Carla Sofia
da Silva Sousa

Combining Neuroprotection and Neuroregeneration for Spinal Cord Injury Repair

UMinho | 2025

Universidade do Minho
Escola de Medicina

Carla Sofia da Silva Sousa

Combining Neuroprotection and Neuroregeneration for Spinal Cord Injury Repair

Janeiro de 2025

Universidade do Minho
Escola de Medicina

Carla Sofia da Silva Sousa

Combining Neuroprotection and Neuroregeneration for Spinal Cord Injury Repair

Tese de Doutoramento em Medicina

Trabalho realizado sob a orientação de:
Doutor Nuno Silva
Doutor António Salgado

janeiro 2025

DIREITOS DE AUTOR E CONDIÇÕES DE UTILIZAÇÃO DO TRABALHO POR TERCEIROS

Este é um trabalho académico que pode ser utilizado por terceiros desde que respeitadas as regras e boas práticas internacionalmente aceites, no que concerne aos direitos de autor e direitos conexos.

Assim, o presente trabalho pode ser utilizado nos termos previstos na licença abaixo indicada.

Caso o utilizador necessite de permissão para poder fazer um uso do trabalho em condições não previstas no licenciamento indicado, deverá contactar o autor, através do RepositóriUM da Universidade do Minho.

Licença concedida aos utilizadores deste trabalho



Atribuição-NãoComercial-SemDerivações
CC BY-NC-ND

https://creativecommons.org/licenses/by-nc-nd/4.0/

Agradecimentos/Acknowledgments:

Ao terminar esta etapa na minha formação académica, surge agora o momento de agradecer às pessoas e entidades que tornaram possível este trabalho. Nesta difícil tarefa de reconhecer todos os que de alguma forma contribuíram para o que hoje é aqui apresentado nesta tese, quero primeiramente agradecer à Escola de Medicina da Universidade do Minho e ao Instituto Ciências da Vida e da Saúde (ICVS), por ter fornecido todas as infraestruturas para a realização do trabalho laboratorial. A jornada aqui descrita só foi possível graças à orientação do Nuno Silva e do António Salgado. Ao António Salgado, gostaria de agradecer todo o apoio no desenvolvimento desta tese. Ao Nuno Silva, agradecer de forma particular: a junção de visão, paciência, dedicação, apoio e estratégia no desenvolvimento deste trabalho. Sem a sua ajuda, não teria sido possível a sua realização. Aos meus colegas da equipa Spinal Cord o meu muito obrigada. Contudo, sinto necessidade de agradecer em particular ao Rui Lima, fulcral na sua participação ativa em todos os trabalhos. To my non-official supervisor Dr. Richard G Fessler and to Rush University Medical Center, I want to thank the opportunity that it was given to do part of the team in your lab and great institution. I learned a lot while I was there. I really enjoyed the scientific discussions with Dr. Fessler, who was always supportive and kind and allowed me to experience a real American Thanksgiving with his family. Um agradecimento particular pelo apoio, estímulo e compreensão durante estes anos ao Dr. Manuel Cunha e Sá, diretor do serviço de Neurocirurgia do hospital onde trabalho e à Dra. Cátia Gradil, neurocirurgiã e orientadora do meu Internato de Neurocirurgia.

Por fim, um agradecimento especial à minha mãe, irmão e amigos especiais (que eles sabem quem são) por estarem a meu lado neste caminho e em especial ao meu pai, a minha inspiração, que certamente estaria muito feliz por me ver completar mais uma etapa. Não tenho palavras para descrever o quão grata estou por todo o apoio. A todos vós, o meu muito obrigada!

The work presented in this thesis was performed in the Life and Health Sciences Research Institute (ICVS), University of Minho. Financial support was provided by grants from the Foundation for Science and Technology (FCT) through the project PTDC/BTM-MAT/29968/2017 and CEECIND/04794/2017(DOI 10.54499/CEECIND/04794/2017/CP1458/CT0031), by Prémios Santa Casa Neurociências–Prize Melo e Castro for Spinal Cord Injury Research (MC-18-2021) and by Wings For Life Spinal Cord Research Foundation (WFL-PT-14/23). Institutional support was also provided by National funds, through the FCT - project UIDB/50026/2020 (DOI 10.54499/UIDB/50026/2020), UIDP/50026/2020 (DOI 10.54499/UIDP/50026/2020) and LA/P/0050/2020 (DOI 10.54499/LA/P/0050/2020).







STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Resumo:

As lesões vertebro-medulares (LVM) são uma das condições mais devastadoras do Sistema Nervoso Central para a qual ainda não há uma resposta curativa.

No sistema nervoso central, a limitada regeneração axonal após lesão permanece como um dos maiores desafios e como um mecanismo fisiopatológico crucial. Assim, nesta tese, avaliámos o efeito terapêutico de um inibidor da PDE4 aprovado pela Food and Drug Administration (FDA), o Roflumilast (Rof), com intuito de promover a regeneração axonal, debelando o efeito inibitório de proteínas da mielina. Foi utilizado um modelo de contusão torácica em ratos e os resultados indicam que o tratamento foi eficaz na recuperação funcional e os animais com sinais de paraplegia demonstraram uma recuperação significativa com suporte de peso ocasional. A avaliação histológica revelou uma diminuição significativa no tamanho da cavidade e densidades celulares de microglia reativa, assim como neuroregeneração nos animais tratados. A análise molecular revelou que a IL-10 e IL-13 séricas estão aumentadas nos animais tratados com Roflumilast, assim como o VEGF. Numa segunda fase e considerando a complexidade da lesão e a multiplicidade de alterações secundárias, tentámos uma abordagem de combinação do Rof e um tratamento neuroprotetor, Levetiracetam (Lev), que reduz a excitotoxicidade do glutamato durante a fase aguda da lesão. Ambos os tratamentos, isoladamente e em combinação, foram eficazes na promoção da recuperação funcional, mas não mostraram efeitos aditivos. Tendo isto em conta, na última parte deste trabalho, explorámos a combinação do Rof com o secretoma de células estaminais derivadas de tecido adiposo (ASCs), que induzem a preservação da substância branca e a modulação da resposta inflamatória nas LVM. Este tratamento só levou a melhorias funcionais quando os tratamentos foram administrados isoladamente. Aliás, a combinação de Rof + secretoma de ASCs teve um resultado prejudicial.

