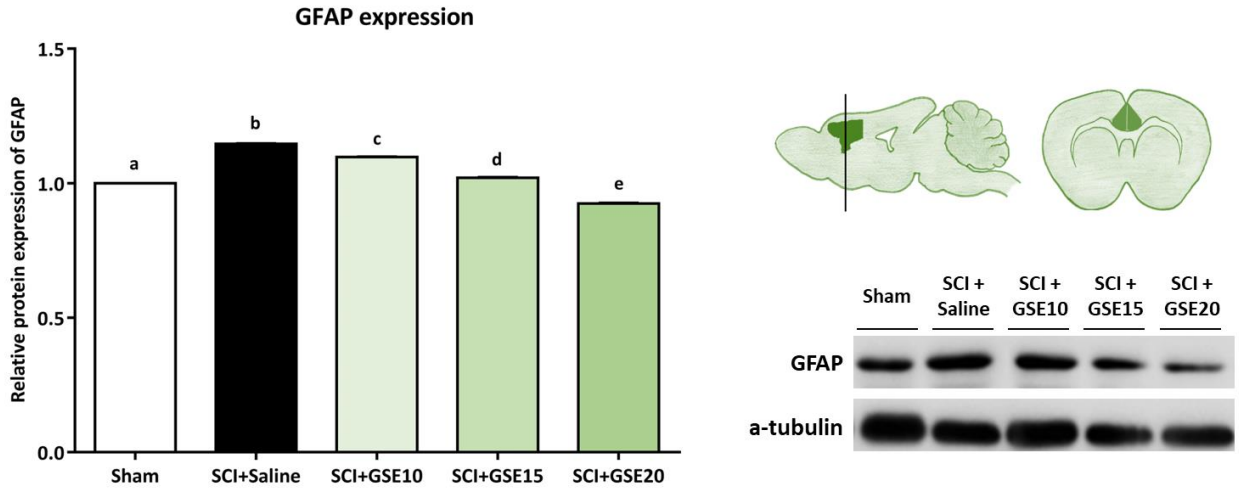


Figure 52. GFAP expression in ACC after preventive GSE treatment (10, 15, 20 mg/kg) on spinal cord injured mice at the end of experimental period. Protein expression was normalized to a-tubulin. Data is expressed as a relative percentage respect to Sham group (mean ± SEM). a–e: Groups not sharing a letter showed significant differences, $p<0.05$. Experimental groups: Sham (n=4), SCI+Saline (n=4), SCI+GSE10 (n=4), SCI+GSE15 (n=4), SCI+GSE20 (n=4).

On the other hand, microgliosis in ACC after GSE treatment on SCI mice was also evaluated. To this end, anti-IBA1 antibody was incubated on histological brain sections. Then, the proportion of IBA1 immunoreactivity area was quantified. Immunohistochemical data for IBA1 immunoreactivity in ACC did not follow a normal distribution according to Shapiro-Wilk test ($p<0.001$). On further Kruskal-Wallis test significant differences between groups were found ($p<0.001$). Concretely, Mann Whitney U test showed a significant increase of ACC IBA1 immunoreactivity proportion in SCI+Saline group when compared with Sham ($p<0.05$). Referring to GSE treatment groups, all tested doses of GSE significantly reduced the proportion of IBA1 immunoreactivity in ACC compared to SCI+Saline group ($p's<0.05$). More specifically, while GSE10 dose showed similar Sham IBA1 immunoreactivity ($p>0.05$), both GSE15 and GSE20 doses showed significant lower IBA1 immunoreactivity proportion, presenting the GSE15 group the lowest proportion of IBA1 immunoreactivity in the ACC compared with the rest of experimental groups ($p's<0.05$) (***Fig. 53. B***).

Additionally, to complement these immunohistochemical results, western blot analysis of the total IBA1 protein in ACC was also performed. Shapiro-Wilk normality test indicated that western blot data for IBA1 in ACC did not follow a normal distribution. Thus, non-parametric tests were applied to elucidate possible between-group differences in IBA1 expression. Kruskal-Wallis test showed significant differences between groups ($p<0.001$) and further Mann Whitney U tests indicated that these differences were between all experimental groups (all $p's<0.05$). Specifically, the highest expression of IBA1 in ACC was shown by the SCI+Saline group, followed by Sham group and GSE10, GSE20 and GSE15 treatment groups, respectively.

Therefore, GSE treatment groups not only prevented microgliosis in ACC compared to SCI+Saline group, but also prevented IBA1 expression even at lower levels than the Sham group (***Fig. 54***).

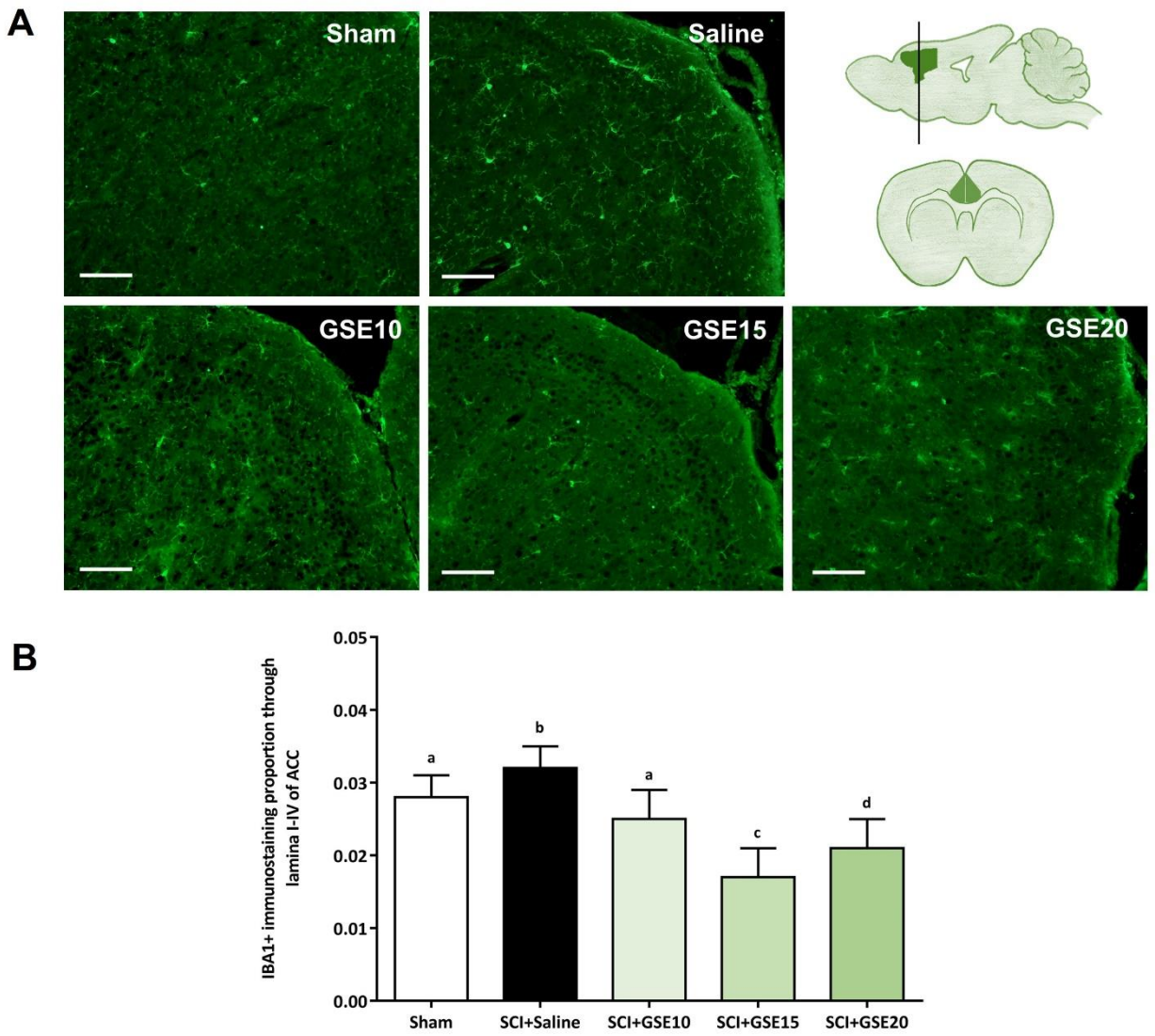


Figure 53. Effects of GSE treatment (10, 15 and 20 mg/kg) on IBA1 immunoreactivity proportion in ACC of SCI mice at the end of experimental period. (**A**) Representative histological images of the ACC immunostained against IBA1 of each group (Scale bar 200 µm). (**B**) Histogram representing the proportion of IBA1-immunoreactivity in ACC. Data is expressed as mean ± SEM. a–d: Groups not sharing a letter showed significant differences, $p<0.05$. Experimental groups: Sham (n=5), SCI+Saline (n=5), SCI+GSE10 (n=5), SCI+GSE15 (n=5), SCI+GSE20 (n=5).

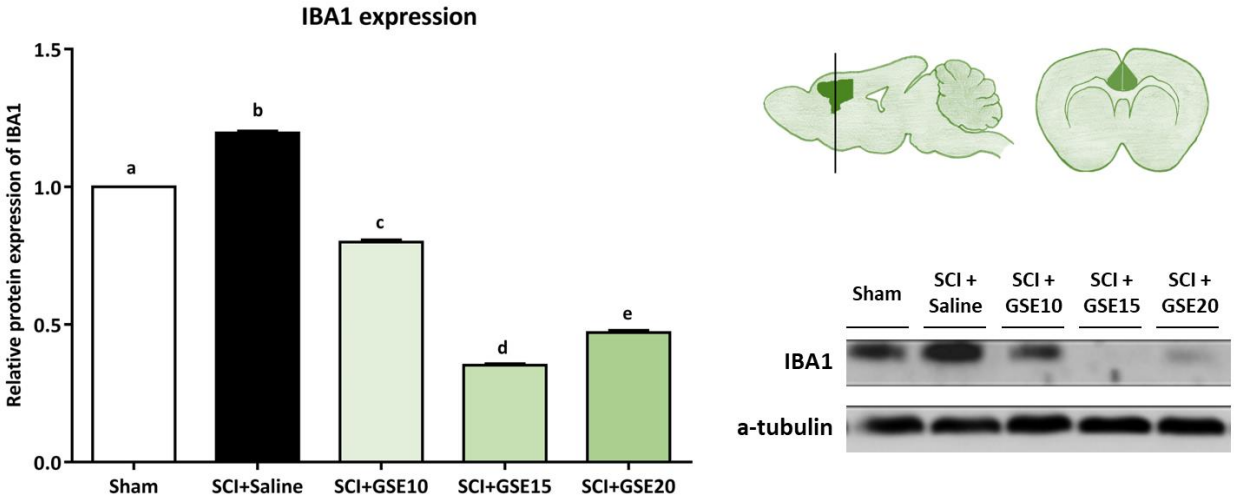


Figure 54. IBA1 expression in ACC after preventive GSE treatment (10, 15, 20 mg/kg) on spinal cord injured mice at the end of experimental period. Protein expression was normalized to a-tubulin. Data is expressed as a relative percentage respect to Sham group (mean ± SEM). a–e: Groups not sharing a letter showed significant differences, p<0.05. Experimental groups: Sham (n=5), SCI+Saline (n=5), SCI+GSE10 (n=5), SCI+GSE15 (n=5), SCI+GSE20 (n=5).

The dose of 15GSE treatment modulates CX3CL1 chemokine and its receptor CX3CR1 expression in the ACC of SCI mice.

To determine whether fractalkine (CX3CL1) and its receptor (CX3CR1) were overexpressed in the ACC after SCI and whether treatment with GSE modulated their expression, western blot analyses against CX3CL1 and CX3CR1 in the ACC were performed. Referring to fractalkine, CX3CL1 data did not follow a normal distribution (Shapiro-Wilk test, $p<0.01$). Thus, parametric tests were used to analyse these data. On the one hand, significant differences between groups were found by Kruskal-Wallis test ($p<0.01$) and further Mann Whitney U tests indicated that these differences were between all experimental groups (all p's<0.05). Concretely, CX3CL1 was significant overexpressed in SCI+Saline group when compared with Sham ($p<0.05$). While GSE10 and GSE20 treatment groups showed slightly increased levels of CX3CL1 expression compared to both Sham and SCI+Saline groups (p's<0.05), the GSE15 dose significantly reduced CX3CL1 expression when compared with SCI+Saline group ($p<0.05$) (***Fig. 55. A***). As for CX3CR1, data followed a normal distribution according to the Shapiro-Wilk test ($p>0.05$) and significant group differences were evidenced by further ANOVA analysis ($p<0.001$). Similar to CX3CL1, post-hoc analysis indicated significant overexpression of CX3CR1 in SCI+Saline group when compared to Sham ($p<0.05$). The GSE15 was the only tested dose that reduced CX3CR1 levels below sham ($p<0.05$). In contrast, both GSE10 and GSE20 doses not significantly reduced CX3CR1 expression in comparison to SCI+Saline levels ($p>0.05$) (***Fig. 55. B***).

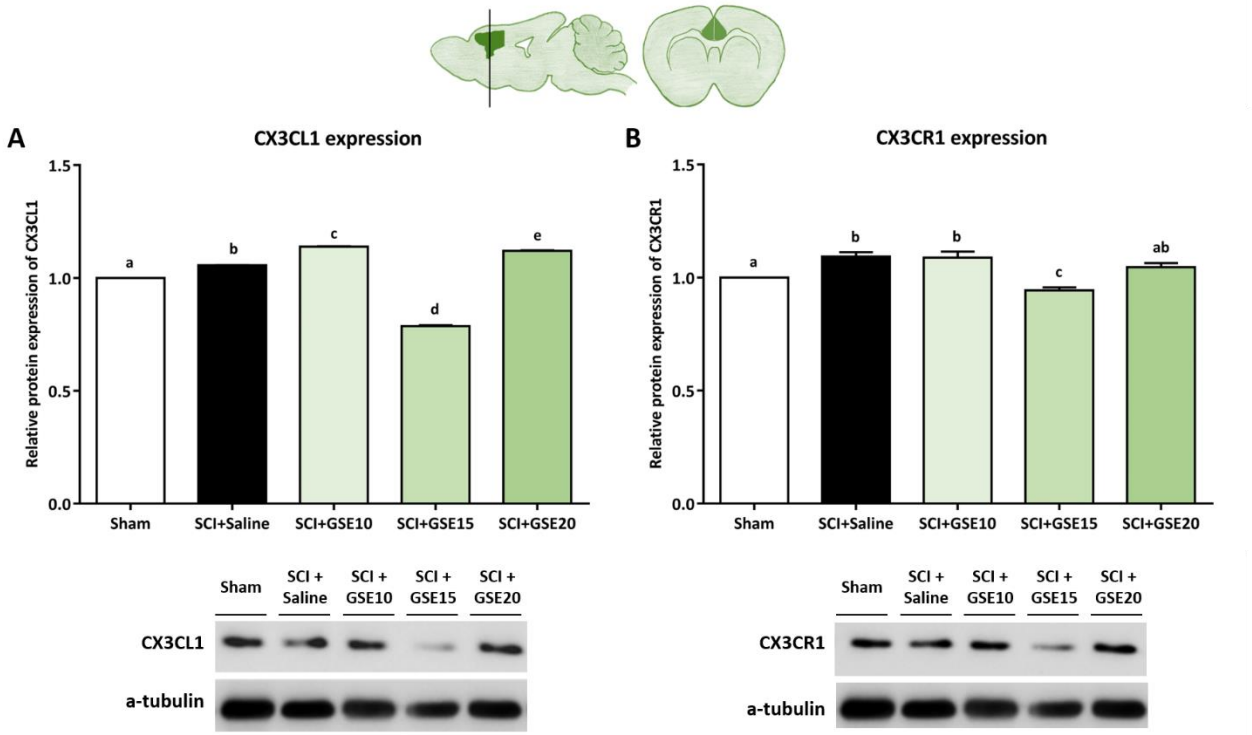


Figure 55. CX3CL1 and CX3CR1 expression in ACC after preventive GSE treatment (10, 15, 20 mg/kg) on spinal cord injured mice at the end of experimental period. Protein expression was normalized to a-tubulin. Data is expressed as a relative percentage respect to Sham group (mean ± SEM). a–e: Groups not sharing a letter showed significant differences, p<0.05. Experimental groups: Sham (CX3CL1 n=4; CX3CR1 n=4), SCI+Saline (CX3CL1 n=4; CX3CR1 n=4), SCI+GSE10 (CX3CL1 n=4; CX3CR1 n=4), SCI+GSE15 (CX3CL1 n=4; CX3CR1 n=4), SCI+GSE20 (CX3CL1 n=4; CX3CR1 n=4).

Preventive GSE treatment reduces astrogliosis and induces microgliosis in the PAG of SCI mice.

As mentioned in the introduction of the present thesis, periaqueductal gray (PAG) is a key component that mediates pain modulation. Indeed, glial activation in PAG has been correlated with neuropathic pain by contributing to descending facilitation (**Ni et al., 2016**). To determine whether SCI induces activation of glial expression in PAG and whether GSE treatment can modulates it, immunofluorescence labelling and western blot techniques were used to detect GFAP and IBA1 in PAG of Sham, SCI and SCI-GSE treated mice.

Referring to astrogliosis, immunohistochemical data for GFAP did not follow a normal distribution (Shapiro Wilk, p<0.001). Thus, data were analysed by Kruskal-Wallis test which detected significant differences on GFAP reactivity in PAG between groups. Specifically, SCI+Saline group showed a significant increase in GFAP immunoreactivity proportion compared to Sham (Mann Whitney U test, p<0.001) indicating that SCI induced astroglial activation in PAG at 21 dpi. Referring to GSE treatment, all tested doses reduced GFAP reactivity in PAG when compared to SCI+Saline group (all p's<0.01) although none of them reached at immunoreactivity Sham levels (all p's<0.05). The dose of GSE15 showed the lowest astroglial reactivity in PAG, followed by the GSE20 and GSE10, respectively (***Fig. 56. B***).

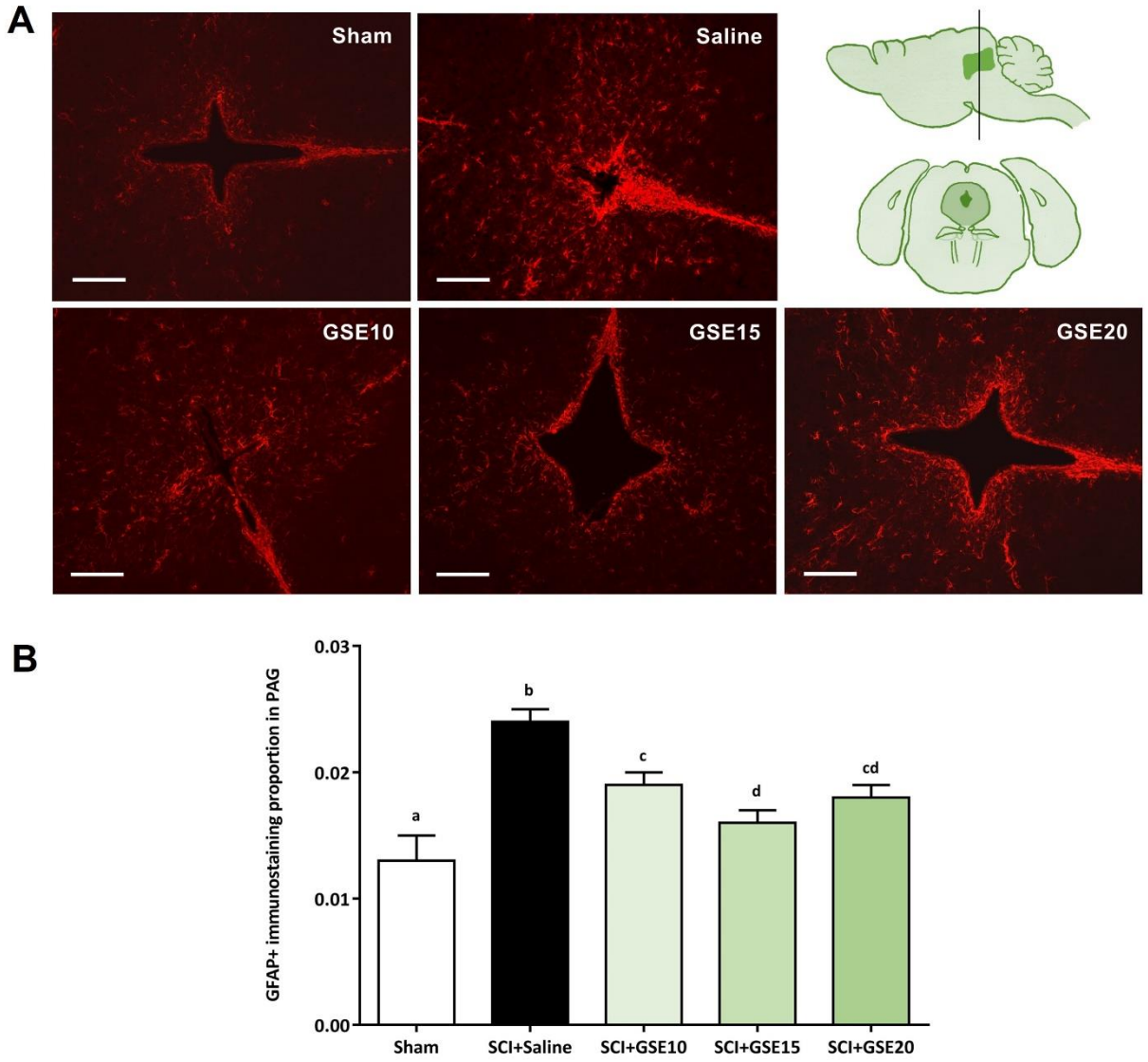


Figure 56. Effects of GSE treatment (10, 15 and 20 mg/kg) on GFAP immunoreactivity proportion in PAG of SCI mice at the end of experimental period. (**A**) Representative histological images of the PAG immunostained against GFAP of each group (Scale bar 200 µm). (**B**) Histogram representing the proportion of GFAP-immunoreactivity in PAG. Data is expressed as mean ± SEM. a–d: Groups not sharing a letter showed significant differences, $p<0.05$. Experimental groups: Sham (n=5), SCI+Saline (n=5), SCI+GSE10 (n=5), SCI+GSE15 (n=5), SCI+GSE20 (n=5).

Western blot analysis of total GFAP expression in PAG were also performed. In this case, data followed a normal distribution according to the Shapiro-Wilk test ($p>0.05$). ANOVA revealed significant group differences in GFAP expression in PAG ($p<0.001$). Similar to immunohistochemical analysis, SCI+Saline group showed a significant overexpression of GFAP compared to Sham ($p<0.05$). GSE20 treatment dose reduced GFAP expression in PAG at Sham levels ($p>0.05$) whereas GSE10 and GSE15 doses reduced it below the levels of Sham ($p's<0.05$). GSE15 treatment group showed less expression of GFAP in PAG compared to GSE10 group ($p<0.05$) (***Figure. 57***).

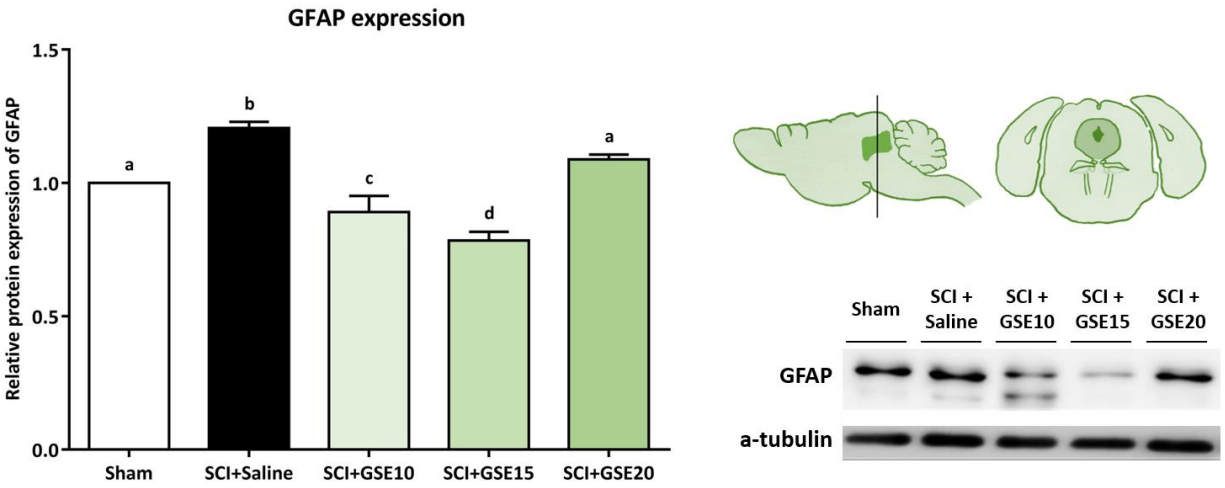


Figure 57. GFAP expression in PAG after preventive GSE treatment (10, 15, 20 mg/kg) on spinal cord injured mice at the end of experimental period. Protein expression was normalized to a-tubulin. Data is expressed as a relative percentage respect to Sham group (mean ± SEM). a–d: Groups not sharing a letter showed significant differences, $p<0.05$. Experimental groups: Sham (n=5), SCI+Saline (n=5), SCI+GSE10 (n=5), SCI+GSE15 (n=5), SCI+GSE20 (n=5).

As to microgliosis in PAG, immunohistochemical data for IBA1 followed a normal distribution (Shapiro-Wilk, $p>0.05$). Thus, ANOVA test was performed, indicating significant group differences in IBA1 immunostained proportion in PAG ($p<0.001$). In contrast to the spinal cord and ACC, the proportion of IBA1 immunoreactivity in PAG did not differ between the Sham and SCI+Saline groups ($p>0.05$). Therefore, these results indicated that SCI in mice did not induce microgliosis in PAG. Surprisingly, all SCI+GSE treatment groups showed a significant increase of IBA1 immunoreactivity when compared with both Sham and SCI+Saline groups (all p's<0.05), thus indicating that all tested doses of GSE in SCI mice induced microgliosis in PAG. The immunoreactivity proportion of IBA1 did not differ between the GSE treatment groups ($p>0.05$) (***Fig. 58. B***).

Western blot data for IBA1 expression in PAG did not follow a normal distribution (Shapiro-Wilk, $p<0.01$). Subsequent Kruskal-Wallis test showed significant group differences in IBA1 expression ($p<0.001$). In this case, IBA1 was downregulated in SCI+Saline group when compared with Sham ($p<0.01$). In contrast, GSE treatment groups showed an IBA1 overexpression compared to Sham (p's>0.05). Concretely, GSE20 dose showed the highest levels of IBA1 expression, followed by GSE15 and GSE10, respectively. In conclusion, these results indicated that SCI induced downregulation of IBA1 in PAG while GSE treatments in SCI animals promoted the overexpression of such protein (***Fig. 59***).

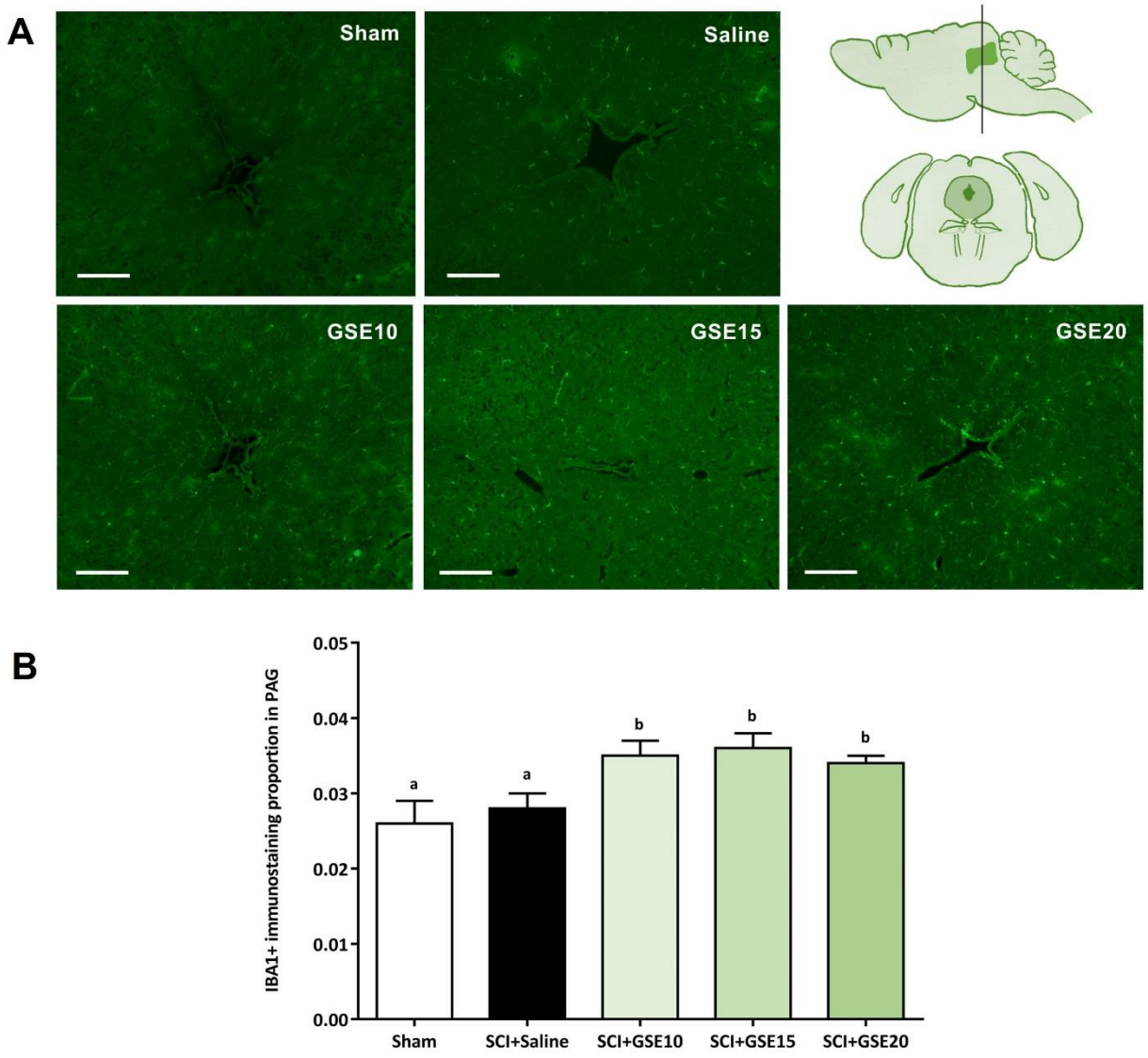


Figure 58. Effects of GSE treatment (10, 15 and 20 mg/kg) on IBA1 immunoreactivity proportion in PAG of SCI mice at the end of experimental period. (**A**) Representative histological images of the PAG immunostained against IBA1 of each group (Scale bar 200 µm). (**B**) Histogram representing the proportion of IBA1-immunoreactivity in PAG. Data is expressed as mean ± SEM. a–d: Groups not sharing a letter showed significant differences, $p<0.05$. Experimental groups: Sham (n=5), SCI+Saline (n=5), SCI+GSE10 (n=5), SCI+GSE15 (n=5), SCI+GSE20 (n=5).

In order to explain the increased levels of microglial marker IBA1 in PAG following GSE treatment, the microglial phenotype was characterized for GSE20 group. Thus, immunohistochemistry for the protein OX-42 and for CD206 markers were performed. OX-42, an antibody designed to detect CD11b beta-integrin, has been the most widely used microglia marker in the brain since constitutively expressed CD11b is upregulated in parallel to the activation-induced morphological transformation of microglia (**Ladeby et al., 2005**). CD206 is widely recognised as a representative microglial marker of the M2 phenotype (**Durafourt et al., 2012; Kobayashi et al., 2013**) due to its involvement in important cellular functions, especially pinocytosis and phagocytosis (**Marzolo et al., 1999; Regnier-Vigouroux, 2003**). It is therefore suggested that

CD206 plays a critical role in the first step of pathogen recognition and capture in neural tissues (**Zimmer et al., 2003**).

The results showed that GSE20 treatment induced a higher percentage of CD206 microglial cells in PAG compared to Sham ($p<0.01$), exhibiting the M2 phenotype and thus suggesting an anti-inflammatory effect (***Fig. 60***).

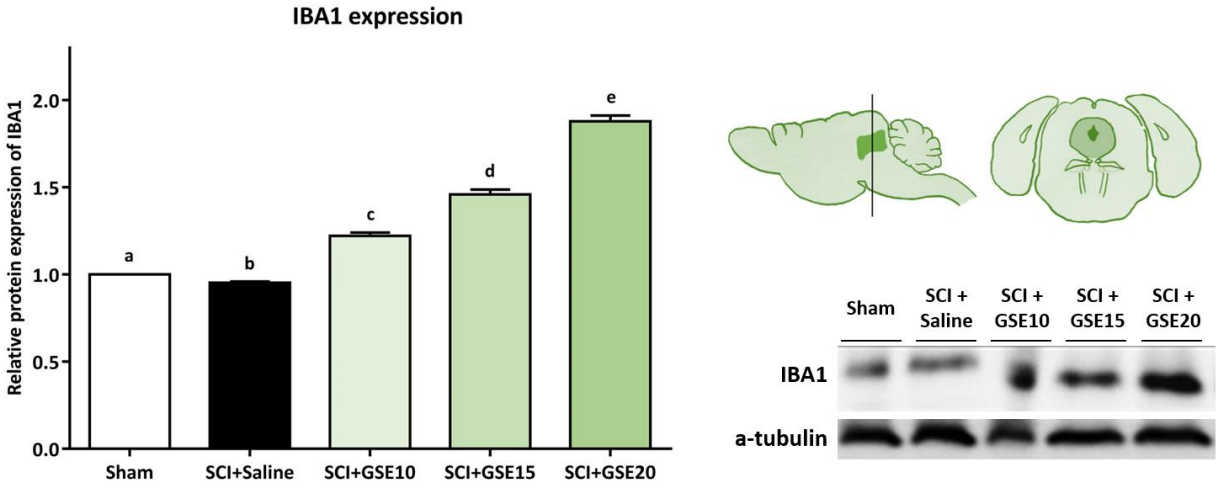
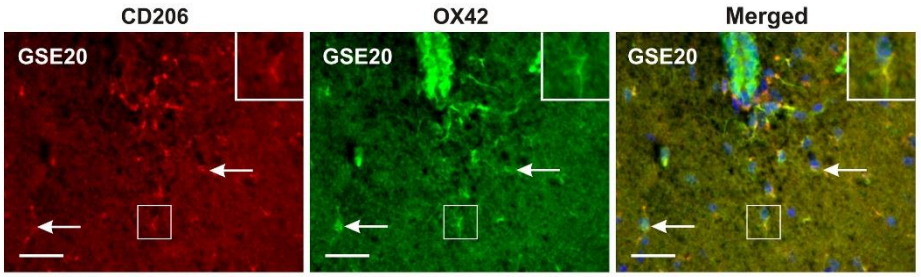


Figure 59. IBA1 expression in ACC after preventive GSE treatment (10, 15, 20 mg/kg) on spinal cord injured mice at the end of experimental period. Protein expression was normalized to a-tubulin. Data is expressed as a relative percentage respect to Sham group (mean ± SEM). a–e: Groups not sharing a letter showed significant differences, $p<0.05$. Experimental groups: Sham (n=5), SCI+Saline (n=5), SCI+GSE10 (n=5), SCI+GSE15 (n=5), SCI+GSE20 (n=5).



Percentage of CD206/OX42 immunopositive microglial cells	
Sham	15.2 ± 2.6
SCI+GSE20	27 ± 1.9*

Figure 60. Percentage of CD206 immunopositive OX42 immunostained microglial cells in PAG of animals with SCI and treated with GSE (20 mg/kg). *$p<0.01$ when compared with Sham. Representative pictures illustrating double immunostaining of OX42 immunopositive microglial cells and CD206 in the section through PAG of SCI mouse and treated with GSE20. Merged green (OX42-FITC) and red (CD206-TRITC) immunofluorescence and blue Hoechst nuclear staining indicate position of OX42+/CD206+ microglial cells. Scale bar: 50µm. Experimental groups: Sham (n=3) and SCI+GSE20 (n=3).

Preventive GSE treatment modulates CX3CL1 chemokine and its receptor CX3CR1 expression in the PAG of SCI mice.

Western blot analyses against CX3CL1 and CX3CR1 in the PAG were performed in order to elucidate whether fractalkine (CX3CL1) and its receptor (CX3CR1) were overexpressed in the PAG after SCI and whether

treatment with GSE modulated their expression. According to Shapiro-Wilk test, CX3CL1 data did not follow a normal distribution ($p<0.01$). For this reason, Kruskal-Wallis test was applied, indicating significant differences between groups ($p<0.001$). Specifically, the subsequent post-hoc analysis indicated that SCI+Saline group showed significant CX3CL1 overexpression in comparison to Sham ($p<0.01$) and all tested doses of GSE treatment significantly reduced this overexpression below sham levels (all p's<0.01). The GSE10 dose showed the lowest CX3CL1 expression levels in PAG, while the CX3CL1 levels of GSE15 and GSE20 groups did not differ significantly from each other ($p>0.05$) (***Fig. 61. A***). Referring to CX3CR1, data followed a normal distribution according to the Shapiro-Wilk test ($p>0.05$). Thus, ANOVA analysis was performed indicating significant differences between groups ($p<0.001$). Subsequent post-hoc analysis showed significant overexpression of CX3CR1 in SCI+Saline group when compared to Sham and all SCI-GSE treated groups (all p's<0.05). While GSE10 and GSE15 doses reduced CX3CR1 levels below Sham ($p<0.05$), GSE20 dose do not significantly differed from Sham ($p>0.05$). The GSE10 dose showed the lowest CX3CR1 expression levels in PAG, followed by GSE15 and GSE20 groups respectively (***Fig. 61. B***).

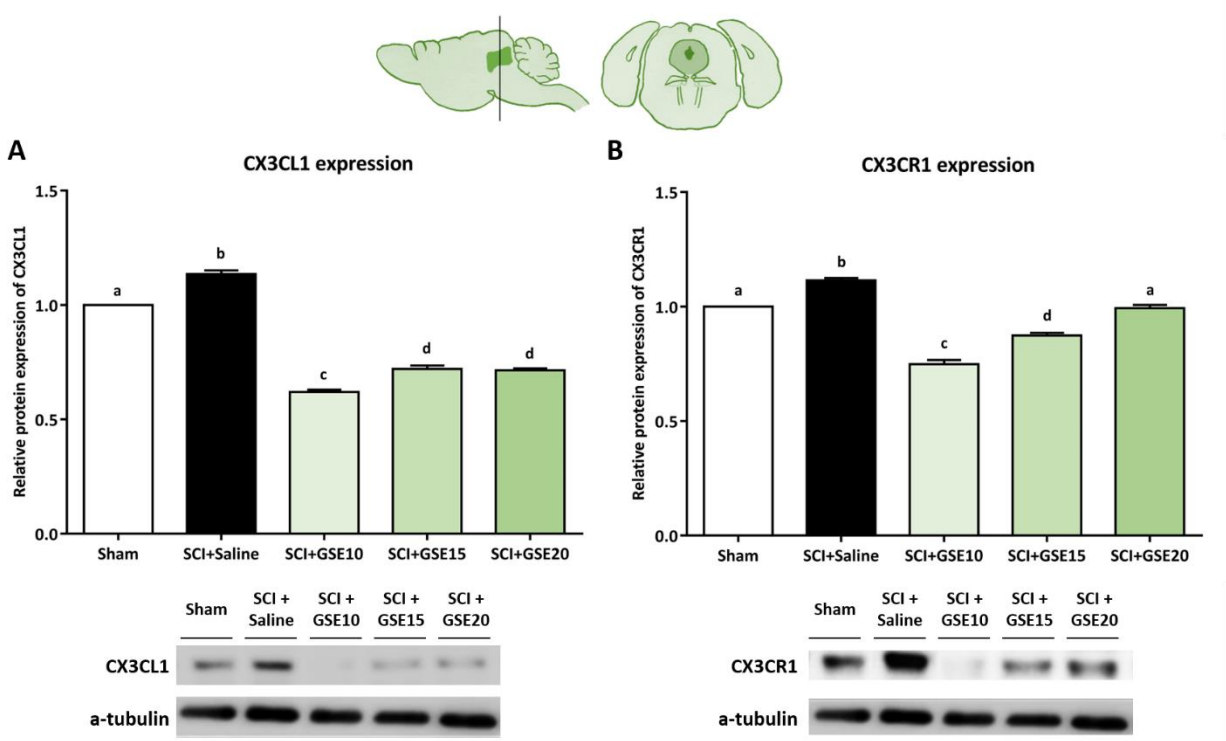


Figure 61. CX3CL1 and CX3CR1 expression in PAG after preventive GSE treatment (10, 15, 20 mg/kg) on spinal cord injured mice at the end of experimental period. Protein expression was normalized to a-tubulin. Data is expressed as a relative percentage respect to Sham group (mean ± SEM). a–d: Groups not sharing a letter showed significant differences, $p<0.05$. Experimental groups: Sham (CX3CL1 n=6; CX3CR1 n=6), SCI+Saline (CX3CL1 n=6; CX3CR1 n=6), SCI+GSE10 (CX3CL1 n=6; CX3CR1 n=6), SCI+GSE15 (CX3CL1 n=6; CX3CR1 n=6), SCI+GSE20 (CX3CL1 n=6; CX3CR1 n=6).

Summary results Chapter II

In the present chapter, we have demonstrated that preventive GSE administration during the first week post SCI, resulted in the inhibition (10 and 15 mg/kg; i.p.) or an attenuation (20 mg/kg; i.p.) of mechanical allodynia, and in the inhibition (15 and 20 mg/kg; i.p.) or an attenuation (10 mg/kg; i.p.) of thermal hyperalgesia development during the acute phase of SCI-derived neuropathic pain.

Another important finding was that all tested doses of GSE treatment were pharmacologically safe, as they did not significantly affect the weight or aspect of the animals and did not alter biomarkers of hepatotoxicity and nephrotoxicity in the animals' serum.

In parallel, immunohistochemical and molecular studies revealed that all doses of GSE modulate astrogliosis and microgliosis in the spinal cord at 21 dpi. Furthermore, GSE treatment prevented the ERK1/2 phosphorylation and the upregulation of CX3CL1, CX3CR1 and CCR2 evidenced in the spinal cord of SCI+Saline mice. Moreover, GSE treatments attenuated and sometimes completely prevented SCI-induced astrogliosis in both supraspinal structures of the ACC and PAG. While GSE treatments prevented microgliosis in the ACC of SCI mice, the same treatments induced microglial activation in PAG characterized by an increase of cells with a CD206-positive M2 phenotype. Finally, although only the 15 mg/kg dose of GSE modulated the expression of CX3CL1/CX3CR1 in ACC, all tested doses of GSE prevented the upregulation of this chemokine and its receptor in PAG.

Therefore, these results suggest that the repeated administration of GSE during the first week after spinal cord contusion may be a suitable therapeutic strategy to prevent the development of SCI-induced neuropathic pain during the acute injury phase (up to 21 days after injury). Such effects would be exerted by modulating central sensitization phenomena present not only at the site of injury but also on pain-related supraspinal structures such as the ACC and PAG.

CHAPTER III

Preventive effects on Spinal Cord Injury-induced neuropathic pain development by repeated administration of Coffee Extract (CE) in CD1 mice.

CHAPTER III. Preventive effects on Spinal Cord Injury-induced neuropathic pain development by repeated administration of Coffee Extract (CE) in CD1 mice.

After elucidating the preventive effects of GSE on the development of SCI-induced neuropathic pain during the acute phase of injury, we began to investigate the preventive effects of another polyphenolic extract obtained from coffee beans (CE). It is known that coffee contains different types of polyphenols (**Moeenfard et al. 2014; Nebesny and Budryn, 2003; Gornas et al. 2016**), mostly different than those found in the GSE, but CE effects on central pathological pain development remains unknown. Hence, considering the data available of the different antihiperalgesic effects exerted by polyphenols (**Hassler et al., 2014; Renno et al., 2014; Boadas-Vaello et. al 2017; Wang et al., 2020; Limcharoen et al., 2021; Rao et al., 2021**), in the present chapter we wanted to evaluate whether an extract of different origin and composition to GSE could exert similar or better effects in the prevention of neuropathic pain development after SCI. To this end, CE was administered to SCI mice during the first week post-injury and both mechanical allodynia and thermal hyperalgesia were weekly assessed. Moreover, we analysed the pharmacological safety of the CE treatment as well as the gliosis, chemokines expression and ERK phosphorylation in spinal cord and cellular and biochemical processes in the supraspinal structures PAG and ACC. Similar to GSE studies, the first step was the CE obtaining, the process of which is described in the Materials and Methods section. As performed in the GSE studies, before administration, the CE was characterized in order to determine its suitability to be used as potential treatment and the quantification and compounds identification is described below.

Characterization of phenolic compounds in CE

Total polyphenol content in CE

Throughout the thesis several batches of CE were obtained to perform the scheduled experimental procedures. According to the Folin-Ciocalteu assay, the total polyphenolic content of GSE batches used in the present thesis are indicated in **Figure 62**. It can be observed that the total CE polyphenolic content obtained after extraction procedure was approximately 2200 mg GAE/L on average. This polyphenols concentration allows preparing suitable volume of administration of experimental doses.