No geral, nesta tese, testámos o efeito terapêutico de diferentes abordagens nas LVM. O tratamento com o Rof foi o medicamento mais promissor estudado e pode desempenhar um papel no tratamento das LVM. As combinações experimentadas não parecem ser benéficas, mas são necessários estudos adicionais para esclarecimento.

Palavras-chave: neuroproteção; neuroregeneração; lesão vertebro-medulares; terapias combinadas.

Abstract

Spinal Cord Injury (SCI) is one of the most devastating conditions of the Central Nervous System (CNS) for which there is still no curative answer besides the extensive research.

In the CNS, axonal failure to regenerate after injury remains one of the greatest challenges facing neuroscience and this is a crucial pathophysiological mechanism. Thus, in this thesis we evaluated the therapeutic effect of a Food and drug administration (FDA)-approved PDE4 inhibitor, Roflumilast (Rof) for promoting axonal regeneration, by overcoming the inhibitory effect of myelin proteins. Rof therapy was tested in a thoracic contusion rat model. Results indicate that the treatment was effective in promoting functional recovery (both gross and fine motor functions) and animals with signs of paraplegia demonstrated a significant recovery by achieving occasional weight-supported plantar steps. The histological assessment revealed a significant decrease in the cavity size and less reactive microglia cell densities, as well as neuroregeneration in treated animals. Molecular analysis revealed that IL-10 and IL-13 are increased in the serum of Roflumilast-treated animals, as well as VEGF.

In a second phase and considering the lesion complexity and the multitude of secondary alterations, we tried a combination approach with Rof and a neuroprotective treatment, Levetiracetam (Lev), which reduce the glutamate excitotoxicity in the acute phase. Both treatments, alone and combined, were effective in promoting functional recovery but did not show additive effects. For this reason, in the last part of this thesis, we explored the combination of Rof with Adipose Stem Cells (ASCs) secretome, which induce white matter preservation and a modulation of the inflammatory response in SCI. This treatment only led to functional improvements when the treatments were administered alone. In fact, the combination of Rof + ASCs secretome had a detrimental outcome.

Overall, in this thesis we tested the therapeutic effect of different approaches to treat SCI. The Rof treatment for neuroregeneration was the most promising drug studied and may have a role in SCI treatment. The experimented combinations do not seem to be beneficial but further studies are needed to clarify these results.

Keywords: neuroprotection; neuroregeneration; spinal cord injury; combinational therapies.

Figures and Tables:

Figure I.1. Representation of Dermatomes Diagram for lesion location in SCI. 5

Figure I. 2. Representation of immune response with the respective pathophysiological events occurring during injury. 8

Figure I.3. Representation of an injured spinal cord with the respective secondary pathophysiological events occurring after injury. 10

Figure I. 4. Schematic representation of possible combinatorial treatments against spinal cord injuries. Cells and scaffolds can be used alone or in combination with pharmacological agents and other biomolecules such as growth factors. Technological strategies such as epidural electrical stimulation are being tested in combination with biomolecules or cells. 26

Figure II.1. Methodological scheme and temporal line for the in vivo study design. 87

Figure II.2. The effect of Roflumilast on cAMP-driven responses in spinal cord neurons. **A)** Experimental outline. **B)**, **C)** Representative photomicrographs of controls and Roflumilast-treated cells, respectively. **D)** Neuronal nuclear CREB area quantification. Unpaired t-test, 6df, p = 0.0441. Controls n = 4, Roflumilast n = 4. **E)** Neuronal cytoplasmic cAMPKA area quantification. * $p < 0.05$; ns = non-significant. 91

Figure II.3. The effect of Roflumilast on axonal regeneration in vitro. **A)** Neurofilament + staining for cultures with Neurobasal + Roflumilast. **B)** Neurofilament + staining for cultures with only Neurobasal. **C)** Neurofilament + area in organotypic cultures. Unpaired t-test, 11df, p = 0.008. Controls n = 4, Roflumilast n = 5. ** $p < 0.01$ 92

Figure II.4. Pre-clinical evaluation of Roflumilast using a contusion SCI model. **A)** In vivo cAMP concentration for Rof-treated animals and controls. Unpaired t-test, 10 df, p = 0.029. Saline n = 6, Roflumilast n = 6. **B)** Functional recovery after spinal cord injury of Rof-treated animals and controls. Two-way ANOVA F (1, 20) = 4.408, p = 0.0487. Saline n = 11; Roflumilast n = 12. **C)** Activity box test—8th

week. Velocity (cm/s) of Rof-treated animals and controls. Unpaired t-test, 21 df, p = 0.0008. Saline n = 11; Roflumilast n = 12. **D)** Gridwalk test of Roflumilast-treated animals and controls—8th week. Unpaired t-test, 21 df, p = 0.0001. Saline n = 11; Roflumilast n = 12. * p < 0.05; *** p < 0.001. 93

Figure II.5. Multiplex analysis. **A)** Serum IL-10 concentration 1-week post-injury. Unpaired t-test, 13 df, p = 0.010. Saline n = 10; Roflumilast n = 5. B) Serum IL-10 concentration 4 weeks post-injury. Unpaired t-test, 14 df, p = 0.013. Saline n = 10; Roflumilast n= 6. **C)** Serum IL-13 concentration 1-week post-injury. Unpaired t-test, 6 df, p = 0.035. Saline n = 4; Roflumilast n = 4. **D)** Serum IL-13 concentration 4 weeks post-injury. Unpaired t-test, 7 df, p = 0.027. Saline n = 5; Roflumilast n = 4. **E)** Serum VEGF concentration 8 weeks post-injury. Unpaired t-test, 9 df, p = 0.040. Saline n = 6; Roflumilast n = 5. * p < 0.05. 95

Figure II.6.A) Cavity Size of Roflumilast-treated animals and controls. Unpaired t-test, 12 df, p = 0.0149. Saline n = 8; Roflumilast n = 6. **B)** Spared tissue in the injury epicenter of Roflumilast-treated animals and controls. Unpaired t-test, 12 df, p = 0.0036. Saline n = 8; Roflumilast n = 6. **C)** Longitudinal GFAP+ staining slice of saline-treated group and **D)** for Rof-treated group. A total of 69 longitudinal slices were analyzed. * p < 0.05; ** p < 0.01. 96

Figure II.7.A) Microglia staining with IBA1 + to assess the percentage of ameboid microglia in Roflumilast-treated animals and controls. Unpaired t-test, 12 df, p = 0.0003. Saline n = 7; Roflumilast n = 7. **B)** Longitudinal slice of injured spinal cord, with IBA1+ staining for microglia reactivity in a Rof-treated animal; **B')** magnification of the dashed square. **C)** IBA1 + staining in an injured spinal cord of a saline-treated animal; **C')** magnification of the dashed square. A total of 68 longitudinal slices were analyzed. *** p < 0.001. 97

Figure II.8. Roflumilast increases GAP-43 expression along the rostro–caudal axis of the injured spinal cord. **A)** and **B)** Microphotographs of the spinal cord of control and Roflumilast-treated animals. **C)** Shows the quantification of GAP-43 fibers along the rostro– caudal axis at 200, 400 and 600 μm from the injury epicenter in both directions. Two-way ANOVA F (DFn:1.409, DFd:18.31) = 4.182. Saline n = 7; Roflumilast n = 8. Insets show zoom magnification GAP-43 labeling. Scale Bars: 250 μm at **A)** and **B)** and 50 μm at **A')** and **B')**. * p < 0.05. 98

Figure II.9. Roflumilast promotes axonal regeneration in vivo. **A)** Fluoro-Gold ratio (Rostral/Caudal) in Roflumilast-treated animals and controls. Unpaired t-test, 12 df, $p < 0.0001$. Saline n = 7; Roflumilast n = 7. **B)** Fluoro-Gold + staining in a Roflumilast-treated animal caudal to the epicenter. **C)** Fluoro-Gold + staining caudal to the epicenter in a saline-treated animal. **D)** Fluoro-Gold + staining in the Roflumilast group rostral to the lesion. **E)** Fluoro-Gold + staining rostral to the lesion in the saline group. A total of 88 transversal slices were analyzed. *** $p < 0.001$. 99

Figure III.1. Schematic experimental design. **A)** Therapeutic synergies between Roflumilast (ROF) and Levetiracetam (LEV). Wistar rats underwent intrathecal catheter placement 3 days before spinal cord injury (SCI). LEV was administered by intraperitoneal injection (IP) 4 hours after injury. ROF treatment was administered locally through the intrathecal catheter on days 2, 4, and 6 of pos-injury and then weekly as highlighted in green. Behavioral assessment was performed through Basso, Beattie, and Bresnahan (BBB) scale, Activity Box Test (ABT), Motor Swimming Test (MST) at the timepoints indicated above. **B)** Therapeutic synergies between Roflumilast (ROF) and secretome of ASCs. Wistar rats undergo secretome administration through intravenous injection 30 minutes after SCI, followed by daily administration until 72 hours post-injury, as highlighted in yellow. Treatment with Rof was administered from week 2 and every week until the seventh week by IP as highlighted in green. Behavioral assessment was performed BBB scale and Von Frey test at the timepoints indicated above. 120

Figure III. 2. Evaluation of functional recovery after spinal cord injury in animals treated with Rof, Lev, Rof+Lev or vehicle. **A)** Locomotor recovery analysis demonstrated that only treated animals presented significant motor recovery when compared to vehicle. No differences were observed between the treatments when administered alone or in combination. **B)** Weight support analysis demonstrated that animals treated with Lev, Rof, and Lev+Rof presented higher recovery body weight support contrasting with the vehicle-treated group. **C)** Coordination analysis demonstrated that vehicle-treated animals did not recover any level of coordination. All treatment groups presented significant improvement in coordination when compared to controls, however, no statistical differences were observed between treatments. Data was analyzed using 2-way ANOVA $p=0.0064$ followed by Tukey's multiple comparisons test. Vehicle n = 10; Lev n = 9; Rof n = 11; Lev+Rof n = 8. Values shown as mean ± SEM. ***$p<0.001$. 123

Figure III. 3. Analysis of exploratory behavior using the Activity Box Test. **A)** No differences were observed between all groups when analyzing total overground distance. **B)** Animals treated with our therapeutic agents presented higher velocity than vehicle-treated animals. A synergistic effect between Lev+Rof was observed but only when compared to the Lev-treated group. **C)** Animals treated with Lev showed a statistically significant difference when compared to the Rof and vehicle-treated groups on the motor swimming test (MST). Values shown as mean ± SEM. *p<0.05; **p<0.01. 124

Figure III. 4. Evaluation of functional recovery after treatment with Rof, Sec, Rof+Sec, or vehicle. **A)** Locomotor recovery analysis demonstrated that the combination of Rof and Sec leads to a significant worse locomotor recovery when compared to animals treated with Rof or Secretome alone. **B)** Body weight support analysis revealed statistical differences between treatments with the combinatory therapy showing again detrimental effects. **C)** Coordination analysis revealed that when treatments are administered alone significant recovery can be achieved when compared with Rof+Sec or vehicle treatment. Differences over time were analyzed using 2-way ANOVA followed by Tukey's multiple comparisons test. Vehicle n=5; Rof n=5; Sec n=6 and Rof+Sec n = 6. Values shown as mean ± SEM. *p<0.05; **p<0.01. 126

Figure III. 5. Results from Frey filaments applied both on the **A)** Left paw and **B)** Right paw. Statistical analysis did not reveal any differences between groups. Normality analysis using the Kolmogorov-Smirnov test revealed that data does not follow a Gaussian distribution, for this reason, the non-parametric Kruskal-Wallis test was performed followed by the Dunn's multiple comparisons test. Vehicle = 5; Rof = 5; Sec = 6; Rof + Sec = 6. Values shown as mean ± SEM. 126