Polyphenol content in CE (mg GAE/L)
First Batch: 1985 ± 124
Second Batch: 2186 ± 137
Third Batch: 2456 ± 153
Fourth Batch: 2480 ± 130

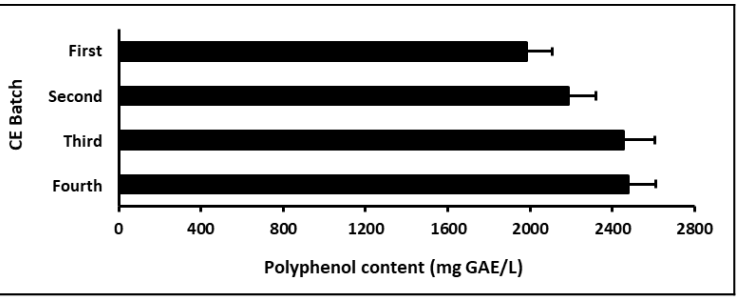


Figure 62. Total polyphenolic content in CE extract calculated according to the Folin-Ciocalteu method. Polyphenol content are expressed as milligrams of gallic acid equivalents (GAE) per litre of extract. All values are presented as mean ± standard deviation (n=6).

Identification and quantitative analysis of polyphenols in CE by HPLC-UV-ESI-FTMS

As mentioned before, coffee is a beverage that has been well characterised due to its multiple properties and applications. Considering such previous knowledge, a literature search was carried out in order to define the major polyphenols that could be found in the coffee extract developed in this thesis. Thus, thanks to studies performed by other authors, it was possible to narrow down the number and type of polyphenols to be identified and quantified in the CE. In ***Table 7*** are indicated the major polyphenols identified in coffee beverages that have been prepared under similar conditions to the extracts developed in the present thesis.

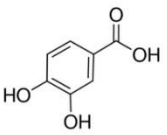
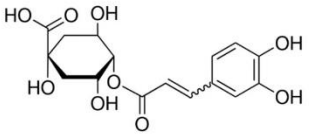
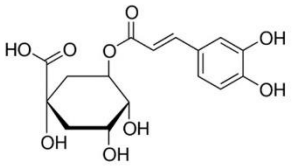
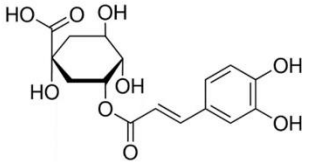
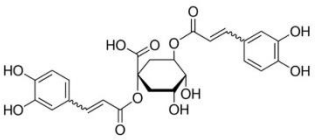
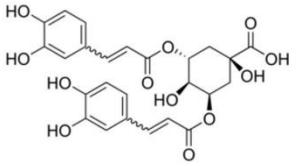
Table 7. Polyphenols identified in coffee beverages prepared under similar conditions to the coffee extract developed in the present thesis.

	Moeenfard et al., 2014	Nebesny and Budryn, 2003	Gornas et al., 2016
Caffeic acid	X	X	X
Ferulic acid	X	X	X
p-Coumaric acid	X		X
o-Coumaric acid			X
Protocatechuic acid			X
Gentisic acid			X
Vanillic acid			X
Salicilic acid			X
Sinapic acid			X
Chlorogenic acid (3-O-Caffeoylquinic acid)	X	X	X
Cryptochlorogenic acid (4-O-Caffeoylquinic acid)	X	X	X
Neochlorogenic acid (Trans-5-O-Caffeoylquinic acid)	X	X	X
4-O-Feruloylquinic acid		X	
5-O-Feruloylquinic acid		X	
3,4-Di-O-caffeoylquinic acid		X	
3,5-Di-O-caffeoylquinic acid		X	
4,5-Di-O-caffeoylquinic acid		X	

After this literature identification, fourteen commercially available standard solutions of these polyphenols were analysed by UPLC-ESI-FTMS (-) to further be able to be detected in the CE extract. Solutions of all

polyphenols at 1000, 100, 10 and 1 mg/L were analysed. The presence of polyphenols in CE were confirmed when were compared with chromatographic peaks obtained from standard analysis (exact mass, error<2mDa, and retention time). The polyphenols detected in our CE are presented in **Table 8**.

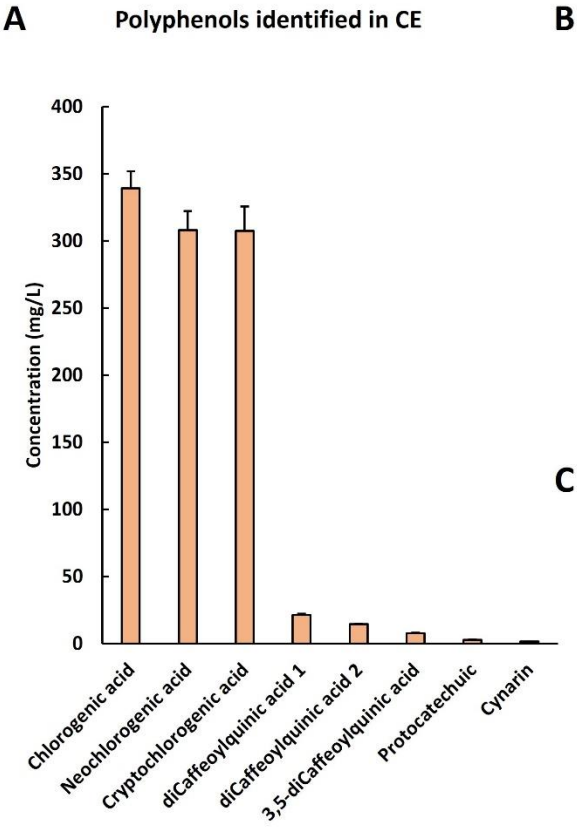
Table 8. Polyphenols identified in the CE by UPLC-ESI-FTMS (-).

Peak	Molecular weigh	Identificaton	Formula	Structure
1	154.12	Protocatechuic acid (3,4-dihydroxybenzoic acid)	$(HO)_2C_6H_3CO_2H$	
2	354.31	Cryptochlorogenic acid (4-O-Caffeoylquinic acid)	$C_{16}H_{18}O_9$	
3	354.31	Clorogenic acid (3-O-Caffeoylquinic acid)	$C_{16}H_{18}O_9$	
4	354.31	Neoclorogenic acid (Trans-5-O-Caffeoylquinic acid)	$C_{16}H_{18}O_9$	
5	516.45	Cynarin (1,3-Dicaffeoylquinic acid or 1,5-Di-O-caffeoylquinic acid)	$C_{25}H_{24}O_{12}$	
6	516.45	3,5-Di-O-caffeoylquinic acid	$C_{25}H_{24}O_{12}$	

Additionally, two diCaffeoylquinic acids which were named “Unknown diCaffeoylquinic acid 1” and “Unknown diCaffeoylquinic acid 2” were also identified. These diCaffeoylquinic acids could probably be 3,4-Di-O-caffeoylquinic acid and 4,5-Di-O-caffeoylquinic acid, which have been identified by other authors (**Nebesny and Budryn, 2003**) in other coffee extracts obtained under conditions similar to those of our coffee extract.

After their identification, the polyphenolic compounds of CE were quantified by ultra-high performance liquid chromatography (UHPLC) in order to avoid isobaric interferences. Thus, standard calibration curves with internal standard (abscisic acid-d_6, 1.3 mg/L) were calculated using the integration of the area of the peak present on the 2 mDa trace chromatogram, centred on the exact mass of each compound. The

calibration curves were obtained by preparing different concentrations of standard compounds within the range 0.048-7.2 mg/L. All the calibration curves were presented a good correlation (r^2>0.99) and precision (80-120%). As a result, three polyphenols resulted as major compounds: Chlorogenic acid, Neochlorogenic acid and Cryptochlorogenic acid (***Fig. 63***). In contrast, the other polyphenols showed much lower levels of quantification (***Fig. 63***).



B

Polyphenol	Concentration (mg/L)	SD
Chlorogenic acid	339,2	12,9
Neochlorogenic acid	308,1	14,3
Cryptochlorogenic acid	307,6	18,2
Unknown diCaffeoylquinic acid 1	21,3	1
Unknown diCaffeoylquinic acid 2	14,5	0,4
3,5-diCaffeoylquinic acid	7,8	0,3
Protocatechuic	2,9	0,1
Cynarin	1,5	0,1

C

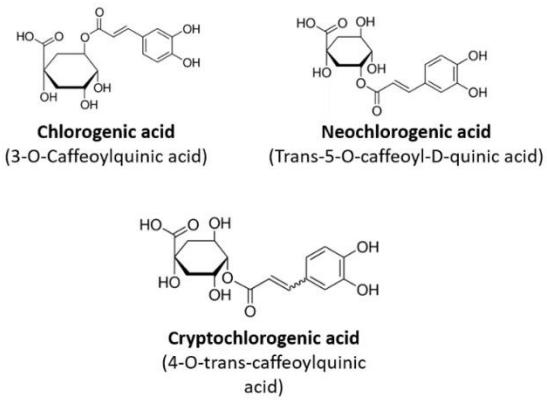


Figure 63. Quantification of phenolic compounds in CE by UHPLC. Results are presented by (A) Histogram and (B) values data table. Quantification of each polyphenol identified in the CE was performed in triplicate and all values are presented as mean ± SD (n=3). In (C) the molecules structures of major compounds are presented.

Once it was observed that CE is characterized by a rich polyphenolic content at concentrations high enough to be administered by a reasonable injection volume, a dose-response study was scheduled. That is, SCI mice were treated with the whole extract (10 and 15 mg/kg; i.p.) during the first week post-injury. Afterwards, similar to GSE studies, mechanical allodynia and thermal hyperalgesia were weekly assessed up to the end of SCI acute phase. At the end of the experimental period, serum levels of hepatotoxicity and nephrotoxicity biomarkers were analysed to assess the pharmacological safety of the GSE treatment. Finally, to explain the potential effects exerted by CE on neuropathic pain behaviours, astrogliosis and microgliosis were analysed in both the spinal cord and supraspinal structures (periaqueductal gray matter, PAG; and anterior cingulate cortex, ACC). Moreover, in order to get some mechanistic insights, the protein

expression of ERK phosphorylation as well as the expression of MCP1/CCL2 and CX3CL1 chemokines and their receptors CCR2 and CX3CR1 were analysed. The obtained results are explained below.

Preventive CE treatment is not associated with systemic toxicity and does not cause hepatotoxic nor nephrotoxic effects.

The general aspect of the animals was normal throughout the whole experimental period since changes in coat and skin, vibrissae of nose, nasal secretions, aggressiveness or signs of autotomy were not observed in any experimental group of mice, according to the protocol animal welfare supervision based on Morton D.B and Griffiths P.H. guidelines (**Morton and Griffiths, 1985**).

The effects of treatment, injury and time on animals' weight were evaluated using different statistical tools. Kolmogorov-Smirnov normality test confirmed a normal distribution of the data corresponding to weight for all experimental time-points (p's>0.05) except for the basal day (p<0.01). On further Kruskal-Wallis test, no significant group differences were found at basal day (p>0.05). MANOVA analysis of the weight data from 7 until 21 dpi indicated that there were significant effects on day factor ($F_{(3,39)}$ = 26.597, p<0.001) and on the day x treatment interaction ($F_{(9,95.066)}$= 2.887, p<0.01) but not on treatment ($F_{(3,41)}$ = 0.794, p>0.05) factor. On further ANOVA, significant group differences were found at 7 dpi (p<0.05). Concretely, Duncan post-hoc test indicated that the weight of the animals in the SCI+Saline group was significantly lower compared to the other experimental groups at 7 dpi(p<0.05). Despite this, no significant weight loss was observed at the following time-points. Actually, all animals showed similar weight recovery suggesting no effects of the treatment on this parameter as ANOVA indicated (***Fig. 64***).

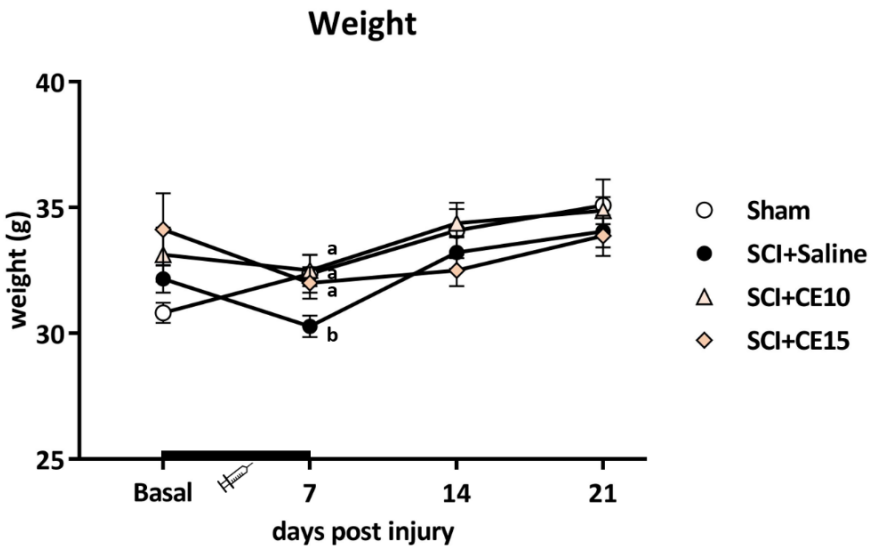


Figure 64. Mice weight control during the injury acute phase of SCI after preventive CE treatment. Each point and vertical line represent the mean ± SEM. Treatment administration week (basal to 7 dpi) is highlighted with a thick black line. Experimental groups: Sham (n=11), SCI+Saline (n=18), SCI+CE10 (n=8), SCI+CE15 (n=8).

As in the GSE experiment, the pharmacological safety of the polyphenolic CE treatment was analysed and compared to the DMSO and EGCG20 administration. To this end, biomarkers of hepatotoxicity (ALT/GPT and AST/GOT) and nephrotoxicity (UREA) in animals' serum were quantified at the end of experimental period. According to Shapiro-Wilk normality test, data corresponding to ALT/GPT and AST/GOT did not follow a normal distribution (p's>0.05) whereas data for UREA followed it (p<0.01). Both the ANOVA test for urea data ($F_{(5,33)}$=0.35, p=0.879) and the Kruskal-Wallis test for AST/GOT data (p=0.111) indicated no significant differences between the experimental groups (***Fig. 65. B, C***). In contrast, Kruskal-Wallis test for ALT/GPT data showed significant group differences (p<0.01). Specifically, SCI animals administered with DMSO and EGCG20 showed significantly higher ALT values compared to the rest of the experimental groups (***Fig. 65. A***). Therefore, these results indicate that there is no systemic toxicity associated with CE administration and neither hepatotoxic nor nephrotoxic effects.

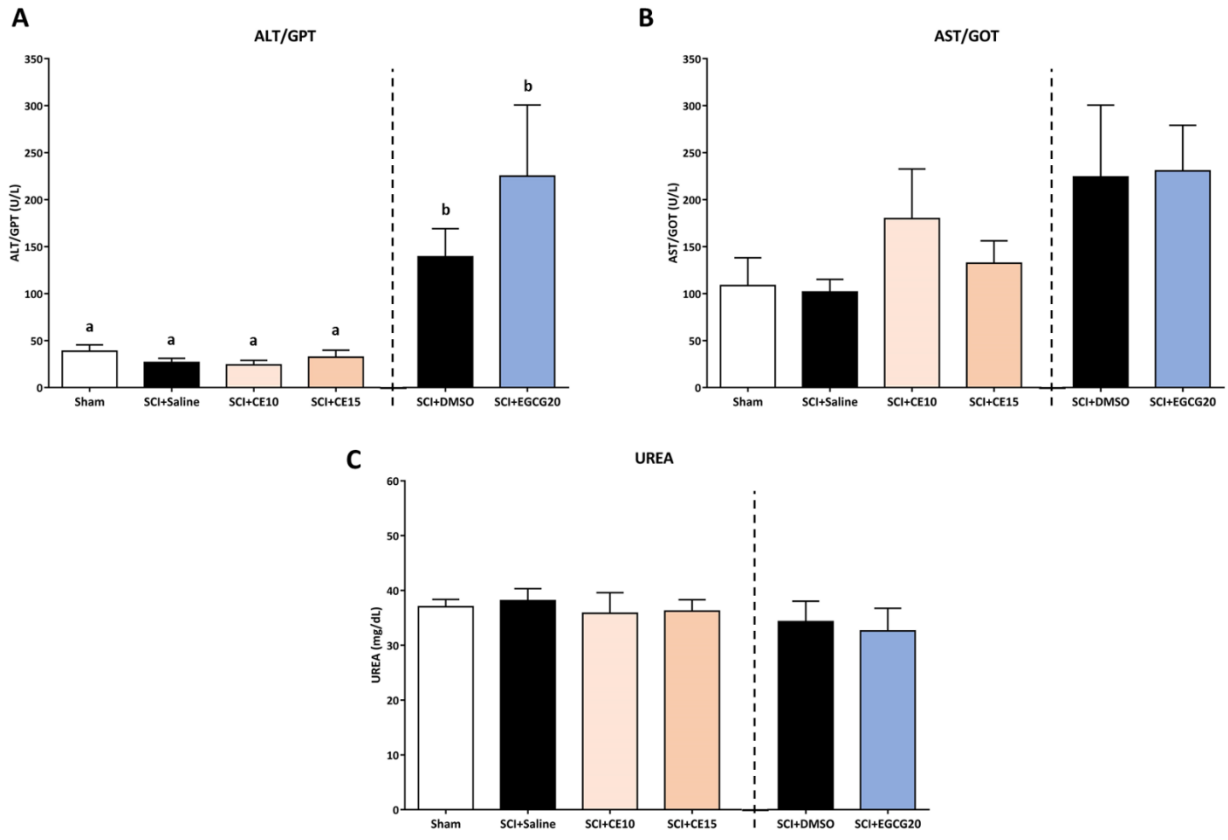


Figure 65. Biomarkers quantification of (A-B) hepatotoxicity and (C) nephrotoxicity, in the serum of each experimental group at the end of experimental period. The results are represented as the mean ± SEM. Experimental groups: Sham (ALT/GTP n=6; AST/GOT n=6; UREA n=6), SCI+Saline (ALT/GTP n=6; AST/GOT n=6; UREA n=6), SCI+CE10 (ALT/GTP n=5; AST/GOT n=5; UREA n=6), SCI+CE15 (ALT/GTP n=6; AST/GOT n=6; UREA n=6), SCI+DMSO (ALT/GTP n=5; AST/GOT n=4; UREA n=9), SCI+EGCG20 (ALT/GTP n=5; AST/GOT n=4; UREA n=6).

Preventive CE treatment attenuates mild spinal cord injury-induced mechanical allodynia and thermal hyperalgesia development.

To make sure that evoked pain responses, such as thermal hyperalgesia and mechanical allodynia, cannot be associated with locomotor impairment, and to assess whether the injury or CE treatment affected the locomotor activity of the animals, this parameter was weekly assessed by Basso Mouse Scale (BMS) assessment. According to Kolmogorov Smirnov test, BMS data did not follow a normal distribution in all experimental time-points (all p's<0.001). For this reason, non-parametric tests were used to analyse the data. Friedman's test detected differences over experimental period (p<0.001) and further Kruskal-Wallis test indicated significant group differences at 14 and 21 dpi (p's<0.05). Specifically, a slight impairment in coordination and locomotor function was detected in SCI animals. While the Sham group showed a significantly higher score compared to the SCI+Saline group at 14 and 21 dpi, the treated groups (CE10 and CE15) showed no significant difference to either the Sham or the SCI+Saline group at 14 dpi; and only the SCI+CE10 group showed no significant difference to Sham at 21 dpi (***Fig. 66. A***). It is worth mentioning that the slight locomotor impairments were only paw position alterations observed in some of the SCI animals, but no horizontal locomotion alterations were detected, indicating that all mice were able to move freely without neither paralysis nor major impairment in the coordination and locomotor functions. Thus, all mice were able to move freely without neither paralysis nor major impairment in the coordination and locomotor functions. These results justify that evoked pain responses, such as thermal hyperalgesia and mechanical allodynia, cannot be associated with locomotor impairment, as no significant locomotor deficits were observed during the study.

After checking that none of the animals had any significant impairment of coordination and locomotor functions, mechanical allodynia and thermal hyperalgesia were assessed. Referring to mechanical allodynia, data did not follow a normal distribution at any of the experimental time points (Kolmogorov-Smirnov test, all p's<0.05). Significant differences in the distribution of the data were found by Friedman's test (p<0.001) and further Kruskal-Wallis test showed significant differences between groups at all post-injury days (7, 14 and 21 dpi) (all p's<0.001). Specifically, subsequent Mann Whitney U test revealed significant mechanical sensitivity increase of SCI+Saline group when compared with the rest of experimental groups (all p's<0.05). As for the CE treatments, the SCI group treated with CE15 showed no significant differences with Sham at all experimental time points (p's>0.05), indicating that the CE15 dose completely prevented the development of mechanical allodynia after SCI throughout the acute phase of the injury. Otherwise, the SCI+CE10 group showed no significant difference in the mechanical paw withdrawal threshold compared to Sham at 7 dpi, but did at 14 and 21 dpi, indicating that the CE10 dose completely prevented the development of mechanical allodynia after SCI at 7 dpi, and attenuated it until the end of the experimental period (***Fig. 66. B***).

As to thermal hyperalgesia, data did not follow a normal distribution for all time-points of functional assessment (all p's<0.05) according to the Kolmogorov-Smirnov normality test. The non-parametric Friedman results significative (p<0.001) and further Kruskal-Wallis test indicated that these significant differences were present between groups at all post-SCI days (7, 14 and 21 dpi; all p's<0.001). Specifically, SCI+Saline group showed significant decrease of thermal paw withdrawal thresholds compared to Sham and CE treated groups (Mann Whitney U, all p's<0.05), thus indicating preventive effects on thermal hyperalgesia development for both CE tested doses. Contrarily to mechanical allodynia results, the SCI+CE10 group showed no significant differences between Sham at any of the experimental time-points (all p's>0.05), indicating that the CE10 dose completely prevented thermal hyperalgesia development after SCI. The dose of CE15 attenuated thermal hyperalgesia after SCI up to 21 dpi, showing significant differences with the Sham group (all p's<0.05; ***Fig. 66. C***).

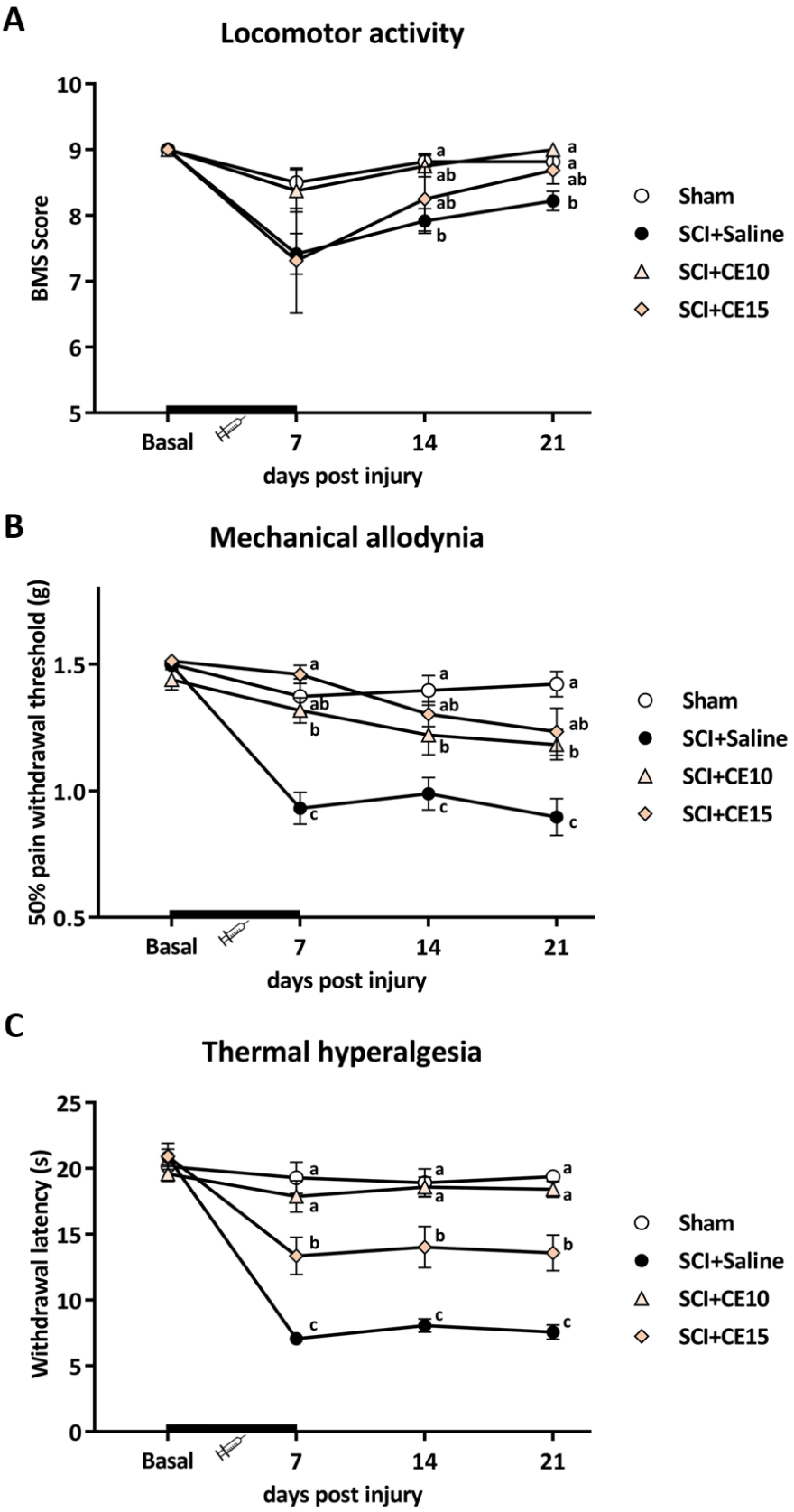


Figure 66. Time-course assessment of locomotor activity (A), mechanical allodynia (B) and thermal hyperalgesia (C) during the injury acute phase of SCI after preventive CE treatment (10 and 15 mg/kg). Each point and vertical line represent the mean ± SEM. Treatment administration week (basal to 7 dpi) is highlighted with a thick black line. Experimental groups: Sham (n=11), SCI+Saline (n=18), SCI+CE10 (n=8), SCI+CE15 (n=8).

Preventive CE treatment modulates astrogliosis and microgliosis in spinal cord of SCI mice.

Immunohistochemical and molecular analyses were performed to study astrogliosis induced by SCI and to assess whether CE treatment could prevent this phenomenon. After the incubation with anti-GFAP antibody on the histological spinal cord sections, the percentage of GFAP immunoreactivity area was quantified.

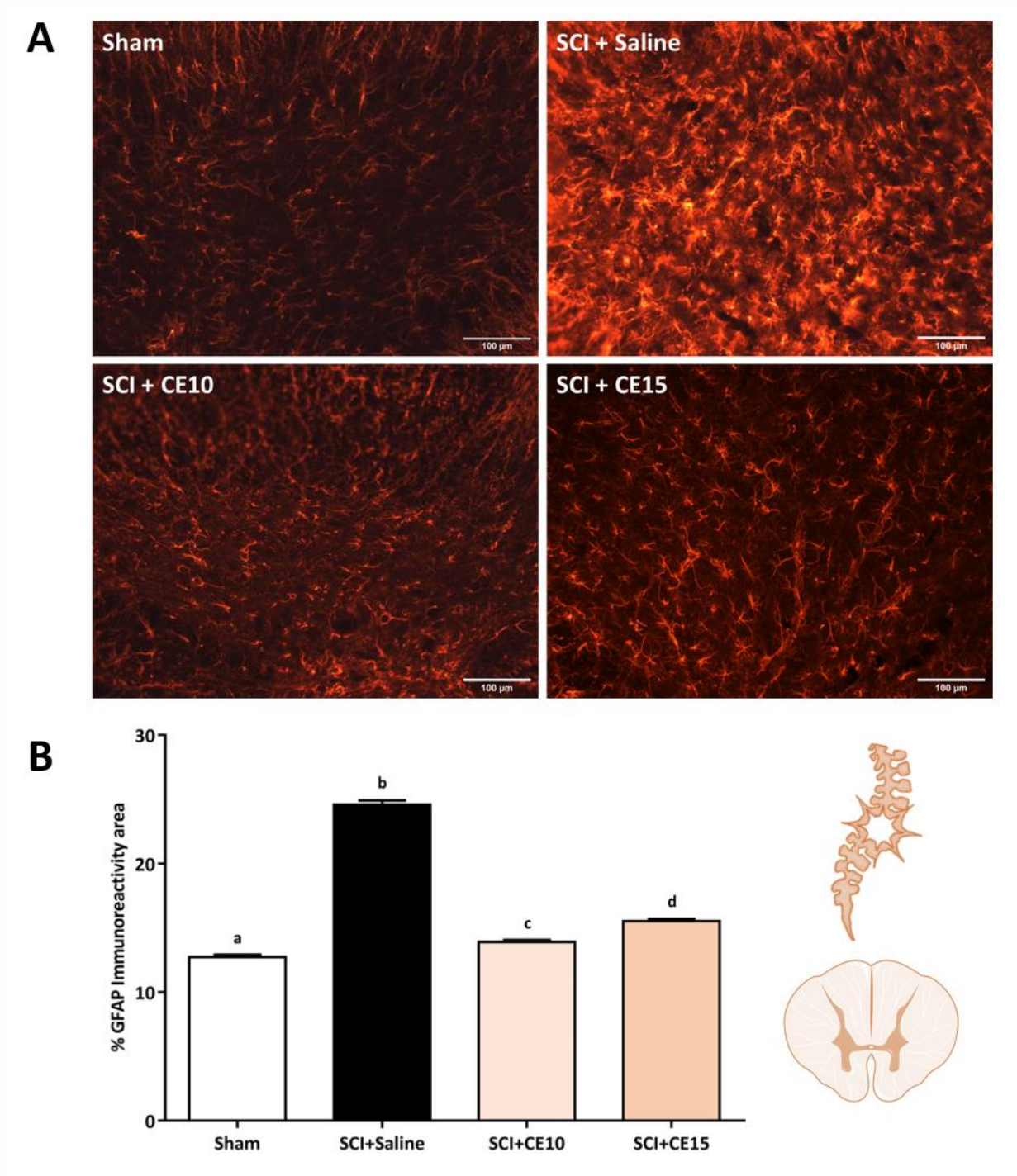


Figure 67. Effects of CE treatment (10 and 15 mg/kg) on spinal GFAP immunoreactivity area in SCI mice at the end of experimental period. (**A**) Representative histological images of the spinal cord immunostained against GFAP of each group (Scale bar 100 µm). (**B**) Histogram representing the percentage of dorsal horn GFAP-immunoreactivity. Data is expressed as mean ± SEM. a–d: Groups not sharing a letter showed significant differences, $p<0.05$. Experimental groups: Sham (n=4) (slices=60), SCI+Saline (n=4) (slices=62), SCI+CE10 (n=5) (slices=58), SCI+CE15 (n=4) (slices=67).

Immunohistochemical data did not follow a normal distribution according to the Shapiro-Wilk test ($p<0.001$). Thus, Kruskal-Wallis test was applied showing significant differences on %GFAP immunoreactivity area between all experimental groups (all p's<0.001). As expected, a significant increase in GFAP immunoreactivity area was found in the SCI+Saline group compared to Sham. Both CE treatment groups (SCI+CE10 and SCI+CE15) showed a significant reduction in GFAP immunoreactivity area compared to SCI+Saline, although none of them reached at Sham levels of astrogliosis. Importantly, CE10 dose significantly attenuated SCI-induced astrogliosis compared with CE15 dose (***Fig. 67. B***). Representative histological sections illustrating spinal GFAP immunostaining (***Fig. 67. A***) clearly show hypertrophy of astrocytic processes and soma and increased astrocytic proliferation in the SCI+Saline group compared to Sham and SCI+CE treatment groups.

To complement the immunohistochemical results beforementioned, western blot analysis of the total spinal GFAP protein was also performed. Non-parametric tests such as Kruskal-Wallis and Mann Whitney U were applied to analyse the obtained data since it did not follow a normal distribution (Shapiro-Wilk, $p<0.001$). As in the immunohistochemical results, the SCI+Saline group showed a significant increase in spinal GFAP expression compared to Sham ($p<0.001$). Both doses of CE completely prevented GFAP overexpression in SCI mice, showing no significant differences with the Sham group (p's>0.05; ***Fig. 68***).

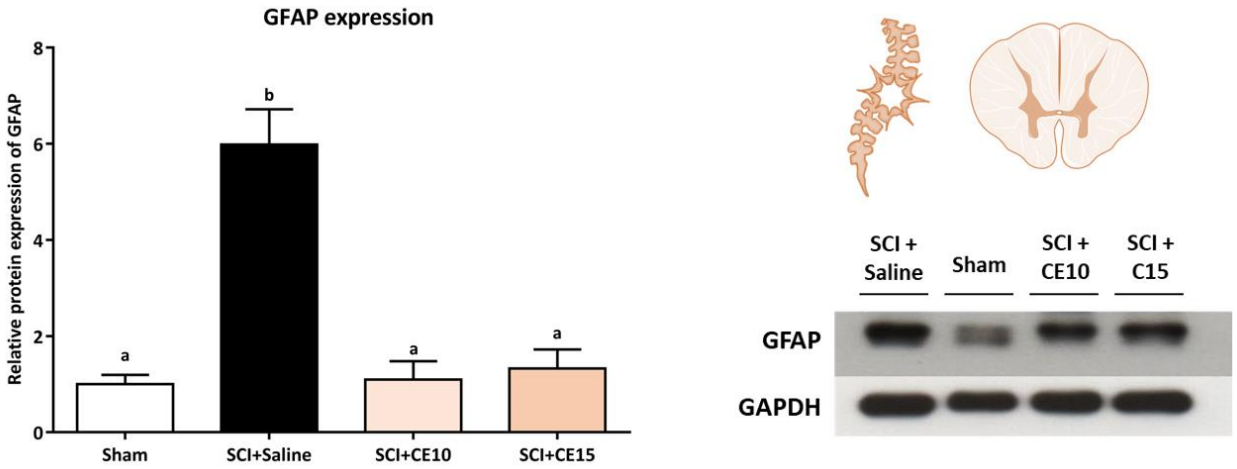


Figure 68. Spinal GFAP expression after preventive CE treatment (10 and 15 mg/kg) on spinal cord injured mice at the end of experimental period. Protein expression was normalized to GAPDH. Data is expressed as a relative percentage respect to Sham group (mean ± SEM). a–b: Groups not sharing a letter showed significant differences, $p<0.05$. Experimental groups: Sham (n=10), SCI+Saline (n=9), SCI+CE10 (n=5), SCI+CE15 (n=5).

In order to evaluate the effects of CE treatment on spinal microgliosis after SCI, immunohistochemical and molecular analysis were performed. After incubation of anti-IBA1 antibody on histological spinal cord sections, reactive and non-reactive microglia cells were counted and expressed as a percentage of two phenotypes. Once it was confirmed that the data did not follow a normal distribution (Shapiro-Wilk, $p<0.001$), non-parametric tests have been applied. Kruskal-Wallis indicated significant differences between

all groups (p<0.001) in both, percentage of reactive and non-reactive microglia cells. Specifically, SCI+Saline group showed a significant increase of percentage of reactive microglia cells compared to Sham (p<0.001). Regarding CE treatment, both doses clearly reduced the percentage of reactive cells compared to non-treated SCI animals (p's<0.05 vs. SCI+Saline) but without reaching the percentage level of the Sham group (p's<0.05 vs. Sham). Importantly, the dose of CE10 showed a significant effect in reducing the percentage of reactive cells compared to CE15 dose (p<0.05). Finally, Kruskal-Wallis test indicated statistical intra-group differences between reactive and non-reactive microglia cells for all experimental groups (all p's<0.001; ***Fig. 69. B***). Moreover, representative histological sections illustrating spinal IBA1 immunostaining (***Fig. 69. A***) show that SCI+Saline group presents more reactive-like microglial cells, with an amoeboid form and less and shorter branching processes, compared to Sham and the SCI groups treated with CE10 or CE15.

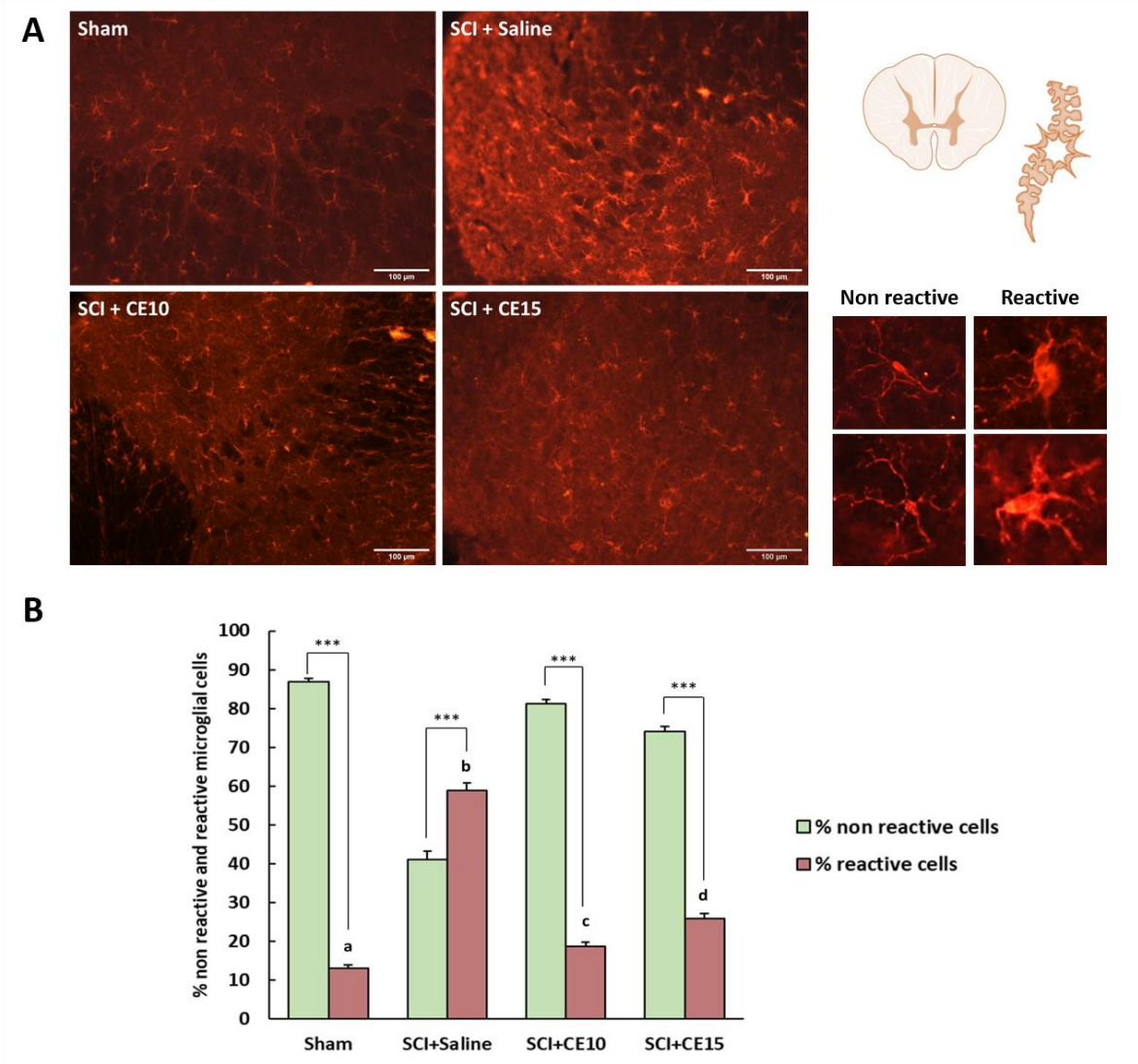


Figure 69. Effects of CE treatment (10 and 15 mg/kg) on spinal IBA1 immunoreactivity in SCI mice at the end of experimental period. (**A**) Representative histological images of the spinal cord immunostained against IBA1 of each group (Scale bar 100 µm) and examples of non-reactive and reactive microglial cells. Note that reactive cells have an amoeboid form, and a larger nucleus and shorter branching processes compared to non-reactive cells. (**B**) Histogram representing the percentage of reactive and non-reactive microglia cells in the spinal dorsal horn. Data is expressed as mean ± SEM. a–d: Groups not sharing a letter showed significant differences in %reactive cells, p<0.05. Intra-groups significant differences: *** p<0.001 %non-reactive vs %reactive. Experimental groups: Sham (n=4) (slices=60), SCI+Saline (n=4) (slices=62), SCI+CE10 (n=5) (slices=67), SCI+CE15 (n=4) (slices=57).

In addition to the immunohistochemical study of microgliosis, molecular analysis of total spinal IBA1 protein was also performed. After Shapiro-Wilk test confirmed that data did not follow a normal distribution ($p<0.01$), Kruskal-Wallis test was applied indicating significant differences in IBA1 protein expression between all groups ($p<0.001$). Similar to the immunohistochemical results, SCI+Saline group showed significant increase in IBA1 expression compared to Sham ($p<0.001$). In parallel, both doses of CE significantly reduced IBA1 overexpression shown in the SCI+Saline group ($p's<0.01$) although neither of the CE-treated groups reached the IBA1 expression levels of Sham ($p's<0.05$; ***Fig. 70***).

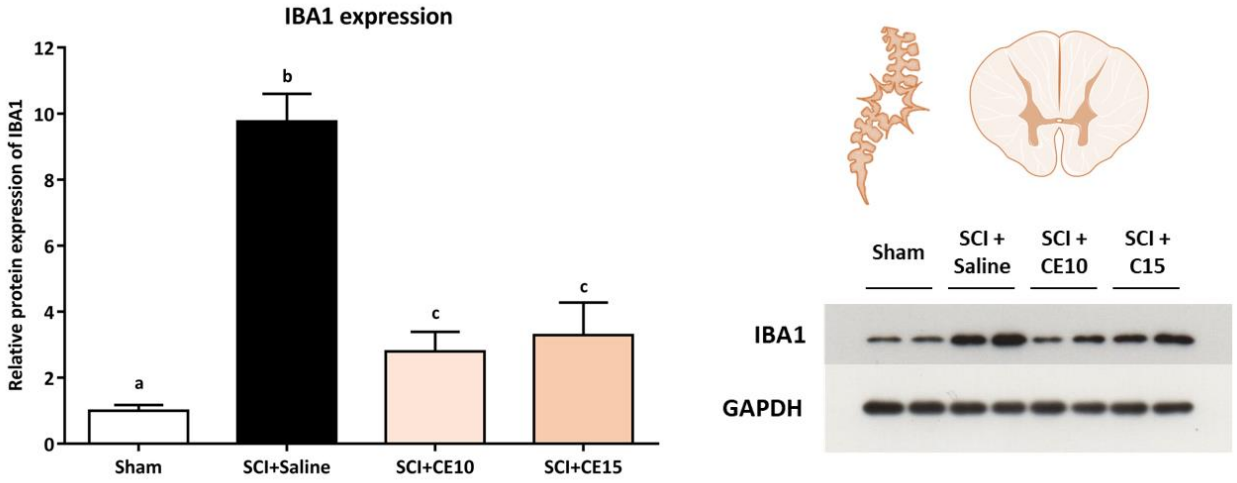


Figure 70. Spinal IBA1 expression after preventive CE treatment (10 and 15 mg/kg) on spinal cord injured mice at the end of experimental period. Protein expression was normalized to GAPDH. Data is expressed as a relative percentage respect to Sham group (mean ± SEM). a–c: Groups not sharing a letter showed significant differences, $p<0.05$. Experimental groups: Sham (n=8), SCI+Saline (n=7), SCI+CE10 (n=4), SCI+CE15 (n=4).

Preventive CE treatment modulates chemokines expression in spinal cord of SCI mice.

The above results indicating that CE administration attenuates spinal gliosis in SCI mice lead to the hypothesis that the same treatment may modulate the release or expression of chemokines and their receptors in the spinal cord. Therefore, molecular experiments were performed to elucidate the expression of chemokines (MCP1/CCL2 and CX3CL1) and their receptors (CCR2 and C3XCR1) in the spinal cord, since, as mentioned before, they are potential candidates for integrating inflammation and central neuropathic pain after SCI.

On the one hand, Shapiro-Wilk test confirmed that western blot data for MCP1/CCL2 expression followed a normal distribution ($p>0.05$). Thus, ANOVA was applied indicating no significant differences in MCP1/CCL2 expression between the experimental groups ($p>0.05$, ***Fig. 71. A***). In contrast, CCR2 data did not follow a normal distribution (Shapiro-Wilk, $p<0.05$) and further Kruskal-Wallis test indicated significant differences in its expression between groups ($p<0.01$). Specifically, CCR2 was significant overexpressed in SCI+Saline

group compared to the rest of experimental groups (p's<0.01) and SCI+CE10 and SCI+CE15 mice significantly reached at least the CCR2 expression levels of Sham animals (p<0.05; ***Fig. 71. B***).