Table I.1. Pre-clinical combinatorial approaches for SCI repair: cells and biomaterials (from 2013 to 2023). 34

Table I.2. Current clinical trials on scaffolds with cells for SCI treatment. Adverse event (AE); Functional Independence Measure (FIM); Motor Evoked Potential (MEP); Magnetic resonance imaging (MRI), Olfactory nerve fibroblasts (ONFs); Somatosensory Evoked Potential (SSEP). 39

Table I.3. Pre-Clinical Combinatorial Approaches for SCI repair: Biomaterials and small molecules. . 42

List of Abbreviations

3-AB	3-Aminobenzamide
4-AP	4-Aminopyridine
5-HT	5-Hydroxytryptamine
ABT	Activity Box Test
aFGF	Acidic Fibroblast Growth Factor
ACSS	Aligned collagen sponge scaffold
ASCs	Adipose Stem Cells
Ad-MSCs	Adipose-Derived Mesenchymal Stem Cells
AANS/CNS	American Association of Neurological Surgeons/ Congress of Neurological Surgeons
ASIA	American Spinal Cord Injury Association
AMPA	Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid
AM-MSC	Amniotic Membrane Mesenchymal Stem Cells
Ang-1	Angiopoietin-1
anti-HMGB1	Anti-High Mobility Group Box-1
AIS	Asia Impairment Scale
bFGF	Basic Fibroblast Growth Factor
BBB	Basso, Beattie and Bresnahan
BSCB	Blood-Spinal Cord Barrier
BM-MSCs	Bone Marrow-Derived Mscs
BDNF	Brain-Derived Neurotrophic Factor
BSI	Brain-Spine Interface
Cab-M	Cabazitaxel Micelles
Ca^{2+}	Calcium
CDH	Cadherins
CI	Confidence Interval
CREB	Camp-Response Element Binding Protein
CO	Carbon Monoxide
CNS	Central Nervous System
CCH	Chitosan-Collagen Hydrogel

CSPGs	Chondroitin Sulfate Proteoglycans
ChABC	Chondroitinase ABC
CLUS	Clusterin
COPD	Chronic Obstructive Pulmonary Disease
CNTF	Ciliary Neurotrophic Factor
cAMP	Cyclic Adenosine Monophosphate
COX	Cyclooxygenase
DAMP	Damage-Associated Molecular Pattern
DCN	Decorin
DPSCs	Dental Pulp Stem Cells
dbcAMP	Dibutyryl Cyclic Adenosine Monophosphate
DPY	Dipyridy
Dpi	Days post-injury
ESCs	Embryonic Stem Cells
ER	Endoplasmic Reticulum
epSPCi	Ependymal Stem/Progenitor Cells of The Spinal Cord After Injury
EPI-NCSCs	Epidermal Neural Crest Stem Cell
EPI-NCSCs	Epidermal Neural Crest Stem Cells
EES	Epidural Electrical Stimulation
EGCG	Epigallocatechin Gallate
Epo B	Epothilone B
EQ-5D	European Quality of Life Five Dimension
ECM	Extracellular Matrix
EVs	Extracellular Vesicles
FBS	Fetal Bovine Serum Exosome-Free
FGM	Fibrin Glue Matrix
FGF2	Fibroblast Growth Factor 2
FDA	Food And Drug Administration
GABA	Gamma-Aminobutyric Acid
GNLs	Gelatin Nanostructured Lipid Carriers
GG	Gellan Gum
GRGDS	Gly-Arg-Gly-Asp-Ser

GDN	Glial-derived nexin
GDNF	Glial Cell Line-Derived Neurotrophic Factor
GLC	Glibenclamide
GM-1	Monosialotetrahexosylganglioside 1
GRASSP	Graded Redefined Assessment of Strength Sensibility and Prehension
G-CSF	Granulocyte Colony-Stimulating Factor
GAP 43	Growth Associated Protein 43
HO-1	Heme Oxygenase-1
HO-2	Hemeoxygenase-2
hADSCs	Human Adipose-Derived Stem Cells
hESCs	Human Embryonic Stem Cells
hEnSCs	Human Endometrial Stem Cells/Schwann Cells
HuCNS-SC	Human Neural Stem Cells
hUCMSCs	human Umbilical Cord Mesenchymal Stromal Cells
INDP	Immunization With Neural Derived Peptides
iPSCs	Induced Pluripotent Stem Cells
iNOS	Inducible Nitric Oxide Synthase
IP3	Inositol Triphosphate
IP	Intraperitoneal Injection
LVM	Lesões vertebro-medulares
Lev	Levetiracetam
LOCS	Linear Ordered Collagen Scaffold
Li	Lithium Chloride
LLLT	Low-Level Laser/Light Therapy
Mg2+	Magnesium
MMPs	Matrix-Metalloproteinase
MAP	Mean Arterial Pressure
MSCs	Mesenchymal Stem Cells
MPSS	Methylprednisolone Sodium Succinate
MAP-2	Microtubule-Associated Protein 2
MASCI	Minocycline In Acute Spinal Cord Injury'
MEP	Motor Evoked Potential

MAG	Myelin-Associated Glycoprotein
MST	Motor Swimming Test
NbA	Neurobasal A
Neurob	Neurobasal
NMDA	N-Methyl D-Aspartate
NHC	Nanofiber-Hydrogel Composite
NASCIS	National Acute Spinal Cord Injury Studies
NIR	Near-Infrared
NGF	Nerve Growth Factor
OMgp	Oligodendrocyte-Myelin glycoprotein
NSCs	Neural Stem Cells
NSPCs	Neural Stem Progenitor Cells
iPSC-NSPCs	Nspcs Derived from Murine And Human Ipscs
NbA	Neurobasal A Medium
NT-3	Neurotrophin-3
NO	Nitric Oxide
NISCI	Nogo Inhibition in Spinal Cord Injury
OR	Odds Ratio
OECs	Olfactory Ensheathing Cells
OMgp	Oligodendrocyte Myelin Glycoprotein
ONFs	Olfactory nerve fibroblasts
OPCs	Oligodendrocyte Precursor Cells
oNPCs	Oligodendrogenic Neural Progenitor Cells
PTX	Paclitaxel
P/S	Penicilin-Streptomycin
PAMs	Pharmacologically Active Microcarriers
PBS	Phosphate-Buffered Saline
PDE4	Phosphodiesterase-4
PRP	Platelet-Rich Plasma
PLLA	Poly (L-Lactic Acid)
PARP-1	Poly(Adp-Ribose)-Polymerase 1
PLGA	Poly(Lactide-Co-Glycolide

PMMA	Poly(Methyl Methacrylate
PPC	Poly(Propylene Carbonate
PA-C	Polyacetal-Curcumin Nanoconjugate
PEG	Polyethylene Glycol
PEDF	Pigment epithelium-derived factor
PVDF-TrFE	Polyvinylidene Fluoride Trifluoroethylene
PGE2	Prostaglandin E2
PKA	Protein Kinase A
RafI	Raf Inhibitor
ROS	Reactive Oxygen Species
rhEPO	Recombinant Human Erythropoietin
rTMS	Repetitive Transcranial Magnetic Stimulation
RGMa	Repulsive Guidance Molecule-A
RhoA	Ras homolog family member A
RISCIS	Riluzole In Acute Spinal Cord Injury Study
Rof	Roflumilast
SR	Scar Removal
SCs	Schwann Cells
SEM	Standard Error of the Mean
SF-36	Short-Form Health Survey-36
STAT3	Signal Transducer and Activator of Transcription 3
Na^+	Sodium
Shh	Sonic Hedgehog
SCIMIII	Spinal Cord Independence Measure
SCI	Spinal Cord Injury
SCAP	Stem Cells of The Apical Papilla
SNC	Sistema Nervoso Central
STASCIS	Surgical Timing in Acute Spinal Cord Injury Study
SV2A	Synaptic Vesicle Protein
TMP	Teramethylpyrazine
TRH	Thyrotropin-Releasing Hormone
TMS	Transcranial Magnetic Stimulation

TGF	Transforming Growth Factor
TBI	Traumatic Brain Injury
TNF	Tumor Necrosis Factor
UEMS	Upper Extremity Motor Score
VEGF	Vascular Endothelial Growth Factor
WMD	Weighted Mean Difference

Contents

CHAPTER I. INTRODUCTION: SPINAL CORD INJURY PATHOPHYSIOLOGY AND CURRENT TREATMENT APPROACHES 1

ABSTRACT 2

1. INTRODUCTION 3

1.1. An Overview of primary injury **4**

1.2. Overview of secondary mechanisms of SCI **5**

2. CURRENT CLINICAL MANAGEMENT 11

3. INNOVATIVE THERAPEUTIC APPROACHES 13

3.1. Molecular treatments 13

3.1.1. Ion channel antagonists 13

3.1.2. Cyclooxygenase (COX) inhibitors: 15

3.1.3. IL-4 cytokine 16

3.1.4. Minocycline 16

3.1.5. Glutamate receptor antagonists 17

3.1.6. Levetiracetam 18

3.1.7. Anti-Nogo-A 18

3.1.8. PDE4 inhibitors 19

3.1.9. Chondroitinase ABC (ChABC) 19

3.2. Cell-based strategies 20

3.2.1. Embryonic stem cells (ESCs) 20

3.2.2. Glial cells 22

3.2.3. Mesenchymal cells 22

3.3. Neuromodulation Technologies 23

3.4. Biomaterials 24

3.5. Combinational strategies in SCI treatments 25

3.5.1. Combination of molecular agents 26

3.5.2. Combination of cells and molecular agents 29

3.5.3. Neural Tissue Engineering 32

CONCLUSIONS 46

CHAPTER II. PRE-CLINICAL ASSESSMENT OF ROFLUMILAST THERAPY IN A THORACIC MODEL OF SPINAL CORD INJURY. 78

Abstract 81
Introduction 82
Materials and methods 83
Results 90
Discussion 100
Conclusions 104
References 106

CHAPTER III. ROFLUMILAST – GOING SOLO OR SEARCHING FOR A FRIEND. 111

Introduction 115
Materials and methods 116
Results 122
Discussion 127
Conclusions 131
References 131

CHAPTER IV. DISCUSSION AND CONCLUSIONS 137

Thesis aims and Layout

The main aim of this thesis was to test a molecular therapy, Roflumilast (Rof), and its combination with another molecular approach, Levetiracetam (Lev), or with a cell-based strategy – secretome, aiming to repair the spinal cord. These strategies were evaluated in the thoracic contusion spinal cord injury (SCI) rat model. This thesis is therefore divided into four chapters:

Chapter I Introduction: SCI Pathophysiology and Current Treatment Approaches: includes the SCI pathophysiology and an overview of the current clinical practice and therapeutic strategies with a special focus given on combinatorial therapies.

Chapter II Pre-Clinical Assessment of Roflumilast Therapy in a Thoracic Model of Spinal Cord Injury: presents the therapeutic effect of Rof administration after SCI. Results indicate that the treatment was effective in promoting functional recovery. Rof-treated animals showed improvements in both gross and fine motor function. Eight weeks post-injury, the animals significantly recovered by achieving occasional weight-supported plantar steps. This work reveals the potential of Rof as an effective treatment for SCI.

Chapter III Roflumilast – Going Solo or Searching for a Friend:

Part 1. Combination of Rof and Lev. A therapy for neuroprotection and neuroregeneration in SCI. This work focuses on the evaluation of functional changes after the combination of Rof and Lev treatment. We determined that Rof + Lev did not promote any significant recovery when compared with the treatment given alone.

Part 2. Combination of Rof with Secretome. We aimed to investigate the therapeutic potential of a combination involving the Adipose Stem Cells (ASCs) secretome and Rof. Treatment only led to functional improvements when administered alone. In fact, the combination of Rof + ASCs Secretome had a worst outcome than vehicle-treated animals.

Chapter IV Discussion and Conclusions: includes a general discussion, incorporating all the findings from the previous chapters. It further discusses the clinical relevance of these treatments and the potential combination of different strategies. Limitations, future works, and the value of this thesis in the context of the SCI research field are also discussed.