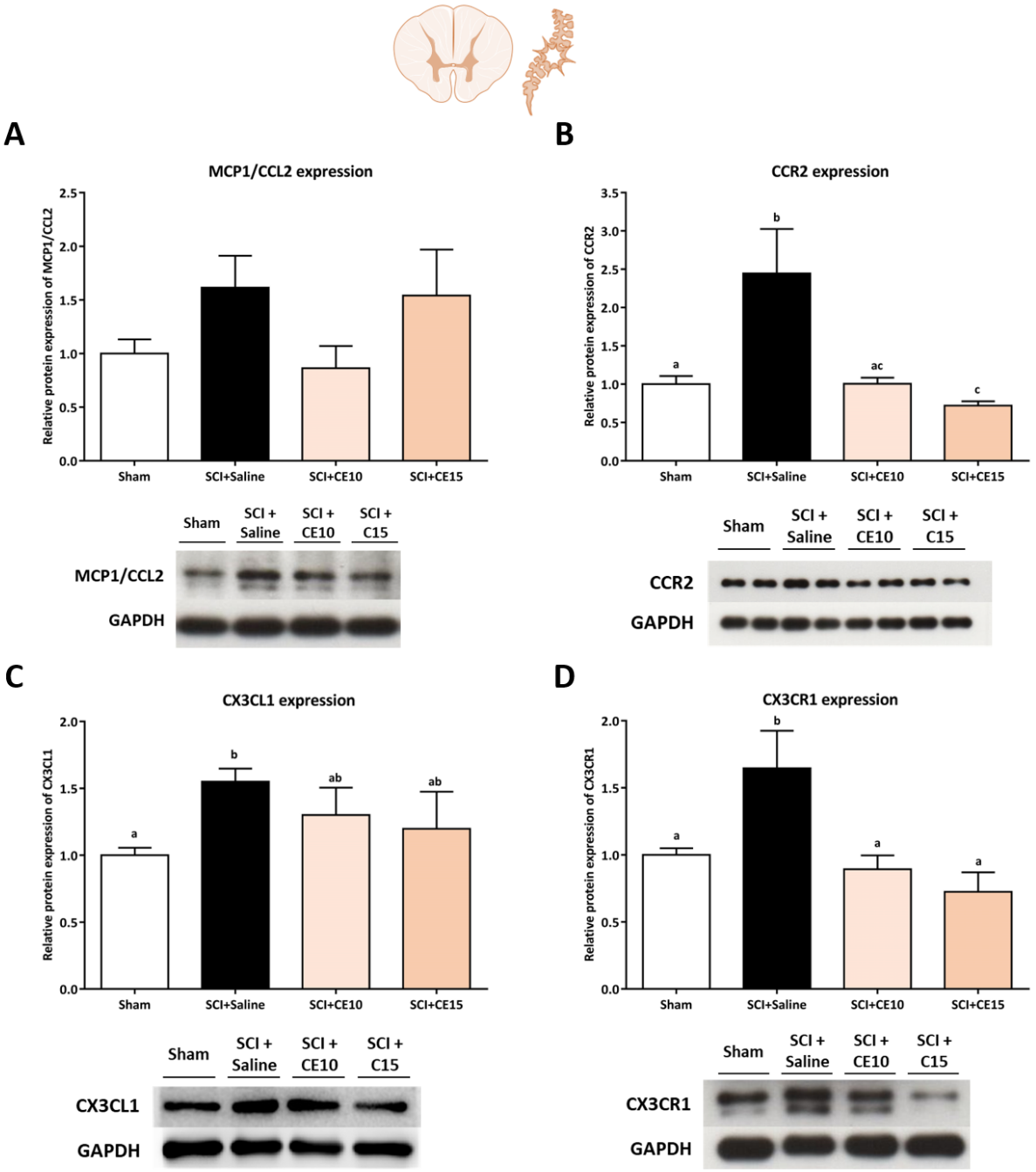


Figure 71. Spinal expression of chemokines (MCP1/CCL2 and CX3CL1) and their receptors (CCR2 and CX3CR1) after preventive CE treatment (10 and 15 mg/kg) on spinal cord injured mice at the end of experimental period. Protein expression was normalized to GAPDH. Data is expressed as a relative percentage respect to Sham group (mean ± SEM). a–c: Groups not sharing a letter showed significant differences, p<0.05. Experimental groups: Sham (MCP1/CCL2 n=9; CCR2 n=10; CX3CL1 n=9; CX3CR1 n=8), SCI+Saline (MCP1/CCL2 n=9; CCR2 n=9; CX3CL1 n=8; CX3CR1 n=7), SCI+CE10 (MCP1/CCL2 n=4; CCR2 n=5; CX3CL1 n=5; CX3CR1 n=5), SCI+CE15 (MCP1/CCL2 n=4; CCR2 n=5; CX3CL1 n=5; CX3CR1 n=5).

On the other hand, data obtained for CX3CL1 expression followed a normal distribution (Shapiro-Wilk, $p>0.05$) and in the subsequent ANOVA, significant differences were found between groups ($p<0.05$). However, these differences were only found between the Sham and SCI+Saline groups, the latter showing a significant increase in CX3CL1 expression. In contrast, both groups of CE treatment showed no significant differences with either the Sham or SCI+Saline groups (all p's>0.05; ***Fig. 71. C***). Data obtained for CX3CR1 expression did not follow a normal distribution (Shapiro-Wilk, $p<0.05$) and subsequent Kruskal-Wallis test indicated significant group differences ($p<0.05$). Specifically, post-hoc analysis indicated a significant overexpression of CX3CR1 in SCI+Saline group when compared with the rest of experimental groups ($p<0.05$). Indeed, both tested doses of CE treatment (CE10 and CE15) significantly prevented CX3CR1 overexpression at Sham levels, showing no significant differences between the control group (p's>0.05; ***Fig. 71. D***).

Preventive CE treatment do not downregulate ERK phosphorylation in spinal cord of SCI mice.

The results obtained in the Chapter 3 of the present thesis indicated that GSE treatment can modulate ERK1/2 phosphorylation in spinal cord after SCI. To elucidate whether the treatment with CE can also modulate the phosphorylation of this central sensitization-related biomarker, western blot analysis against spinal ERK1/2 and pERK1/2 were performed.

Data obtained for ERK1/2 and pERK1/2 expression followed a normal distribution according to the Shapiro-Wilk test ($p>0.05$). On further ANOVA, significant group differences were detected ($p<0.05$) and post-hoc analysis indicated that these differences were present between the Sham and SCI+Saline groups. In particular, SCI+Saline group showed a significant increase in pERK1/2 in comparison to Sham group ($p<0.05$). However, none of the CE treatments succeeded in reducing ERK1/2 phosphorylation to Sham levels, showing no significant differences with either the Sham or SCI+Saline groups (all p's>0.05; ***Fig. 72***).

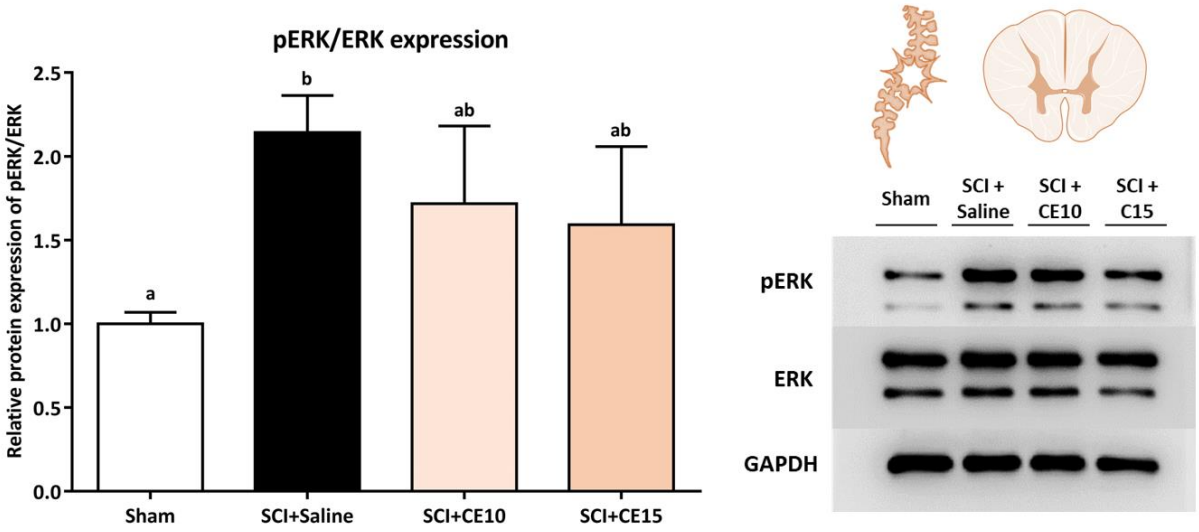


Figure 72. Spinal levels of ERK phosphorylation after preventive CE treatment (10 and 15 mg/kg) on spinal cord injured mice at the end of experimental period. Protein expression was normalized to GAPDH. Data is expressed as a relative percentage respect to Sham group (mean ± SEM). a–b: Groups not sharing a letter showed significant differences, $p<0.05$. Experimental groups: Sham (n=9), SCI+Saline (n=8), SCI+CE10 (n=5), SCI+CE15 (n=4).

Preventive CE treatment modulates astrogliosis and microgliosis in ACC of SCI mice.

To elucidate whether CE treatment can prevent gliosis in the ACC, which as mentioned before is an important contributor to the development and maintenance of severe neuropathic pain after SCI, immunohistochemical and western blot analyses were performed. After incubation with anti-GFAP antibody on histological sections of ACC, the proportion of GFAP immunoreactivity area was quantified. According to the Shapiro-Wilk normality test, data obtained did not follow a normal distribution ($p<0.05$) and subsequent Kruskal-Wallis test indicated significant differences between groups on %GFAP immunoreactivity area ($p<0.001$). As expected, SCI+Saline group showed a significant increase in GFAP immunoreactivity proportion compared to Sham ($p<0.001$), thus indicating that SCI induces astrogliosis in ACC during the acute phase of SCI-induced neuropathic pain. As to CE treatments, both doses (CE10 and CE15) significantly reduced GFAP immunoreactivity proportion in ACC compared to SCI+Saline group. Moreover, CE10 treatment was successful in preventing astrogliosis in ACC at sham levels, showing no significant differences between the control group ($p>0.05$ vs. Sham; ***Fig. 73. B***).

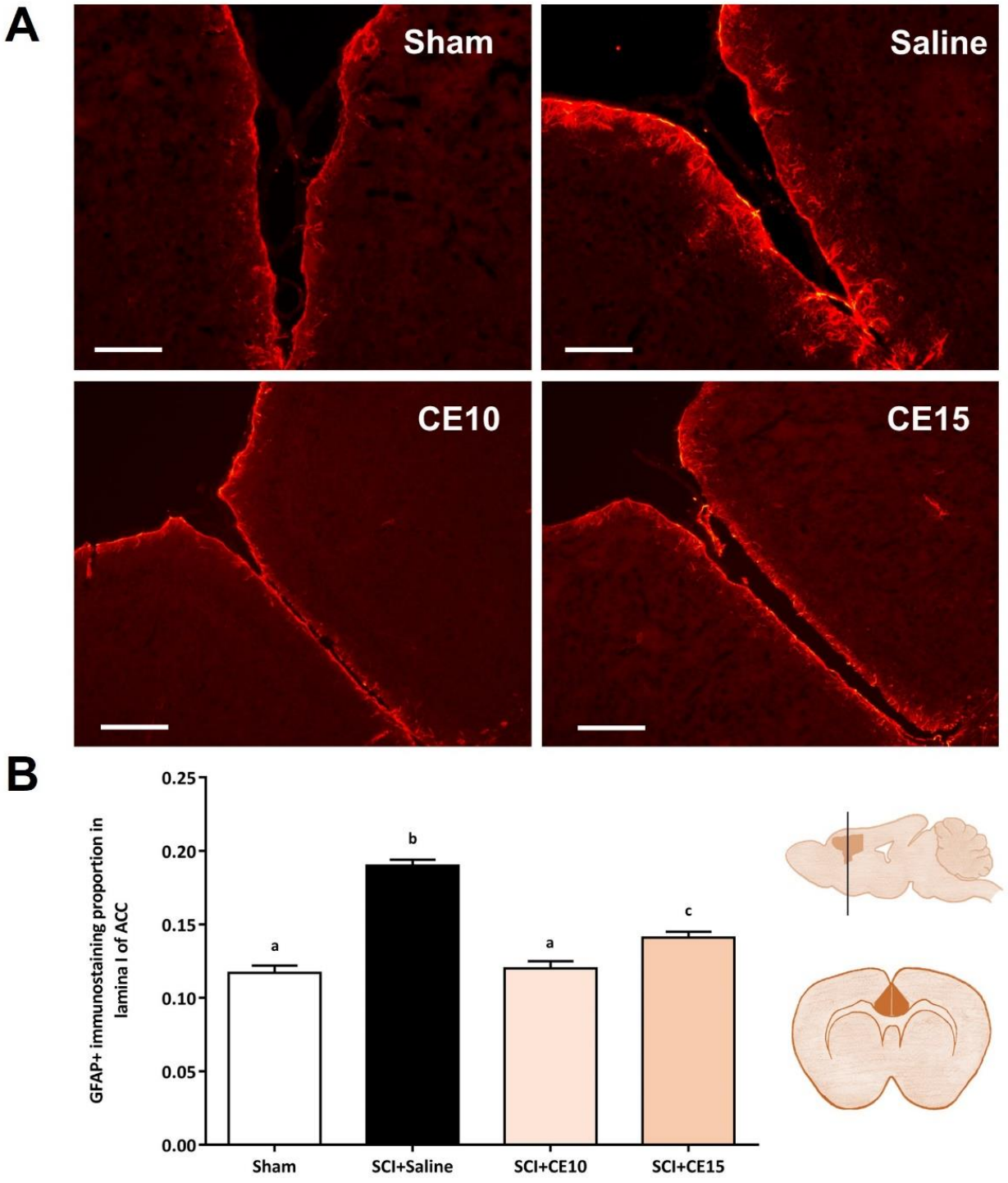


Figure 73. Effects of CE treatment (10 and 15 mg/kg) on GFAP immunoreactivity proportion in ACC of SCI mice at the end of experimental period. (**A**) Representative histological images of the ACC immunostained against GFAP of each group (Scale bar 200 µm). (**B**) Histogram representing the proportion of GFAP-immunoreactivity in ACC. Data is expressed as mean ± SEM. a–c: Groups not sharing a letter showed significant differences, $p<0.05$. Experimental groups: Sham (n=5), SCI+Saline (n=5), SCI+CE10 (n=5), SCI+CE15 (n=5).

In addition, western blot analysis was performed to elucidate total GFAP protein in ACC. The Shapiro-Wilk test showed that the data obtained did not follow a normal distribution ($p<0.05$) and the Kruskal-Wallis test

indicated significant differences between groups in GFAP expression ($p<0.01$). As in the immunohistochemical results, the SCI+Saline group showed a significant GFAP overexpression compared to Sham ($p>0.05$) and both CE treatments significantly reduced this expression ($p's<0.05$ vs. SCI+Saline). On this occasion, CE15 treatment reduced GFAP expression to Sham levels ($p>0.05$) and the SCI+CE10 group showed even lower GFAP expression than the Sham group ($p<0.05$) (***Fig. 74***).

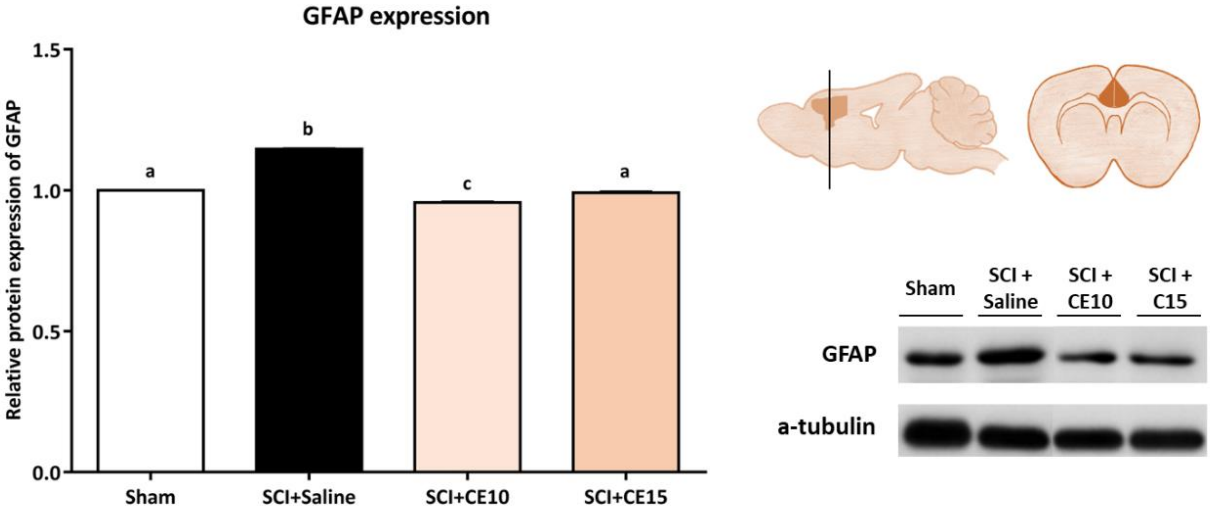


Figure 74. GFAP expression in ACC after preventive CE treatment (10 and 15 mg/kg) on spinal cord injured mice at the end of experimental period. Protein expression was normalized to a-tubulin. Data is expressed as a relative percentage respect to Sham group (mean ± SEM). a–c: Groups not sharing a letter showed significant differences, $p<0.05$. Experimental groups: Sham (n=4), SCI+Saline (n=4), SCI+CE10 (n=4), SCI+CE15 (n=4).

To study the microgliosis, anti-IBA1 antibody was incubated on histological sections of ACC and the proportion of IBA1 immunoreactivity area was then quantified. Significant differences between groups on IBA1 immunoreactivity in ACC were found by Kruskal-Wallis test ($p<0.001$) since data did not follow a normal distribution (Shapiro-Wilk, $p<0.001$). Specifically, SCI+Saline group showed a significant increase of ACC IBA1 immunoreactivity proportion compared to Sham ($p<0.05$) indicating that SCI induced microgliosis in ACC. Both doses of CE treatment reduced this microgliosis ($p<0.05$ vs. Sham; ***Fig. 75. B***).

To complete the study of microgliosis in ACC, western blot analysis of total IBA1 protein was also performed. According to the Shapiro-Wilk test, data did not follow a normal distribution. Thus, the non-parametric test of Kruskal-Wallis was applied indicating significant differences between groups ($p<0.01$) in IBA1 expression. As in immunohistochemical results abovementioned, SCI+Saline group showed a significant IBA1 overexpression in ACC compared to Sham ($p<0.05$) and both SCI+CE-treated groups significantly reduced this overexpression at least at Sham levels (***Fig. 76***).

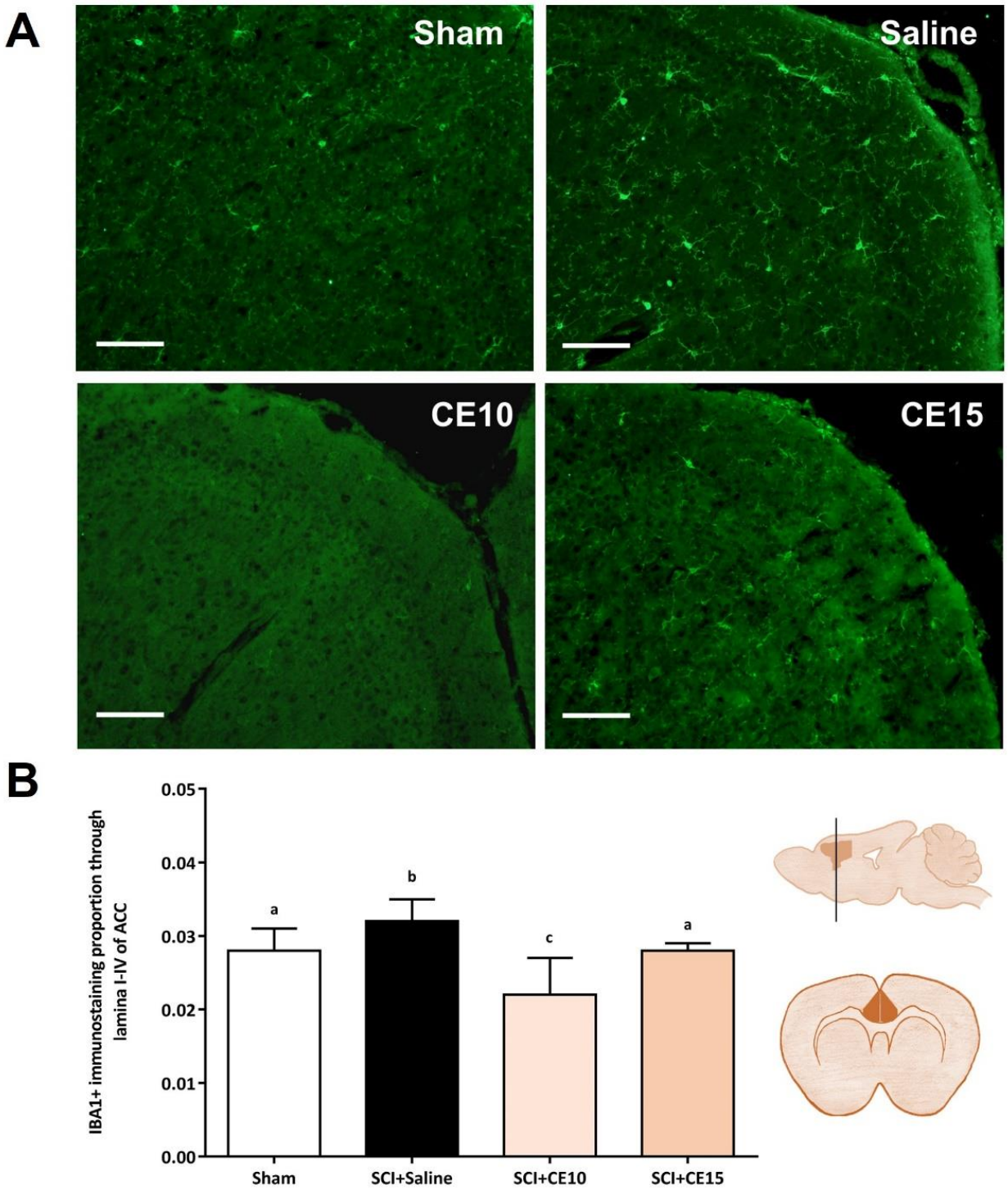


Figure 75. Effects of CE treatment (10 and 15 mg/kg) on IBA1 immunoreactivity proportion in ACC of SCI mice at the end of experimental period. (**A**) Representative histological images of the ACC immunostained against IBA1 of each group (Scale bar 200 µm). (**B**) Histogram representing the proportion of IBA1-immunoreactivity in ACC. Data is expressed as mean ± SEM. a–c: Groups not sharing a letter showed significant differences, $p<0.05$. Experimental groups: Sham (n=5), SCI+Saline (n=5), SCI+CE10 (n=5), SCI+CE15 (n=5).

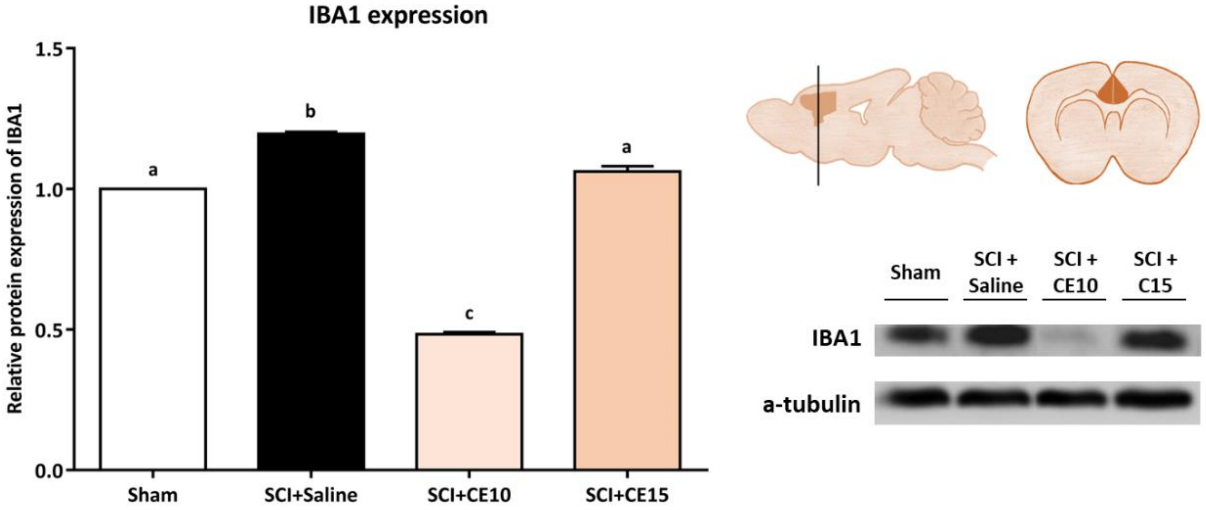


Figure 76. IBA1 expression in ACC after preventive CE treatment (10 and 15 mg/kg) on spinal cord injured mice at the end of experimental period. Protein expression was normalized to a-tubulin. Data is expressed as a relative percentage respect to Sham group (mean ± SEM). a–c: Groups not sharing a letter showed significant differences, $p<0.05$. Experimental groups: Sham (n=5), SCI+Saline (n=5), SCI+CE10 (n=5), SCI+CE15 (n=5).

The dose of CE15 treatment modulates CX3CL1 expression in ACC of SCI mice.

The expression of fractalkine CX3CL1 and its receptor CX3CR1 in ACC was also analyzed to determine whether CE treatment could modulate their expression. According to the Shapiro-Wilk test, data obtained for CX3CL1 expression in ACC did not follow a normal distribution ($p<0.05$) whereas data for CX3CR1 followed a normal distribution ($p>0.05$). Referring to fractalkine, Kruskal-Wallis test indicated significant differences between groups ($p<0.01$). CX3CL1 was significant overexpressed in ACC of SCI+Saline animals compared to Sham ($p<0.05$) and only the CE15 dose was able to reduce this expression below Sham levels (***Fig. 77. A***). As to CX3CR1, significant group differences were evidenced by ANOVA analysis ($p<0.01$) and post-hoc Duncan test indicated that these differences were between Sham and the rest of experimental groups (p's<0.05). Thus, SCI induced CX3CR1 overexpression in ACC and none of the CE treatment groups were able to reduce its expression (***Fig. 77. B***).

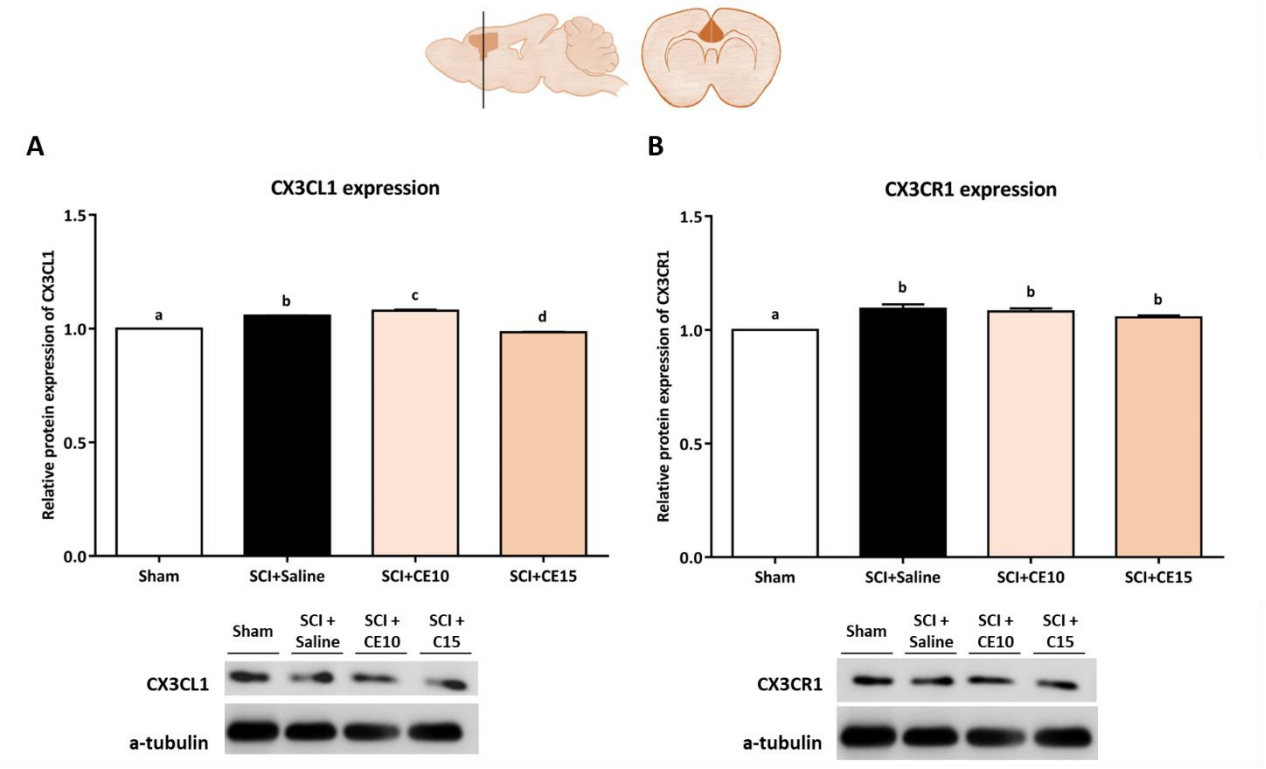


Figure 77. CX3CL1 and CX3CR1 expression in ACC after preventive CE treatment (10 and 15 mg/kg) on spinal cord injured mice at the end of experimental period. Protein expression was normalized to a-tubulin. Data is expressed as a relative percentage respect to Sham group (mean ± SEM). a–d: Groups not sharing a letter showed significant differences, $p<0.05$. Experimental groups: Sham (CX3CL1 n=4; CX3CR1 n=4), SCI+Saline (CX3CL1 n=4; CX3CR1 n=4), SCI+CE10 (CX3CL1 n=4; CX3CR1 n=4), SCI+CE15 (CX3CL1 n=4; CX3CR1 n=4).

Preventive CE treatment reduces astrogliosis and induces microgliosis in PAG of SCI mice.

After elucidating that SCI induces astroglial activation in PAG in the previous chapter, we assessed whether CE treatment was able to modulate it. For this purpose, immunohistochemical and molecular techniques such as immunofluorescence labelling and western blotting were used to detect GFAP in the PAG of animals.

Immunohistochemical data for astrogliosis in PAG did not follow a normal distribution according to the Shapiro-Wilk test ($p<0.001$). Thus, Kruskal-Wallis test was applied indicating significant differences between groups ($p<0.001$). Both doses of CE treatment (CE10 and CE15) significantly prevented the astrogliosis observed in the SCI+Saline group. In particular, the SCI+CE10 group showed a lower GFAP immunoreactivity than the SCI+CE15 group, although neither treatment group reached Sham levels of GFAP immunoreactivity (***Fig. 78. B***).

As to western blot results of the total GFAP protein expression in PAG, data followed a normal distribution (Shapiro-Wilk, $p>0.05$). ANOVA test indicated significant differences between groups ($p<0.001$) and further post-hoc analysis of Duncan showed that CE10 treatment dose reduced GFAP expression in PAG at Sham levels ($p>0.05$) whereas CE15 dose did not differed from SCI+Saline levels ($p>0.05$; ***Fig. 79***).

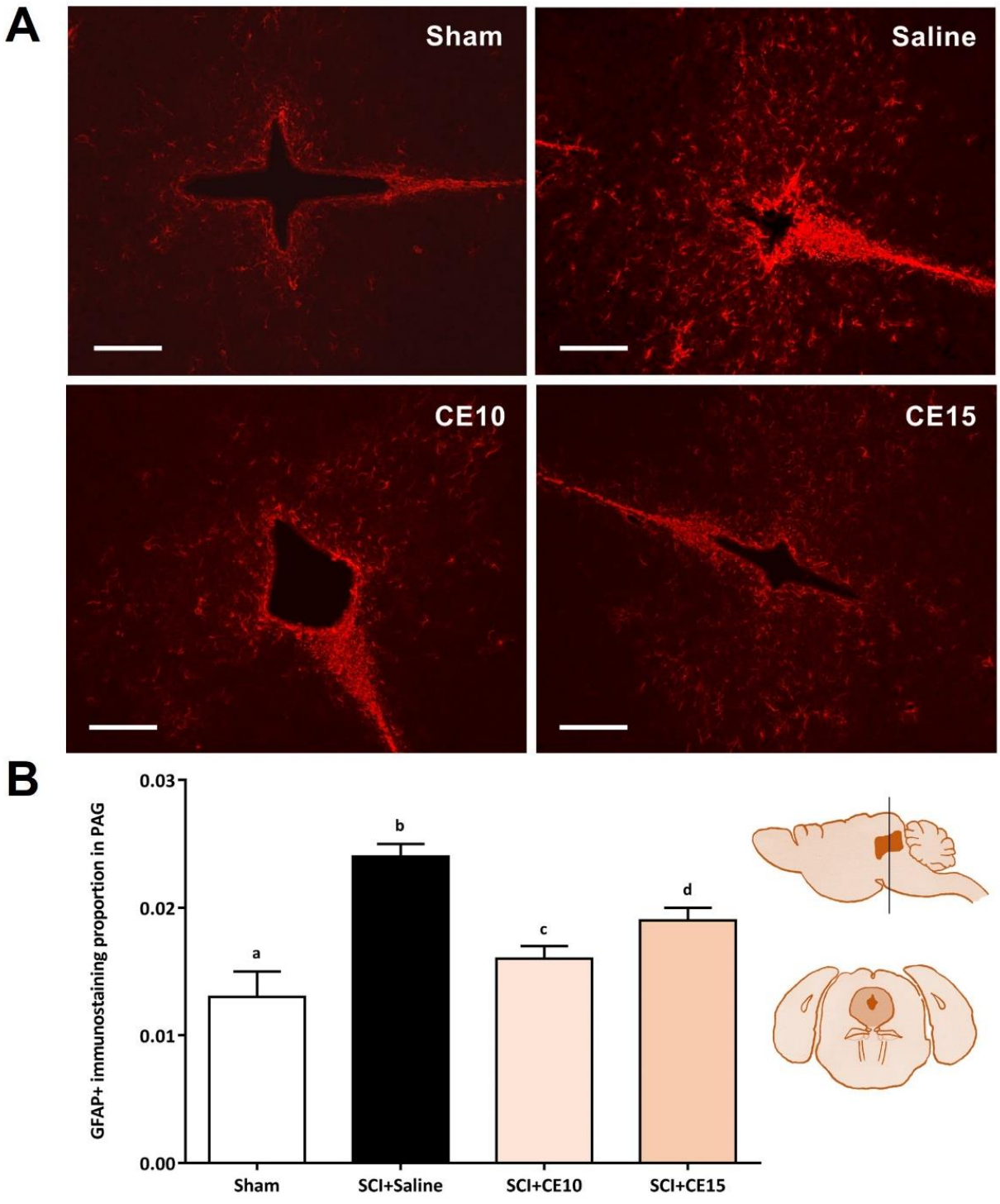


Figure 78. Effects of CE treatment (10 and 15 mg/kg) on GFAP immunoreactivity proportion in PAG of SCI mice at the end of experimental period. (**A**) Representative histological images of the PAG immunostained against GFAP of each group (Scale bar 200 µm). (**B**) Histogram representing the proportion of GFAP-immunoreactivity in PAG. Data is expressed as mean ± SEM. a–d: Groups not sharing a letter showed significant differences, $p<0.05$. Experimental groups: Sham (n=5), SCI+Saline (n=5), SCI+CE10 (n=5), SCI+CE15 (n=5).

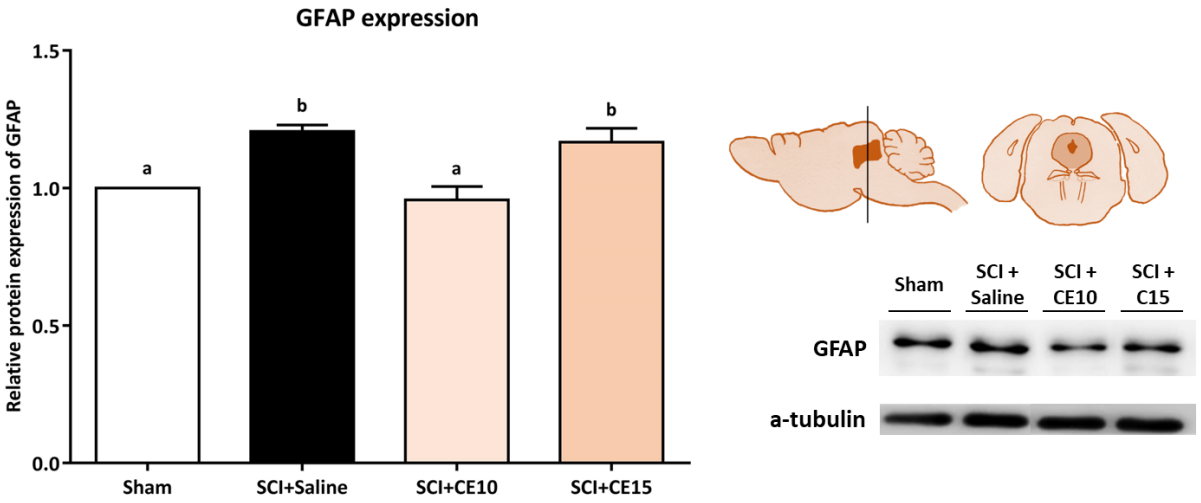


Figure 79. GFAP expression in PAG after preventive CE treatment (10 and 15 mg/kg) on spinal cord injured mice at the end of experimental period. Protein expression was normalized to a-tubulin. Data is expressed as a relative percentage respect to Sham group (mean ± SEM). a–b: Groups not sharing a letter showed significant differences, $p<0.05$. Experimental groups: Sham (n=5), SCI+Saline (n=5), SCI+CE10 (n=5), SCI+CE15 (n=5).

Referring to microgliosis in PAG, immunohistochemical data for IBA1 did not follow a normal distribution (Shapiro-Wilk, $p>0.05$). Thus, Kruskal-Wallis test was applied, indicating significant group differences in IBA1 immunostained proportion in PAG ($p<0.001$). As we proved in the previous Chapter, SCI did not induce microgliosis in PAG since the proportion of IBA1 immunoreactivity of SCI+Saline group did not differ to the Sham group ($p>0.05$). In contrast, the two SCI+CE groups showed a significant increase in IBA1 immunoreactivity compared to the control groups (p's<0.05 vs. Sham and SCI+Saline), indicating that both CE10 and CE15 treatments induced microgliosis in the PAG of SCI mice (***Fig. 80. B***).

Western blot data for IBA1 expression in the PAG were analysed by non-parametric tests, as they did not follow a normal distribution (Shapiro-Wilk, $p<0.01$). Kruskal-Wallis test showed significant group differences ($p<0.001$) and Mann Whitney U test indicated a significant downregulation of IBA1 expression in SCI+Saline group compared to the rest of experimental groups (all p's<0.01). Moreover, both CE10 and CE15 doses induced overexpression of IBA1 in PAG of SCI mice compared to both Sham and SCI+Saline groups (p's<0.001). It is worth mentioning that SCI+CE10 group showed significantly higher levels of IBA1 in PAG compared to SCI+CE15 group ($p<0.05$). In summary, SCI induced downregulation of IBA1 protein in PAG while CE treatments promoted the overexpression of such protein in SCI animals (***Fig. 81***).

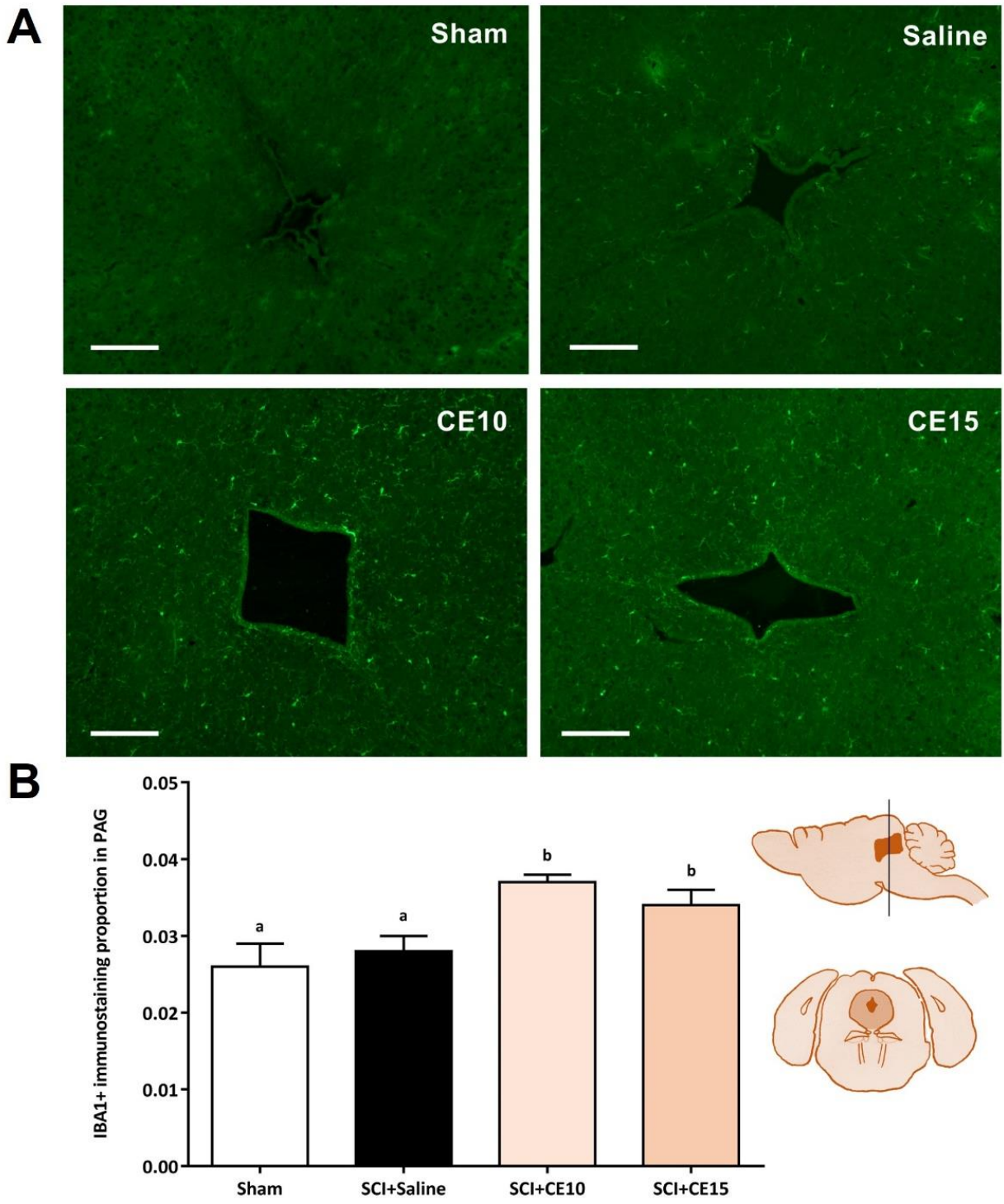


Figure 80. Effects of CE treatment (10 and 15 mg/kg) on IBA1 immunoreactivity proportion in PAG of SCI mice at the end of experimental period. (**A**) Representative histological images of the PAG immunostained against IBA1 of each group (Scale bar 200 µm). (**B**) Histogram representing the proportion of IBA1-immunoreactivity in PAG. Data is expressed as mean ± SEM. a–b: Groups not sharing a letter showed significant differences, $p<0.05$. Experimental groups: Sham (n=5), SCI+Saline (n=5), SCI+CE10 (n=5), SCI+CE15 (n=5).

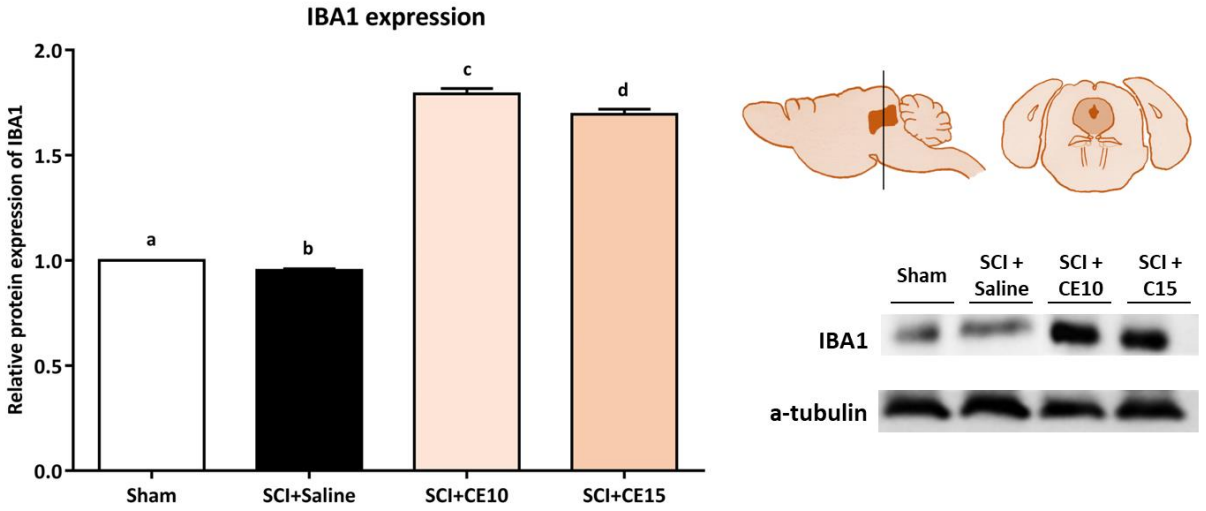
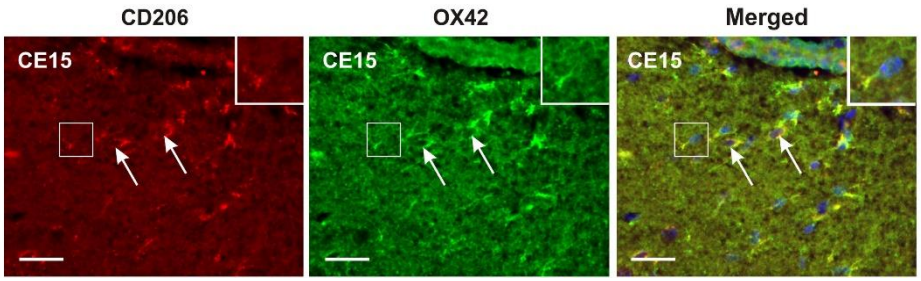


***Figure 81. IBA1 expression in ACC after preventive CE treatment (10 and 15 mg/kg) on spinal cord injured mice at the end of experimental period*.** Protein expression was normalized to a-tubulin. Data is expressed as a relative percentage respect to Sham group (mean ± SEM). a–d: Groups not sharing a letter showed significant differences, p<0.05. Experimental groups: Sham (n=5), SCI+Saline (n=5), SCI+CE10 (n=5), SCI+CE15 (n=5).

Moreover, immunohistochemical analysis for OX-42 and CD206 markers were performed in order to identify the phenotype of microglial cells that were increased in PAG. Thus, the microglial phenotype was characterized for CE15 group, and the results showed that the percentage of CD206-positive cells in the PAG of animals treated with CE15 was significantly higher than in the PAG of Sham-operated animals (p<0.01). Therefore, CE15 induced microglial activation characterized by an increase in OX-42 positive cells and a large proportion of cells with a CD206-positive M2 phenotype (***Fig. 82***).



Percentage of CD206/OX42 immunopositive microglial cells	
Sham	15.2 ± 2.6
SCI+CE15	23 ± 3.7*

Figure 82. Percentage of CD206 immunopositive OX42 immunostained microglial cells in PAG of animals with SCI and treated with CE (15 mg/kg). *p<0.01 when compared with Sham. Representative pictures illustrating double immunostaining of OX42 immunopositive microglial cells and CD206 in the section through PAG of SCI mouse and treated with CE15. Merged green (OX42-FITC) and red (CD206-TRITC) immunofluorescence and blue Hoechst nuclear staining indicate position of OX42+/CD206+ microglial cells. Scale bar: 50µm. Experimental groups: Sham (n=3) and SCI+CE15 (n=3).

Preventive CE treatment modulates CX3CL1/CX3CR1 expression in PAG of SCI mice.

To elucidate whether treatment with CE can modulate the expression of fractalkine (CX3CL1) and its receptor (CX3CR1) in PAG of SCI mice, western blot analyses were performed. Data for both CX3CL1 and CX3CR1 expression in PAG did not follow a normal distribution (Shapiro-Wilk, $p<0.05$). For this reason, they were analysed by Kruskal-Wallis test, indicating significant differences between groups ($p<0.001$). Both CX3CL1 and CX3CR1 were overexpressed in SCI+Saline group compared to Sham ($p's<0.05$) and both doses of CE treatment reduced their expression in comparison to SCI+Saline group ($p's<0.05$). On the one hand, CE15 dose reduced CX3CL1 expression to the Sham level, while CE10 dose reduced it below the Sham level. On the other hand, the CE15 dose reduced CX3CR1 expression compared to the SCI+Saline group, but did not reach Sham expression level, while the CE10 dose reduced it to the Sham level. Therefore, these results indicated that SCI induced overexpression of both CX3CL1 and CX3CR1 in PAG, while CE treatments prevented overexpression of these proteins (***Fig. 83. A, B***).

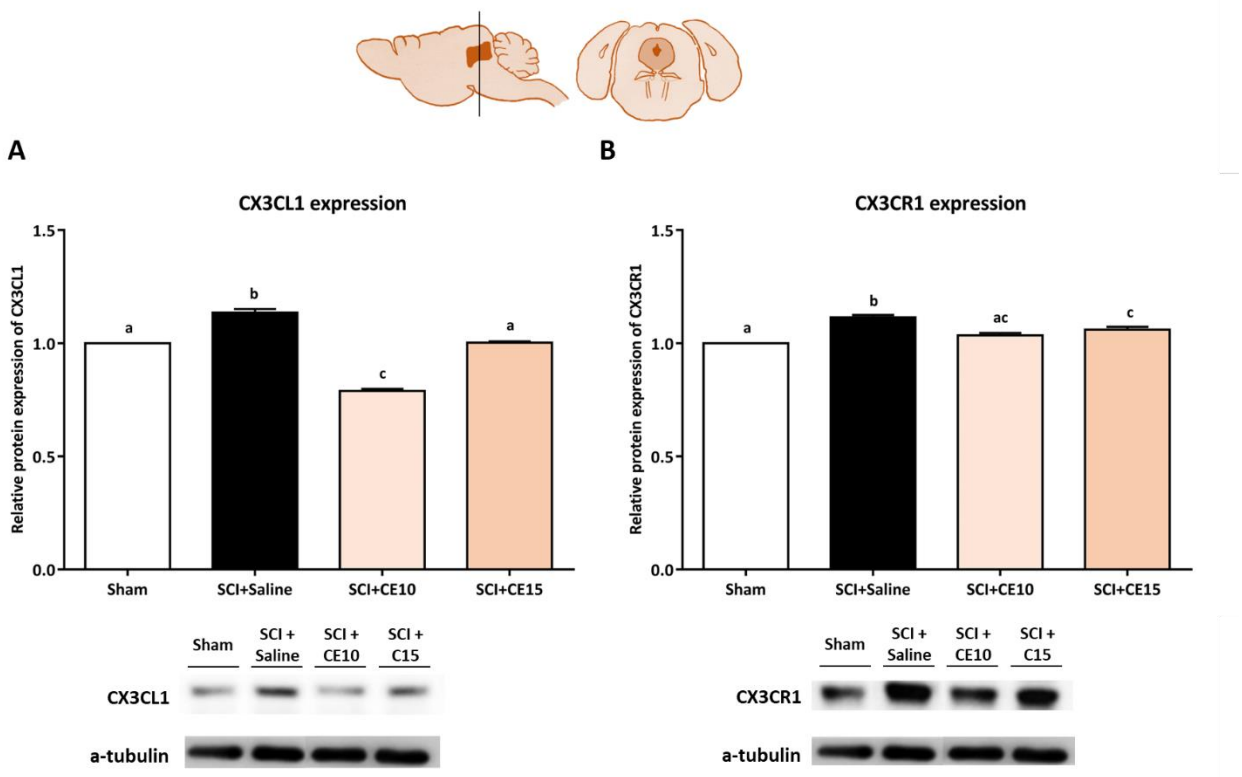


Figure 83. CX3CL1 and CX3CR1 expression in PAG after preventive CE treatment (10 and 15 mg/kg) on spinal cord injured mice at the end of experimental period. Protein expression was normalized to a-tubulin. Data is expressed as a relative percentage respect to Sham group (mean ± SEM). a–c: Groups not sharing a letter showed significant differences, $p<0.05$. Experimental groups: Sham (CX3CL1 n=6; CX3CR1 n=6), SCI+Saline (CX3CL1 n=6; CX3CR1 n=6), SCI+CE10 (CX3CL1 n=6; CX3CR1 n=6), SCI+CE15 (CX3CL1 n=6; CX3CR1 n=6).

Summary results Chapter III

In the present chapter, we have demonstrated that the two tested doses of the other polyphenolic extract developed in this thesis (CE) were pharmacologically safe since they did not significantly affect the weight or aspect of the animals and did not alter the biomarkers of hepatotoxicity and nephrotoxicity in the serum of the animals.

Referring to evoked pain responses, preventive CE administration during the first week post SCI, resulted in the inhibition (15 mg/kg; i.p) or an attenuation (10 mg/kg; i.p) of mechanical allodynia development, and in the inhibition (10 mg/kg; i.p) or an attenuation (15 mg/kg; i.p) of thermal hyperalgesia development during the acute phase of SCI-derived neuropathic pain.

In addition, immunohistochemical and molecular studies revealed that both doses of CE modulated astrogliosis and microgliosis and prevented the upregulation of CX3CR1 and CCR2 but not ERK1/2 phosphorylation and CX3CL1 overexpression detected in the spinal cord of SCI+Saline mice at 21 dpi. Furthermore, SCI-induced astrogliosis in ACC and PAG and microgliosis in ACC were attenuated and sometimes completely prevented by both doses of CE treatment. Although SCI did not induce microgliosis in PAG, both doses of CE significantly induced microglial activation characterized by an increase of cells with a CD206-positive M2 phenotype. Finally, both doses of CE modulated the expression of CX3CL1 and its receptor CX3CR1 in the PAG, while only the 15 mg/kg dose of CE prevented the upregulation of this chemokine in the ACC.

Therefore, these results suggest that the repeated administration of CE during the first week after spinal cord contusion may be a suitable therapeutic strategy to prevent the development of SCI-induced neuropathic pain during the acute injury phase (up to 21 days after injury). These effects would be associated with gliosis activation modulation and chemokines release reduction, not only at the site of injury but also on pain-related supraspinal structures such as the ACC and PAG.

CHAPTER IV

Antinociceptive effects on SCI-induced neuropathic pain by repeated administration of EGCG and Polyphenolic Extracts in CD1 mice during the intermediate phase of injury.

Chapter IV. Antinociceptive effects on SCI-induced neuropathic pain by repeated administration of EGCG and Polyphenolic Extracts in CD1 mice during the intermediate phase of injury.

After elucidating the preventive effects of EGCG and the two polyphenolic extracts on the development of CNP after SCI during the acute phase of the injury, we began to investigate whether they could have an analgesic effect once the acute phase of SCI had passed. Certainly, the clinical protocol of action after SCI consists of the immediate administration of drugs to stabilize the spinal cord by preventing the neuroinflammatory and excitotoxic processes that will eventually lead to the neuropathic pain development. However, due to the lack or limited efficacy of these drugs, there are many patients who suffer from CNP after the acute phase of SCI is over. It is therefore interesting to investigate not only the preventive but also the analgesic effects of these compounds. To this end, the doses of EGCG, GSE and CE that exerted the best preventive effects in the previous experiments were selected and daily administered during the third week post-injury. To this end, EGCG (15 and 20 mg/kg; i.p.), GSE (15 mg/kg; i.p.) and CE (10 mg/kg; i.p.) were daily administered to SCI mice during the third week post-surgery (end of the acute phase of SCI). On the one hand, mechanical allodynia and thermal hyperalgesia were weekly assessed. On the other hand, animals treated with either GSE and CE were also evaluated for non-reflexive pain responses such as anhedonic-, anxiolytic- and depressive-like behaviours. Moreover, serum levels of hepatoxicity and nephrotoxicity biomarkers were analysed to assess the pharmacological safety of both GSE and CE treatments.

IV.I. Antinociceptive effects on SCI-induced neuropathic pain by repeated administration of EGCG in CD1 mice during the intermediate phase of injury.

Repeated EGCG administration during the third week post-injury does not trigger weight loss in spinal cord injured mice.

Importantly, the general aspect of the animals was normal and changes in coat and skin, vibrissae of nose, nasal secretions, signs of autotomy or aggressiveness were not detected in any experimental group of mice throughout the whole experimental period according to the protocol animal welfare supervision based on Morton D.B and Griffiths P.H. guidelines (**Morton and Griffiths, 1985**).

The influence of time and treatment on animal's weight were evaluation by means of different statistical tools. First, Shapiro-Wilk test indicated that data corresponding to weight did not follow a normal distribution for all experimental time-points ($p's<0.05$). Then, while the Friedman test revealed that the

distribution of weight data significantly varied during the experiment period (p<0.001), the Kruskal-Wallis test indicated no significant differences between groups for all time-points (p's>0.05) (***Fig. 84***).

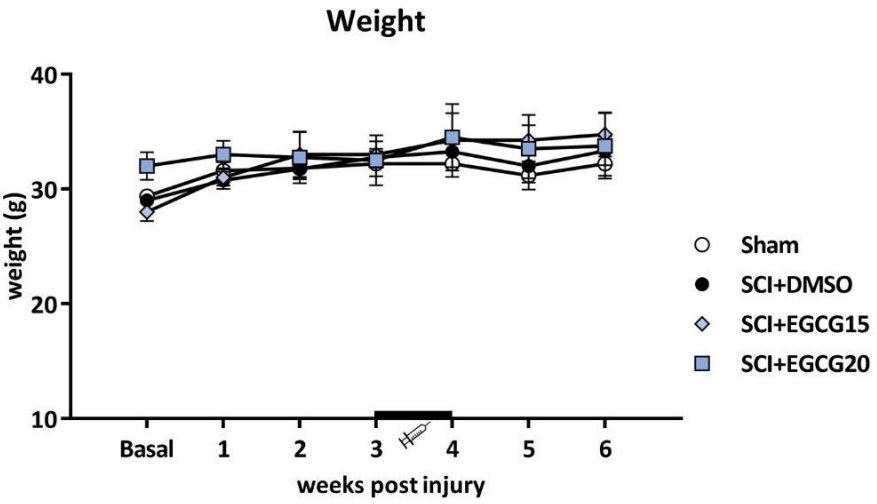


Figure 84. Mice weight control during the intermediate phase of SCI-induced neuropathic pain after EGCG administration at the third week post-injury. Each point and vertical line represent the mean ± SEM. Treatment administration week (3 to 4 wpi) is highlighted with a thick black line. Experimental groups: Sham (n=5), SCI+DMSO (n=4), SCI+EGCG15 (n=4), SCI+EGCG20 (n=4).

Repeated administration of EGCG during the third week post-injury does not modulate either mechanical allodynia or thermal hyperalgesia induced by mild spinal cord injury.

The locomotor activity of the mice was assessed to see how the injury or EGCG treatment had affected this parameter. BMS data did not follow a normal distribution for all experimental time-points according to the Shapiro-Wilk test (all p's<0.01). Although Friedman's test detected differences over experimental time on BMS scores (p's<0.001), no significant group differences were found on any of the post injury days (p>0.05) by the application of Kruskal-Wallis test (***Fig. 85. A***). Moreover, these mild BMS alterations only were referred to altered paw position but not to altered horizontal locomotion. Therefore, all mice were able to move freely without neither paralysis nor major impairment in the coordination and locomotor functions.

Referring to pain responses, both mechanical allodynia and thermal hyperalgesia data did not follow a normal distribution (p's<0.01). Thus, Friedman test was applied showing that distribution of the data was not the same throughout the experimental period (p<0.001) and further Kruskal-Wallis test revealed that these significant differences were between groups at all post-injury days (all p's<0.01). Specifically, both untreated spinal cord injured mice (SCI+Saline) and mice treated with EGCG during the third week after injury (SCI+EGCG15 and SCI+EGCG20) showed both a significant increase in mechanical sensitivity and a significant decrease in thermal paw withdrawal thresholds compared to the Sham group (all p's<0.05). Moreover, none of the EGCG treated groups showed significant differences compared to SCI+Saline group at any of all post-injury days (***Fig. 85. B, C***). Therefore, neither mechanical allodynia nor thermal hyperalgesia developed after SCI were modulated by any of the EGCG treatment doses.

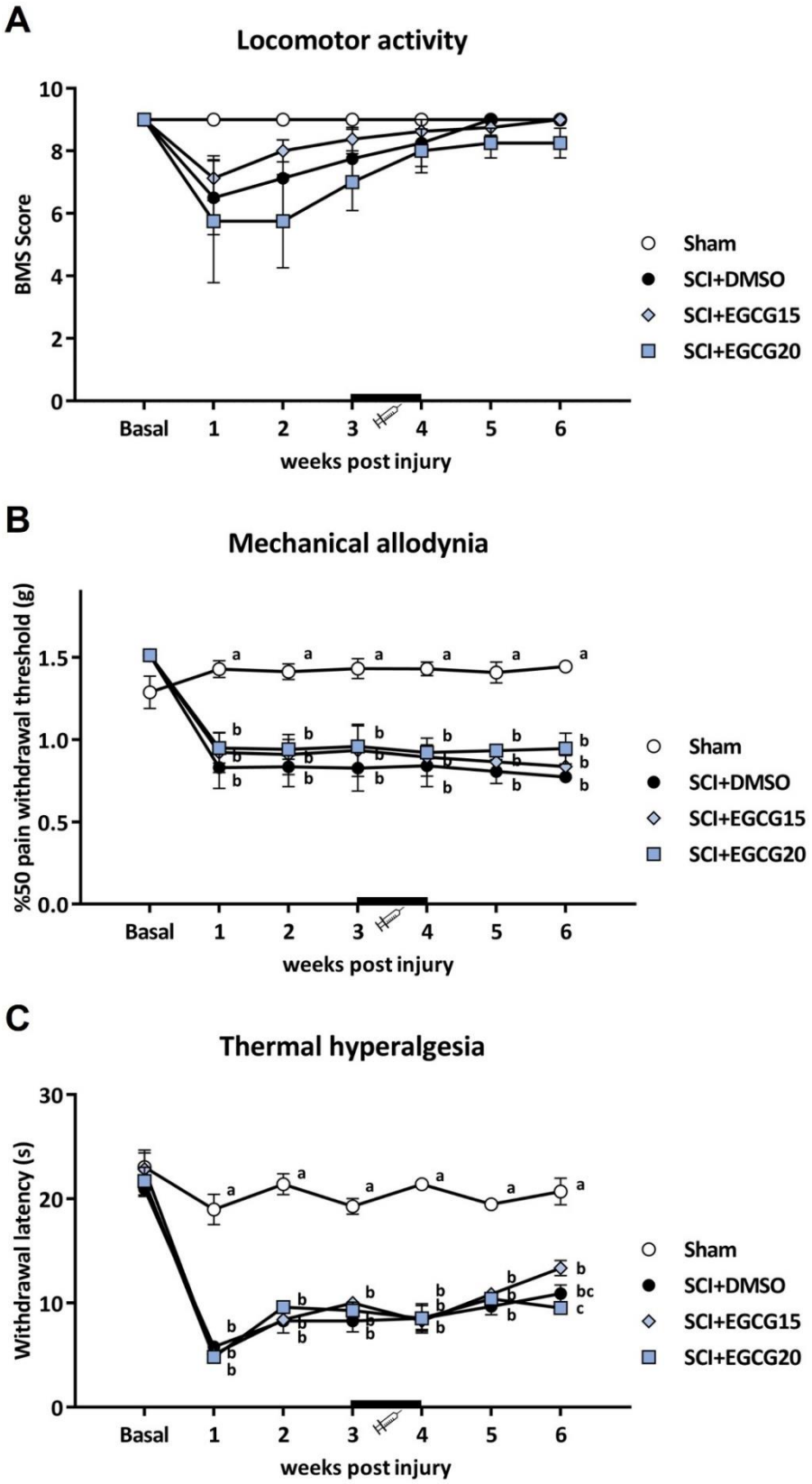


Figure 85. Time-course assessment of locomotor activity, mechanical allodynia and thermal hyperalgesia during the intermediate phase of SCI-induced neuropathic pain after EGCG treatment at the third week post-injury. Each point and vertical line represent the mean ± SEM. Treatment administration week (3 to 4 wpi) is highlighted with a thick black line. a-c: groups not sharing a letter are significantly different, $p<0.05$, by post-hocs' test. Experimental groups: Sham (n=5), SCI+DMSO (n=4), SCI+EGCG15 (n=4), SCI+EGCG20 (n=4).

IV.II. Antinociceptive effects on neuropathic pain development by repeated administration of GSE15 and CE10 in CD1 mice during the intermediate phase of spinal cord injury.

Repeated GSE15 or CE10 administration during the third week post-injury does not trigger weight loss or hepatotoxic or nephrotoxic effects in spinal cord injured mice.

During the whole experimental period, the general aspect of the animals was normal and changes in coat and skin, vibrissae of nose, nasal secretions, signs of autotomy or aggressiveness were not detected in any of the mice following a protocol animal welfare supervision based on Morton D.B and Griffiths P.H. guidelines (**Morton and Griffiths, 1985**).

Regarding the weight control, Shapiro-Wilk normality test revealed that data did not follow a normal distribution ($p's<0.05$). Thus, Friedman test was applied, which revealed that the distribution of weight data significantly varied over the experiment period ($p<0.001$), but the Kruskal-Wallis test indicated no significant differences between groups for all time-points ($p's>0.05$) (***Fig. 86***). Therefore, neither the surgeries nor the treatments had any significant effect on the animals' weight.

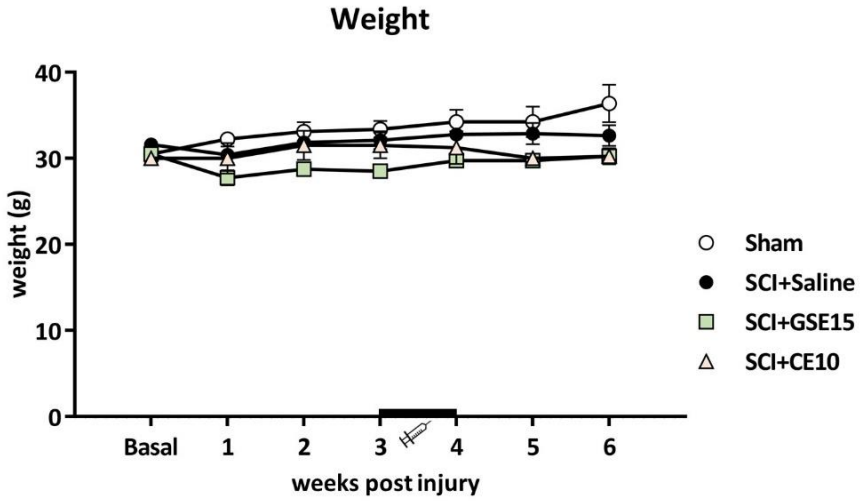


Figure 86. Mice weight control during the intermediate phase of SCI-induced neuropathic pain after GSE15 and CE10 treatments at the third week post-injury. Each point and vertical line represent the mean ± SEM. Treatment administration week (3 to 4 wpi) is highlighted with a thick black line. Experimental groups: Sham (n=8), SCI+Saline (n=8), SCI+GSE15 (n=6), SCI+CE10 (n=6).

Although the pharmacological safety of GSE15 and CE10 was already evaluated in the previous chapters of this thesis, biomarkers of hepatotoxicity (ALT/GPT and AST/GOT) and nephrotoxicity (UREA) in animals' serum were quantified at the end of experimental period to assess whether a different administration pattern could have altered the levels of these biomarkers. Kruskal-Wallis test for ALT/GPT, AST/GOT and UREA data indicated no significant differences between the experimental groups (***Fig. 87. A-C***). Hence,

these results indicated that there are no hepatotoxic or nephrotoxic effects associated with the administration of GSE15 or CE10 during the third week post SCI.

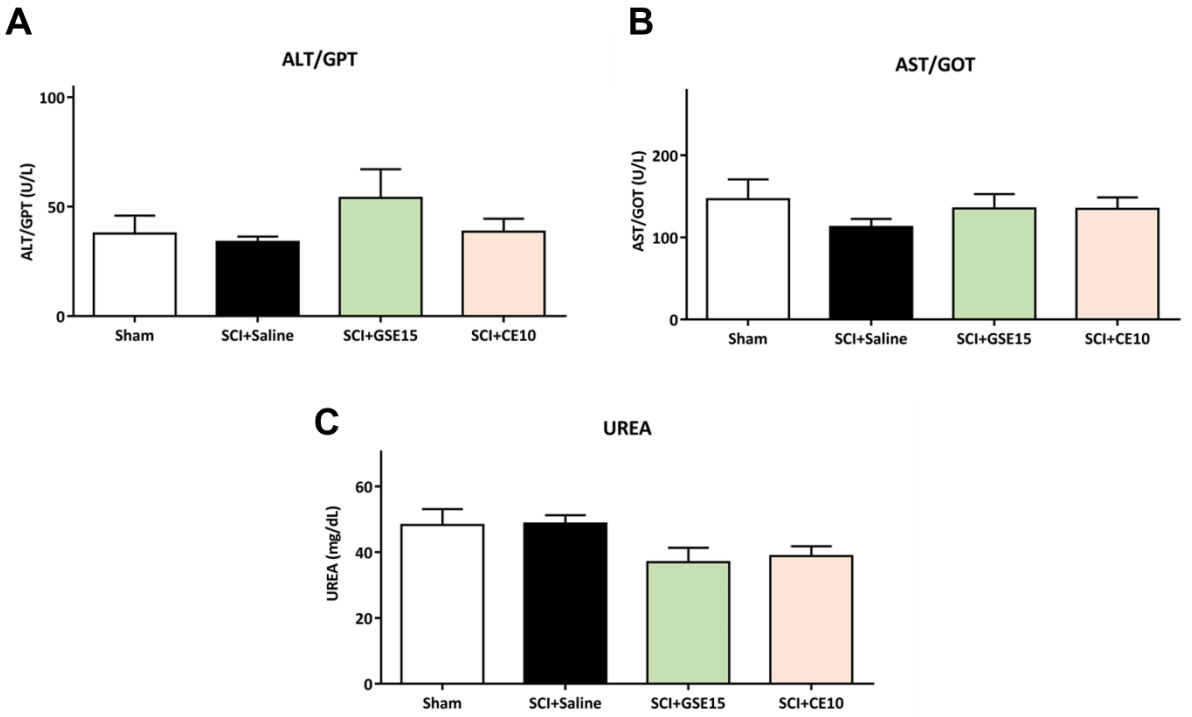


Figure 87. Biomarkers quantification of (A-B) hepatotoxicity and (C) nephrotoxicity, in the serum of each experimental group at the end of experimental period. The results are represented as the mean ± SEM. Experimental groups: Sham (ALT/GTP n=4; AST/GOT n=5; UREA n=5), SCI+Saline (ALT/GTP n=4; AST/GOT n=4; UREA n=4), SCI+GSE15 (ALT/GTP n=4; AST/GOT n=4; UREA n=4), SCI+CE10 (ALT/GTP n=4; AST/GOT n=4; UREA n=4).

Repeated administration of GSE15 or CE10 during the third week post-injury modulates both mechanical allodynia and thermal hyperalgesia induced by mild spinal cord injury.

According to the Shapiro-Wilk test, locomotor activity data did not follow a normal distribution for all experimental time-points (all p's<0.001). Although Friedman's test detected differences over experimental time (p's<0.001) and BMS scores decreased slightly in the weeks following the injury, no significant group differences were found on any of the experimental time-points (p>0.05) by the application of Kruskal-Wallis test (***Fig. 88. A***). Moreover, these BMS alterations only were referred to altered paw position but not to altered horizontal locomotion. Therefore, all mice were able to move freely without neither paralysis nor major impairment in the coordination and locomotor functions.

Mechanical allodynia data did not follow a normal distribution for all experimental time-points (Shapiro-Wilk, p's<0.05). Significant differences in the distribution of the data were found by Friedman's test (p<0.05)

and further Kruskal-Wallis test indicated significant differences between groups at all post-injury weeks (all p's<0.01). Specifically, spinal cord injured mice without treatment (SCI+Saline) showed significant decrease of paw withdrawal mechanical thresholds at all post injury weeks when compared to the Sham group (p's<0.05). As expected, SCI+GSE15 and SCI+CE10 groups did not significantly differed to SCI+Saline group before the start of the corresponding treatments, that is at 1-, 2- and 3-wpi. On the one hand, GSE15 treatment significantly attenuated mechanical allodynia at 4 wpi. However, this effect was lost from 5-wpi until the end of experimental period since SCI+GSE15 group did not significantly differed to SCI+Saline group at 5- and 6-wpi. On the other hand, CE10 treatment significantly attenuated mechanical allodynia to Sham group levels from 4 wpi until the end of experimental period. While it is true that the SCI+CE10 group did not differ significantly from Sham at 6wpi, it did not differ from the SCI+Saline group either (***Fig. 88. B***).

Referring to thermal hyperalgesia, Shapiro-Wilk normality test showed that data did not follow a normal distribution (p's<0.001). Significant differences in the distribution of the data were found by Friedman's test (p<0.001) and further Kruskal-Wallis test indicated significant differences between groups at all post-injury weeks, i.e., from 1 to 6 wpi (all p's<0.01). Concretely, the SCI+Saline group showed a significant decrease in thermal paw withdrawal thresholds compared to the Sham group, thus indicating SCI-induced thermal hyperalgesia development up to 6 wpi. Similar to the mechanical allodynia results, the SCI+GSE15 and SCI+CE10 groups did not differ significantly from the SCI+Saline group at 1, 2 and 3 wpi, i.e. before the start of the treatments. At 4 wpi, although the paw withdrawal thresholds of the SCI+GSE15 and SCI+CE10 groups increased slightly, they did not differ significantly from the SCI+Saline group. On the one hand, GSE15 treatment significantly attenuated mechanical allodynia at 5 wpi. However, this effect was lost after a week since SCI+GSE15 group did not significantly differed to SCI+Saline group at 6 wpi. On the other hand, treatment with CE10 significantly attenuated mechanical allodynia from 5 wpi until the end of the experimental period, showing no significant differences with the Sham group at 5 and 6 wpi (***Fig. 88. C***).

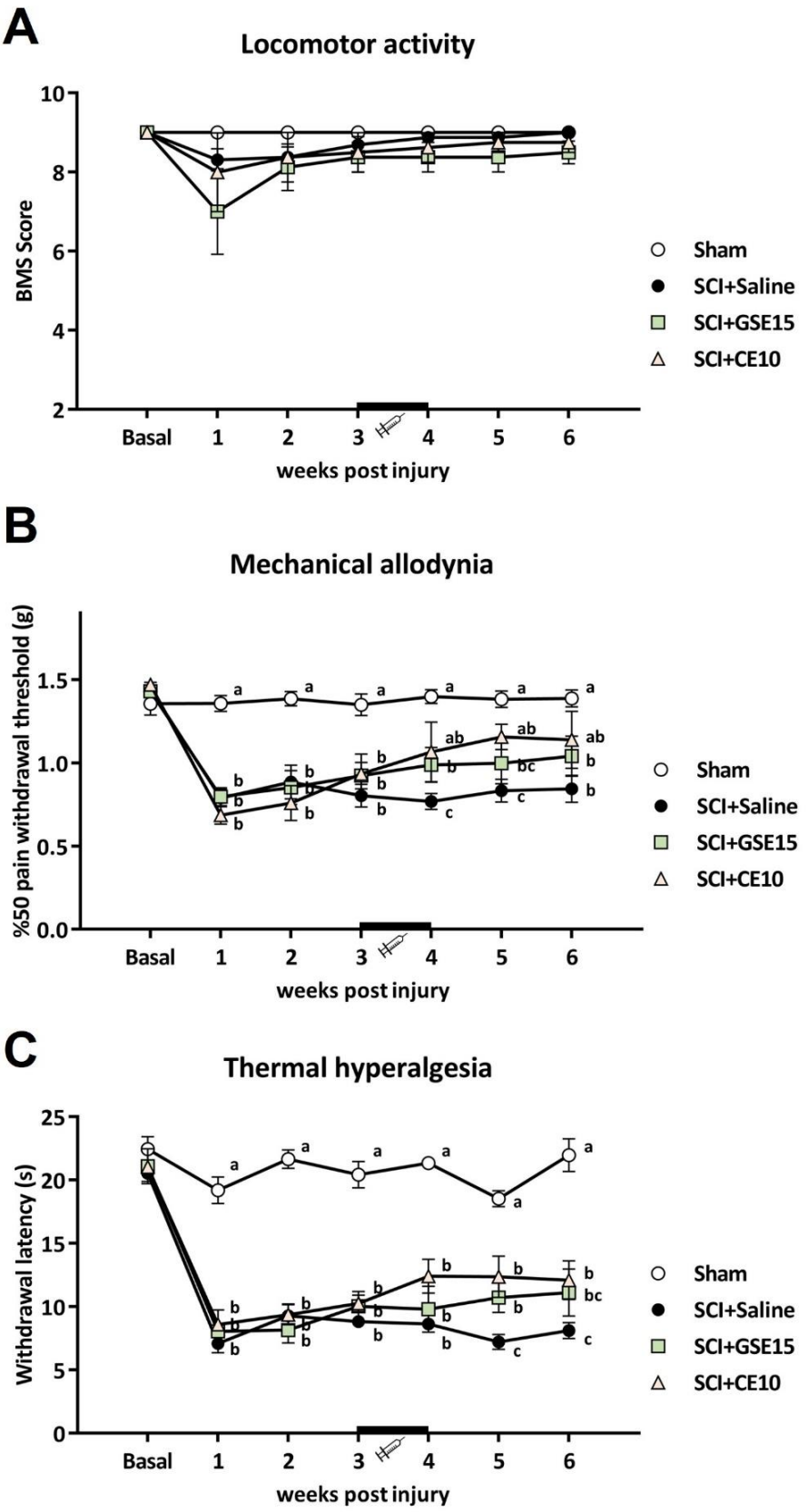


Figure 88. Time-course assessment of locomotor activity, mechanical allodynia and thermal hyperalgesia during the intermediate phase of SCI-induced neuropathic pain after GSE15 and CE10 treatments at the third week post-injury. Each point and vertical line represent the mean ± SEM. Treatment administration week (3 to 4 wpi) is highlighted with a thick black line. a-c: groups not sharing a letter are significantly different, $p<0.05$. Experimental groups: Sham (n=8), SCI+Saline (n=8), SCI+GSE15 (n=6), SCI+CE10 (n=6).

No disturbances on motivational behaviour associated with mild spinal cord injury-induced neuropathic pain at 6 weeks post-injury

In the Reward-Seeking Behaviour (RSB) test, Kruskal-Wallis revealed no significant group differences in any of the parameters studied: amount of chocolate consumed, eating duration, latency to eat and number of approaches to reward (all p's>0.05). However, although none of the experimental groups showed statistical significance for any of these parameters, it is worth mentioning that the Sham group showed a higher eating duration, amount consumed and number of approaches to chocolate and a shorter latency to eat compared to the SCI+Saline group (***Fig. 89. A-D***). While treatment with GSE15 did not seem to have any positive effect on any of the parameters, SCI animals treated with CE10 ate longer (***Fig. 89. B***) and approached to chocolate more times (***Fig. 89. D***) compared to SCI+Saline animals.

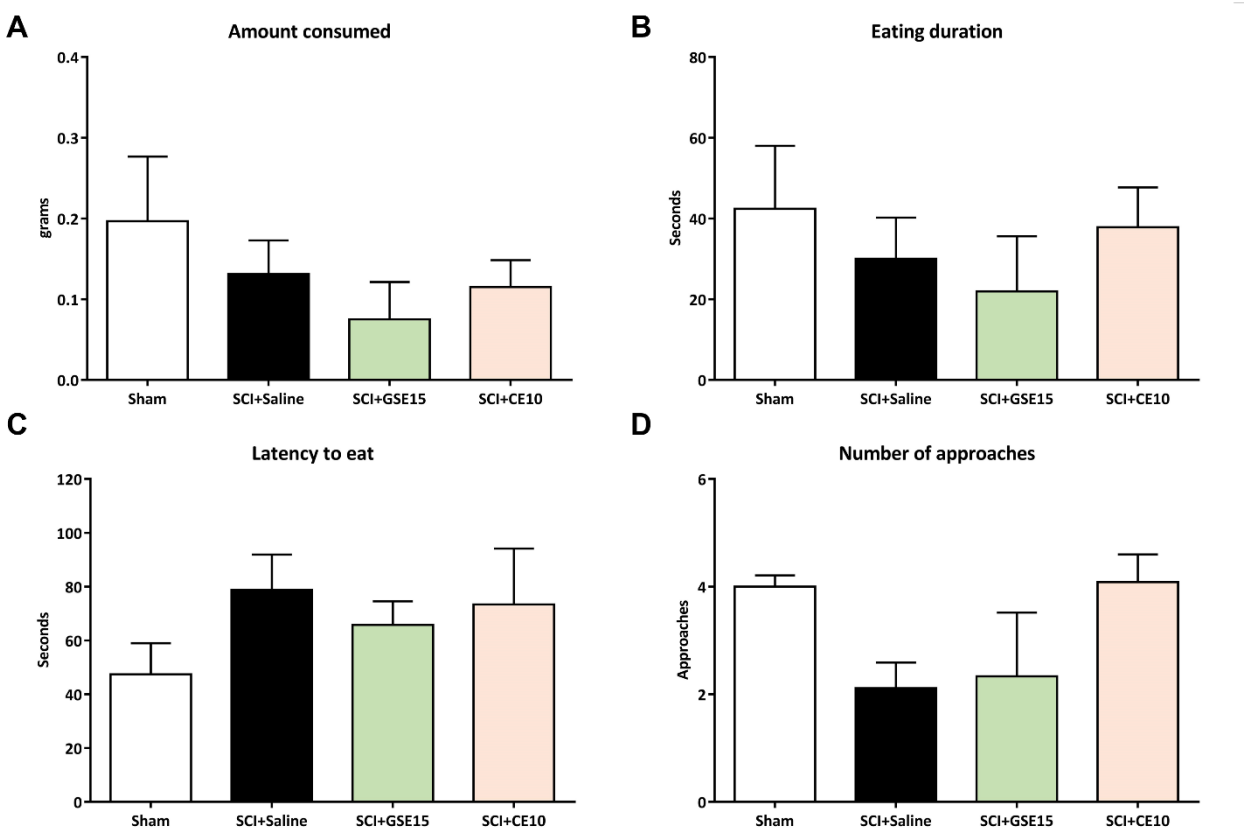


Figure 89. Reward-Seeking Behaviour (RSB) test at the intermediate phase of SCI-induced neuropathic pain after GSE15 and CE10 treatments at the third week post-injury. Results are the mean ± SEM. Experimental groups: Sham (n=5), SCI+Saline (n=4), SCI+GSE15 (n=4), SCI+CE10 (n=4).

No anxiety-like behaviour development associated with mild spinal cord injury-induced neuropathic pain at 6 weeks post-injury

In the light and dark box test, Kruskal-Wallis revealed no significant group differences in the global activity, the number of crossings between chambers (zone transition) and the number of entries in the light chamber (all p's>0.05; ***Fig. 90. B-D***). Nevertheless, although no significant group differences were found in the percentage of time spent in the light chamber and in the middle zone, spinal cord injured animals treated with GSE15 (SCI+GSE15) spent less time in the dark chamber compared to the SCI+Saline group (p<0.05). The rest of the experimental groups showed no significant differences in the time spent in the dark chamber (***Fig. 90. A.***)

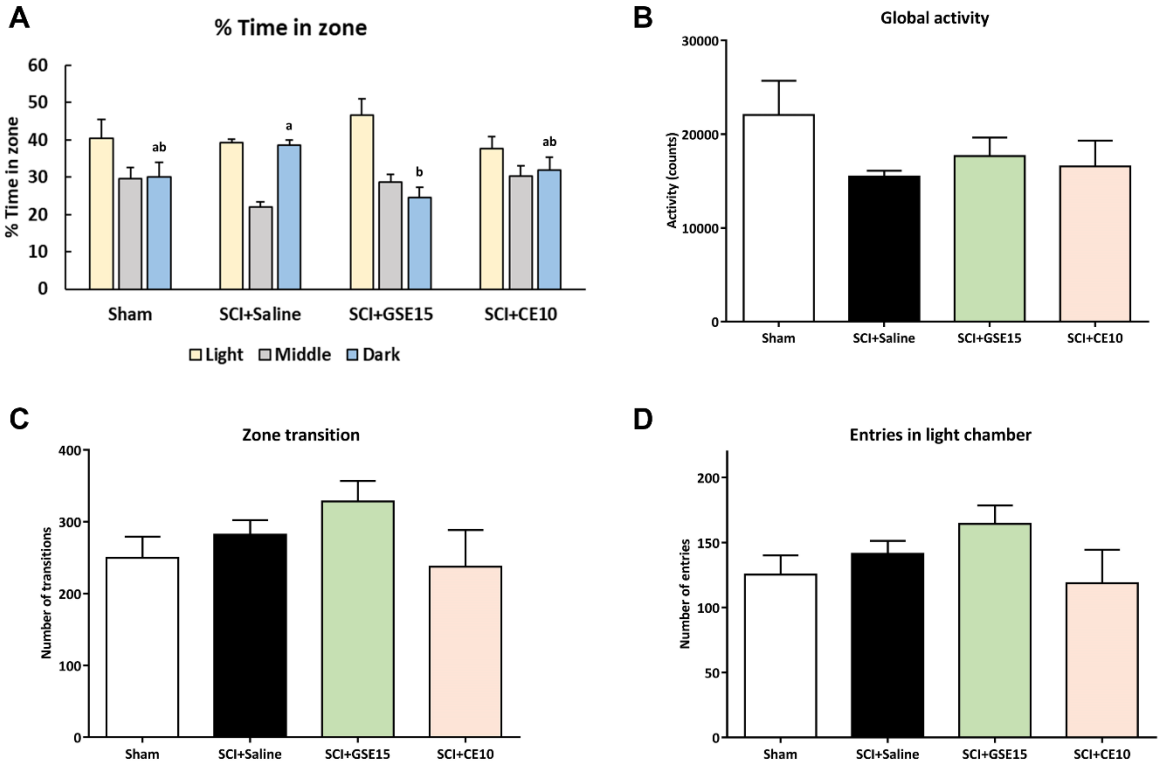


Figure 90. Light and dark box test at the intermediate phase of SCI-induced neuropathic pain after GSE15 and CE10 treatments at the third week post-injury. Results are the mean ± SEM. a-b: groups not sharing a letter are significantly different, p<0.05, by posthocs' test. Experimental groups: Sham (n=5), SCI+Saline (n=4), SCI+GSE15 (n=4), SCI+CE10 (n=4).

In the Open Field (OF) test, Kruskal-Wallis revealed no significant group differences in any of the parameters investigated: percentage of time spent in each zone (peripheral, middle and central), latency to entry in zone, entries in zone, zone transition and global activity (all p's>0.05; ***Fig. 91. A-D***). Therefore, neither the treatment nor the injury altered the animal's activity or behaviour in response to novelty and an anxiogenic environment such as the open field.

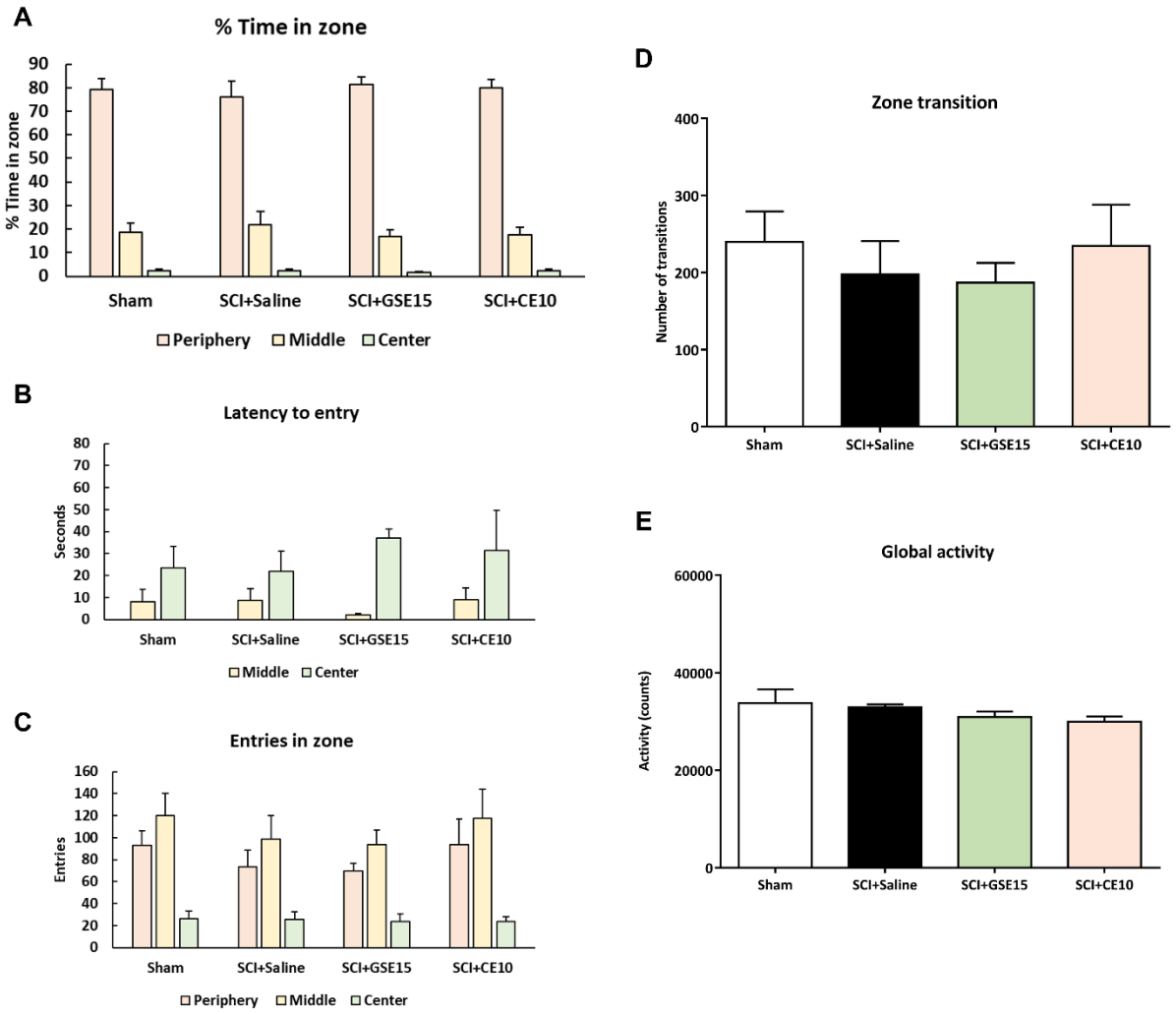


Figure 91. Open Field test at the intermediate phase of SCI-induced neuropathic pain after GSE15 and CE10 treatments at the third week post-injury. Results are the mean ± SEM. Experimental groups: Sham (n=5), SCI+Saline (n=4), SCI+GSE15 (n=4), SCI+CE10 (n=4).