Chapter I

Introduction: Spinal Cord Injury Pathophysiology and Current Treatment Approaches

Chapter one is currently accepted as a review article:

Carla S Sousa, et al. Combinatorial Therapies for Spinal Cord Injury Repair.

Neural Regeneration Research.

REVIEW ARTICLE

Combinatorial Therapies for Spinal Cord Injury Repair

Carla S. Sousa[1,2,3]; António J. Salgado[1,2]; Nuno A. Silva[1,2,*]

1. Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Campus de Gualtar - Gualtar, 4710-057 Braga, Portugal;

2. ICVS/3B's Associate Lab, PT Government Associated Lab, Campus de Gualtar - Gualtar, 4710-057 Braga, Portugal;

3. Department of Neurosurgery, Hospital Garcia de Orta, Av. Torrado da Silva, 2805-267 Almada, Portugal;

* Corresponding author: nunosilva@med.uminho.pt; Tel.: +351253604875.

Abstract

Spinal cord injuries (SCI) have profound detrimental effects on individuals, regardless of whether they are caused by trauma or non-traumatic events. The compromised regeneration of the spinal cord is primarily attributed to damaged neurons, inhibitory molecules, dysfunctional immune response, and glial scarring. Unfortunately, there are no effective treatments available that can fully repair the spinal cord and improve functional outcomes. Nevertheless, numerous pre-clinical approaches have been study for SCI recovery, including using biomaterials, cells, drugs, or technological-based strategies. Combinatorial treatments, which target various aspects of SCI pathophysiology, have been extensively tested in the last decade. These approaches aim to synergistically enhance repair processes by addressing various obstacles faced during spinal cord regeneration. Thus, this review intended to provide scientists and clinicians with an overview of pre-clinical combinatorial approaches that have been developed toward the solution of spinal cord regeneration as well as update the currently knowledge about SCI pathophysiology with an emphasis on the current clinical management.

Keywords: Spinal cord injury; Combinatorial treatment; Neuroprotection; Neuroregeneration; Multitherapy.

1. Introduction

SCI can be a devastating neurological condition with significant socioeconomic impact on patients and the healthcare system. Surgery decompression and stabilization of mean arterial blood pressure are the current standards of care for SCI. Steroid therapy is decidedly controversial and is no longer used as a standard of care. The complexity of this event and the lack of an effective treatment turn SCI into a worldwide problem with many research teams trying to solve it.

Based on a large study, The Global Burden of Diseases estimates that there are 0.93 million new cases of spinal cord injury worldwide every year, with a prevalence of 27.04 million persons affected [1]. Depending on the region, there are 10 to 90 incidents of SCI per million people each year [2,3]. For example, North America has an estimated 17.900 new SCI per year according to the National Spinal Cord Injury Statistical Center [4].

The mechanisms for these injuries can be traumatic or nontraumatic [5]. Etiologically, more than 90% of SCI cases are traumatic and caused by incidences such as vehicle accidents, falls, or sports and recreation activities. The average age at injury is 43 years, and, since 2015, about 78% of new SCI cases are male [4].

The clinical results of SCI are variable. The severity can range from partial to complete loss of sensory and/or motor function below the injury and according to the anatomic location, cervical lesions can provoke quadriplegia while lower thoracic lesions can cause paraplegia [6]. The majority of spinal cord injuries occur in the cervical region (50%) followed by the thoracic region (35%), and the lumbar region (11%) [7].

Recent advances in medical procedures and patient care have allowed SCI patients to survive these traumatic injuries and live for decades [8]. A study of SCI patients in Australia between 1955 and 2006 showed that tetraplegia patients survived at 91.2% and paraplegia patients survived at 95.9%, while the 40-year survival rate was 47 and 62%, respectively [8]. The life expectancy of SCI patients depends on the severity of the injury and preserved functions. For instance, the life expectancy of patients with an American Spinal Cord Injury Association (ASIA) Impairment Scale (AIS) grade D who need a wheelchair for daily activities is approximately 75%, whereas patients who do not require a wheelchair or catheterization can live 90% of a healthy individual [9].

Additionally, SCI has also a high socioeconomic impact on patients and the healthcare system. Currently, a SCI patient's lifetime cost is estimated to be $2.35 million [7]. Due to these reasons, multiple studies have been conducted in preclinical and clinical SCI with the hope of finding new therapeutic targets.

1.1. An Overview of primary injury

SCI usually occurs when a vertebra is fractured or dislocated by an abrupt, traumatic, and direct impact. Primary injury is caused by mechanical forces transmitted to the spine at the time of injury. Displaced bone fragments, disc materials, and/or ligaments bruise or tear the spinal cord at this point [10–12]. Remarkably, the spinal cord is rarely completely severed in most injuries [13].

Primary injury can be attributed to four distinct mechanisms: (1) Impact plus persistent compression; (2) Impact alone with transient compression; (3) Distraction and (4) Laceration or transection [11],[14]. They are most caused by impact plus persistent compression caused by burst fractures with bone fragments compressing the spinal cord or fracture-dislocation injuries [13],[15,16]. An isolated impact with transient compression occurs less frequently, but most often during hyperextension traumas [11]. A distraction injury occurs when two vertebrae are pulled apart, causing axial stretching and tears in the spinal column [11,16]. Last but not least, laceration and transection injuries can be caused by severe dislocations or sharp fractures of bone fragments dislocations. They can range from minor injuries to complete transections [11]. These types of forces directly damage ascending and descending pathways in the spinal cord and cell membranes, regardless of the form of the primary injury [14,17]. Similarly, nearby blood vessels are damaged, resulting in hemorrhaging and edema and peripheral inflammatory cells infiltrate the nervous tissue as a result of the disruption of the blood-spinal cord barrier (BSCB). These initial events occur immediately after injury (seconds to minutes) and are considered the primary injury [18], causing spinal shock, hypotension, vasospasm, ischemia, ionic imbalance, and accumulations of neurotransmitters.