Repeated CE10 administration during third week post-injury modulates depressive-like behaviour induced by mild spinal cord injury at 6 weeks post-injury.

In the Forced swimming test, Kruskal-Wallis indicated significant differences between groups in the percentage of immobility and high activity time, and in the global activity (p's<0.05; ***Fig. 92. A, B, E***). In contrast, no significant differences were found between groups in the number of immobility and percentage of low activity time by the Kruskal-Wallis test (p's>0.05; ***Fig. 92. C, D***). In terms of activity level, SCI+Saline animals spent more time immobile compared to Sham animals (p<0.05). Although SCI animals treated with GSE15 or CE10 remained immobile for less time compared to SCI+Saline animals, both SCI+GE15 and SCI+CE10 did not differ significantly from either SCI+Saline or Sham (p's>0.05; ***Fig. 92. A***). In addition, the percentage of time Sham animals spent in high activity was significantly higher compared to SCI+Saline animals (p<0.05). While the GSE15 treatment failed to increase the percentage of high activity of the SCI

animals, the CE10 treatment succeeded in increasing this parameter to sham levels since these two groups did not differ statistically (p>0.05; ***Fig. 92. B***). Referring to the global activity, as expected, Sham animals showed a higher activity compared to the SCI+Saline group (p<0.05). As to the polyphenolic treatments, although GSE15 administration increased the global activity levels of the SCI animals, it did not differ statistically from the SCI+Saline group (p>0.05). In contrast, CE10 treatment increased the global activity of the SCI animals, showing no significative differences with the Sham group (p>0.05; ***Fig. 92. E***).

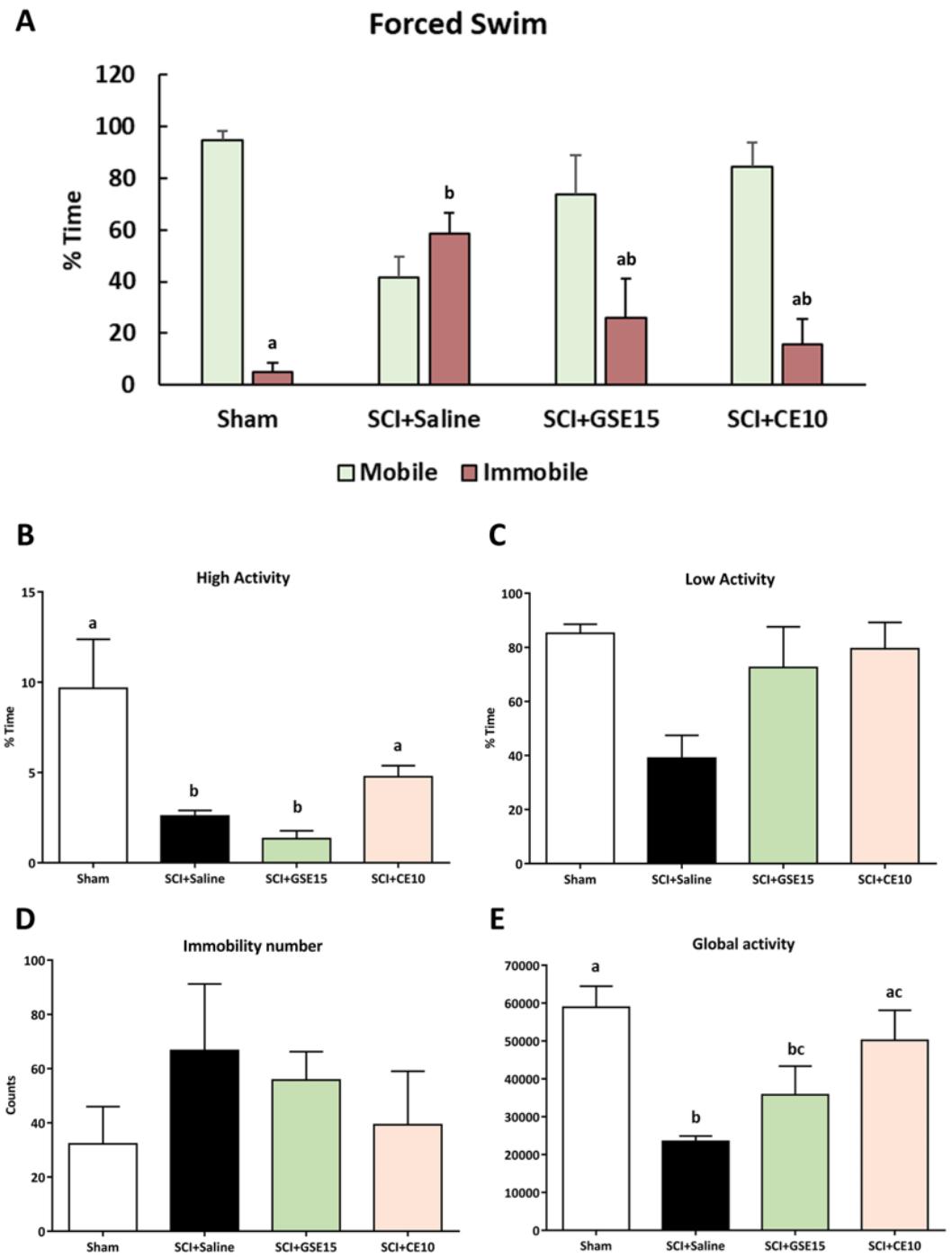


Figure 92. Forced swim test at the intermediate phase of SCI-induced neuropathic pain after GSE15 and CE10 treatments at the third week post-injury. Results are the mean ± SEM. a-c: groups not sharing a letter are significantly different, p<0.05, by posthocs' test. Experimental groups: Sham (n=5), SCI+Saline (n=4), SCI+GSE15 (n=4), SCI+CE10 (n=4).

Summary results Chapter IV

In the present chapter, we have demonstrated that neither mechanical allodynia nor thermal hyperalgesia developed after SCI were modulated by repeated administration of EGCG (15 and 20 mg/kg; i.p) during the third week post SCI. In contrast, the same administration pattern with the two polyphenolic extracts (GSE15 mg/kg and CE10 mg/kg; i.p) modulated both mechanical allodynia and thermal hyperalgesia induced by mild spinal cord injury. In addition, no systemic toxicity, hepatotoxic or nephrotoxic effects were associated with the administration of GSE15 or CE10 during the third week post SCI.

In parallel, animals treated with either GSE and CE were also evaluated for non-reflexive pain responses such as anhedonic-, anxiolytic- and depressive-like behaviours. On the one hand, no anhedonic or anxious-like behaviours were observed in SCI animals at 6wpi. On the other hand, the depressive-like behaviour detected in SCI animals were modulated by the CE (10 mg/kg; i.p) administration.

Altogether, these results indicated that while EGCG administration during the third wpi had no analgesic effect, both GSE and CE administration exerted an analgesic effect for both reflexive and non-reflexive neuropathic pain responses once the acute phase of SCI was over. Therefore, these results suggest that treatment with polyphenolic extracts may be a suitable therapeutic strategy to modulate not only the reflexive neuropathic pain responses but also the non-reflexive ones, at the intermediate phase of SCI.

CHAPTER V

Preventive effects on SCI-induced neuropathic pain development by repeated administration of Polyphenolic Extracts in CD1 mice during the acute, intermediate, and chronic phase of injury.

Chapter V. Preventive effects on SCI-induced neuropathic pain development by repeated administration of Polyphenolic Extracts in CD1 mice during the acute, intermediate, and chronic phase of injury.

At this point, we showed that repeated administration of GSE and CE during the first week post SCI had preventive effects on CNP development during the acute phase of injury and repeated administration of these polyphenolic extracts during the third week post SCI exerted analgesic effects during the intermediate phase of injury. In this context, a new administration schedule was proposed, consisting of repeated administration of the polyphenolic extracts during the first week post injury to exert preventive effects on the CNP development, and two others repeated administrations during the third- and sixth-week post injury in order to reinforce these preventive effects. This administration protocol was designed to test whether treatment with these polyphenolic extracts could modulate reflexive and non-reflexive CNP responses over time until the chronic phase of the injury was established.

Repeated GSE15 or CE10 administration during the first-, third- and sixth-week post-injury does not trigger weight loss or hepatotoxic or nephrotoxic effects in spinal cord injured mice.

Throughout the study and following a protocol animal welfare supervision based on Morton D.B and Griffiths P.H. guidelines (**Morton and Griffiths, 1985**) the general aspect of the animals was normal and changes in coat and skin, vibrissae of nose, nasal secretions, signs of autotomy or aggressiveness were not detected in any experimental group of mice.

Different statistical tests were used to evaluate the role of both time and treatment on animal's weight. After Kolmogorov-Smirnov normality test revealed that data did not follow a normal distribution ($p's<0.05$), the Friedman analysis indicated that the distribution of weight data significantly varied during the experimental period ($p<0.001$). Nevertheless, the subsequent Kruskal-Wallis test showed no significant differences between the groups for any of the experimental time points (all $p's>0.05$), thus indicating that neither surgeries nor treatments had significant effects on the animals' weight (***Fig. 93***).

Biomarkers of hepatotoxicity (ALT/GPT and AST/GOT) and nephrotoxicity (UREA) were quantified in the serum of the animals at the end of the experimental period to assess whether the administration pattern followed for GSE15 and CE10 could trigger hepatotoxic or nephrotoxic effects. After Kolmogorov-Smirnov test confirmed a normal distribution of the data (all $p's>0.161$), the ANOVA analysis indicated no significant differences between experimental groups for any of the biomarkers: ALT/GPT ($F_{(3,29)}= 0.731$, $p=0.543$), AST/GOT ($F_{(3,29)}= 0.614$, $p=0.612$) and UREA ($F_{(3,33)}= 1.743$, $p=0.179$) (***Fig. 94***).

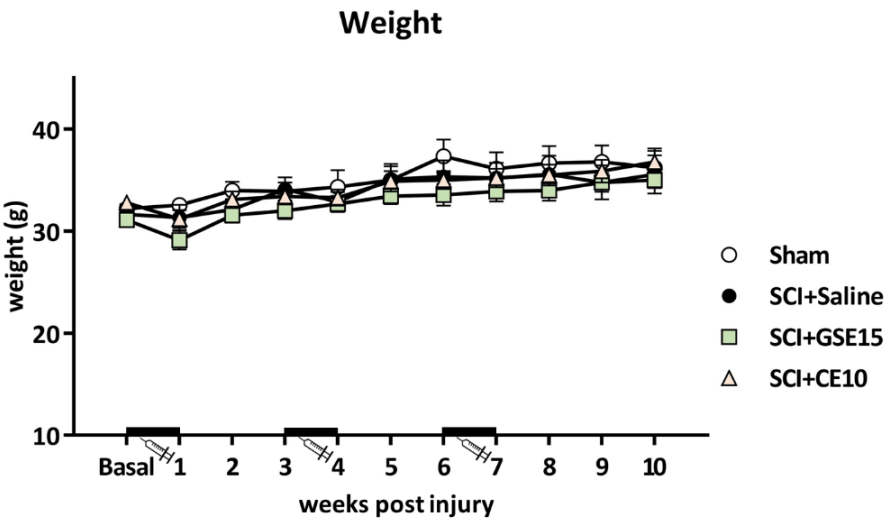


Figure 93. Time-course assessment of mice weight during the acute, intermediate and chronic phase of SCI-induced neuropathic pain after GSE15 and CE10 treatments at the first-, third- and sixth-week post-injury. Each point and vertical line represent the mean ± SEM. Treatment administration weeks (basal to 1 wpi, 3 to 4 wpi and 6 to 7 wpi) are highlighted with a thick black line. Experimental groups: Sham (n=8), SCI+Saline (n=9), SCI+GSE15 (n=9), SCI+CE10 (n=10).

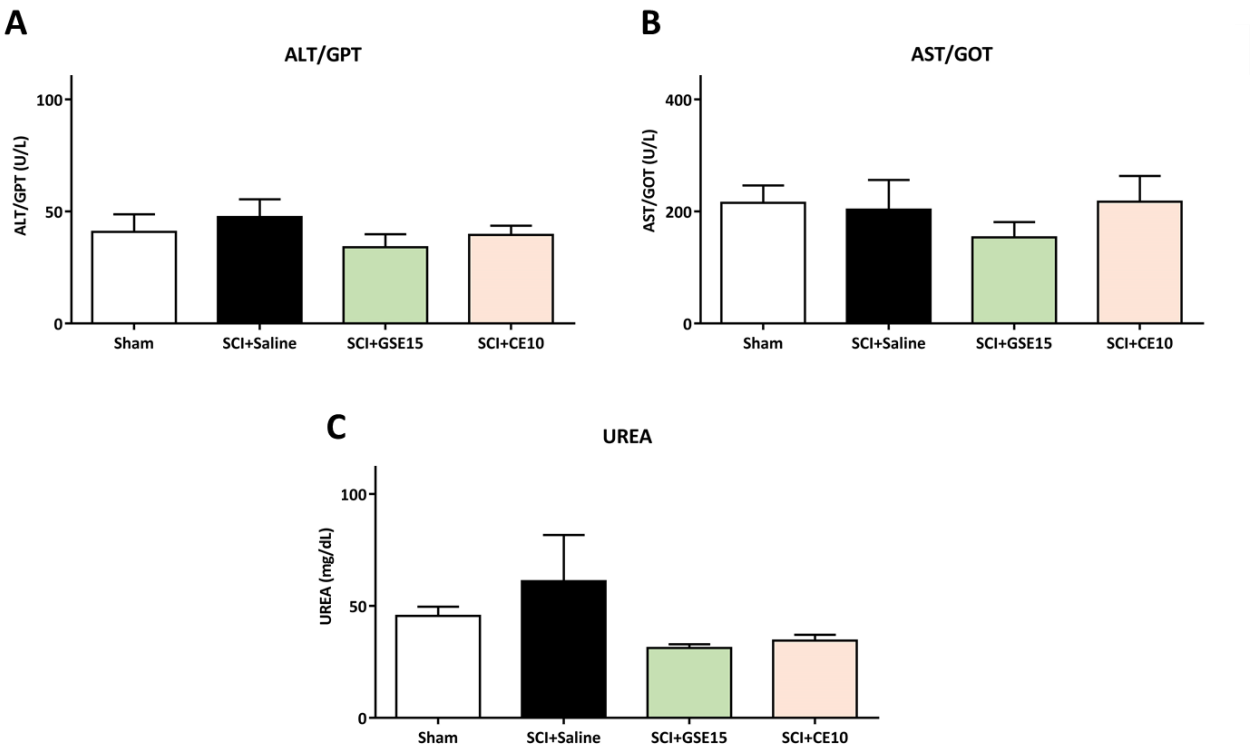


Figure 94. Biomarkers quantification of (A-B) hepatotoxicity and (C) nephrotoxicity, in the serum of each experimental group at the end of experimental period. The results are represented as the mean ± SEM. Experimental groups: Sham (ALT/GTP n=7; AST/GOT n=8; UREA n=8), SCI+Saline (ALT/GTP n=8; AST/GOT n=6; UREA n=8), SCI+GSE15 (ALT/GTP n=8; AST/GOT n=8; UREA n=8), SCI+CE10 (ALT/GTP n=7; AST/GOT n=8; UREA n=10).

Repeated GSE15 or CE10 administration during the first-, third- and sixth-week post-injury modulates both mechanical allodynia and thermal hyperalgesia induced by mild spinal cord injury.

Firstly, locomotor activity was evaluated to rule out locomotor disturbances that may override the reflexive-pain responses evaluation. Kolmogorov-Smirnov test revealed that the data did not follow a normal distribution (all p's<0.001) and, although the Friedman test showed significant differences throughout the experimental period (p<0.001), no significant differences were found between the groups in any of the weeks post injury by the Kruskal-Wallis test (all p's>0.268) (***Fig. 95. A***). Moreover, all alterations were referred to paw position but not to horizontal locomotion, indicating that all mice were able to move freely without neither paralysis nor major impairment in the coordination and locomotor functions.

Referring to mechanical allodynia, Kolmogorov-Smirnov normality test showed that data did not follow a normal distribution (p's<0.05). Significant differences in the distribution of the data were found by Friedman's test (p<0.001) and further Kruskal-Wallis test indicated significant differences between groups at all weeks post-injury (all p's<0.038) except at 10 wpi (p=0.128). Specifically, subsequent Mann Whitney U tests revealed significant mechanical sensitivity increase of SCI+Saline group when compared with the Sham group up to 9 wpi (all p's<0.05). Although SCI+Saline group showed significant decrease of paw withdrawal mechanical thresholds when compared with Sham up to 9 wpi, from 7 wpi until the end of the experimental period an upward trend of these withdrawal mechanical thresholds was observed. Referring to polyphenolic treatments, both GSE15 and CE10 significantly prevented mechanical allodynia development after SCI up to 9 wpi. While SCI+CE10 group did not significantly differed to Sham at all experimental time points, SCI+GSE15 group showed significant differences with Sham at 4wpi (p=0.031). Moreover, due to the loss of mechanical sensitivity of the SCI+Saline group, SCI animals treated with GSE15 showed no significant differences with either SCI+Saline or Sham animals at 8 wpi (***Fig. 95. B***).

In contrast to mechanical allodynia, thermal hyperalgesia data followed a normal distribution (p's>0.05) according to the Kolmogorov-Smirnov test. For this reason, MANOVA was applied revealing significant effects on treatment ($F_{(3,21)}$ = 18.988, p<0.001), week ($F_{(10,12)}$ = 3.491, p=0.022), and interaction week x treatment ($F_{(30,35.898)}$ = 3,071, p=0.001) factors. On subsequent ANOVA, significant group differences were found on all post-injury weeks from 1 to 10 (all p's<0.001). Specifically, the SCI+Saline group showed significant decrease of paw withdrawal latency to thermal stimulation when compared to the Sham group. Post-hoc test also revealed that GSE15 and CE10 treatments significantly prevented thermal hyperalgesia development in the SCI animals from 1 to 10 wpi. On the one hand, the SCI+CE10 group showed no significant differences to the Sham group for all post injury weeks except for 3 wpi. On the other hand, the SCI+GSE15 did not significantly differed from Sham except at 3 and 4 wpi (***Fig. 95. C***).

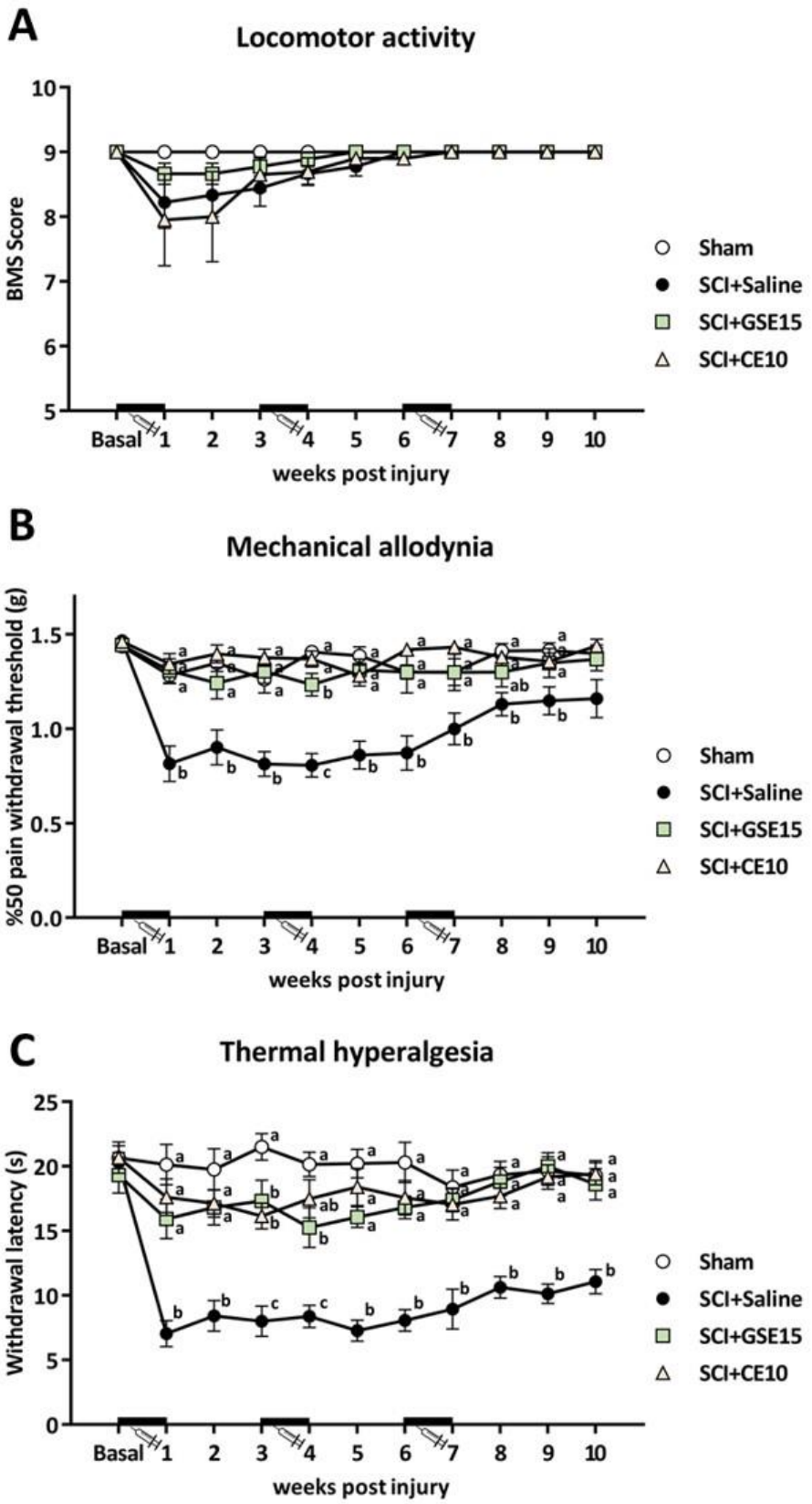


Figure 95. Time-course assessment of locomotor activity, mechanical allodynia and thermal hyperalgesia during the acute, intermediate and chronic phase of SCI-induced neuropathic pain after GSE15 and CE10 treatments at the first-, third- and sixth-week post-injury. Each point and vertical line represent the mean ± SEM. Treatment administration weeks (basal to 1 wpi, 3 to 4 wpi and 6 to 7 wpi) are highlighted with a thick black line. a-c: groups not sharing a letter are significantly different, $p<0.05$, by post-hocs' test. Experimental groups: Sham (n=8), SCI+Saline (n=9), SCI+GSE15 (n=9), SCI+CE10 (n=10).

Repeated GSE15 or CE10 administration during the first-, third- and sixth-week post-injury modulates motivational behaviour disturbances induced by mild spinal cord injury at 10 weeks post-injury.

In the Reward-Seeking Behaviour (RSB) test, Kruskal-Wallis revealed significant group differences in both the latency to eat the reward ($p<0.01$) and the number of approaches to the reward ($p<0.01$) (***Fig. 96. C, D***). Specifically, post-hoc analysis indicated that spinal cord injured animals without treatment (SCI+Saline) had a significant increased latency to eat the reward and a significant decreased number of approaches when compared with Sham and spinal cord injured animals treated with GSE15 or CE10 (SCI+GSE and SCI+CE10). Moreover, the SCI+GSE15 and the SCI+CE10 groups showed no significant differences compared to the Sham group. These results suggest that mild spinal cord injury-induced neuropathic pain results in reward-seeking (motivational) behaviour decrease at the SCI chronic phase indicating long-lasting motivational behaviour alteration and polyphenol-based treatments may prevent it. In contrast, no effects on consumption behaviour were detected, as no significant differences were found in either the amount of chocolate consumed ($p=0.119$) or the eating duration ($p=0.063$) (***Fig. 96. A, B***). In addition, it should be noted that no neophobic behaviour against white chocolate was detected, because all animals were habituated to the reward prior to testing.

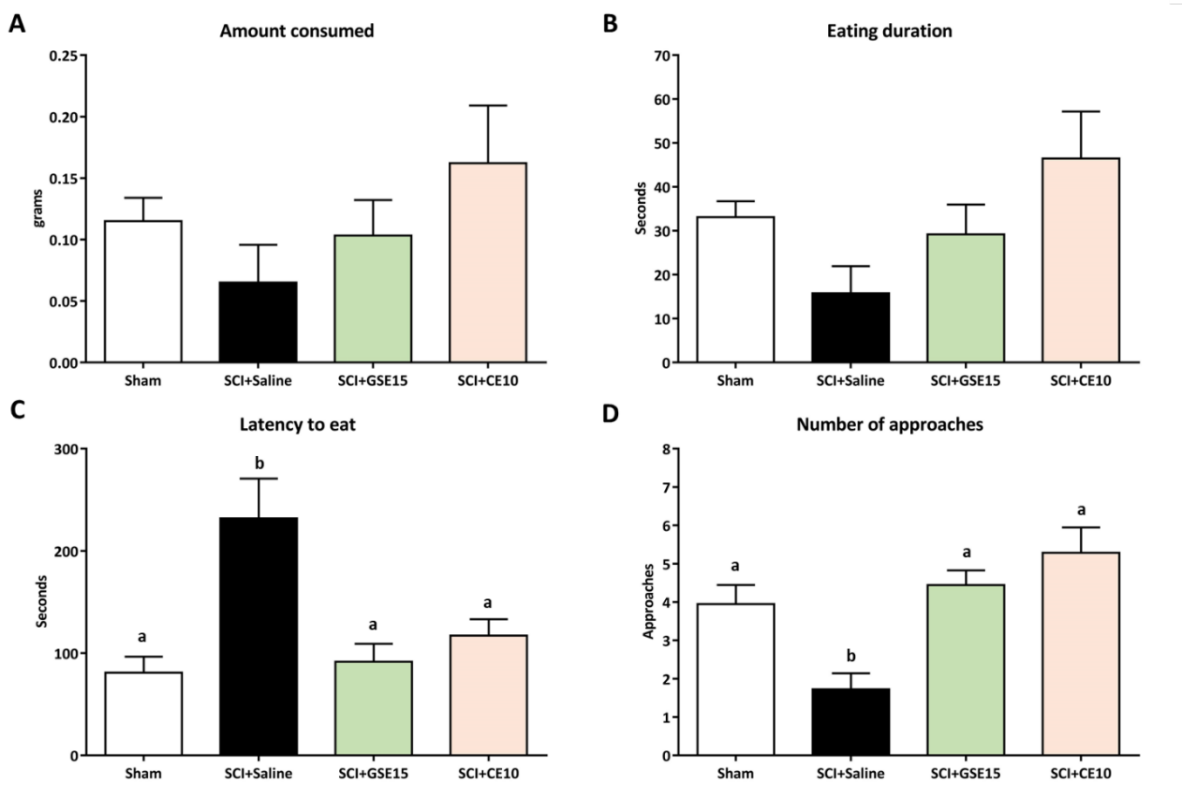


Figure 96. Reward-Seeking Behaviour (RSB) test at the chronic phase of SCI-induced neuropathic pain after GSE15 and CE10 treatments at the first-, third- and sixth-week post-injury. Results are represented as mean ± SEM. a-c: groups not sharing a letter are significantly different, $p<0.05$, by post-hocs' test. Experimental groups: Sham (n=8), SCI+Saline (n=9), SCI+GSE15 (n=9), SCI+CE10 (n=10).

Repeated GSE15 or CE10 administration during the first-, third- and sixth-week post-injury modulates slightly impairments related to anxiety-like behaviour induced by mild spinal cord injury at 10 weeks post-injury.

According to the Kolmogorov-Smirnov test, the parameters analysed in the light and dark test followed a normal distribution (p's>0.05) except for global activity (p<0.001). ANOVA analysis revealed significant group differences in the percentage of time spent in the light ($F_{(3,32)}$=3.916, p=0.017), dark ($F_{(3,32)}$= 8.355, p<0.001) and intermediate zone ($F_{(3,32)}$= 4.674, p=0.008). Specifically, post-hoc analysis indicated that Sham animals spent more time in the light chamber and less time in the dark chamber compared to SCI+Saline group. Regarding to the time spent in the light chamber, both SCI+GSE15 and SCI+CE10 groups did not significantly differed from SCI+Saline group. However, both GSE15 and CE10 treatments reduced the time spent in the dark chamber of SCI animals to Sham levels. As a result, SCI animals treated with either GSE15 or CE10 spent more time in the intermediate zone than SCI+Saline animals, like the Sham group (***Fig. 97. A***).

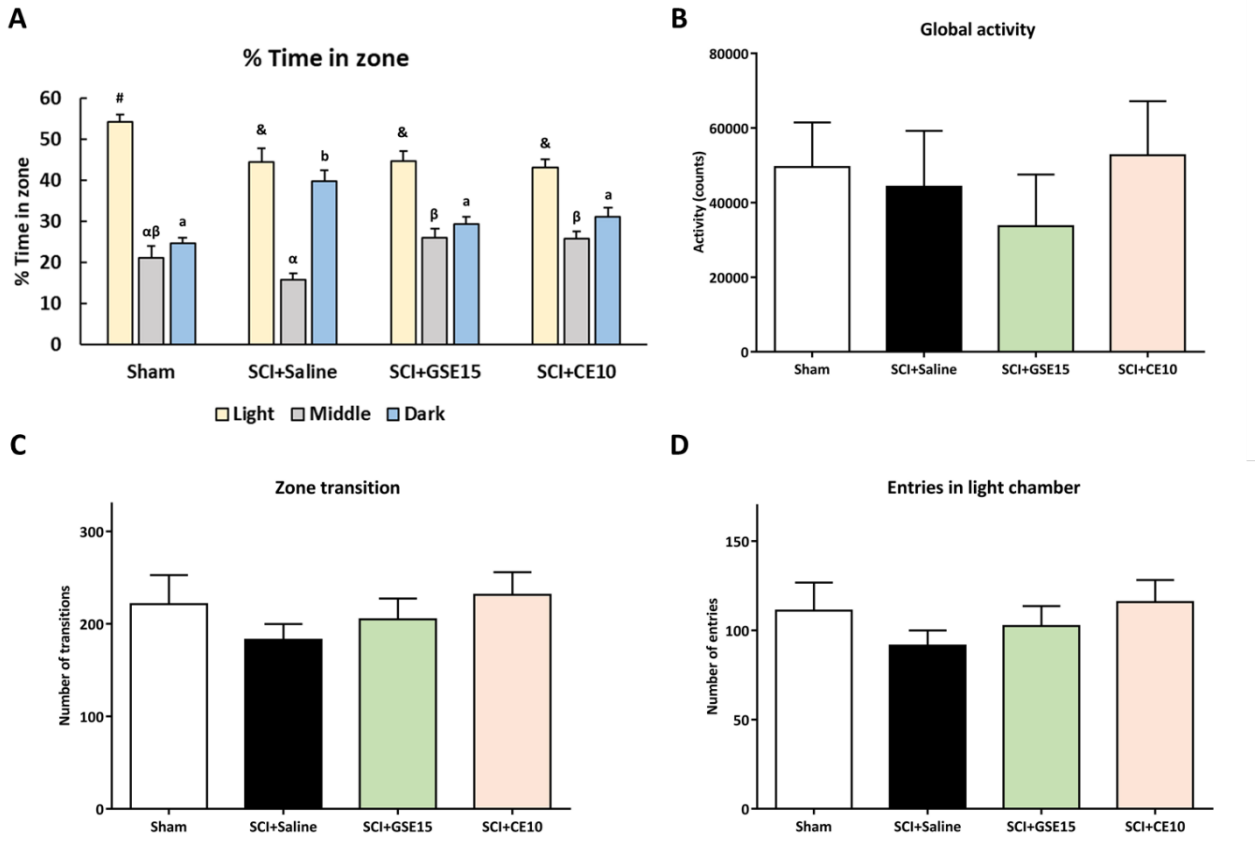


Figure 97. Light and dark box test at the chronic phase of SCI-induced neuropathic pain after GSE15 and CE10 treatments at the first-, third- and sixth-week post-injury. Results are represented as mean ± SEM. #-& / α-β / a-b: groups not sharing a symbol/letter are significantly different, p<0.05, by post-hocs' test. Experimental groups: Sham (n=8), SCI+Saline (n=9), SCI+GSE15 (n=9), SCI+CE10 (n=10).

In contrast, although a tendency towards statistical significance was observed, no significant group differences were found in zone transition ($F_{(3,32)}$= 0.797, p=0.504) and in entries in light chamber ($F_{(3,32)}$= 0.804, p=0.501). However, it is worth mentioning that the Sham group showed a positive trend in transitions and entries in the light zone compared to the SCI+Saline group and SCI animals treated with either GSE15 and CE10 presented a greater number of transitions and more entries in the light chamber than non-treated SCI animals (***Fig. 97. C, D***). Finally, no significant group differences were found in the global activity of the animals (Kruskall-Wallis, p>0.578). This result helps to rule out any false-positive results in transition parameters because some drugs can affect general motor function by increasing or decreasing the global activity and thus the number of transitions (***Fig. 97. B***).

In the Open Field (OF) test, the parameters analysed followed a normal distribution (p's>0.05) except for latency to entry in the middle and center zones (p's<0.01) according to Kolmogorov-Smirnov test. Subsequent ANOVAs and Kruskal-Wallis tests indicated no significant group differences in any of the parameters analysed: percentage of time spent in peripheral ($F_{(3,32)}$= 0.373, p=0.773), middle ($F_{(3,32)}$= 0.335, p=0.8) and central ($F_{(3,32)}$= 0.582, p=0.631) zone, latency to entry in middle (p=0.744) and central (p=0.429) zone, entries in middle ($F_{(3,32)}$= 2.527, p=0.075) and central ($F_{(3,32)}$= 0.617, p=0.609) zone, zone transition ($F_{(3,32)}$= 2.51, p=0.076) and global activity ($F_{(3,32)}$= 0.477, p=0.7), except for entries in peripheral zone ($F_{(3,32)}$= 3.301, p=0.033) (***Fig. 98. A-E***). In particular, SCI+GSE15 group showed fewer entries in the periphery compared to the SCI+Saline and SCI+CE10 groups, while it did not differ significantly from the Sham group (***Fig. 98. C***). Overall, although some anxiety related parameters seem to be affected, taken together dark and light and open field tests it can be suggested no general anxiety development in the SCI chronic phase

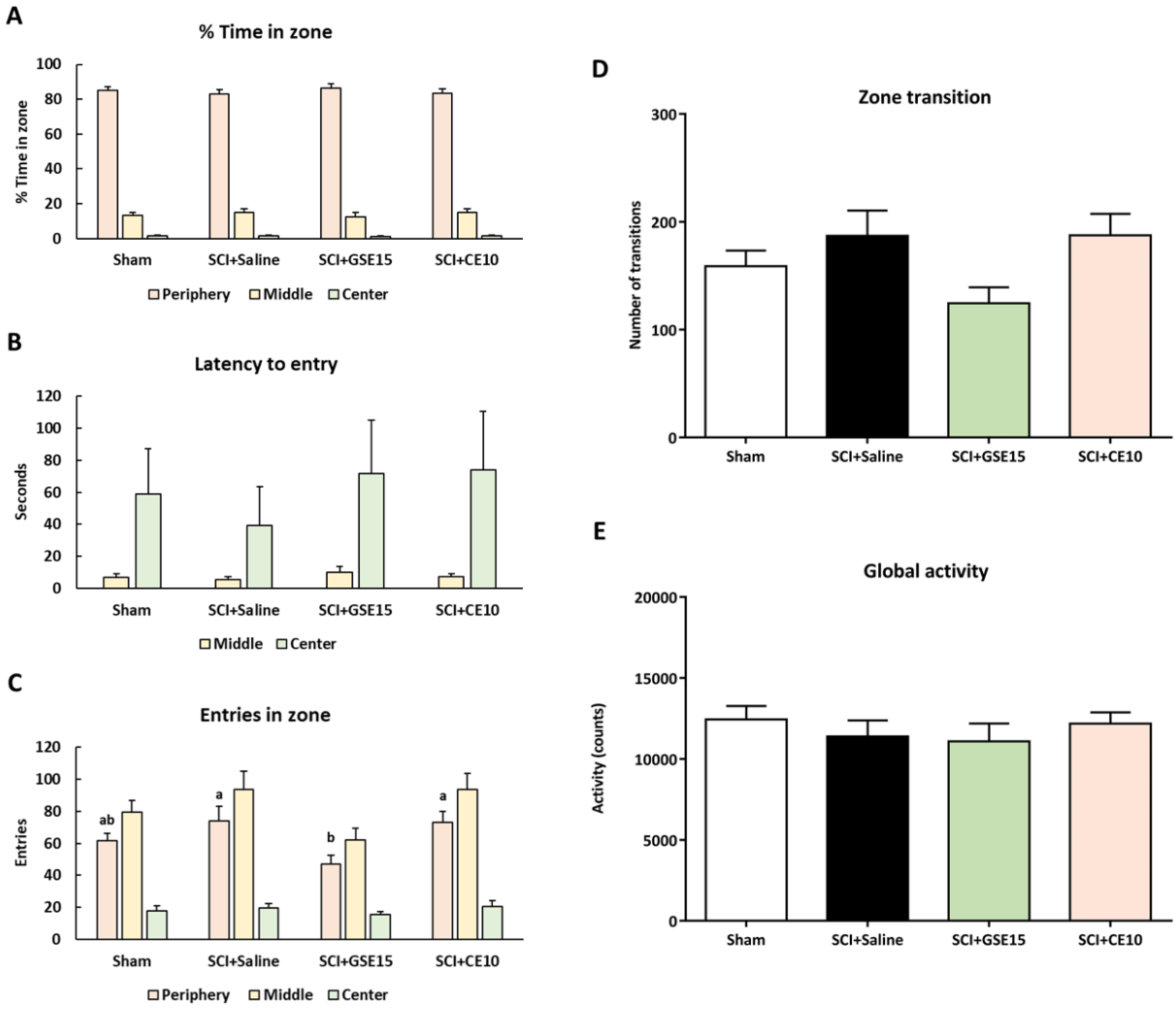


Figure 98. Open Field test at the chronic phase of SCI-induced neuropathic pain after GSE15 and CE10 treatments at the first-, third- and sixth-week post-injury. Results are represented as mean ± SEM. a-b: groups not sharing a letter are significantly different, $p<0.05$, by post-hocs' test. Experimental groups: Sham (n=8), SCI+Saline (n=9), SCI+GSE15 (n=9), SCI+CE10 (n=10).

Repeated GSE15 or CE10 administration during the first-, third- and sixth-week post-injury modulates depressive-like behaviour induced by mild spinal cord injury at 10 weeks post-injury.

In the depressive-like behaviour evaluation, Kolmogorov-Smirnov normality test confirmed a normal distribution of the data for percentage of mobility and immobility time, number of immobility and low activity (p's>0.05). In contrast, the high and global activity parameters did not follow a normal distribution (p's<0.05). For the percentage of immobility time, ANOVA analysis revealed significant group differences ($F_{(3,32)}$=12.344, p<0.001). Specifically, SCI+Saline animals spent more time immobile compared to both Sham and spinal cord injured animals treated with either GSE15 or CE10 ($p<0.05$). Treatment with CE10 was able to reduce the immobility time of SCI animals to Sham levels, while treatment with GSE15 was able to reduce it significantly without reaching Sham values (***Fig. 99. A***).

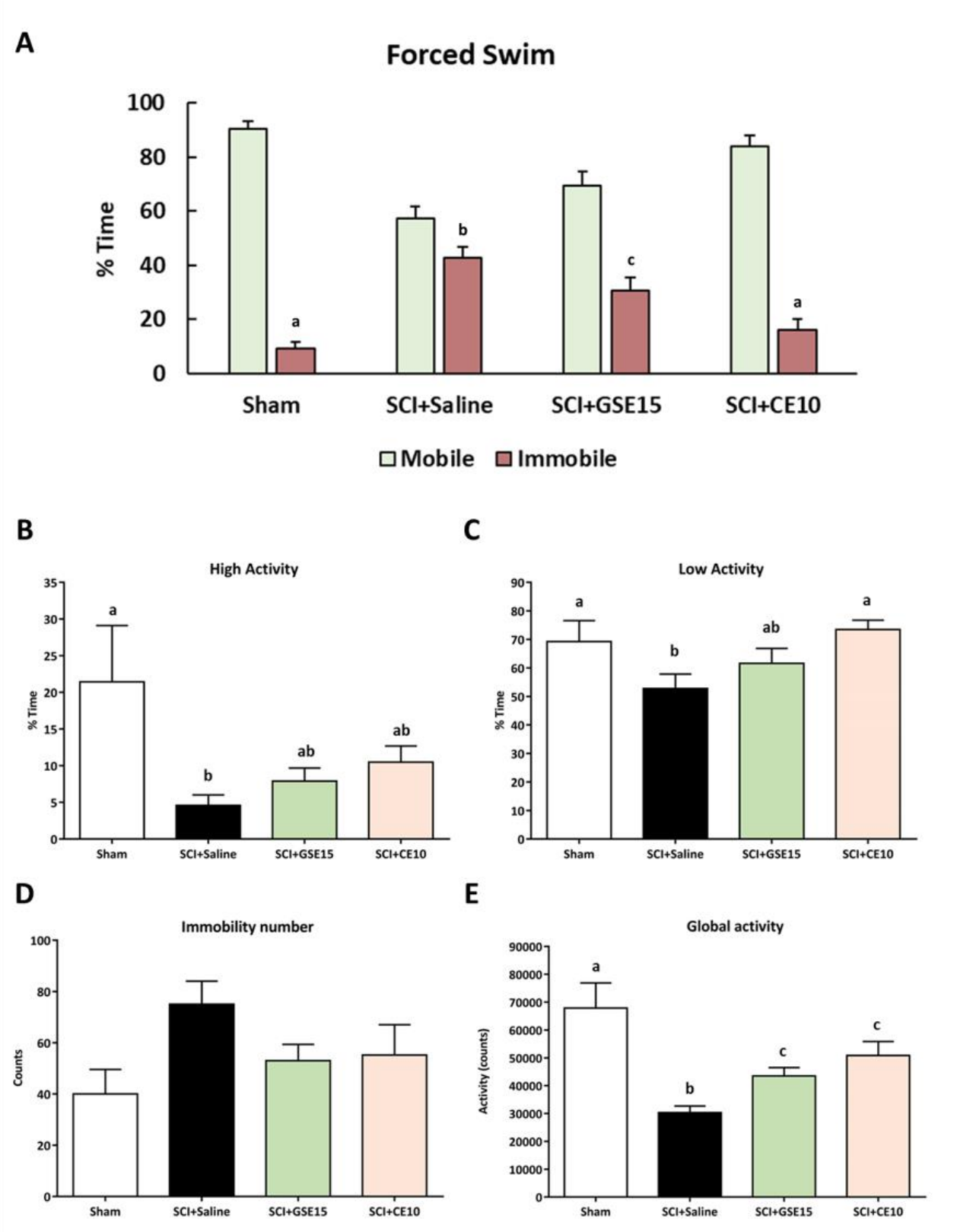


Figure 99. Forced swim test at the chronic phase of SCI-induced neuropathic pain after GSE15 and CE10 treatments at the first-, third- and sixth-week post-injury. Results are represented as mean ± SEM. a-c: groups not sharing a letter are significantly different, $p<0.05$, by post-hocs' test. Experimental groups: Sham (n=8), SCI+Saline (n=9), SCI+GSE15 (n=9), SCI+CE10 (n=10).

Referring to the global activity, Kruskal-Wallis test indicated significant differences between groups ($p<0.05$). As expected, Sham animals showed a higher global activity compared to the SCI+Saline group ($p<0.05$). As to the polyphenolic treatments, although both GSE15 and CE10 administration significantly increased the global activity levels of the SCI animals, they did not reach at Sham activity levels (***Fig. 99. E***). Continuing with activity levels, significant group differences were detected in the percentage of high (Kruskal-Wallis, $p=0.016$) and low (ANOVA, $F_{(3,32)}=3.116$, $p=0.04$) activity time (***Fig. 99. B, C***). Specifically, post-hoc analysis indicated that the percentage of time Sham animals spent in both high and low activity was significantly higher compared to SCI+Saline animals ($p<0.05$). On the one hand, although SCI animals treated with either GSE15 or CE10 spent more time in high activity than the SCI untreated animals, they did not differ significantly from either SCI+Saline or Sham groups ($p's>0.05$; ***Fig. 99. B***). On the other hand, SCI animals treated with either GSE15 or CE10 spent more time in low activity compared to SCI+Saline group. While the SCI+GSE15 group did not differ significantly from either the SCI+Saline or the Sham group, the SCI+CE10 group did not differ from the Sham group but did differ from the SCI+Saline group (***Fig. 99. C***). With regard to immobility number, ANOVA analysis revealed no significant differences between the experimental groups ($F_{(3,32)}=2.152$, $p=0.113$) (***Fig. 99. D***).

Repeated GSE15 or CE10 administration during the first-, third- and sixth-week post-injury modulates social interaction disturbances induced by mild spinal cord injury at 10 weeks post-injury.

As explained in the "materials and methods" section of this thesis, the Social Interaction test consisted in two evaluation trials: social affiliation aspect (session I) and social novelty/preference session (session II). In the session I, Kruskal-Wallis analysis revealed significant group differences in the direct interaction ($p=0.003$). Specifically, untreated spinal cord injured mice (SCI+Saline) showed a significant decrease in direct interaction with stranger mouse 1 compared to Sham. Although GSE15 treatment increased the number of interactions of SCI mice with the stranger mouse, the SCI+GSE15 group did not differ significantly from the SCI+Saline group. In contrast, treatment with CE10 significantly increased the number of interactions of SCI mice with the stranger mouse but did not reach the levels of Sham (***Fig. 100. A***). The rest of the parameters analysed in session 1 of the test showed no significant differences between groups: latency to the first interaction ($p=0.765$), preference time in the stranger mouse 1 area ($F_{(3,18)}=1.299$, $p=0.305$) and entries in stranger mouse 1 area ($F_{(3,18)}=0.488$, $p=0.695$) (***Fig. 100. B-D***). In addition, intra-group analysis of the preference time in zones indicated that all experimental groups spent significantly more time in the stranger mouse 1 area than in the empty area (***Fig. 100. C***).

In the social novelty/preference session (session II), ANOVA analysis indicated significant group differences in the direct interaction with the stranger mouse 2 ($F_{(3,18)}$=3.33, p=0.043). Specifically, Sham animals showed a significantly higher number of interactions with the stranger mouse 2 compared to SCI+Saline animals. On the one hand, treatment with GSE15 increased the number of interactions of SCI mice with the stranger mouse 2, although it did not differ significantly from either the SCI+Saline or Sham group. On the other hand, CE10 treatment significantly increased the number of interactions of SCI mice with the stranger mouse 2 at Sham levels, showing no significant differences with the Sham group. In addition, intra-group analysis indicated that the only experimental groups that showed a significantly higher number of interactions with stranger mouse 2 than with stranger mouse 1 were the Sham group ($F_{(1,6)}$=158.897, p<0.001) and the SCI+CE10 group ($F_{(1,12)}$=9.919, p=0.008). In contrast to the direct interaction with the stranger mouse 2, Kruskal-Wallis test revealed no significant differences between groups in the number of direct interactions with the stranger mouse 1 (p=0.174) (***Fig. 100. E***).

Similar to the session I, the rest of the parameters analysed in the session II showed no significant differences between groups. Data were analysed by ANOVA or Kruskal-Wallis test depending on their distribution: latency to the first interaction with stranger 1 mouse (p=0.27) and with stranger 2 (p=0.726), preference time in the stranger mouse 1 area ($F_{(3,18)}$=0.568, p=0.643) and in the stranger mouse 2 area (p=0.247), and entries in stranger mouse 1 area ($F_{(3,18)}$=1.352, p=0.289) and in stranger mouse 2 area ($F_{(3,18)}$=1.207, p=0.336) (***Fig. 100. F-H***). Intra-group analysis of the latency to the first interaction indicated that the only group that took significantly longer to interact with stranger mouse 1 than with stranger mouse 2 was Sham ($F_{(3,18)}$=6.269, p=0.046) (***Fig. 100. F***). Finally, intra-group analysis of the preference time in zones indicated that the only experimental groups that preferred to spend more time in the stranger mouse 2 area than in the stranger mouse 1 area were the Sham ($F_{(1,6)}$=65.102, p<0.001) and SCI+GSE15 ($F_{(1,10)}$=10.52, p=0.009) groups (***Fig. 100. G***).

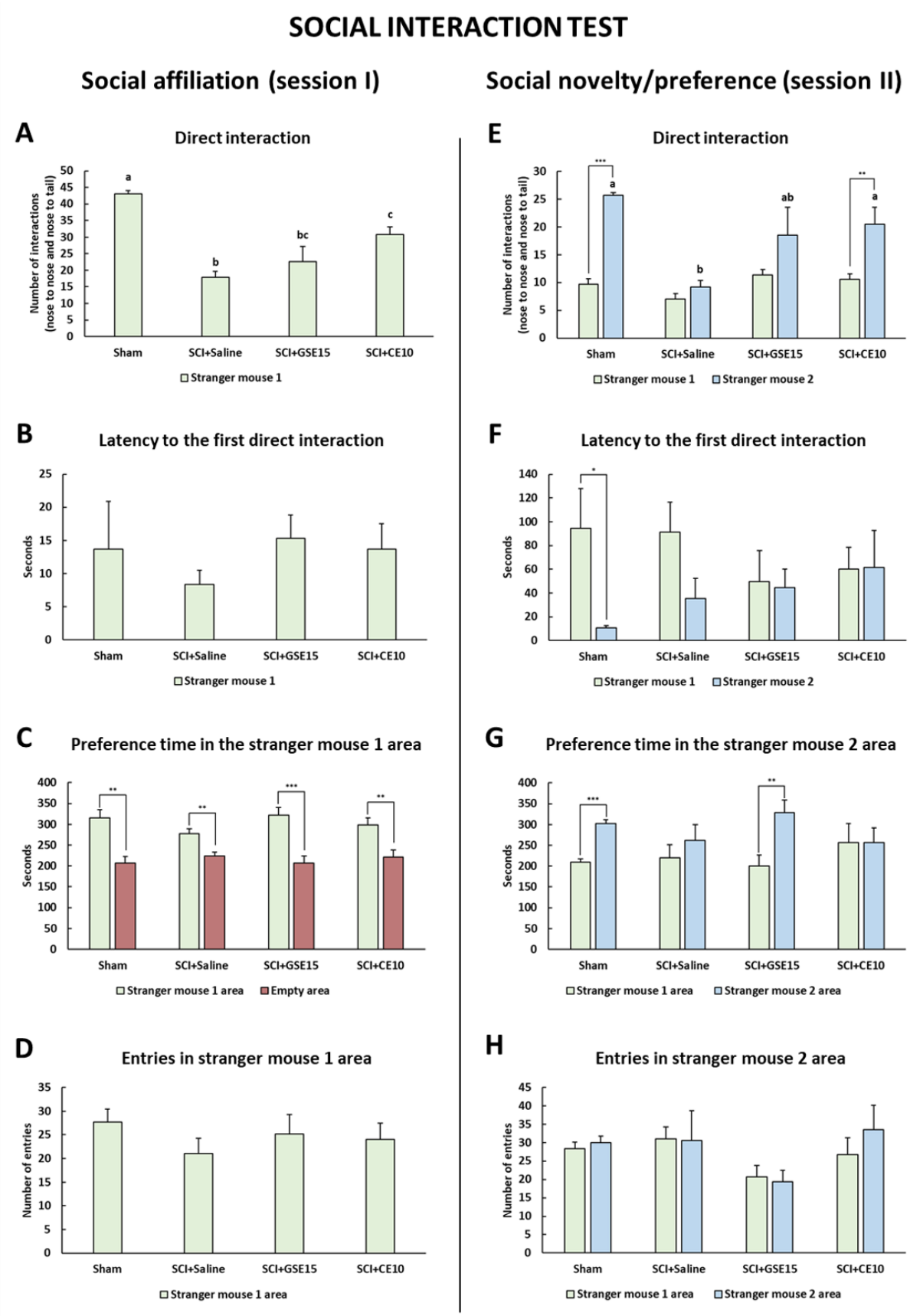


Figure 100. Social interaction test at the chronic phase of SCI-induced neuropathic pain after GSE15 and CE10 treatments at the first-, third- and sixth-week post-injury. Results are represented as mean ± SEM. a-c: groups not sharing a letter are significantly different, p<0.05, by post-hocs' test. Intra-groups significant differences: *** p<0.001, ** p<0.01, * p<0.05. Experimental groups: Sham (n=4), SCI+Saline (n=5), SCI+GSE15 (n=6), SCI+CE10 (n=7).

Repeated GSE15 or CE10 administration during the first-, third- and sixth-week post-injury slightly modulates microgliosis in spinal cord at 10 weeks post-SCI.

To gain some mechanistic insights into why these polyphenolic treatments were able to prevent the development of reflexive and non-reflexive neuropathic pain responses, the protein expression of both GFAP and IBA1 was analysed in the spinal cord. According to the Shapiro-Wilk normality test, none of the markers followed a normal distribution (all p's<0.001). Kruskal-Wallis's post-tests indicated significant differences between groups for IBA1 protein expression ($p<0.05$), but not for GFAP expression ($p=0.371$). Specifically, SCI+Saline group showed significant increase of IBA1 expression when compared to Sham group ($p<0.01$). As for GSE15 and CE10 treatment groups did not showed significant differences with either Sham or SCI+Saline groups ($p>0.05$) suggesting a slight modulation without reaching the same Sham's IBA1 expression (***Fig. 101***).

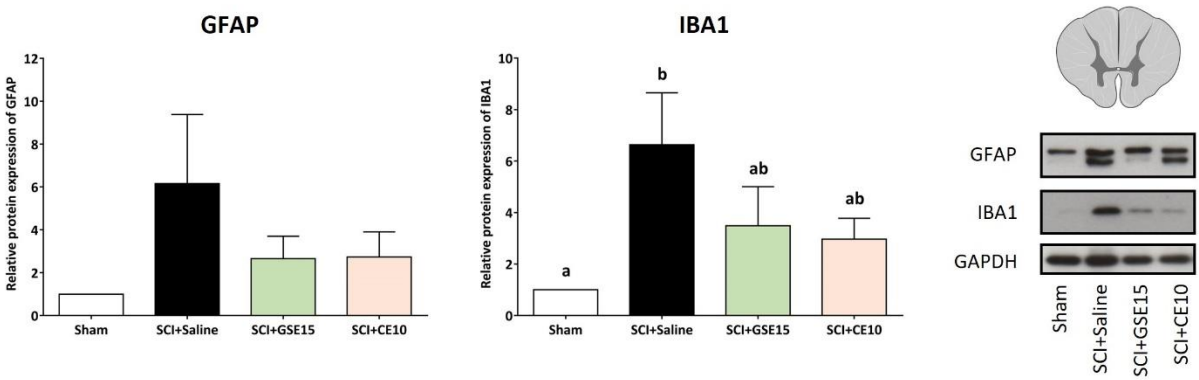


Figure 101. Expression of GFAP and IBA1 in spinal cord at the chronic phase of SCI-induced neuropathic pain after GSE15 and CE10 treatments at the first-, third- and sixth-week post-injury. Protein expression was normalized to GAPDH. Data is expressed as a relative percentage respect to Sham group (mean ± SEM). a–b: Groups not sharing a letter showed significant differences, $p<0.05$. Experimental groups: Sham (GFAP n=8; IBA1 n=6), SCI+Saline (GFAP n=7; IBA1 n=6), SCI+GSE15 (GFAP n=6; IBA1 n=5), SCI+CE10 (GFAP n=9; IBA1 n=6).

Repeated GSE15 or CE10 administration during the first-, third- and sixth-week post-injury reduces astrogliosis and modulates CCL2/CCR2 and CX3CL1/CX3CR1 signalling in ACC and amygdala at 10 weeks post-SCI.

The anterior cingulate cortex (ACC) and amygdala (AMG) are considered to be key neural substrates underlying pain affect (**Gao et al., 2004**). Anatomically, the ACC has strong connections with amygdala, which is implicated in in the processing of mood, fear, emotional memory, and the emotional-affective dimension of pain (**Veinante et al., 2013**). For this reason, gliosis (protein expression of GFAP and IBA1), expression of the chemokines CCL2 and CX3CL1 and their receptors, CCR2 and CX3CR1, as well as Cathepsin

S (CatS), responsible for the proteolytic release of soluble CX3CL1 (**Clark et al., 2007**), were analysed in these two supraspinal structures.

Regarding to the ACC and according to the Shapiro-Wilk normality test, none of the markers followed a normal distribution (all p's<0.05). Thus, Kruskal-Wallis tests were applied and showed significant differences between groups for all markers analysed (all p's<0.001). Specifically, Mann Whitney U tests indicated that GFAP, IBA1, CCL2/CCR2, CX3CL1/CX3CR1 and CatS were overexpressed in the SCI+Saline group compared to the Sham group (all p's<0.01). On the one hand, GSE15 treatment reduced astrogliosis (GFAP) and CCR2 expression in ACC compared to SCI+Saline animals (p's<0.01). While GSE15 treatment reduced CCR2 expression below Sham levels (p<0.01), astrogliosis observed in SCI animals treated with GSE15 was still significantly higher than that observed in Sham animals (p<0.01). For the rest of the markers, SCI animals treated with GSE15 showed similar (IBA1, p=0.485) or even higher expression levels (CCL2, CX3CL1, CX3CR1 and CatS; p<0.01) than those observed in saline-treated SCI animals. On the other hand, CE10 treatment reduced astrogliosis (GFAP) and expression of CCL2/CCR2, CX3CR1 and CatS compared to SCI+Saline animals (all p's<0.01). Despite this reduction, the expression levels of all markers were still significantly higher than those observed in Sham animals (all p's<0.01), except for CX3CR1, which did not differ from the Sham group (p=0.065) (***Fig. 102***).

As for amygdala, Shapiro-Wilk normality test revealed that data did not follow a normal distribution for all the markers (p's<0.05) and further Kruskal-Wallis tests indicated significant differences on protein expression between groups (p<0.01). In particular, Mann Whitney U tests indicated that while GFAP, CCR2, CX3CL1/CX3CR1 and CatS were overexpressed in the SCI+Saline group compared to the Sham group (all p's<0.01), the expression levels of IBA1 and CCL2 in the amygdala were lower in saline-treated SCI animals compared to Sham animals (p's<0.01). Regarding to polyphenolic treatments, both GSE15 and CE10 reduced the expression of GFAP, CCR2, CX3CL1, CX3CR1 and CatS compared to non-treated SCI animals (all p's<0.01). As for grape stalk extract, GSE15 treatment reduced astrogliosis below the Sham levels (p<0.01), decreased CCR2, CX3CL1 and CatS expression to levels significantly equal to those registered in the Sham group (p's>0.05) and although it reduced CX3CR1 expression, it remained significantly higher than that observed in the Sham group (p<0.01). Referring to CE10 treatment, it downregulated the expression of CCR2, CX3CL1, CX3CR1 and CatS to below levels of the Sham group (p's<0.01) and reduced the astrogliosis to levels similar to those observed in the Sham group (p=0.394) (***Fig. 103***).

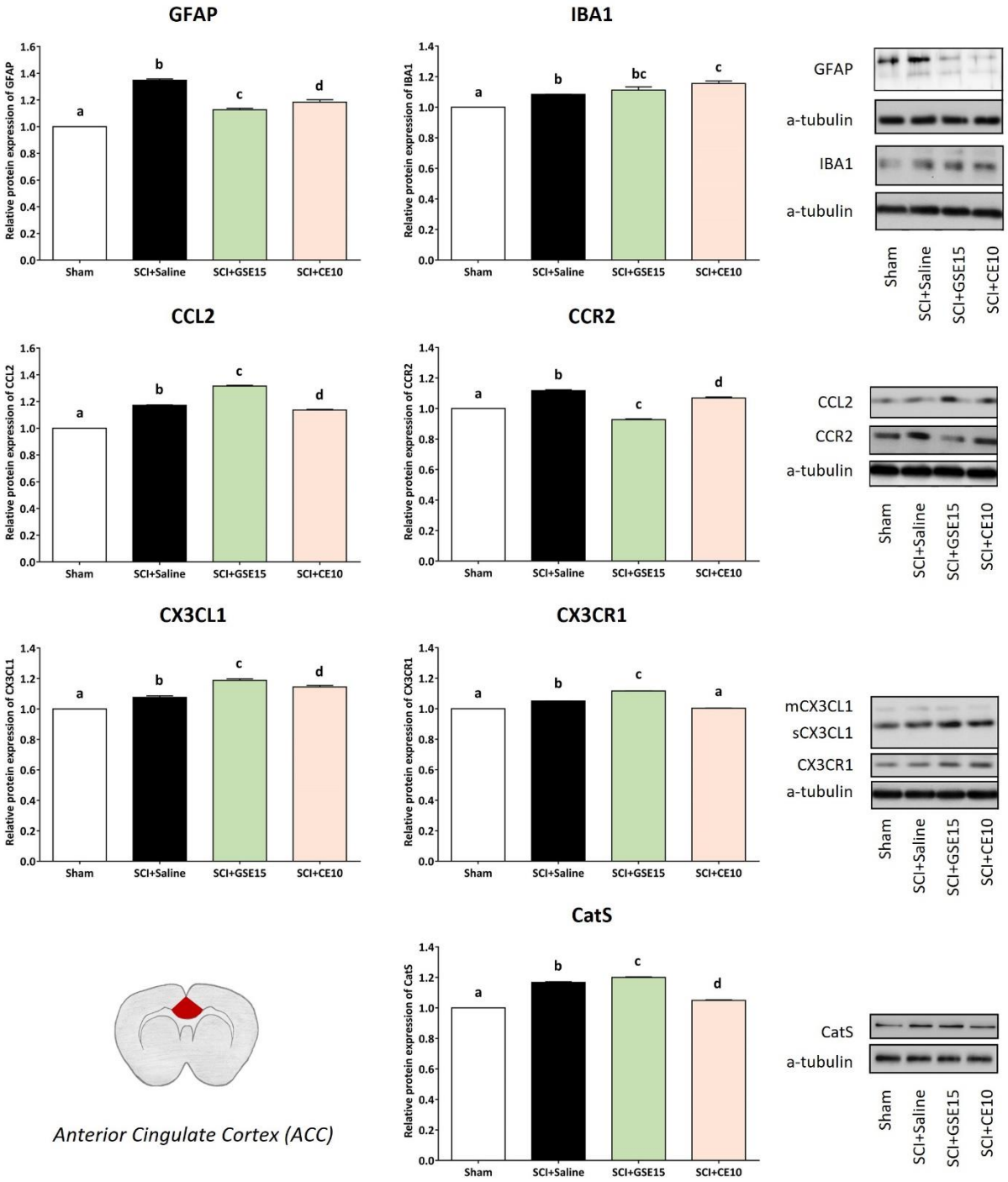


Figure 102. Expression of GFAP, IBA1, CCL2, CCR2, CX3CL1, CX3CR1 and CatS in ACC at the chronic phase of SCI-induced neuropathic pain after GSE15 and CE10 treatments at the first-, third- and sixth-week post-injury. Protein expression was normalized to a-tubulin. Data is expressed as a relative percentage respect to Sham group (mean ± SEM). a–d: Groups not sharing a letter showed significant differences, $p<0.05$. Experimental groups: Sham (GFAP n=6; IBA1 n=6; CCL2 n=6; CCR2 n=7; CX3CL1 n=6; CX3CR1 n=6; CatS n=6), SCI+Saline (GFAP n=6; IBA1 n=6; CCL2 n=6; CCR2 n=7; CX3CL1 n=6; CX3CR1 n=6; CatS n=6), SCI+GSE15 (GFAP n=6; IBA1 n=6; CCL2 n=6; CCR2 n=7; CX3CL1 n=6; CX3CR1 n=6; CatS n=6), SCI+CE10 (GFAP n=6; IBA1 n=6; CCL2 n=6; CCR2 n=7; CX3CL1 n=6; CX3CR1 n=6; CatS n=6).

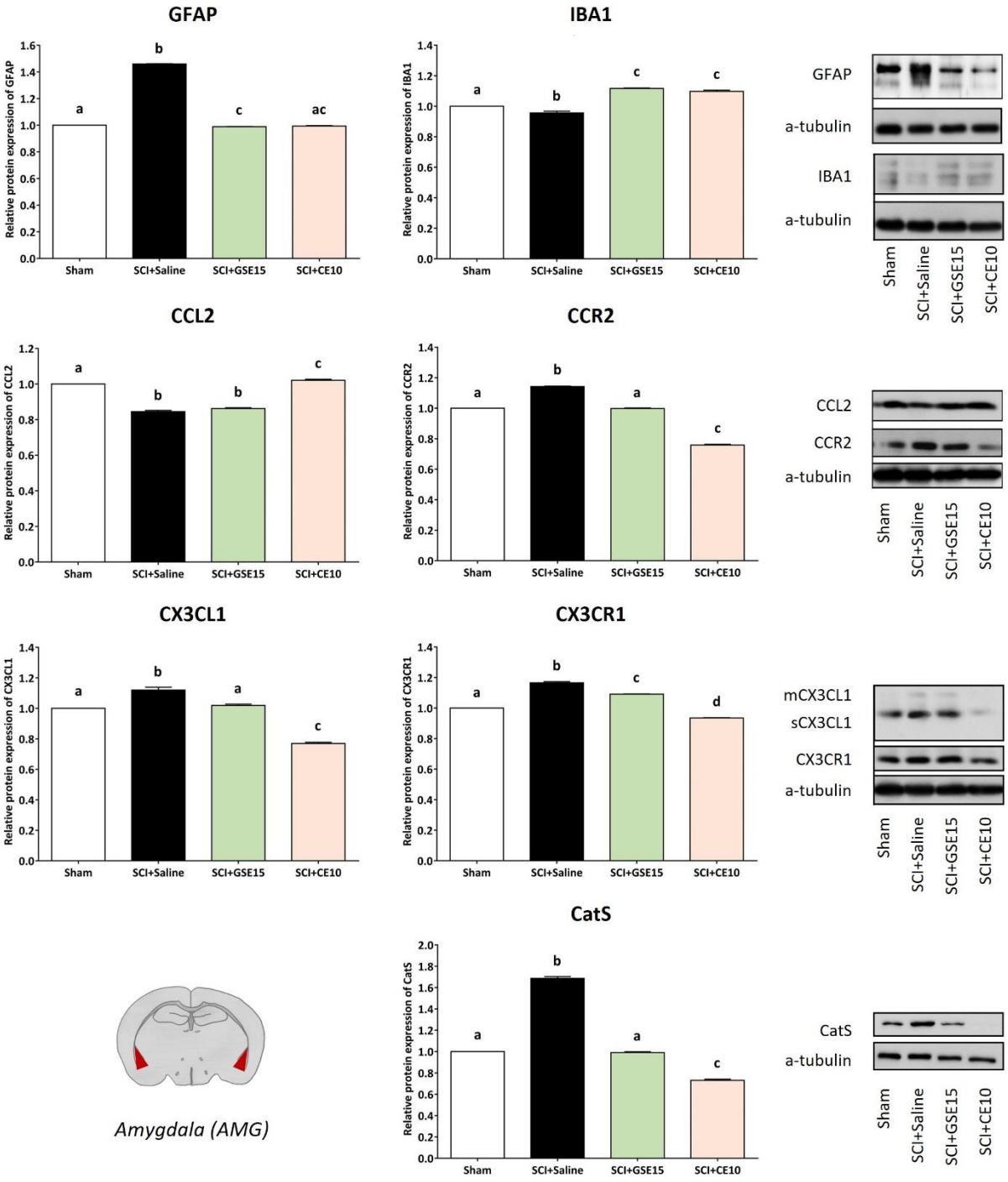


Figure 103. Expression of GFAP, IBA1, CCL2, CCR2, CX3CL1, CX3CR1 and CatS in AMG at the chronic phase of SCI-induced neuropathic pain after GSE15 and CE10 treatments at the first-, third- and sixth-week post-injury. Protein expression was normalized to a-tubulin. Data is expressed as a relative percentage respect to Sham group (mean ± SEM). a–d: Groups not sharing a letter showed significant differences, $p<0.05$. Experimental groups: Sham (GFAP n=6; IBA1 n=6; CCL2 n=6; CCR2 n=7; CX3CL1 n=6; CX3CR1 n=6; CatS n=6), SCI+Saline (GFAP n=6; IBA1 n=6; CCL2 n=6; CCR2 n=7; CX3CL1 n=6; CX3CR1 n=6; CatS n=6), SCI+GSE15 (GFAP n=6; IBA1 n=6; CCL2 n=6; CCR2 n=7; CX3CL1 n=6; CX3CR1 n=6; CatS n=6), SCI+CE10 (GFAP n=6; IBA1 n=6; CCL2 n=6; CCR2 n=7; CX3CL1 n=6; CX3CR1 n=6; CatS n=6).

Repeated GSE15 or CE10 administration during the first-, third- and sixth-week post-injury reduces astrogliosis and modulates CCL2/CCR2 and CX3CL1/CX3CR1 signalling in PAG and RVM at 10 weeks post-SCI.

The periaqueductal grey (PAG) and rostral ventromedial medulla (RVM) have a pivotal role in nociceptive modulation (**Fields et al., 2006**). While PAG receives inputs from higher brain centres and can activate a potent analgesic effect, RVM can both facilitate or inhibit nociceptive inputs and acts as a final relay in the control of descending pain facilitation (**Ossipov et al., 2014**). The PAG has a columnar functional organisation with ventrolateral PAG (vPAG) and dorsolateral PAG (dPAG) columns that have differences in antinociceptive effects with respect to their dependence on opioid mechanisms (**Lane et al, 2004; Lovick and Bandler, 2005; Eidson and Murphy, 2013; Wilson-Poe et al., 2013**). Given that different subregions of the PAG may play different or specific roles in the descending modulation of pain (**McMullan and Lumb, 2006; Eidson and Murphy, 2013**), it is interesting to investigate the expression of nociceptive markers separately in the dPAG and vPAG after SCI, and whether these changes appear in parallel in the RVM.

Therefore, the levels of gliosis (GFAP and IBA1), chemokines CCL2 and CX3CL1 and their receptors, CCR2 and CX3CR1, as well as CatS were analysed in the dPAG and vPAG respectively. According to the Shapiro-Wilk normality test, none of the markers analysed in both dPAG and vPAG followed a normal distribution (all p's<0.05) and further Kruskal Wallis tests indicated significant differences on protein expression between groups (all p's<0.001). Regarding dPAG, all the markers were overexpressed in SCI saline-treated animals in comparison to Sham animals (all p's<0.01) except CCL2, which showed the same expression levels in both Sham and SCI+Saline groups (p=0.065). Both GSE15 and CE10 treatments reduced GFAP, CX3CL1/CX3CR1 and CatS in dPAG compared to saline-treated SCI animals (all p's<0.01). GSE15 reduced the expression of CX3CL1 and CatS below the Sham levels (p's<0.01) and although it also decreased the expression of GFAP and CX3CR1, these were significantly higher than those observed in the Sham group (p's<0.01). As for CE, it also reduced CX3CL1 and CatS below the Sham levels (p's<0.01), decreased CX3CR1 expression to levels significantly equal to those observed in the Sham group (p=0.394) and although it reduced astrogliosis, GFAP expression remained significantly higher than that observed in the Sham group (p<0.01). Finally, animals treated with either GSE15 or CE10 showed an increase in CCL2 expression at dPAG in comparison to saline-treated animals (p's<0.01). The same happened for the marker IBA1 in CE10-treated animals, which showed more microgliosis than animals in the SCI+Saline group (p<0.01). In contrast, animals treated with GSE15 showed IBA1 expression levels significantly equal to those observed in the SCI+Saline group (p=0.699) (***Fig. 104***). Concerning vPAG, all the markers were overexpressed in SCI saline-treated animals compared to Sham animals (all p's<0.01) except CCR2, whose expression levels were lower in saline-treated animals compared to Sham animals (p<0.01). Both GSE15 and CE10 treatments were able to reduce

the expression of GFAP, IBA1, CCL2, CX3CL1, CX3CR1 and CatS below the levels observed in the SCI+Saline group (all p's<0.01). On the one hand, GSE15 reduced the expression of CX3CL1 and CatS below Sham levels (p's<0.01), decreased the expression of IBA1 and CCL2 to levels significantly equal to those registered in the Sham group (p's>0.05) and decreased the expression of GFAP and CX3CR1 but without reaching the expression levels observed in the Sham group (p's<0.01). On the other hand, CE10 treatment reduced CCL2, CX3CL1, CX3CR1 and CatS expression below Sham levels (all p's<0.01). Moreover, although CE10 treatment modulated both astrogliosis and microgliosis in the vPAG, GFAP and IBA1 expression remained significantly higher than that observed in the Sham group (p's<0.01) (***Fig. 105***).

Referring to RVM, none of the markers analysed followed a normal distribution (all p's<0.01) according to Shapiro-Wilk normality test. Further Kruskal-Wallis tests indicated significant differences on protein expression between groups (p<0.01). Concretely, Mann Whitney U tests indicated that all markers analysed were overexpressed in the SCI+Saline group compared to the Sham group (all p's<0.01). Regarding to polyphenolic treatments, both GSE15 and CE10 reduced GFAP, CX3CL1/CX3CR1 and CatS expression compared to the SCI+Saline group (all p's<0.05). Despite this reduction, the expression levels of these markers in GSE15 and CE10 treated animals were still significantly higher than those observed in Sham animals (all p's<0.01), except for CX3CR1 in CE10 treated animals, which did not differ from the Sham group (p=0.065). Finally, the expression levels of IBA1 and CCL2/CCR2 were significantly higher in both GSE15 and CE10 treated animals compared to non-treated SCI animals (all p's<0.05), except for IBA1 in CE10 treated animals, which did not differ from the SCI+Saline group (p=1) (***Fig. 106***).

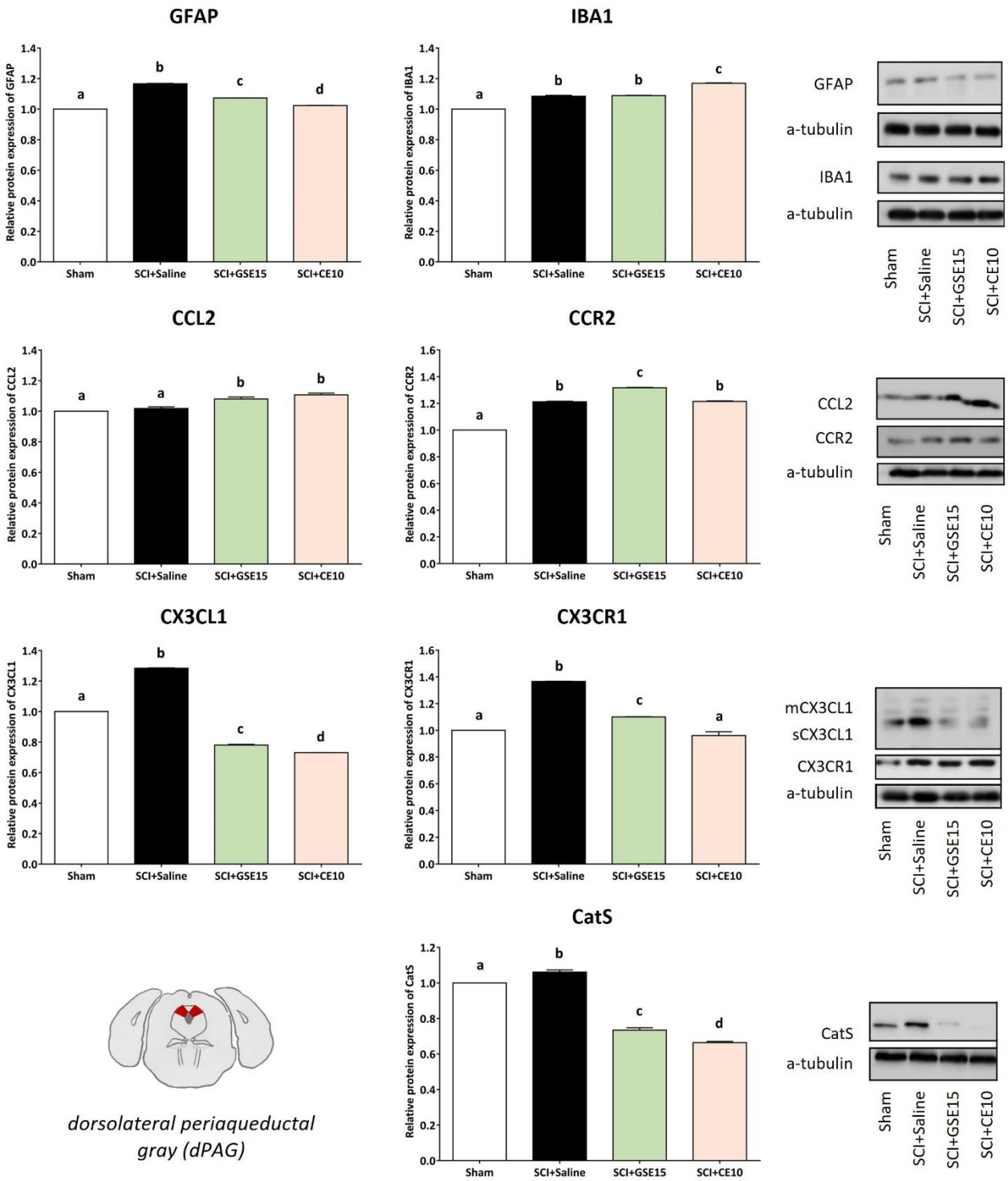


Figure 104. Expression of GFAP, IBA1, CCL2, CCR2, CX3CL1, CX3CR1 and CatS in dPAG at the chronic phase of SCI-induced neuropathic pain after GSE15 and CE10 treatments at the first-, third- and sixth-week post-injury. Protein expression was normalized to a-tubulin. Data is expressed as a relative percentage respect to Sham group (mean ± SEM). a–d: Groups not sharing a letter showed significant differences, $p<0.05$. Experimental groups: Sham (GFAP n=6; IBA1 n=6; CCL2 n=6; CCR2 n=7; CX3CL1 n=6; CX3CR1 n=6; CatS n=6), SCI+Saline (GFAP n=6; IBA1 n=6; CCL2 n=6; CCR2 n=7; CX3CL1 n=6; CX3CR1 n=6; CatS n=6), SCI+GSE15 (GFAP n=6; IBA1 n=6; CCL2 n=6; CCR2 n=7; CX3CL1 n=6; CX3CR1 n=6; CatS n=6), SCI+CE10 (GFAP n=6; IBA1 n=6; CCL2 n=6; CCR2 n=7; CX3CL1 n=6; CX3CR1 n=6; CatS n=6).

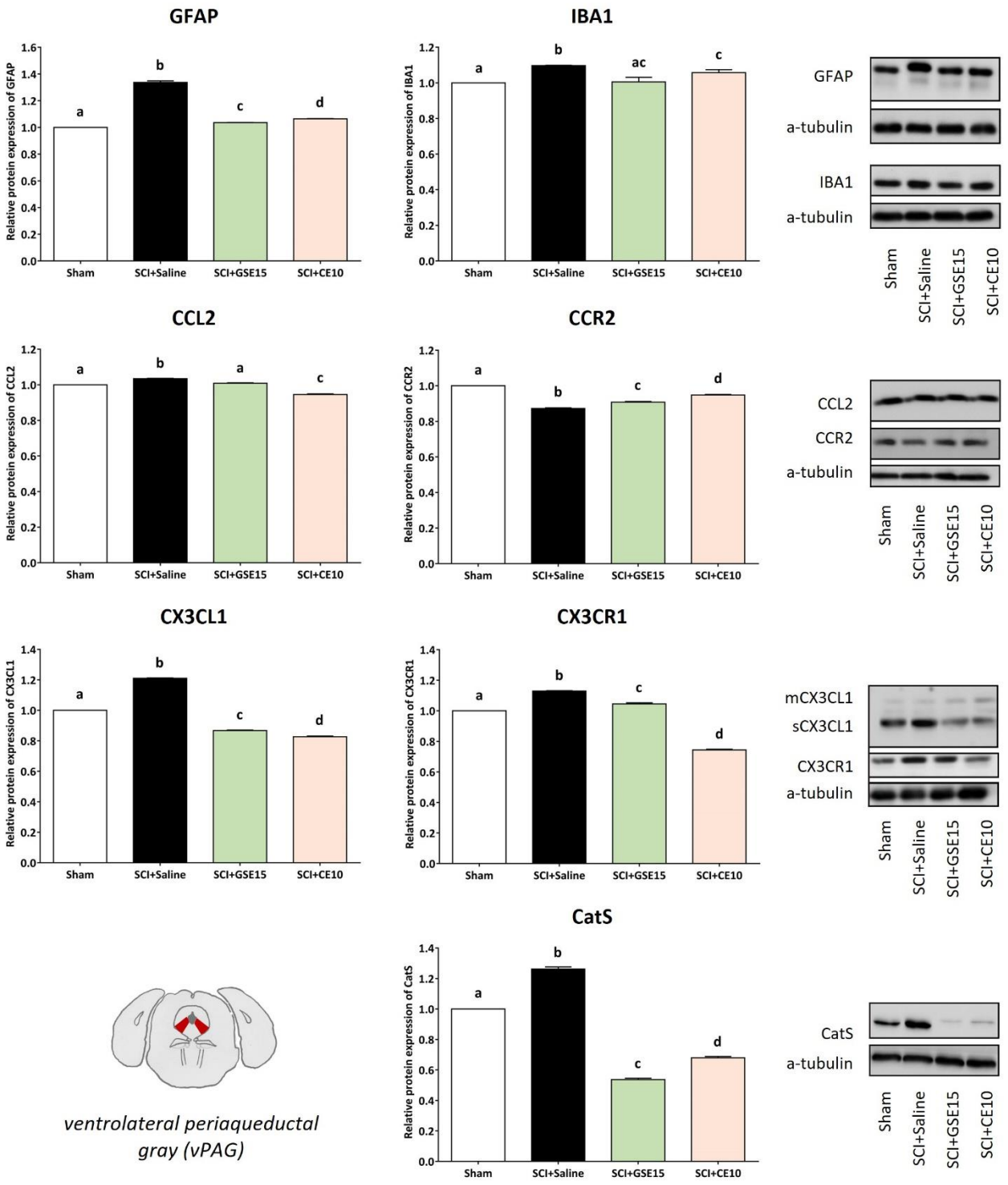


Figure 105. Expression of GFAP, IBA1, CCL2, CCR2, CX3CL1, CX3CR1 and CatS in vPAG at the chronic phase of SCI-induced neuropathic pain after GSE15 and CE10 treatments at the first-, third- and sixth-week post-injury. Protein expression was normalized to a-tubulin. Data is expressed as a relative percentage respect to Sham group (mean ± SEM). a–d: Groups not sharing a letter showed significant differences, $p<0.05$. Experimental groups: Sham (GFAP n=6; IBA1 n=6; CCL2 n=6; CCR2 n=7; CX3CL1 n=6; CX3CR1 n=6; CatS n=6), SCI+Saline (GFAP n=6; IBA1 n=6; CCL2 n=6; CCR2 n=7; CX3CL1 n=6; CX3CR1 n=6; CatS n=6), SCI+GSE15 (GFAP n=6; IBA1 n=6; CCL2 n=6; CCR2 n=7; CX3CL1 n=6; CX3CR1 n=6; CatS n=6), SCI+CE10 (GFAP n=6; IBA1 n=6; CCL2 n=6; CCR2 n=7; CX3CL1 n=6; CX3CR1 n=6; CatS n=6).

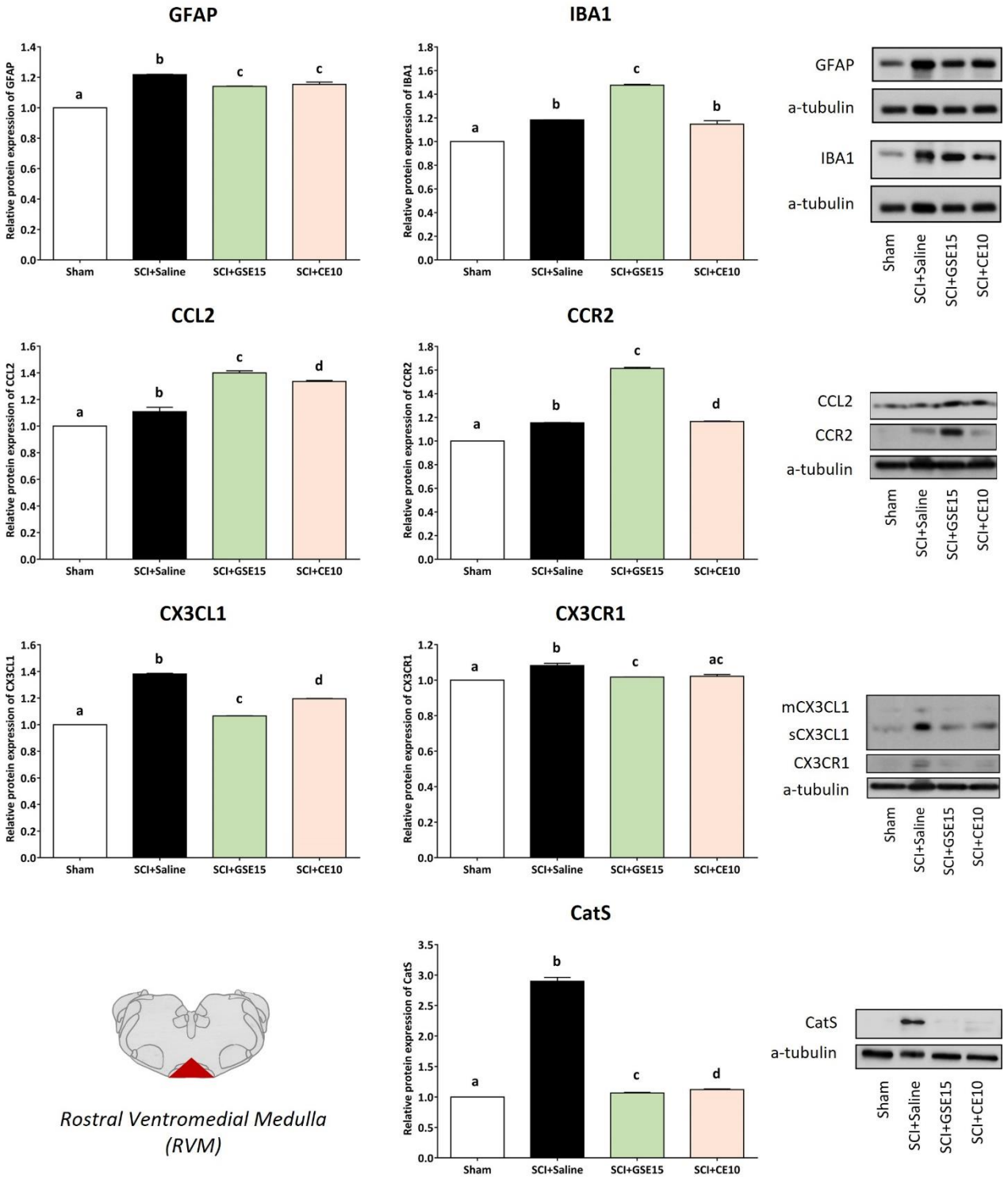


Figure 106. Expression of GFAP, IBA1, CCL2, CCR2, CX3CL1, CX3CR1 and CatS in RVM at the chronic phase of SCI-induced neuropathic pain after GSE15 and CE10 treatments at the first-, third- and sixth-week post-injury. Protein expression was normalized to a-tubulin. Data is expressed as a relative percentage respect to Sham group (mean ± SEM). a–d: Groups not sharing a letter showed significant differences, $p<0.05$. Experimental groups: Sham (GFAP n=6; IBA1 n=6; CCL2 n=6; CCR2 n=7; CX3CL1 n=6; CX3CR1 n=6; CatS n=8), SCI+Saline (GFAP n=6; IBA1 n=6; CCL2 n=6; CCR2 n=7; CX3CL1 n=6; CX3CR1 n=6; CatS n=8), SCI+GSE15 (GFAP n=6; IBA1 n=6; CCL2 n=6; CCR2 n=7; CX3CL1 n=6; CX3CR1 n=6; CatS n=8), SCI+CE10 (GFAP n=6; IBA1 n=6; CCL2 n=6; CCR2 n=7; CX3CL1 n=6; CX3CR1 n=6; CatS n=8).

Summary results Chapter V

In the present chapter, we have demonstrated that there is no systemic toxicity associated with repeated administrations of GSE (15 mg/kg) and CE (10 mg/kg; i.p) during the first-, third- and sixth-week post- SCI and neither hepatotoxic nor nephrotoxic effects, as they did not significantly affect the weight or aspect of the animals and did not alter the biomarkers of hepatotoxicity and nephrotoxicity in the animals' serum.