Depending on the spinal cord level affected (**Figure I.1**), different symptoms and neurological systems can be damaged. Among all injuries, cervical injuries are the deadliest. According to the severity of the lesion (complete or incomplete injury), they could lead to paralysis of all body below the injury site (tetraplegia), compromising patients' autonomy. A variety of vital body homeostasis systems can also be affected along with motor problems. Respiratory insufficiency and cardiac problems are the most obvious ones, threatening the survival of SCI patients. However, other complications might arise from cervical injuries, such as autonomic dysreflexia, and changes in bladder and bowel control. Patient's quality of life is drastically reduced by all these alterations, leaving them in a constant need for caregiving. A thoracic or lumbar injury may also result in motor deficits (paraparesis to paraplegia), along with reduced or no control of bowel and bladder function. Generally, the magnitude of the primary injury determines the severity and outcome of SCI [19].

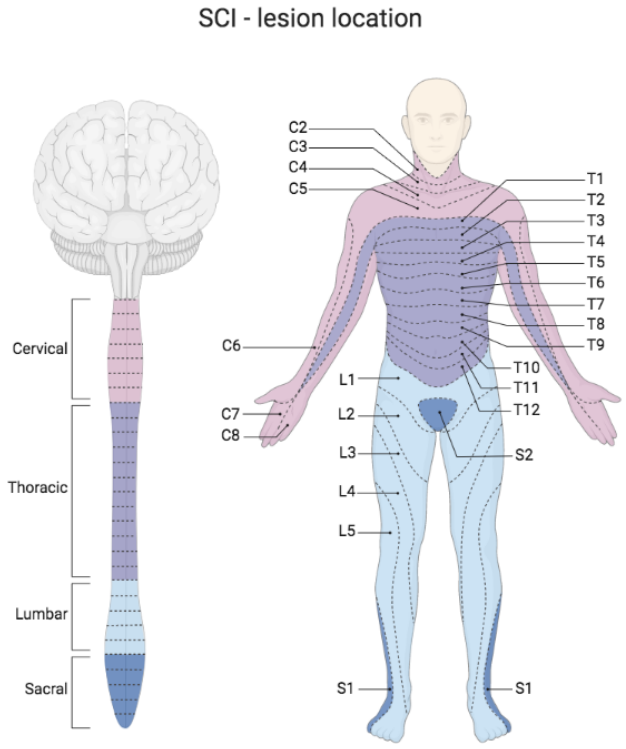


Figure I.1. *Representation of Dermatomes Diagram for lesion location in SCI.*

1.2. Overview of secondary mechanisms of SCI

Secondary injury consists of multiple pathophysiological changes and is subdivided into the acute phase (<2 days), the subacute phase (2 days to 2 weeks), the intermediate phase (2 weeks to 6 months), and the chronic phase (>6 months) [14]. Within minutes and hours after the initial insult, there is a progressive process of post-traumatic damage, which is proportional to the magnitude of the initial insult [18]. This comprises permeability and vascular changes, free radical formation and lipid peroxidation, ionic disruption and glutamate excitotoxicity, dysfunctional inflammatory reaction, apoptosis, and glial scarring.

Vascular changes: After the primary hemorrhage, the vascular changes include vasospasm and thrombosis, which leads to hypoperfusion and consequently to ischemia [20]. Ultimately, this biological phenomenon leads to necrosis, which becomes deprived of oxygen and glucose [21]. Moreover, post-injury hemorrhage and ischemia also impact BSCB permeability, which promotes the infiltration of critical pro-inflammatory mediators that enhance the inflammatory environment and edema at the injured spinal cord [22,23].

Disruption of ionic balance of K^+, Na^+, Ca^{2+}: After the initial insult, the homeostatic ionic balance is severely compromised. Anoxia and ischemia cause rapid energy depletion, failure of the (Na^+- K^+) - ATPase with accumulation of axoplasmic Na^+ and severe K^+ depletion. This disruption leads to depolarization of cell membranes and an increase of intracellular Ca^{2+}, which enhances neuronal and glial cell death [24,25]. The excessive accumulation of Ca^{2+} in turn triggers various Ca^{2+}-dependent enzymes, such as calpain, phospholipases, and protein kinase C, resulting in irreversible injury [24,25].

Free oxygen radical-induced lipid peroxidation: In response to rapid increases in intracellular Ca^{2+} after the primary mechanical injury to the spinal cord, posttraumatic oxygen radical reactions begin. When superoxide radicals are produced, they can potentially react with other molecules to form more reactive, and thus more potentially damaging, radical species [26]. Radical-induced oxidative damage is caused by oxidative attacks on polyunsaturated fatty acids in the cell membrane. This leads to lipid peroxidation, resulting in disruptions in cellular and membrane integrity. It is also important to note that lipid peroxidation produces aldehyde products that affect key metabolic enzymes, such as (Na^+- K^+) - ATPase [27], which is crucial for maintaining neuronal excitability, and its failure leads to loss of neuronal function and ultimately tissue dissolution [28].

Glutamate excitotoxicity: after SCI there is an increased release of an extracellular amino acid, namely glutamate, a well-described excitatory neurotransmitter, regulated by Ca^{2+} flux at the synaptic cleft. Excessive activation of glutamate receptors [N-methyl D-aspartate (NMDA) and amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)] leads to an increase in Na^+ and Ca^{2+} influx. Ionic dysregulation has a direct impact on neuronal and glial cells, especially on oligodendrocytes and neurons, leaving them vulnerable to cell death [25,29]. In addition, axonal degeneration is mediated by Ca^{2+} influx from the endoplasmic reticulum (ER) through the inositol triphosphate (IP3) receptor that promotes mitochondrial permeability [30]. Globally, glutamate excitotoxicity disturbs ionic homeostasis and normal mitochondrial functioning resulting in axonal demyelination and neuronal cell death at the injury site [31,32].

Inflammatory response: inflammation plays a central role in the secondary mechanisms of SCI (**Figure I.2**), and it can persist for several weeks or months following SCI [33]. Cellular debris are cleared through the inflammatory immune response, which can have a role in neural tissue repair by secreting

trophic factors and anti-inflammatory cytokines. [34] Nevertheless, in the acute phase, overactivation of the inflammatory response can damage healthy tissues and worsen functional outcomes.