On the one hand, regarding evoked pain responses, both GSE15 and CE10 treatments prevented thermal hyperalgesia development from the first to the tenth week after injury and prevented mechanical allodynia development for as long as this evoked response was manifested in the SCI animals, which was up to week 9. On the other hand, disturbances in the affective behaviours such as anhedonia, depression, anxiety, and social interaction impairment were also modulated by the GSE15 and CE10 treatments.

In addition, molecular studies revealed that both GSE15 and CE10 slightly modulated IBA1 overexpression detected in the spinal cord of SCI+Saline mice at 10 wpi. Moreover, such treatments also modulated astrogliosis and CCL2/CCR2 and CX3CL1/CX3CR1 signalling in the supraspinal structures of ACC, amygdala, dorsal and ventral PAG and RVM, as well as microgliosis in ventral PAG, at 10 weeks post-SCI.

Altogether, these results suggest potential role of both GSE and CE treatments in preventing the SCI-induced reflexive and non-reflexive neuropathic pain responses until the chronic phase of injury. Such effects would be exerted by modulating central sensitization phenomena present not only at the site of injury but also on pain-related supraspinal structures such as the ACC, amygdala, dPAG, vPAG and RVM.

DISCUSSION

V. DISCUSSION

Spinal cord injury (SCI) results in severe motor, sensory and autonomic dysfunction frequently accompanied by neuropathic pain (**Ding et al., 2005; Nees et al., 2017**). Neuropathic pain after SCI has a negative impact on the patient's quality of life and rehabilitation, being a major specific health problem in its own right (**Baastrup and Finnerup, 2008**). Approximately 40% to 60% of all SCI patients develop neuropathic pain at or below the level of injury (**Ataoğlu et al., 2013; Finnerup et al., 2014; Burke et al., 2017**) and half of them report pain levels as moderate to severe (**Siddall et al., 2003; Finnerup et al., 2014**). Neuropathic pain following SCI usually develops within the first year and tends to become chronic and life-long, leading to severe psychosocial and functional consequences (**Budh et al., 2003; Siddall et al., 2003; Werhagen et al., 2004; Widerström-Noga et al., 2017**).

Since SCI neuropathic pain comprises several pathological mechanisms, current treatments are often ineffective because they target only one or two of these mechanisms (**Nijs, 2019**). Nowadays, to design a personalized therapeutic approach, treatment consists in a “trial-and-error” of different strategies, including pharmacological and non-pharmacological (**Martínez-Salio, 2012; Attal, 2019**). Pharmacological treatment includes antidepressants, antiepileptics, GABA-antagonists, local anaesthetics, NMDA-antagonists, cannabinoids and opioids (**Dworkin, 2007; Baron, 2010; Baastrup & Finnerup, 2012; Finnerup, 2015; Colloca, 2017; Zilliox, 2017; Attal, 2019**). However, these drugs are usually inadequate and only one third of patients respond to pharmacological treatments when compared with placebo (**Attal et al., 2011; Attal, 2019**). Moreover, the best pharmacological strategy results in a reduction of only 20–30% in pain intensity, frequently accompanied by severe side effects (**Dworkin et al., 2007; Finnerup et al., 2010**).

In this context, the lack of effective treatments is one of the main reasons for the chronicity of neuropathic pain after SCI (**Finnerup, Sindrup and Jensen, 2010; Vranken, 2015**) and nowadays most of SCI patients are in the chronic phase (**Tashiro et al., 2017**). Moreover, central neuropathic pain implies a high probability of severe disabilities in daily life and is commonly associated with an increased vulnerability to develop emotional disorders, especially when this pain becomes chronic (**Arango-Lasprilla et al., 2011; Lazzaro et al., 2013**).

Thus, given the lack of effective treatments and the increasing number of patients to SCI chronic phase, it is necessary to develop new pharmacological strategies not only for the relief of neuropathic pain but also for the prevention of its chronification. Current strategies aimed at modulating pathological pain include the use of polyphenols, as preclinical evidence of their antinociceptive effects can be found in the literature (**Boadas-Vaello, 2017; Rao et al., 2021**). Among the properties that can be attributed to polyphenols that may explain pain modulation are free radical scavenging/antioxidant, immunomodulatory,

neuroprotective, anti-apoptotic and autophagy-regulating activities (**Boadas-Vaello, 2017**). However, although several studies have been specifically aimed at elucidating the effects of polyphenolic treatments on the development of neuropathic pain, most of them have been conducted in preclinical models unrelated to SCI, such as peripheral neuropathic pain (**Çivi et al., 2016; Renno et al., 2017; Wang et al., 2020; Limcharoen et al., 2021**), diabetic neuropathic pain (**Raposo et al., 2015; Li et al., 2018; Cui et al., 2020; Dhaliwal et al., 2020**) or alcoholic neuropathy (**Tiwari et al., 2011; Raygude et al., 2012;**), among others. Furthermore, although studies on polyphenol treatment after SCI are available, most of them have focused on motor recovery or spinal cord regeneration (**Cao et al., 2010; Song et al., 2013; Tian et al., 2013; Çiftçi et al., 2016; Zhang et al., 2016; Wang et al, 2018**), leading to a lack of information despite promising results on its effects on modulating pathophysiological processes that may also be related to the induction of neuropathic pain. Thus, while there is a wealth of data on the beneficial effects of polyphenolic treatments, fewer studies have demonstrated the modulation of SCI-induced neuropathic pain by polyphenols (**Hassler et al., 2014; Renno et al., 2014; Álvarez-Pérez et al., 2016; Ma et al., 2018**).

In this context, the present thesis was conceived to evaluate new polyphenolic treatments as novel pharmacological strategies to address SCI neuropathic pain health concern. To this end, a suitable animal model was used, in which experimental studies may be properly conducted according to previous experimental data (**Álvarez-Pérez et al., 2016; Castany et al., 2018, 2019**). To be consistent, female CD1 mice were used since such strain is wildly used for pharmacological assessments (**Mogil et al., 1996, 1999; Leo et al., 2008**) and is characterised by low levels of anxiety, which is important for assessing pain behaviours in animal models (**Bailey and Crawley, 2009**). Moreover, epidemiological data suggest that females have a higher prevalence of chronic pain and a higher vulnerability in the development of pathological pain-related comorbidities, including emotional disorders (**Miller and Cano, 2009; Goesling et al., 2013**). Although the prevalence and intensity of neuropathic pain following SCI are reported to be equivalent between male and female patients (**Budh, 2003; Cardenas, 2004**), pathological pain following SCI has been mainly studied in young male rodents (**Kramer, 2017**). For all the reasons above-mentioned, it is clear that female animal models are needed in the field of SCI neuropathic pain research. Regarding type of SCI, it should be noted that severe SCI cause significant locomotor disturbances, which may mask the evaluation of neuropathic pain and hinder behavioural assessments that require free movement activity of the animals when performed. Hence, a mild spinal cord contusion was performed following procedures explained elsewhere (**Álvarez-Pérez et al., 2016; Castany et al., 2018**) in order to obtain mice with central neuropathic pain development without locomotor paralysis.

One of the most studied polyphenols in the field of neuropathic pain is EGCG. Its antinociceptive effects have been demonstrated in different models of neuropathic pain including spinal cord injury (SCI) (**Álvarez-Pérez, 2016; Renno, 2014**), chronic constriction injury (CCI) (**Xifró, 2015; Kuang, 2012; Bosch-Mola, 2017;**

Renno, 2017), spinal nerve ligation (SNL) (**Choi, 2012; Krupkova, 2014, An, 2014**) and diabetic neuropathic pain (**Morgado, 2012; Raposo, 2015**), among others. Thus, EGCG has become one of the reference polyphenols in the field of neuropathic pain treatment research (**Bimonte et al., 2017**). However, the pharmacological safety of this polyphenol remains controversial since both hepatotoxic (**Wang et al., 2015**) and nephrotoxic (**Rasheed et al, 2017**) effects have been suggested in different animal models. Furthermore, the use of DMSO as a solvent, the organic compound employed to dilute EGCG, have been reported to cause cellular toxicity and neuronal death even at low concentrations such as <10% (v/v) (**Hanslick et al., 2009; Galvao et al., 2014; Asyura et al., 2016**). Despite this, the use of this solvent is so ubiquitous that it is often used as a control vehicle for *in vitro* and *in vivo* studies (**Matsumura et al., 2008; Xifró et al., 2015; Li and Zhang, 2015; McDonnell et al, 2019**). Therefore, it would be interesting not only to study the antinociceptive effects of other polyphenolic compounds without associated toxicity, but also to use a physiological solvent such as saline for these new treatments.

Before starting to study other polyphenolic treatments, it was first considered relevant to elucidate the preventive effects of EGCG on our model of SCI-induced neuropathic pain throughout the whole acute phase of injury. That is, while acute SCI phase in mice lasts 21 days, EGCG antinociceptive effects were only available up to 14 days after SCI contusion in mice (**Álvarez-Pérez, 2016**). As a results, in the present thesis we have shown that EGCG administration during the first week post SCI attenuate both mild spinal cord injury-induced mechanical allodynia and thermal hyperalgesia development up to the end of the SCI acute phase. Such attenuation may be associated with gliosis modulation since the highest EGCG doses (15 and 20 mg/kg) modulated both astrogliosis and microgliosis in the spinal cord of SCI animals. Actually, these results are consistent with the short-term effects of EGCG on the development of neuropathic pain after SCI (**Álvarez-Pérez et al., 2016**) and our results are showing that this modulation will be effective, at least, during the whole acute phase. Regarding the mechanistic insights, Álvarez-Pérez *et al*. demonstrated that, at short time, EGCG reduces thermal hyperalgesia associated not only with reduced glial reactivity but also with RhoA down-regulation in the spinal cord (**Álvarez-Pérez et al., 2016**). In parallel, it has been demonstrated that RhoA/Rho kinase inhibition alleviates neuropathic pain (**Ohsawa et al., 2016**) and RhoA/ROCK signalling pathway is also implicated in inflammatory (**Wong et al 2019**) and diabetic pain (**Ohsawa et al., 2011**), as well as, in microglial reactivation (**Tatsumi et al., 2015**). Another reason that may explain the antinociceptive activity of EGCG is that this polyphenol binds to the epithelial growth factor receptor (EGFR) inhibiting its associated intracellular cascade (**Sah et al., 2004**). Actually, it has been reported that in SCI mice, the administration of an EGFR receptor inhibitor, localised to both astrocytes and microglia, reduce the proliferation of both cells in the injured spinal cord parenchyma (**Zhang et al., 2016**). Taken together, all these findings suggest that EGCG treatment may reduce SCI-derived glial proliferation by blocking the EGFR receptor. In addition, EGCG also binds to the 67 kDa laminin receptor (**Fujimura et al.,**

2012, Gundimeda et al., 2015), which is involved in the development of microgliosis (**Wang et al., 2006**) and in the migration of these glial cells (**Ren et al., 2014**). On the other hand, EGCG administration has been shown to reduce TRL4 receptor expression following chronic sciatic nerve constriction (CCI), accompanied by relief of thermal hyperalgesia and mechanical allodynia (**Kuang et al., 2012**). The TRL4 receptor is expressed on astrocytes and microglial cells, and its activation leads to the synthesis of proinflammatory mediators (**Rahimifard et al., 2017**). Thus, all this evidence indicates that EGCG may exerts its effects by binding to different receptors such as EGFR, 67 kDa-Laminin, and TRL4, all of which are expressed on glial cells. EGCG may act by blocking these receptors or inhibiting their expression, and thus the production of inflammatory mediators and proliferation and reactivity of glial cells.

Although these findings suggest potential role of EGCG treatment in modulating the SCI-induced central neuropathic pain during the acute phase of injury, it is worthy to mention that systemic toxicity would be associated with such treatment since all tested doses of EGCG resulted in significant weight loss of the animals during the whole experimental period. In addition, SCI animals administered with a DMSO/saline solution (1:9; v/v) or 20 mg/kg EGCG showed significantly higher serum ALT and AST activity levels compared to untreated SCI animals. Increased serum ALT and AST levels are indicative of liver injury and they are considered a highly sensitive and fairly specific preclinical and clinical biomarkers of hepatotoxicity (**Ozer et al., 2008, 2010**). Actually, previous studies have also used these two biomarkers to detect hepatotoxicity of EGCG treatment (**Bun et al. 2006, Galati et al. 2006, Takami et al. 2008, Kapetanovic et al. 2009, Hsu et al 2011, Church et al. 2015**). Therefore, considering that animals treated with 20mg/kg EGCG showed approximately 4 times more ALT and 2 times more AST in serum compared to untreated animals, although they did not reach maximum levels established as undoubtedly toxic, it can be suggested that this treatment may trigger hepatotoxicity in female CD1 mice, especially in longer-term treatments. Indeed, there is some evidence of EGCG toxicity available in the literature. While the use of EGCG-based supplements has been shown to be implicated in liver failure (**Molinari et al., 2006**), other studies have shown that high doses of EGCG induce hepatic, renal and intestinal toxicity in animals (**Lambert et al., 2010; Inoue et al., 2011**). Moreover, a study performed by Goodin and Rosengren (**Goodin and Rosengren, 2003**) showed that EGCG (50 mg/kg, i.p) administration for 7 days in female Swiss mice resulted in extensive hepatic necrosis, significant weight loss, elevated plasma ALT activity and mortality in 67% of the mice.

Although only the serum ALT, AST and UREA/bun levels of the animals treated with DMSO and the 20 mg/kg EGCG dose were analysed in this thesis, it should be noted that all treated animals (including the 10 and 15 mg/kg doses) showed a significant weight loss. In addition, although lower doses of EGCG administration may result in lower serum ALT and AST activities, this type of treatment may require repeated administrations over time to avoid SCI-induced neuropathic pain chronification. Related to this, gradually increasing doses of EGCG have been shown to cause liver failure in mice (**Wang et al., 2019**) and to reduce

hepatocyte activity in *in vivo* studies (**Kucera et al., 2015**). Therefore, with increasing EGCG administrations, the likelihood of developing more toxicity also increases, compromising the pharmacological safety of the treatment.

Considering that EGCG would exert antinociceptive effects but the use of this polyphenol and DMSO as a vehicle may be associated with adverse side effects, new polyphenolic treatments may be considered for both prevention and treatment of SCI-derived neuropathic pain. According to the literature, polyphenols alleviate neuropathic pain by numerous mechanisms, including modulation of serotonergic, noradrenergic, GABAergic, glutamatergic (NMDAR-dependent) and peptidergic neurotransmission, regulation of several cell membrane receptors and ion channels (P2X3, CX3CR1, TRL4, TRPV1, Na+ channels), inhibition of intracellular MAPK pathways (ERK1/2, JNK, p38MAPK), protein-kinases (PKA, PKC), phosphodiesterases (PDE2), phospholipases (PLC), apoptotic pathways (Bax/Bcl2, caspases), enzymes (iNOS, COX-2, FASN), and transcription factors (NF-κB, STAT) (**Boadas-Vaello, 2017**). Moreover, among their cellular targets there are not only neurons but also astrocytes and microglia, that contribute to activation/modulation of nociceptive information (**Gosselin et al.,2010; Ji and Nedergaard, 2013**). This variety of action mechanisms may be explained by the fact that, although polyphenols belong to the same family, several structural differences can be found between them (**Boadas-Vaello, 2017**) with which could exert different pharmacological effects interacting with different molecular targets associated with pathological pain development. Nevertheless, while the antinociceptive activities of many different polyphenols have been reported, none has yet been described that completely prevents the development of central neuropathic pain on its own. On this basis, it is not unreasonable to hypothesise that the combination of different polyphenols may act synergistically to modulate central neuropathic pain. Moreover, if this mixture of polyphenols would be dissolved in an injectable physiological solution, would makes this potential treatment even more suitable.

In this context, the main objective of the present thesis was to obtain two new plant based polyphenolic extracts to be tested in the SCI-induced neuropathic pain model. Concretely, the two polyphenolic extracts were obtained from different plant sources, grape residual material and roasted coffee bean, using physiological serum as solvent. Both grapevine and coffee are known to be rich natural sources of polyphenols (**Balík et al., 2009; Xia et al., 2010; Hečimović et al., 2011; Rajha et al., 2014; Król et al., 2020**). On the one hand, the polyphenolic content of coffee has been extensively studied taking into account the degree of roasting, the origin and even the effect of storage (**Król et al., 2020**). On the other hand, although there are many studies on the polyphenolic content of grapes (**Xia et al., 2010; Bunea et al., 2012**), grape seeds (**Nawaz et al., 2006; Bucić-Kojić et al., 2007**), and leaves (**Balík et al., 2009; Borai et al., 2017**), lesser is known about the polyphenol content in grape stalk, which may be affected by numerous factors, such as vine variety or vineyard environmental factors like climate, soil or sanitary stage (**Fang et al., 2008**). Therefore, considering the lack of this information, it was considered appropriate to determine the total

amount of polyphenols, as well as to identify and quantify the polyphenolic compounds in both extracts before the pharmacological assessments.

Following the chemical extraction methodology described in the proper thesis section, we obtained a total amount of polyphenols ranging from 960-1285 mg/L for GSE and 2186-2480 mg/L for CE, using physiological serum as extractor agent. These concentrations were sufficiently high to allow intraperitoneal administration of an adequate injection volume according to the established doses of polyphenols, without using any organic solvent as an extracting agent that would be incompatible for administration to animals. In addition, the polyphenols of both extracts were identified and quantified by analytical chemistry techniques such as HPLC. On the one hand, the major polyphenols in grape stalk extract were gallic acid (39.55mg/L), protocatechuic acid (30.5mg/L), catechin (29.2mg/L), miquelianin or quercetin 3-O-beta-D-glucuronopyranoside (18.12mg/L) and caftaric acid (11.5mg/L). Although none of these polyphenols have yet been tested against neuropathic pain resulting from SCI, it is worth mentioning that some of them have shown antinociceptive effects in other animal models of pathological pain. For instance, it has been reported that administration of gallic acid, protocatechuic acid or catechin reduces induced nociception in different animal models such as CCI-induced peripheral neuropathic pain (**Trevisan et al., 2014**), spinal nerve ligation (**Quiñonez-Bastidas et al., 2018**), paclitaxel-induced neuropathic pain (**Kaur and Muthuraman, 2019**), diabetic neuropathy (**Raafat and Sami, 2014**) and inflammatory pain (**Santos et al., 1999; Lende et al., 2011; Trevisan et al 2014; Quiñonez-Bastidas et al., 2018; Dikmen et al., 2019; Islam et al., 2019**). In contrast, although antinociceptive effects of different extracts containing miquelianin (**Hanifah and Tristantini, 2019; Nho et al., 2019; Ribeiro et al., 2019**) or caftaric acid (**Conea et al., 2017**) have been described, none of these polyphenols have shown such effects on their own.

On the other hand, the major polyphenols identified in coffee extract were chlorogenic acid or 3-caffeoylquinic acid (339.2mg/L), neochlorogenic acid or 5-O-caffeoylquinic acid (308.1mg/L) and 4-O-caffeoylquinic acid (307.6mg/L). Again, while the antinociceptive effects of these polyphenols on central neuropathic pain have not been reported, chlorogenic acid has been shown to modulate inflammatory and peripheral neuropathic pain in different animal models (**Bagdas et al., 2013; 2014; 2020; Hará et al., 2014**). As for neochlorogenic acid and 4-O-caffeoylquinic acid, pharmacological effects on pathological or specific-neuropathic pain have not yet been described. However, some extracts containing neochlorogenic acid, among other polyphenols, have shown antinociceptive activity (**Kalegari et al., 2014; Lim et al., 2015**).

Once the two polyphenolic extracts (GSE and CE) were obtained and characterised, pharmacological assessments were designed in order to study their preventive effects on SCI-induced neuropathic pain development during the acute phase of the injury. The results of the present thesis showed that either GSE or CE administration during the first week post SCI, resulted in the modulation of both mechanical allodynia

and thermal hyperalgesia development during the acute phase of SCI-induced neuropathic pain. Specifically, the doses that were most effective in preventing the development of pathological pain were 15mg/kg of GSE, that completely inhibited both mechanical allodynia and thermal hyperalgesia development, and 10mg/kg of CE that resulted in the prevention of thermal hyperalgesia and in the attenuation of mechanical allodynia development. Importantly, these results related to hind paw withdrawal cannot be associated with locomotor disturbances since no significant locomotor deficits were observed during the experimental period and therefore, the animals maintained the capacity for voluntary movement of the hind limbs. In addition, none of the tested doses of GSE or CE treatments triggered any apparent side effects, showing no alterations in weight, neither in general appearance of the animals nor biomarkers of hepatotoxicity or nephrotoxicity in the serum. These results are very significant because, unlike EGCG, both polyphenolic extracts are not associated with systemic toxicity, showing a more promising preventive effects against central neuropathic pain development than EGCG.

In order to explain the effects exerted by polyphenolic extracts in the neuropathic pain behaviours, different molecular and histological analysis in the spinal cord were performed, including protein expression of some pain biomarkers which are pERK, chemokines CCL2 and CX3CL1 and their receptors CCR2 and CX3CR2, and glial reactivity. Regarding the latter, it is important to note that glial cells comprise about 70% of the central nervous system and play an important role in maintenance and homeostasis. After SCI, astrocytes and microglial cells are chronically activated in the spinal cord (**Schmitt et al., 2000; Chang, 2007; Hulsebosch et al., 2009**) releasing inflammatory mediators (**Mika et al., 2013**). These pro-inflammatory factors cause depolarization and sensitization of the nociceptive neurons, leading to plastic changes (**Ji, 2003; Kuner et al., 2010; Babos et al, 2013**) and showing an important feedback or neuron-glia crosstalk, which maintains and/or enhances central pathological pain (**Austin and Moalem-Taylor, 2010**). Hence, it is well known that astrogliosis is characterized by production of proinflammatory chemokines and cytokines (**Zamanian et al 2012, Brambilla et al 2005**), generation of Ca^{2+} signals (**Kuchibhotla et al 2009**), upregulation of aquaporin 4 (AQP4) (**Huang et al 2019**) and elevation of glutamate levels (**Oh et al 2012, Takano et al 2005**); while microgliosis preferentially causes the production of inflammatory cytokines and chemokines and other inflammatory mediators (e.g. nitric oxide, prostaglandins) that stimulate nociceptive neurons, but also trigger astrocyte reactivation (**Hulsebosch et al., 2009, Gensel and Zhang, 2015, Rezvan et al., 2020**). All these factors lead to further inflammation, cytotoxic oedema, and ischemia, which contribute to the excitation and sensitization of spinal nociceptive neurons that have survived injury, and ultimately to potentiation of central neuropathic pain after SCI. These evidences can explain the results observed in the present work, where untreated SCI animals show a significant increase in gliosis together with thermal hyperalgesia and mechanical allodynia, compared to control animals (Sham). These results are supported by other published studies where thermal hyperalgesia and mechanical allodynia after SCI in mice were also

observed (**Hoschouer et al., 2010, Watson et al., 2014, Álvarez-Pérez et al., 2016, Castany et al., 2018, Li et al., 2018, McFarlane et al., 2020, Cheng et al., 2021**). Our results also showed that GSE and CE treatment modulates both astrogliosis and microgliosis in spinal cord of SCI mice accompanied with an attenuation of SCI-induced mechanical allodynia and thermal hyperalgesia development. Therefore, considering the SCI related pathophysiological events above mentioned, these results suggest potential role of both GSE and CE treatment in modulating the SCI-induced central sensitization during the acute phase of injury by modulating the spinal cord gliosis.

In addition to what has been said so far, it is worth mentioning that growing evidence indicates that the pathogenesis of pathological pain is not simply limited to changes in the activity of neuronal systems, but also involves interactions between neurons, inflammatory immune cells and immune-like glial cells, as well as a raft of immune cell-derived inflammatory cytokines and chemokines (**Austin and Moalem-Taylor, 2010**). Spinal glial cells are activated by a variety of additional mediators, among them chemokines are attracting significant attention as candidates for recruitment and activation. In fact, chemokines increase pain sensitivity by the attraction of immune cells, activation of microglia and excitation of nociceptive neurons (**Chang, 2007**). Chemokines that have been suggested to be pivotally involved in the generation of neuropathic pain after SCI include CCL2 (also known as MCP-1) and CX3CL1 (also called fractalkine) (**Cruz-Almeida et al., 2009**). While CCL2-mediated signalling is critical for the activation of spinal glia and the developing of neuropathic pain (**Thacker et al., 2009; Gao et al., 2009**), CX3CL1 is involved in microglial activity and promotes the synthesis of proinflammatory cytokines (**Verge et al., 2004; Lindia et al., 2005**). In this context, the results of the present thesis showed that, although no significant upregulation of CCL2 were detected, CX3CL1, CX3CR1 and CCR2 were significant overexpressed in spinal cord of SCI animals at 21 dpi.

Both behavioural and electrophysiological evidence show that CCL2 plays a critical role in pain facilitation via its preferred receptor, CCR2 (**Gao et al., 2009; Jung et al., 2009; Gao and Ji, 2010**). For instance, intrathecal administration of CCL2 induces microglial activation, heat hyperalgesia and mechanical allodynia in WT mice, but not in CCR2 KO mice (**Gao et al., 2009; Thacker et al., 2009; Moreno et al., 2014**). Overexpression of CCL2 in astrocytes enhances nociceptive responses in mice (**Menetski et al., 2007**). Furthermore, mechanical allodynia development after peripheral nerve injury is totally abrogated in CCR2 KO mice (**Abbadie et al., 2003; Zhang et al., 2007**). In isolated spinal cord slice preparation, application of CCL2 immediately increases the frequencies of sEPSCs in lamina II neurons of the dorsal horn (**Gao et al., 2009**), suggesting a presynaptic mechanism of CCL2 to enhance glutamate releases (**Baba et al., 2003; Kohno et al., 2005**). In addition, CCL2 increases the amplitudes of sEPSCs and enhance NMDA and AMPA-induced current (**Gao et al., 2009**), suggesting a postsynaptic mechanism of CCL2 to enhance glutamate receptor function (**Kohno et al., 2005**). Altogether, these findings show that CCL2 enhances sensitivity to

pain by direct action on CCR2, suggesting that CCL2/CCR2 signalling plays an important role in the establishment and/or persistence of pathological pain.

In physiological conditions, CCL2 is constitutively expressed in DRG neurons (**Dansereau et al., 2008**) and in astrocytes of the spinal cord (**Gao et al., 2009**). It has been demonstrated that CCL2 can be transported and subsequently secreted from neuronal nerve terminals to spinal cord (**Van Steenwinckel et al., 2011**). In fact, double-labelling experiments showed that CCL2 colocalized with substance P and CGRP positive primary afferents in the superficial dorsal horn of spinal cord (**Dansereau et al., 2008**). Further, CCR2 is constitutively expressed in dorsal horn neurons (**Gosselin et al., 2005; Gao et al., 2009**). However, the expression and distribution of CCL2 and CCR2 in animal models of pathological pain shows a lesion-dependent pattern. Thus, CCL2 and CCR2 can be expressed in both neurons and/or glial cells (astrocytes and microglia) and can be up- or down-regulated depending on the animal model of neuropathic pain and the time elapsed after the specific lesion. Specifically, in SCI model, CCL2 and CCR2 are mainly expressed in astrocytes and granulocytes at injury level. Moreover, in dorsal horn, CCL2 is also co-expressed with transmitters and receptors that are involved in nociceptive processing like CGRP, Substance P, TRPV1 and its activated phosphorylated form (**Knerlich-Lukoschus et al., 2008**). On the other hand, besides of lesion localization particularities, the expression of CCL2/CCR2 in such models shows a time-dependent pattern. Hence, although most studies using animal models of neuropathic pain have reported an overexpression of spinal CCL2 after injury (**Tanaka et al., 2004; White et al., 2005; Zhang and Koninck 2006; Jung et al., 2008; Knerlich-Lukoschus et al., 2008**), its overexpression follows a temporal pattern (**Mukhamedshina et al., 2017**). For instance, several studies have shown that CCL2 expression in the spinal cord increases dramatically a few days after injury, reaching a maximum peak of expression at 3 dpi and thereafter it gradually decreases over time (**Zhu et al., 2014**), even showing no significant difference with the non-injured group at 14 dpi (**Mukhamedshina et al., 2017**). Although our results show an increased expression of CCL2 in the spinal cord of SCI animals at 21 dpi, it is not significant compared to sham animals. Based on the literature, it is not unreasonable to think that CCL2 was overexpressed during the days following injury and that by 21 dpi its expression was stabilising. In order to complement our results, it could be interesting study the temporal pattern of CCL2 expression in spinal cord to see if this chemokine is overexpressed at any time-point after injury. In addition, further immunohistochemical studies would be crucial to identify the distribution pattern of this chemokine in the spinal cord following our SCI model.

In contrast to CCL2, the results obtained in the present thesis showed a significant overexpression of CCR2 in the spinal cord of SCI mice at 21 dpi. Moreover, all tested doses of CE and GSE treatments, except for 15 mg/kg of GSE, prevented the overexpression of CCR2. Our results showing an upregulation of CCR2 in SCI animals are supported by previous studies reporting spinal CCR2 overexpression in spinal cord injured rats (**Knerlich-Lukoschus et al., 2008**) and in other animal models of pathological pain such as spinal nerve injury

(**Gao et al., 2009; Thacker et al., 2009**), inferior alveolar nerve and mental nerve transection-induced trigeminal neuropathic pain (**Zhang et al., 2012**) and lumbar disc herniation-induced neuropathic pain (**Zhu et al., 2014**). One of the targets of CCR2 signalling is mitogen-activated protein kinase (MAPK) (**Wain et al., 2002**), an important intracellular signalling involved in the regulation of neuronal plasticity and inflammatory responses (**Ji et al., 2009**), indicating that CCL2/CCR2 signalling may be involved in neuroinflammation and pathological pain development. Regarding to preventive effect on CCR2 overexpression of both GSE and CE treatments, there are few studies about some polyphenolic compounds, including caffeic acid (**Zhao et al., 2012**), chlorogenic acid (**Shi et al., 2013**) and curcumin (**Zhang et al., 2012**), that modulate CCL2/CCR2 signalling pathway. It should be noted that chlorogenic acid is the major polyphenol identified in coffee extract, and its ability to inhibit the CCL2 overexpression has previously been reported (**Shi et al., 2013**). Considering that polyphenols mentioned above can modulate the CCL2/CCR2 pathway, is it not unreasonable hypothesize that other polyphenols, such as those present in GSE and CE extracts, may also exert the same effect.

As for CX3CL1/CX3CR1 pathway, in the spinal cord, CX3CL1 is predominantly expressed on the cell surface of neurons (**Kim et al., 2011**). This chemokine exists in both membrane-bound and soluble forms. Membrane-bound form has an adhesion function in the vascular immune system and soluble form possesses functions as a chemoattractant for monocytes, natural killer cells (NK), B cells and T cells, and is essential for the transendothelial migration of monocytes that express CX3CL1 receptor, CX3CR1 (**Auffray et al., 2007; Corcione et al., 2009**). Regarding CX3CR1, in the spinal cord it is only expressed by microglial cells (**Jung et al., 2000; Cardona et al., 2006; Clark, Old, and Malcangio, 2013**) and is exclusively activated by CX3CL1 (**Schall, 1997; Hesselgesser and Horuk, 1999**). Pronociceptive effects attributed to CX3CL1 are associated specifically with cleaved or soluble form. Indeed, following peripheral nerve injury, whilst the expression of membrane-bound CX3CL1 remains invariable, the expression of soluble CX3CL1 in the cerebrospinal fluid (CSF) increases significantly (**Clark et al., 2009; Nieto et al., 2016**). It has been demonstrated that activated microglia releases the lysosomal cysteine protease cathepsin S (CatS) which is the responsible for the proteolytic liberation of soluble CX3CL1 in the spinal cord under neuropathic pain conditions (**Clark et al., 2007**). Actually, it has been reported that the expression of CatS in dorsal horn microglial cells is correlated with the maintenance of pain hypersensitivity (**Abbadie et al., 2009**). Altogether, the high fidelity of CX3CL1/CX3CR1 signalling, as well as the unique expression pattern of both ligand and receptor in the spinal cord, make this signalling pair and intuitive candidate for the mediation of changes in neuronal-microglial crosstalk as well as a potentially ideal therapeutic target for central neuropathic pain.

In the same context, a growing body of evidence supports the involvement of CX3CL1/CX3CR1 signalling in neuropathic pain. For instance, intrathecal administration of CX3CL1 induces dose-dependent mechanical

allodynia and thermal hyperalgesia in naive rats and mice (**Milligan et al., 2004; Clark et al., 2007**) and intrathecal administration of CX3CR1-neutralising antibody attenuates the development of neuropathic pain and blocks CX3CL1-induced mechanical allodynia and thermal hyperalgesia in naïve animals (**Milligan et al., 2004; 2005**). This pronociceptive effect of CX3CL1 is exerted through activation of CX3CR1, which in turn induces intracellular phosphorylation of microglial p38 MAPK (**Clark et al., 2007; Staniland et al., 2010; Nieto et al., 2016**), leading to the release of proinflammatory mediators such as TNF-α, IL-1β, and IL-6 (**Jin et al., 2003; Milligan et al., 2005**), which can activate neighbouring neurons and contribute to increased neuronal excitability (**Gao and Ji, 2010; Yan and Weng, 2013; Clark et al., 2015**). Focusing on injured nervous system, following injury or inflammation, microglia and astrocytes become reactive and increase the expression of chemokines and chemokine receptors (**Ambrosini and Aloisi, 2004; Abbadie et al., 2009**), and it has been reported that several animal models of pathological pain, such as sciatic inflammatory neuropathy (SIN) (**Verge et al., 2004**), partial nerve ligation (PNL) (**Clark et al., 2009**), chronic constriction injury (CCI) (**Verge et al., 2004**), spared nerve injury (SNI) (**Holmes et al., 2008**), spinal nerve ligation (SNL) (**Lindia et al., 2005; Zhuang et al., 2007**) and paclitaxel-induced neuropathic pain (**Li et al., 2015**) showed overexpression of CX3CL1 and/or CX3CR1. Moreover, it has been demonstrated that CX3CL1 can be induced in spinal astrocytes by spinal nerve ligation, in addition to spinal neurons (**Lindia et al., 2005**).

Despite of all these evidences, only few studies have investigated the expression pattern of CX3CL1 and CX3CR1 in animal models of SCI. On the one hand, Detloff and colleagues showed that spinal mRNA levels of CX3CL1 presented no changes after 7, 21 and 35 days following the SCI, while mRNA expression levels for CX3CR1 were overexpressed compared to control groups (**Detloff et al., 2008**). On the other hand, Donnelly and collaborators demonstrated that while CX3CL1 expression remained unchanged for 6 weeks after SCI, CX3CR1 expression was gradually increased starting on the 3 day after SCI (**Donnelly et al., 2011**). Similarly, Cizkova et al. revealed that the CX3CR1 was overexpressed after 3 days post SCI when compared with sham-operated group (**Cizkova et al., 2014**). Finally, Blomster et al. showed that SCI mice presented a slightly elevated CX3CL1 concentration in the serum at 7 dpi compared to non-injured and sham-operated control groups (**Nicolini and Fahnestock, 2018**). In the present thesis we have demonstrated that mild SCI in CD1 female mice causes overexpression of CX3CL1 in the spinal cord at 21 dpi. Moreover, while both doses of CE treatment did not significantly exert CX3CL1 expression modulation compared to untreated SCI animals, all GSE tested doses prevented CX3CL1 upregulation up to Sham levels. The overexpression of CX3CL1 observed in SCI animals could be associated with microglial cells activation, which was also revealed in the present thesis. Hence, it may be suggested that reactive microglia would release CatS which would mediate proteolytic cleavage of membrane-bound CX3CL1, thereby increasing the overall expression of soluble CX3CL1. Also, these results would be associated with SCI-induced changes in the intracellular signalling pathways of nociceptive neurons, which in turn lead to changes in gene expression, resulting in increased

expression of membrane-bound CX3CL1 in the neurons. Finally, other animal models of neuropathic pain, such as SNL, have shown CX3CL1 expression in the spinal astrocytes (**Lindia et al., 2005**). Therefore, it is not unreasonable to hypothesise that the SCI model may also induce CX3CL1 expression in astrocytes. This hypothesis could explain the increase in spinal CX3CL1 since astrogliosis has also been observed in the spinal cord of injured animals, and such overexpression of CX3CL1 could be directly correlated with the previously reported spinal astrogliosis. To elucidate whether CX3CL1 overexpression is associated with microgliosis, astrogliosis or neurons plasticity, and how polyphenols could prevent this overexpression, further experiments would be necessary, such as immunohistochemical analyses revealing the localisation of CX3CL1 as well as its density in different cell types or molecular analyses of CatS expression.

As for CX3CL1 receptor, the obtained results for CX3CR1 expression revealed that it was overexpressed in the spinal cord of SCI CD1 female mice at 21 dpi and all tested doses of CE and GSE treatments (except for 15 mg/kg of GSE) prevented such overexpression. The overall increase of CX3CR1 in SCI animals could be either a result of an increase in its own expression within microglial cells or an increase in the number of microglial cells, which in turn would be leading to an increase in the overall expression of CX3CR1. Moreover, polyphenolic treatments could exert changes in the intracellular signalling pathways of microglial cells, leading to CX3CR1 less expression, or by reducing the number of reactive microglial cells. In summary, an increase in both soluble-CX3CL1 and CX3CR1 and thus heightened spinal CX3CL1/CX3CR1 signalling is a consistent feature of preclinical neuropathic pain models and the two polyphenolic treatments tested for the first time in the present thesis modulate CX3CL1/CX3CR1 signalling by preventing overexpression of both CX3CL1 and CX3CR1 molecules.

In parallel to glial activation and the expression of pivotal chemokines involved in the generation of neuropathic pain after SCI, the expression and activation/phosphorylation of ERK1/2 have been also analysed since is known, together with other protein kinases, to be involved in central sensitization. ERK has a major role in regulating neuronal plasticity in pathological pain (**Yang, 2004; Liu and Zhou, 2015**) with its phosphorylation and the consequent activation of some transcription factors such as cAMP-response-element binding protein (CREB), NF-kβ, Elk-1 and ATF-2, among others (**Woolf 2001; Ji 2003; Liu and Zhou, 2015**). These transcription factors will induce the transcription of different nociceptor genes, including cell membrane receptors (NK1, TrkB) (**Ji, 1997; Dubner, 1992; McCarson, 1994**), ion channels (AMPAR, NMDAR), enzymes (COX-2), cytokines (TNF-α, IL-1β) and other proteins and peptides (c-fos, BDNF, dynorphin, CGRP) (**Samad, 2001; Hunt, 19987**). The activation of these intracellular pathways take place in the second order neurons but also in glial cells, contributing to the hypersensitivity state of the secondary nociceptive neurons (**Gwak 2017**). Therefore, the involvement of MAPKs in the regulation of inflammatory mediator synthesis, both at the transcriptional and translational levels, makes them potential targets for novel anti-inflammatory therapeutics.

The results of the present thesis showed a significant increase of pERK1/2 in spinal cord of SCI mice compared to Sham animals. Accordingly, other authors have been reported ERK phosphorylation in dorsal horn nociceptive neurons (**Obata and Noguchi, 2004; Ma and Quirion, 2005; Zhuang et al., 2005; Xu et al., 2014**) and in reactive astrocytes (**Seltzer et al., 1990; Ma and Quirion, 2002; Ciruela et al., 2003; Zhuang et al., 2005**) accompanied by increased pain hypersensitivity after peripheral nerve and spinal cord injuries (**Yu and Yezierski, 2005; Crown et al., 2006; Cruz et al., 2006**). Moreover, all tested doses of GSE treatment (10, 15 and 20mg/kg) completely prevented the pERK upregulation detected at 21 dpi after SCI. In contrast, although animals treated with CE also showed a significant reduction of mechanical and thermal hypersensitivity development, CE treatment did not prevent ERK phosphorylation in the spinal cord. Hence, our results suggest that polyphenols in GSE and CE extracts may exert their effects via different molecular mechanisms to prevent SCI-derived neuropathic pain development, being only GSE polyphenols the ones modulating ERK signalling pathway. Actually, several phenolic compounds have been shown to modulate the MAPK pathway by acting at various steps of the activation cascade and consequently on downstream effectors (**Soobrattee et al., 2005**). Specifically, several *ex vivo* studies have shown the ability of gallic acid (the most abundant polyphenol in the GSE) to inhibit ERK phosphorylation in human ovarian carcinoma cells (**Sánchez-Carranza et al., 2018**), retinal capillary endothelial cells (**Shanmuganathan and Angayarkanni, 2018**) and breast cancer cells (**Chen et al., 2016**).

Altogether, our results showed that mild SCI in CD1 female mice induces mechanical allodynia and thermal hyperalgesia without major locomotor disturbances during the acute phase of injury. These changes in reflexive pain responses have been correlated with increased pro-inflammatory factors, that cause depolarization and sensitization of the nociceptive neurons of the dorsal horn of the spinal cord (**Kawasaki et al 2008, Hulsebosch et al 2009, Gao and Ji 2010, Nishio et al 2013, Castany et al 2018, Kloc et al 2019, Kim et al 2021**), secreted by reactive microglial cells and astrocytes observed in the injured spinal cord parenchyma. Furthermore, we have shown that polyphenols can prevent neuropathic pain development after SCI by modulating glial activation, phosphorylation of ERK1/2, and consequently, regulation of inflammatory mediator synthesis such as several chemokines and their receptors in the spinal cord.

Regarding the mechanism of action by which the extracts obtained in this thesis exert their analgesic effects, some molecular targets of the polyphenols contained in GSE and CE have been found in the literature. In particular, gallic acid, the most abundant polyphenol in GSE, binds to the estrogen receptor beta (**Ye and Shaw, 2020**) and the purinergic P2X7 receptor (**Yang et al., 2021**). Estrogen receptor beta is expressed in reactive astrocytes (**Carbonaro et al., 2009; Sakuma et al., 2009**) and microglial cells (**Wu et al., 2013**). The binding of gallic acid to this receptor causes its activation, leading to reduced gliosis and thus reduced synthesis of inflammatory cytokines (**Cvoro et al., 2008, Brown et al., 2010, Smith et al., 2011, Guo et al., 2020**). One of the functions of the P2X7 receptor is to mediate the activation and proliferation of microglia

(**Monif et al., 2010**) and thereby the synthesis and release of inflammatory cytokines that contribute to the hyperexcitability and sensitisation of dorsal horn nociceptive neurons (**Zhang et al., 2020**), as well as the synthesis and release of cathepsin-S (**Clark et al., 2010**), a protease that promotes the generation of soluble fractalkine (**Clark and Malcangio, 2012**). It has been demonstrated that gallic acid can bind to the P2X7 receptor, inhibiting activation of the TNF-α/STAT3 signalling pathway (**Yang et al., 2021**). Previous studies have already shown that the use of P2X7 receptor inhibitors relieves pain (**Dell'Antonio et al., 2002, Honore et al., 2009**). Some mechanisms whereby protocatechuic acid, the second most abundant polyphenol in GSE, develops its analgesic effect have also been proposed. Several studies indicate that central antinociceptive action of protocatechuic acid is mediated by spinal opioid and cholinergic receptors, spinal and supraspinal noradrenergic modulation (**Arslan et al., 2018**), as well as modulation by cannabinoids (**Dikmen et al., 2019**). Moreover, this polyphenol inhibits microglial activation (**Xi et al., 2021**) and the inflammatory response associated with microglial reactivation via regulating NF-κB and MAPKs signalling pathways (**Wang et al., 2015**) and SIRT1/NF-κB pathway (**Kaewmool et al., 2020**). In addition, catechin, the third most abundant polyphenol in GSE, has also been shown to increase SIRT1 levels/activity in vitro (**Fusi et al., 2018; Cheng et al., 2019**) and in vivo in the CNS (**Ramis et al., 2020**). As for CE polyphenols, chlorogenic acid, the most abundant polyphenol in the extract, may mediate glial anti-inflammatory effects by blocking different intracellular cascades such as MAPK, p38, ERK, JNK and NF-κB. Moreover, it also blocks the generation of prostaglandins, modulating the activity of cyclooxygenases (**Bagdas et al., 2020**). At neuronal level, chlorogenic acid modulates the activity of voltage-gated potassium channels (**Liu et al., 2016**) and acid-sensing ion channels (**Qu et al., 2014**), which may contribute to its analgesic effect. Finally, neochlorogenic and cryptochlorogenic acids, the second and third most abundant polyphenol in the CE, could attenuate glial anti-inflammatory effects via upregulation of the Nrf2/HO-1 signalling pathway, suppression of JNK, ERK and p38 MAPK phosphorylation as well as inhibition of intracellular ROS generation (**Park et al., 2018; Zhao et al., 2020**). For the rest of the polyphenols contained in coffee and grape stalk extracts, the mechanisms by which they potentially may exert analgesic effects have not yet been described.

Besides the local spinal cord neuroplastic processes occurred after SCI, it is known that such processes may facilitate the generation and conduction of action potentials through ascending pathways to supraspinal structures, contributing to the sensory and behavioural alterations of pain behaviours (**Yezierski, 2009**). Among these supraspinal structures or brain areas, the anterior cingulate cortex (ACC) plays an important role in both sensory and affective component of pain since it receives nociceptive projections from the thalamus and somatosensory cortices, as well as emotional fear-anxiety-depression information from the amygdala (**Johansen et al., 2001; Shackman et al., 2011; Bushnell et al., 2013; Bentley et al., 2016**). Moreover, considering that ACC converges emotional and sensory information, it is also probable that the top-down pathways contribute not only to the regulation of pain intensity, but also the interaction between

pain and emotional changes (**Tsuda et al., 2017**). In this context, the findings of the present thesis showed that SCI resulted in a significant increase of both microgliosis and astrogliosis in the ACC at 21 dpi. Similarly, Georgieva et al. demonstrated that spinal cord injured rats presented both microglial and astrocyte activation in ACC compared to Sham animals (**Georgieva et al., 2019**). Importantly, Widerström-Noga and colleagues reported that increased glial activation in ACC is an important contributor to the development and maintenance of severe neuropathic pain after SCI in humans (**Widerström-Noga et al., 2013**). In addition, different studies have also shown astrocyte and microglial activation in the ACC in animal models of chronic, neuropathic and inflammatory pain (**Narita et al., 2006; Kuzumaki et al., 2007; Lu et al., 2011; Chen et al., 2012; Yamashita et al., 2014; Miyamoto et al., 2017; Fan et al., 2018**). The current data suggest that the activation of glial cells in the ACC probably is time-dependent and type of pathological pain model-dependent. Moreover, neuronal hyperexcitability changes in the ACC have been described following several experimental models of pathological pain (**Boadas-Vaello et al., 2017**). Although there are yet no studies on the subject, it is conceivable that the gliosis observed in the ACC may contribute to this neuronal hyperexcitability, and thus to the contribution of pathological pain development. SCI primarily produces neuronal hyperexcitability in the spinal dorsal horn but that changes in neuronal properties extend up to the supraspinal level, especially in the ventral posteriorlateral thalamus (VPL), which integrates somatosensory information, including pain information (**Hubscher and Johnson, 2006**). Since a bidirectional flow of nociceptive information between the thalamus and ACC has been found in rodents (**Wang et al., 2007**), thalamic changes following SCI would be expected to induce plastic changes in the ACC. Related to this, diabetic rats with mechanical allodynia (**Fischer et al., 2009**) and rats with spinal cord hemisection (**Gwak et al., 2010**) or spinal nerve ligation (**Patel and Dickenson, 2016**) presented neuronal hyperexcitability in the VPL neurons in parallel with hyperexcitability of dorsal horn nociceptive neurons. Furthermore, spinothalamic tract lesions induce thermal hyperalgesia and mechanical allodynia accompanied by neuronal hyperexcitability in the VPL thalamic nucleus (**Wang and Thompson, 2008**). Moreover, after spinal cord contusion, remote activation of microglial cells has been observed in the VPL, together with neuronal hyperexcitability and overexpression of CCL21 in this thalamic nucleus (**Zhao et al., 2007**). Taken together all these evidences, it can be suggested that the changes observed in the ACC after SCI may be triggered by trans-synaptic pain propagation from the spinal cord through the thalamus to the ACC.

In parallel, our results also showed a significant overexpression of CX3CL1 and its receptor, CX3CR1, in ACC of the spinal cord injured animals. Although little is known about the CX3CL1/CX3CR1 expression in the ACC of animals with neuropathic pain resulting from SCI, the expression of CX3CL1-CX3CR1 has been investigated in some supraspinal structures from naïve mice and rats (**Hughes et al., 2002**). Current data show that CX3CL1 is constitutively expressed in human, macaque, rat and mouse neurons *in vitro* and *in*

vivo, with high expression levels in cerebral cortex, caudate putamen, hippocampus, amygdala, thalamus and olfactory bulb (**Harrison et al., 1998; Meucci et al., 1998; Nishiyori et al., 1998; Schwaeble et al., 1998; Tarozzo et al., 2003; Sunnemark et al., 2005**). Hughes et al. found that naive rodent brain neurons are strongly immunoreactive for CX3CL1 and CX3CR1, both showing a perinuclear staining pattern. CX3CR1 was also highly expressed in resident microglia throughout the rodent brain, in the parenchyma, choroid plexus and meninges. (**Hughes et al., 2002**). Other studies have shown the presence of CX3CR1 in brain neurons, including hippocampal and thalamic neurons (**Meucci et al., 1998**) and brain microglia (**Harrison et al., 1998; Jung et al., 2000**). Whereas neuronal CX3CL1 mRNA expression after neuronal injury remained relatively stable in vitro (**Maciejewski-Lenoir et al., 1999; Chapman et al., 2000; Hatori et al., 2002; Erichsen et al., 2003; Mizuno et al., 2003**) and during neuroinflammation *in vivo* (**Schwaeble et al., 1998**), the expression of CX3CL1/CX3CR1 in the brain is upregulated in different diseases or/and in toxic-inflammatory conditions (**Jones et al., 2012; Astorri et al., 2013; Ferretti et al., 2014; Flierl et al., 2015; Huo et al., 2015; Shah et al., 2015; Zhang et al., 2015; Chen et al., 2016**). Therefore, we have demonstrated for the first time in this thesis that mild SCI resulted in an up-regulation of both CX3CL1 and CX3CR1 in ACC, accompanied by glial activation, in the acute phase of spinal cord injury. These supraspinal changes in the ACC were correlated with neuropathic pain responses.

Regarding to polyphenolic treatments effects on ACC, both grape stalk and coffee extracts modulated glial activation in this supraspinal structure, accompanied by reduction of mechanical allodynia and thermal hyperalgesia. In contrast, only GSE15 dose modulated CX3CL1 and CX3CR1 expression, whereas CE15 modulated CX3CL1 expression in the ACC of SCI mice. These results suggest that certain doses of specific polyphenols may exert pharmacological effects in the ACC preventing glial activation and modulating inflammatory mediators such as CX3CL1-CX3CR1. Other polyphenols, such as resveratrol, have shown their antihyperalgesic and antiallodynic effects mediated by different subtypes of α-adrenoceptors of ACC (**Mirasheh et al., 2020**) or supraspinal 5-HT1A receptors (**Zhao et al., 2014**). Focusing on the present thesis results, we have shown that the polyphenolic extracts can modulate glial activation, phosphorylation of ERK1/2 and regulation of inflammatory mediators in the spinal cord, which may lead to spinal nociceptive neurons hyperexcitability modulation, and consequently leading to reduce their discharges through ascending pain pathways to the ACC. Therefore, the downregulation of action potentials reaching the ACC may contribute to modulate the neuroplastic changes observed in this supraspinal structure in SCI-treated animals and consequently preventing sensory pathological pain disturbances.

Besides ACC, the periaqueductal gray (PAG) is a supraspinal structure playing also pivotal role in the physiological processes of pain. That is, PAG and its descending projections to the rostral ventromedial medulla (RVM), locus coeruleus, and spinal cord constitute an important endogenous pain-modulatory system (**Basbaum and Fields, 1978; Fields and Basbaum, 1978; Fields et al., 2006**). Moreover, as PAG

receives ascending pain inputs directly from the dorsal horn as well as from limbic forebrain structures such as the anterior cingulate, insular cortex, and amygdala, it is perfectly situated to integrate multiple environmental and emotional factors into the ultimate perception of pain. Under neuropathic pain circumstances, the hyperexcitability of nociceptive ascending neurons induces sensitization of PAG neurons causing overexpression of several receptors, including NMDA/AMPA and SP/NK1, and overexpression of glutamate and BDNF (**Guo et al., 2006**) that will ultimately enhance descending pain facilitation. In addition, it has been described that glial activation in the PAG promotes descending facilitation of neuropathic pain through the p38 MAPK signalling pathway (**Ni et al., 2016**). In fact, preclinical studies of nerve injury models demonstrated glial activation in PAG (**Mor et al., 2010; Ni et al., 2016; Dubový et al., 2018**) associated with changes in cytokines/chemokines (**Wei et al., 2008; Norman et al., 2010; Chu et al., 2012; Guo et al., 2012; Dubový et al., 2018**). However, the cellular and molecular processes occurring in PAG as a consequence of SCI are not yet fully understood. To this aim, glial activation and expression of CX3CL1/CX3CR1 in PAG were also analysed and our findings revealed that mild SCI induced astroglial activation but not microgliosis in this supraspinal structure at 21 dpi. Moreover, SCI animals showed an increased expression of both CX3CL1 and CX3CR1 in PAG. Taken together, these findings suggest a role for activated astroglial cells as well as CX3CL1/CX3CR1 signalling in the modulation of PAG activity with respect to descending facilitation of nociceptive input following SCI. However, further immunohistochemical studies of the cellular distribution of CX3CL1 and CX3CR1 would be necessary to elucidate whether CX3CL1/CX3CR1 signalling is involved in the neuron-glia and glia-glia interactions in PAG after SCI which may be associated with neuropathic pain development and maintenance.

As for polyphenolic treatments effects on PAG, all doses of both GSE and CE extracts modulated astroglial activation and CX3CL1/CX3CR1 overexpression. These results were correlated with the attenuation of both mechanical allodynia and thermal hyperalgesia development, suggesting that polyphenols may exert effects at PAG level modulating descending facilitation of nociceptive input following SCI. In contrast, all tested doses of GSE and CE resulted in the activation of microglial cells in the PAG of SCI animals. This result may be controversial, as other preclinical models of pathological pain describe microglial activation in the PAG correlated with increased pain responses (**Chu et al., 2012; Ni et al., 2016**). However, microglial activation has been shown to be a polarised process that is divided into two phases in which microglia can display two phenotypes that have opposing effects on surrounding structures: the rapidly inducing pro-inflammatory M1 phase and the relatively smaller and transient anti-inflammatory M2 phase (**Kigerl et al., 2009; David and Kroner, 2011; Crain et al., 2013**). Whereas M1 microglial phenotype has been associated with the release of proinflammatory mediators such as IL-1β, IL-6, IL-18, and iNOS (**Nishio et al., 2009; López-Vales et al., 2010; Chhor et al., 2013; Rojewska et al., 2014; Orihuela et al., 2016**), M2 microglial phenotype involves the release of anti-inflammatory cytokines, such as IL-10, and tissue inhibitor of metalloproteinases (TIMP), which promote repair of tissue (**Popiolek-Barczyk et al., 2015**). Our results showed that

polyphenolic treatment with the higher doses of GSE (20 mg/kg) and CE (15 mg/kg) induced microglial activation in PAG, characterized by an increase in the proportion of CD206-positive cells. Therefore, most cells exhibited M2 phenotype suggesting an anti-inflammatory effect. In consonance with that, Bispo da Silva et al. revealed that flavonoid rutin induced microglial proliferation *in vitro* characterized by an increase in OX-42 positive cells and a large proportion of cells with a CD150/CD206-positive M2 phenotype. Rutin also induced a decrease in the mRNA levels of TNF, IL1β, IL6 and iNOS, reduced the production of IL6, TNFα, and nitric oxide, and increased production of the M2 regulatory cytokine IL10 and arginase (**Bispo da Silva et al., 2017**). In addition, Popiolek-Barczyk et al. demonstrated that parthenolide, a natural compound that has been shown to relieve migraine, alleviates both allodynia and hyperalgesia in a rat model of neuropathy. These functional results were correlated with an overall increase in Iba-1 protein concentration in the spinal cord. However, further analysis showed that overall protein concentrations of M1 phase factors were reduced, while M2 factors were increased (**Popiolek-Barczyk et al., 2015**). Finally, it has been demonstrated that protocatechuic acid suppresses microglia activation and facilitates M1 to M2 phenotype switching in intracerebral hemorrhage mice (**Xi et al., 2021**). Thus, microglia do not constitute a homogeneous cell population, but can display a range of phenotypes. Whether they adopt a form that promotes or decreases inflammation probably depends on the nature of signalling within their microenvironment. In view of the above, it is not unreasonable to hypothesise that polyphenols present in the extracts may exert effects on PAG by promoting M2 microglia polarization, which could release anti-inflammatory factors with antinociceptive properties that would promote descending modulation of nociceptive input.

Although our findings clearly suggest supraspinal effects of GSE and CE, it remains unknown how the extracts' polyphenols may reach these supraspinal structures when the latter are not damaged maintaining the blood-brain barrier intact. There are several hypotheses that may explain the results observed in the supraspinal structures: (i) SCI causes disruption of the blood-spinal cord or blood-brain barrier (BBB) (**Noble and Maxwell, 1983; Noble and Wrathall, 1987; McKenzie et al., 1995; Jaeger and Blight, 1997; Banks et al., 1998; Sharma, 2003; Maikos and Shreiber, 2007; Garcia et al., 2016**), allowing polyphenolic extracts to enter the injured spinal cord parenchyma. Once in the parenchyma, the extracts may exert their effects by reducing gliosis and excitability of nociceptive neurons, thereby decreasing the ascending transmission of nociceptive information, and thus inhibiting gliosis and neuronal hyperexcitability in supraspinal structures; (ii) due to disruption of the BBB, polyphenolic extracts may reach the cerebrospinal fluid (CSF) within the central spinal canal and travel to supraspinal regions where they exert their effects; (iii) following the disruption of the BBB, polyphenolic extracts may diffuse rostro-caudally through the brain interstitial fluid (ISF) reaching some brainstem structures (e.g. PAG) where they exert their effects; (iv) due to disruption of the BBB, polyphenolic extracts may diffuse through the perivascular spaces of the glymphatic system of the spinal cord reaching the supraspinal structures. The abovementioned hypotheses about how polyphenolic

extracts reach the supraspinal structures would be supported by several studies. For instance, Liu and collaborators showed that injection of fluorescent tracer into the spinal cord interstitium diffused rostro-caudally two to three spinal cord segments from the injection site. In 50% of grey matter injections, these tracers reached the central canal of the spinal cord where they could be distributed to supraspinal structures (**Liu et al., 2018**). Also, when tracers are injected into the ventricle, they appear in the central canal at 13-15 minutes post-injection, suggesting a flow of CSF between the ventricles and the central canal of the spinal cord (**Cifuentes et al., 1992**). Moreover, when tracers are injected into the cisterna magna, they are observed in the subarachnoid space, and subsequently in the perivascular spaces of the vessels that penetrate the nerve parenchyma, and in the central canal of the spinal cord. At the spinal cord level, the tracers are distributed from the perivascular spaces into the surrounding spinal cord parenchyma (**Lam et al., 2017**). The injection of tracers into the cisterna magna or the subarachnoid space of the tenth thoracic vertebra, allows their rapid observation in the brain and spinal cord perivascular spaces. In the spinal cord, the tracer is localised in the perivascular spaces of the grey matter surrounding the central canal (**Stoodley et al., 1996**). Taken together, these experimental findings support the idea that polyphenolic extracts may be able to reach supraspinal structures via fluid flow through the central canal of the spinal cord and the perivascular spaces of the spinal cord grey matter.

Once the preventive effects on central neuropathic pain development of GSE and CE treatments were studied in the SCI acute phase, we wondered whether polyphenolic extracts might also have analgesic effects when neuropathic pain is already established. Certainly, the protocol for action in the clinic is to administer treatment immediately after injury to avoid as much as possible the pathophysiological processes that lead to the development of neuropathic pain, as well as other SCI secondary diseases, such as motor dysfunction (**Fehlings, 2017**). However, given that current treatments are often ineffective (**Meacham et al, 2017; Attal et al, 2011; 2019**), most SCI patients suffer neuropathic pain beyond the acute phase of the lesion and, this current scenario results in most SCI patients reaching the chronic phase of SCI (**Tashiro et al., 2017**). Thus, given the lack of effective treatments and the increasing number of patients in the chronic phase of SCI, new approaches and treatments focused on the relief of neuropathic pain resulting from SCI are urgently needed in order to improve the quality of life of patients. To this end, a new experiment was performed to evaluate the possible analgesic effect of repeated EGCG, GSE or CE administration during the third week post injury, when the acute phase of SCI ends and the intermediate phase begins. For this experiment, the doses of EGCG (15 and 20 mg/kg; i.p.), GSE (15 mg/kg; i.p.) and CE (10 mg/kg; i.p.) that exerted the best preventive effects in the acute phase of SCI were selected. Our results showed that repeated administration of EGCG (15 and 20 mg/kg; i.p.) during the third week post-injury did not modulate neither mechanical allodynia nor thermal hyperalgesia. Therefore, although EGCG had a preventive effect on the neuropathic pain development during the acute phase of the injury, it had no

analgesic effect once the acute phase of SCI was established. In contrast, the results obtained with polyphenolic extracts were more promising since the administration of GSE (15 mg/kg; i.p.) and CE (10 mg/kg; i.p.), under the same conditions, modulated both mechanical allodynia and thermal hyperalgesia induced by mild SCI. It is worth mentioning that although both polyphenolic treatments, GSE and CE, did not achieve complete pain inhibition, they exerted a significant analgesic effect compared to the SCI control. Therefore, repeated administration of the polyphenolic extracts GSE and CE during the third week post-injury have an analgesic effect on reflexive pain responses in the intermediate phase of SCI. Importantly, this new administration pattern was not associated with systemic toxicity, as the weight and appearance of the animals and the biomarkers of hepatotoxicity and nephrotoxicity in the animals' serum were not altered when compared with control animals.

To understand these results, it is necessary to consider the most important pathological processes that take place in the intermediate phase of SCI, which are the progressive formation and maturation of the glial scar around the injured area, the abortive growth of the surviving axons and the collateral branching of the surviving axons (**Anjum et al., 2020**). On the one hand, reactive glial scar astrocytes secrete chondroitin sulphate proteoglycans (CSPG), which limit axonal growth (**Li et al., 2020**) and block axonal conduction (**Petrosyan et al., 2013**). On the other hand, microglial cells surrounding the astrocyte glial scar secrete inflammatory cytokines and chemokines, ATP, NO, and growth factors such as NGF, NT3, BDNF, bFGF (**Kim and de Vellis, 2005; Gaudet and Fonken, 2018**), which contribute to increase the hyperexcitability of dorsal horn nociceptive neurons, to enhance pain neurotransmission between nerve afferents and spinal neurons, and to sensitise spinal nociceptive neurons. Furthermore, reactive microglial cells of the glial scar secrete IGF-1, a growth factor that maintains astrocyte reactivity (**Bellver-Landete et al., 2019**), and extracellular matrix metalloproteinases (e.g., MMP3 and MMP9; **Yenari et al., 2010**), which are proteases that degrade components of the basal lamina, leading to disruption of the spinal BBB. Disruption of the BBB leads to perivascular astrogliosis (**Alvarez et al., 2015**), as well as invasion of hematogenous cells that induce astrocyte reactivity and glial scar formation around the injury site (**Kawano et al., 2012**). In this context, it has been reported that EGCG can reduce the expression of MMP9 (**Park et al., 2009**) and inflammatory cytokines/chemokines (**Khalatbary and Ahmadvand, 2011; Machova Urdzikova et al., 2017**), and enhance the action of NGF (**Gundimeda et al., 2010**) and the expression of BDNF and GDNF (**Tian et al., 2013**). In contrast, no effect of EGCG on CSPG expression has been reported. Contrastingly, gallic acid reduces CSPG expression (**Siddiqui et al., 2019**) and attenuates BBB disruption (**Park et al., 2020**). Protocatechuic acid also has a protective effect on the BBB (**Park et al., 2019; Xi et al., 2020**) and reduces the expression of inflammatory cytokines (**Li et al., 2021**). Resveratrol reduces MMP9 levels (**Gao et al., 2006**), attenuates BBB disruption (**Wei et al., 2015**) and promotes the release of BDNF and GDNF by astrocytes (**Zhang et al., 2012**). In addition, gallic acid and resveratrol maintain normal IGF-1 levels (**Bagriyanik et al., 2014; Maya et**

al., 2018). The above findings indicate that some of the polyphenols contained in GSE and CE have effects on the BBB disruption, the generation of glial scar and the expression of proteoglycans, all of them phenomena that occur in the intermediate phase of SCI. Therefore, the analgesic effects of polyphenolic extracts would be expected to be more significant than EGCG treatment if they were administered during the intermediate phase of SCI.

Along with reflexive neuropathic pain responses, published data reveal that more than 40% of SCI patients develop long-term cognitive impairments (**Davidoff, Roth and Richards, 1992; Jensen et al., 2007; Lazzaro et al., 2013**) and emotional disorders (**Fullerton et al., 1981; Arango-Lasprilla et al., 2011; Lazzaro et al., 2013**), which may be considered non-reflexive pain responses. These behavioural alterations have already been described in animal models of SCI (**Luedtke et al., 2014; Wu et al., 2014a,b; Maldonado-Bouchard et al., 2016**; **do Espírito Santo et al., 2019**), but most of them have been assessed after moderate or severe spinal cord contusion or compression, and the interpretation of non-reflexive pain responses could be misled by motor deficits. In addition, most of these studies have been performed during the acute or chronic phase of SCI, leading a lack of information on non-reflexive neuropathic pain responses during the intermediate phase of SCI. For this reason, it was decided to analyse whether SCI animals could present alterations in affective behaviour at sixth weeks after the injury and whether the administration of GSE or CE during the third week post-injury could be beneficial in modulating these potential alterations. Specifically, reward-seeking behaviour (**de la Puente et al., 2015**), light and dark box (**J. Crawley & Goodwin, 1980**), open field (**Hall, 1934; Walsh and Cummins, 1976**) and forced swim (**Porsolt et al., 1977**) tests were performed to evaluate anhedonic-, anxiety-, and depressive-like behaviours, respectively. Our results showed that while no anhedonic neither anxiety-like behaviours were developed in the SCI animals at sixth weeks pots injury (wpi), they exhibited a depressive-like behaviour. These alterations are consistent with other study in which SCI by clip-compression induced depressive-like behaviour in female rats at intermediate phase of injury. However, these results may be attributable to locomotor disturbances since SCI caused impairment in hind limb motor function and the authors classified the SCI as severe (**do Espírito Santo et al., 2019**). Regarding the treatment, our results showed that the CE (10 mg/kg; i.p) administration during the third wpi modulated the depressive-like behaviour detected in SCI animals at sixth wpi. This antidepressant effect of CE could be attributed to chlorogenic acid, the most abundant polyphenol in CE, as its antidepressant activity was previously demonstrated. On the one hand, chlorogenic acid isolated from *Artemisia capillaris* Thunb. significantly reduced the immobility period in both tail suspension test and forced swim test in an experimental mice depression model (**Park et al., 2010**). On the other hand, chlorogenic acid from Hawthorn Berry (Crataegus pinnatifida fruit) prevented stress hormone-induced depressive behaviour, through monoamine oxidase B-reactive oxygen species signalling in hippocampal astrocytes of mice (**Lim et al., 2018**). Several studies describe that non-evoked pain responses associated

with anxiety and depression are mediated by changes in the hippocampus (**Mutso et al., 2012; Dellarole et al., 2014**), ACC (**Cao et al., 2009; Zhong et al., 2012; Luo et al., 2015**) and amygdala (**Semenova et al., 2012**). The results of the present thesis indicate that both GSE and CE reduce gliosis in the ACC of SCI animals in the acute phase of injury. Therefore, it is not unreasonable to think that these polyphenolic extracts may modulate these pathophysiological processes also during the intermediate phase of SCI in the ACC as well as in other supraspinal structures such as the hippocampus or amygdala.

In summary, repeated administration of polyphenolic extracts (15 mg/kg of GSE and 10 mg/kg of CE; i.p) during the third week post injury modulates the reflexive pain responses of thermal hyperalgesia and mechanical allodynia during the intermediate phase of SCI and the dose of 10 mg/kg of CE also modulates the non-reflexive pain response expressed at this time point, that is depressive-like behaviour. These results are very encouraging because they open the door to a promising treatment for all those patients who already have developed neuropathic pain and who have been refractory to treatments administered during the acute phase of the SCI.

One we confirmed that repeated administration of 15mg/kg GSE and 10 mg/kg CE exerted significant preventive effects when they are administered during the first week after injury and significant analgesic effects when administered during the third week after injury, without apparent toxicity or adverse effects, a new experimental protocol was designed with the aim of study preventive effects of polyphenols up to the chronic SCI phase. That is, a new administration protocol was proposed consisting of different repetitive administrations over time, including preventive administration during the first week after the injury and reinforcing these beneficial effects during the third week (beginning of the SCI intermediate phase) and the sixth week after the injury (beginning of the SCI chronic phase) in order to evaluate whether either GSE or CE treatments could modulate over time and up to the chronic phase (10 weeks post SCI) the SCI-related reflexive and non-reflexive pain responses.

During this new experimental period, reflexive pain responses were weekly evaluated, and our results showed that both polyphenolic treatments significantly prevented thermal hyperalgesia development until 10 weeks post-SCI, that is, up to the chronic phase of the injury. Regarding mechanical allodynia modulation, both polyphenolic extracts prevented its development for as long as this evoked response was manifested in the SCI animals, which was up to week 9. Although SCI animals developed both thermal hyperalgesia and mechanical allodynia, it is true that they showed increased paw withdrawal thresholds during the chronic SCI phase, when compared to the acute phase. This finding suggest that SCI animals showed a progressive decrease of reflexive neuropathic pain responses along the experimental period up to late-chronic phase. However, it is difficult to elucidate whether these higher withdrawal thresholds are associated with lower hypersensitivity or, on the contrary, may be attributable to other reasons such as habituation to the tests

or other behavioural alterations that may mask pain responses. Other studies have been showed evoked or reflexive pain responses during the chronic phase of the SCI in mice (**Hendricks et al., 2006; Hulsebosch et al., 2009; Watson et al., 2014; Wu et al., 2013, 2014a,b, 2015, 2016a,b; McFarlane et al., 2020**). However, most of them were conducted in male mice and below 60 days post-injury, showing significant locomotor disturbances.

The withdrawal response elicited by a threshold stimulus is the most common endpoint in laboratory pain assessment (**Barrot, 2012**). Withdrawal to thermal or mechanical force is an evoked response that is primarily reflexive and is largely a function of spinal cord circuitry based on motor responses to nociceptive input (**Treede et al., 1992; Jensen, 2002; Backonja, 2003; Sandkuhler, 2009**). The evoked response component of pain needs an ascending input and is influenced by higher order inputs from the central nervous system through descending modulation to spinal cord circuits. However, conscious decision-making about pain or discomfort as part of its character or occurrence is likely to be absent (**Woolf, 1984; Ruscheweyh et al., 2007; King and Porreca, 2014**). Therefore, considering that chronic pain may be more related to the spontaneous and persistent component of pain (**Backonja and Stacey, 2004; Negus et al., 2006; Rice et al., 2008**), evaluation of evoked-reflexive pain responses is not sufficient to make a complete and comprehensive assessment of pain during the chronic phase of SCI. Hence, spontaneous or non-reflexive pain responses are likely to provide different information about nociceptive input and its impact to the whole animal, more along the lines of the subjective description of actual pain (**IASP; Tappe-Theodor and Kuner, 2014; Treede et al., 2019**). This emerging consideration has led to increased use of novel approaches to measure the extent to which nociceptive information changes non-evoked behavioural outputs in freely behaving animals (**LaBuda and Fuchs, 2000; Martin et al., 2004; King et al., 2009; Langford et al., 2010; Mogil et al., 2010; Cobos et al., 2012; De Felice et al., 2013**). For this reason, it was decided to evaluate disturbances in social and seeking-reward natural behaviours as well as anxiety- and depressive-like behaviours in SCI animals as non-reflexive pain responses; and whether treatment with polyphenolic extracts could modulate these affective components of pathological pain. Specifically, reward-seeking behaviour (**de la Puente et al., 2015**), light and dark box (**J. Crawley & Goodwin, 1980**), open field (**Hall, 1934; Walsh and Cummins, 1976**), forced swim (**Porsolt et al., 1977**) and social interaction (**J. N. Crawley, 2003**) tests were performed during the chronic phase of the injury, concretely between the ninth and tenth week post SCI.

Focusing on the present thesis results, our findings indicated that SCI in female CD1 mice induced anhedonia, anxiety and depressive-like behaviours, as well as significant alterations in their sociability in the chronic phase of the injury. These results are consistent with other studies in which SCI has been associated with some emotional and cognitive impairments such as depression-like behaviour (**Luedtke et al., 2014; Wu et al., 2014; Li et al., 2017; Boadas-Vaello et al., 2018; Li et al., 2020**), anhedonia (**Luedtke et al., 2014;**

Li et al., 2020) and social disturbances (**Luedtke et al., 2014; do Espírito Santo et al., 2019; Li et al., 2020**) among others. In addition, another study has shown that anxiety/depression profiles are associated with brain/spinal inflammation resulting from SCI (**Maldonado-Bouchard et al., 2016**). However, most of these studies refer to severe SCI associated with significant locomotor deficits that may mask behavioural disturbances of the animals. In contrast, the results of the present thesis, which show that mild SCI resulted in affective disturbances at the chronic phase of injury, cannot be attributed to locomotor deficits, as the BMS test did not detect significant alterations in locomotor activity. In addition, other parameters analysed in the behavioural tests confirmed this result. For example, in the light and dark box test or in the open field test, no differences in the number of zone transitions or global activity of the animals were detected between any of the experimental groups.

In order to gain mechanistic insights by which these polyphenolic treatments were able to prevent the development of these reflexive and non-reflexive neuropathic pain responses, molecular studies of gliosis and chemokine expression in the spinal cord and supraspinal structures of PAG, RVM, ACC and AMG were performed. Our results revealed that, although GFAP levels were higher in SCI animals compared to both Sham-operated and SCI-treated animals, no significant astrogliosis in the spinal cord were detected. In contrast, spinal microgliosis were detected in SCI mice at 10 wpi, which was slightly modulated by GSE (15 mg/kg) and CE (10 mg/kg) treatments. In relation to this, Hains and Waxman demonstrated that selective pharmacological inhibition of microglial activation results in a return to the resting morphological phenotype as well as reductions in electrophysiologic and behavioural concomitants of pain, suggesting the role of microglia in the active modulation of ongoing pain after SCI (**Hains and Waxman, 2006**). In addition, it has been identified a mechanism by which activated microglia after SCI utilize PGE2 as a signalling molecule to induce dorsal horn sensory neurons to undergo changes that underlie chronic pain (**Zhao et al., 2007**). In this regard, the spinal microgliosis observed in the chronic phase of SCI leads to the release of inflammatory factors, such as interleukin-1β and prostaglandin E2, which are involved in inducing central sensitisation of spinal neurons (**Hains and Waxman, 2006; Hulsebosch et al., 2009**). This central sensitisation has been attributed as one of the causes of evoked pain responses (**Marchand et al., 2005; Chen et al., 2018**). Thus, spinal microgliosis could explain why SCI animals exhibit both thermal hyperalgesia and mechanical allodynia at 10 weeks post-injury. Finally, the modulation of spinal microgliosis by polyphenolic extracts could be explained by some of the previously mentioned properties of the polyphenols contained in them, such as the ability of gallic acid to bind to the estrogen receptor beta (**Ye and Shaw, 2020**) and the purinergic P2X7 receptor (**Yang et al., 2021**) or the ability of chlorogenic acid to block different intracellular cascades such as MAPK, p38, ERK, JNK and NF-κB as well as the generation of prostaglandins (**Bagdas et al., 2020**). All these properties are related to the inhibition of microgliosis and

could explain the effectiveness of polyphenolic extracts in reducing spinal IBA1 expression as well as mechanical allodynia and thermal hyperalgesia responses.

As for supraspinal structures, our molecular results showed that both GSE and CE treatments modulated astrogliosis as well as CCL2/CCR2 and CX3CL1/CX3CR1 signalling in the ACC, amygdala, dorsal and ventral PAG and RVM, and microgliosis in the ventral PAG at 10 weeks post-SCI. Previous studies in rodents have linked supraspinal neuroinflammatory processes to behavioural alterations following SCI (**Walker et al., 2013; Wu et al., 2014b; Maldonado-Bouchard et al., 2016**), with a particular focus on the hippocampus and cerebral cortex. For instance, Wu and colleagues showed that depressive-like behaviour after SCI was marked by microglial hyperactivity with an M1-polarised phenotype in the cerebral cortex and hippocampus (**Wu et al., 2014**). In other study, both hippocampal and spinal cord levels of pro-inflammatory cytokines were upregulated in anxiety/depressed SCI rats (**Maldonado-Bouchard et al., 2016**). In addition, Jure and collaborators demonstrated that SCI impairs neurogenesis and induces glial reactivity in the rat hippocampus (**Jure et al., 2017**). Finally, neuronal changes in opioid receptor expression in the caudate-putamen (**Thompson et al., 2018**), changes in ventral tegmental area neurons (**Markovic et al., 2021**) and alterations in the nucleus accumbens and prefrontal cortex (**Fang et al., 2020**) have also been associated with anhedonic pain responses. However, lesser is known about abnormalities in other brain areas after SCI such as the NAc, the mPFC, the amygdala or the PAG-RVM axis (**Kuner and Kuner, 2021**).

Regarding the RVM and PAG, they are the main structures of the endogenous modulatory system, which regulates the processing of sensory input in the spinal dorsal horn (**Heinricher et al., 2009**). Despite increasing evidence indicating the role of the PAG and RVM in descending pain modulation, the precise underlying cellular and molecular mechanisms involved in descending facilitation/inhibition of the dorsal horn remain elusive. In addition to descending modulation, vPAG columns have projections to many CNS structures critical for the normal expression of sleep-wake cycles and social behaviours (**Monassi et al., 2003; Mor et al., 2011**). Astrogliosis in vPAG was detected mainly in a subset of rats showing both neuropathc pain (allodynia and hyperalgesia) and disability (changes in social and sleep-wake behaviours) after sciatic nerve CCI (**Mor et al., 2011**). In contrast, dPAG plays a role in modulation of autonomic responses to pain (**Mai and Paxinos, 2011**) and fear-conditioned analgesia (**Butler et al., 2011**) and actually descending modulation from dPAG has different effects on nociceptive reflexes evoked by activation of C- and A-delta fibers (**McMullan and Lumb, 2006**). Our results showing activated astrocytes and microglial cells in ventral and dorsal PAG after SCI suggest the role for activated glial cells in the descending facilitation of nociceptive input expressed as evoked pain responses, such as allodynia and hyperalgesia, as well as behavioural alterations, such as social disturbances. As to RVM, it is a relay in the pathways from both vPAG and dPAG columns (**Hudson and Lumb, 1996**) and provides the major common output of descending modulatory system, which is critical in the maintenance of chronic pain states (**Pertovaara et al., 1996;**

Porreca et al., 2002; Dubner and Ren, 2004; Gebhart, 2004; Vanegas and Schaible, 2004; Vera-Portocarrero et al., 2006; Bee and Dickenson, 2007). Our results showing glial activation in RVM after SCI are in concordance with other studies that previously showed that glial activation in RVM promotes descending facilitation to mediate inflammatory hypersensitivity (**Roberts et al., 2009**), mechanical hyperalgesia after CCI (**Wei et al., 2008**) or cancer-induced bone pain (**Liu et al., 2012**).

In addition, the results of the present thesis also showed that chemokines CCL2 and CX3CL1, and their receptors, CCR2 and CX3CR1, as well as CatS, the protease responsible for sCX3CL1 secretion, were overexpressed in PAG and RVM. It is not surprising to find these markers overexpressed, as activated glial cells are known to up-regulate the synthesis and secretion of numerous chemokines involved in the exchange of signals between neurons and glial cells, as well as in the modulation of nociceptive transmission (**White et al., 2007; Hulsebosch et al., 2009; Gao and Ji, 2010; Liou et al., 2013**). To our knowledge, no other studies have shown an increase in these chemokines and their receptors in both PAG and RVM at the chronic phase of SCI, except for one study showing CCL2 overexpression in PAG after severe SCI, accompanied by a reduction of cannabinoid receptor type 1 (CB1) and prolonged neuropathic pain behaviour (**Knerlich-Lukoschus et al., 2011**). Along the same lines, Dubový and collaborators showed CCL2/CCR2 upregulation accompanied with glial activation in both PAG and RVM following sciatic nerve injury (**Dubový et al., 2018**). Furthermore, intra-RVM injection of CCL2 induced a dose-dependent hyperalgesia which was prevented by pre-treatment with an CCL2 inhibitor (**Guo et al., 2012**). Finally, it has been reported that intra-PAG injection of CX3CL1 before administration of mu, delta and kappa opioid agonists significantly reduces the antinociception induced by each of these peptides, thus demonstrating the role of CX3CL1 in the inhibition of opioid anti-nociceptive effect in PAG (**Chen et al., 2007**). Therefore, our results supported by all the findings abovementioned suggest that expression in PAG-RVM axis of CCL2/CX3CL1 chemokine network and the endogenous cannabinoid system, which were shown to interact which each other, are potential key elements in the cascades underlying central neuropathic pain development after SCI.

Regarding amygdala nucleus (AMG), it is known to be involved in the processing of mood, fear, emotional memory, and the emotional-affective dimension of pain (**Veinante et al., 2013**). Neuroimaging studies have reported pain-related changes in the AMG in several pain conditions (**Bornhövd et al., 2002; Paulson et al., 2002**). In particular, the CeA was termed the "nociceptive amygdala" (**Neugebauer et al., 2004**) and increased number and excitability of CeA neurons was associated with depressive-like behaviour in NP (**Gonçalves et al., 2008**). Dependent on environmental conditions and affective states, the amygdala appears to play a dual facilitatory and inhibitory role in the modulation of pain behaviour and nociceptive processing at different levels of the pain neuraxis (**Neugebauer et al., 2004**). In addition to the AMG, the ACC has also been reported to be implicated in both anxiety and pain in human and animal studies (**Etkin**

et al., 2011; Barthas et al., 2015). Anatomically, ACC has strong connections with other cortical and subcortical areas, such as the amygdala, mediodorsal thalamic nuclei, hypothalamus, hippocampal formation, secondary visual cortex, secondary auditory cortex, entorhinal, perirhinal cortex, and orbital cortex, which are all implicated in emotion, cognition, memory, behavioural control, and pain (**Berendse and Groenewegen, 1991; Hsu and Shyu, 1997; Mcdonald and Mascagni, 1996**). Taken together, these data provide a morphological substrate for contribution of the ACC and amygdala to pain affect. Focusing on present thesis results, our findings showed an increase in glial reactivity, chemokine expression of CCL2/CX3CL1 and their receptors CCR2/CX3CR1, as well as CatS in ACC. In contrast, only astrogliosis and overexpression of CX3CL1/CX3CR1, CatS and CCR2 were detected in AMG of SCI animals at 10 weeks post SCI. All these molecular changes in these supraspinal structures were accompanied with depression-, anxious- and anhedonic-like behaviours at the chronic phase of the SCI. The changes in ACC associated with neuropathic pain, in particular gliosis and CX3CL1/CX3CR1 expression, have already been discussed earlier in this thesis. However, there are no studies yet describing the CCL2/CCR2 expression pattern in the ACC of neuropathic pain models. Therefore, according to our knowledge, this doctoral thesis describes for the first time an increase of the chemokine CCL2 and its receptor CCR2 in ACC after SCI. Regarding to the changes in the AMG, other study has reported remarkable GFAP increase in the AMG of rats subjected to spared nerve injury (SNI) of the sciatic nerve (**Marcello et al., 2013**). Along the same lines, Hengjian et al. reported astroglial activation as well as IL-1β and TNFα overexpression in AMG in rat model of peripheral inflammatory pain (**Hengjian et al., 2008**). CCR2 upregulation in the amygdala associated with neuropathic pain has not yet been reported, although it has been showed an overexpression of CCL2 in the CeA of PSNL mice that presented anxiety-like behaviour and hypersensitivity to mechanical stimuli (**Sawada et al., 2014**). Regarding to CX3CL1/CX3CR1 signalling, Cho et al. have recently demonstrated an increased CX3CR1 expression in both hippocampus and AMG in a rat model of post operative pain (**Cho et al., 2021**). All these findings are in concordance with our results and all of them support the idea that non-reflexive pain responses such as anxiety and depression are mediated by changes in the limbic system including hippocampus (**Mutso et al., 2012, Dellarole et al., 2014**), anterior cingulate cortex (**Cao et al., 2009, Zhong et al., 2012, Luo et al., 2015**) and amygdala nuclei (**Semenova et al., 2012**).

As for molecular results in supraspinal structures, they also revealed that both GSE15 and CE10 modulated astrogliosis and CCL2/CCR2 and CX3CL1/CX3CR1 signalling in ACC, AMG, dorsal and ventral PAG and RVM as well as microgliosis in ventral PAG at 10 weeks post-SCI. Therefore, the polyphenols contained in these extracts are not only able to modulate pain behaviours during the acute phase of the injury but also up to the chronic phase of the injury when they are administered repeatedly over time, coinciding with the beginning of acute, intermediate and chronic phases. In the similar line, other authors have used repeated polyphenol treatment to attenuate both evoked and non-evoked neuropathic pain responses, such as Zhao

et al. who demonstrated that chronic resveratrol treatment in animals with CCI decreases thermal hyperalgesia and co-morbid depressive like behaviours, but not mechanical allodynia (**Zhao et al., 2014**). Hence, these molecular and behavioural results are very important as they open the door to a preventive treatment of emotional disorders associated with central neuropathic pain after SCI.

Overall, considering the results of the present thesis, we have demonstrated that a mixture of polyphenols present in natural extracts may be a suitable pharmacological strategy to prevent the development of SCI-induced neuropathic pain up to the chronic phase by modulating not only the reflexive pain responses (more related to the sensory-discriminative dimension of pain) but also the non-reflexive pain responses (included in the affective-motivational dimension of pain). These compounds would not only exert modulatory effects at the site of injury by modulating gliosis and the expression of markers related to central sensitisation but also would play pivotal roles on supraspinal structures closely related to expression and modulation of central neuropathic pain. Hence, to our knowledge this is the first study showing that both GSE and CE may be suitable pharmacological treatment to modulate both reflexive and non-reflexive pain responses development up to the SCI chronic phase, without displaying systemic toxicity and they may be also useful for the translation into the clinics for novel therapeutic strategies.

CONCLUSIONS

VI. CONCLUSIONS

The main conclusions of the present thesis can be summarized as follows:

1. Preventive EGCG administration during the first week post spinal cord injury (SCI) results in inhibition of both mechanical allodynia and thermal hyperalgesia development during the acute phase of SCI, accompanied by attenuation of spinal gliosis detected in the spinal cord of injured mice. However, all tested doses of EGCG mixed with DMSO caused significant weight loss of mice throughout the experimental period, indicating that systemic toxicity may be associated with such treatment.

2. Preventive Grape Stalk polyphenolic extract (GSE) administration during the first week post SCI, results in the inhibition of both mechanical allodynia and thermal hyperalgesia development during the acute phase of SCI without altering either weight or biomarkers of hepatotoxicity and nephrotoxicity in the serum of the animals. Antinociceptive effects of GSE are associated with the prevention of both gliosis and upregulation of central sensitization-related algogens (pERK, CX3CL1, CX3CR1 and CCR2) in the spinal cord of GSE-treated injured mice. In addition, while GSE treatment modulates CX3CL1/CX3CR1 signalling and attenuates astrogliosis in both supraspinal structures of the ACC and PAG, as well as microgliosis in the ACC of injured mice, such treatment induces microglial activation in PAG. The latter may correspond to microglial cells with an M2 anti-inflammatory phenotype.

3. Preventive Coffee polyphenolic extract (CE) during the first week post SCI, results in the inhibition of both mechanical allodynia and thermal hyperalgesia development during the acute phase of SCI without altering either weight or biomarkers of hepatotoxicity and nephrotoxicity in the serum of the animals. Antinociceptive effects of CE are associated with the prevention of gliosis and upregulation of chemokines receptors in the spinal cord of CE-treated injured mice. Furthermore, CE treatment modulates CX3CL1/CX3CR1 signalling and attenuates astrogliosis in the ACC and PAG, as well as microgliosis in the ACC of injured mice. Finally, CE treatment also promotes microglial activation likely characterized by an increase of M2 anti-inflammatory phenotype in PAG.

4. Repeated administration of both GSE and CE during the third week post SCI modulates mechanical allodynia and thermal hyperalgesia induced by mild SCI during the intermediate phase of injury, with no associated systemic toxicity, hepatotoxic or nephrotoxic effects. In contrast, the same administration pattern for EGCG does not modulate these reflexive pain responses. In addition, CE administration during the third week after injury modulates depressive-like behaviour detected in spinal cord injured mice at intermediate phase of injury.

5. Repeated administration of GSE and CE during the first-, third- and sixth-week post SCI prevents thermal hyperalgesia and mechanical allodynia development up to the SCI chronic phase as long as these reflexive pain responses are manifested in the spinal cord injured animals, with no associated systemic toxicity effects. In addition, GSE and CE treatments also modulate disturbances in the affective-motivational behaviours such as anhedonia, depression, anxiety, and social interaction impairment detected in spinal cord injured animals. Antinociceptive effects of GSE and CE can be associated not only with the modulation of spinal microgliosis but also with the modulation of astrogliosis and CCL2/CCR2 and CX3CL1/CX3CR1 signalling in the supraspinal structures of ACC, amygdala, dorsal and ventral PAG and RVM as well as microgliosis in ventral PAG.

6. Overall, the present thesis results suggest that a mixture of polyphenols present in natural extracts of grape stalk and coffee may be a suitable pharmacological strategy to prevent the development or attenuate SCI-induced neuropathic pain by modulating not only the reflexive pain responses (more related to the sensory-discriminative dimension of pain) but also the non-reflexive pain responses (included in the affective-motivational dimension of pain). These compounds not only exert their effects at the site of injury by modulating gliosis and the expression of markers related to central sensitisation but also on supraspinal structures closely related to expression and modulation of central neuropathic pain.

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VII. REFERENCES

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