After trauma, the immune resident cells, microglia, is almost immediately start producing proinflammatory cytokines, such as interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α). Also, rapidly infiltrating neutrophils (the first wave of infiltrating leukocytes) begin to release more cytokines, reactive oxygen species (ROS) and matrix-metalloproteinase (MMPs) [35]. This will facilitate higher extravasation of leukocytes, a higher extension of inflammation, and further tissue damage [36]. The release of MMP-9 and MMP-2 by neutrophils, macrophages, and endothelial cells can further damage the BSCB and increase leukocyte infiltration [37].

Proteins, DNA, and lipids are then oxidized and nitrosylated by reactive oxygen radicals, which can increase secondary damage [38]. In addition, reactive B- and T-cells also infiltrate the spinal cord and are thought to contribute to axonal injury, demyelination, and functional loss in the sub-acute/chronic phase of SCI [39,40].

Despite intensive research, it is unclear how inflammation contributes to the treatment of SCI. The results of many studies indicate that both anti-inflammatory [41–44] and proinflammatory treatments [45,46] can make a positive difference in the outcome of SCI. Moreover, SCI also impacts the autonomic information from the central nervous system to the peripheral sympathetic postganglionic neurons innervating lymphoid organs, affecting their function and how the immune response is mounted [47]. To clarify the exact benefits and drawbacks of the inflammatory process after SCI more research is needed. It is however clear that the immune response after SCI is highly disfunction and damaging and future research should focus on how we can regulate the immune cells in order to have the adequate response over time (inflammation, proliferation, remodeling and resolution).

Apoptosis: Apoptogenic and necrotogenic mechanisms determine the size of spinal cord lesions during the secondary injury phase, after a primary physical impact [48]. Parenchymal cells of the spinal cord, including astrocytes, microglia, oligodendrocytes, and neurons undergo apoptosis. The mediators of apoptosis after SCI are not well understood, but there is a close relationship between microglia and dying oligodendrocytes and demyelination of axons, suggesting that microglial activation may be involved [49].

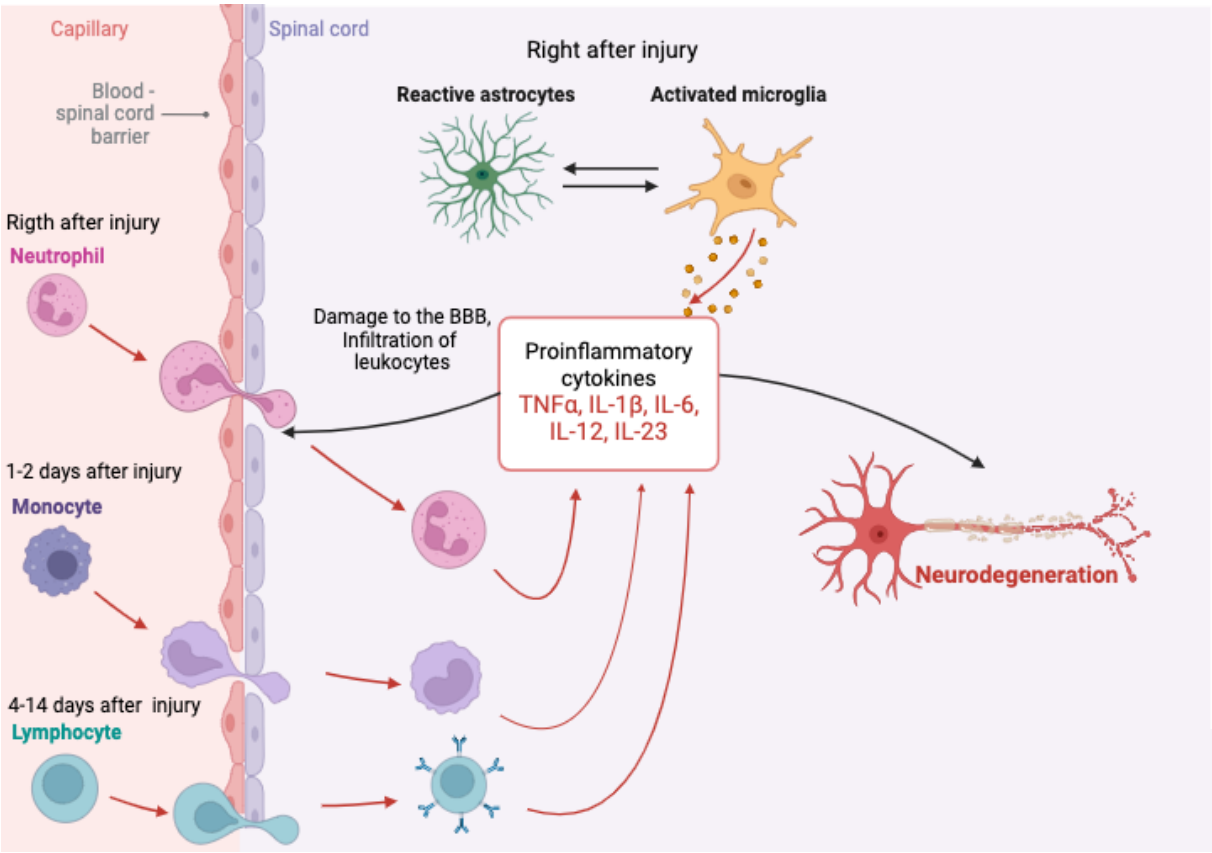


Figure I. 2. *Representation of immune response with the respective pathophysiological events occurring during injury.*

Glial scarring: SCI activates astrocytes, pericytes, and fibroblasts promoting the development of a fibrotic scar. The formation of the cystic cavity is caused by bleeding, necrosis, exudation, and liquefaction of necrotic substances filled by astrocytes and microglia. This cavity is part of scarring and tends to be stable over time [50]. Astrocytes are the main cell type in glial scars [51], and they play a significant role in post-SCI processes, such as inflammatory reactions and tissue reconstruction [50]. The increased presence of transforming growth factor (TGF)-β intensifies microglia/macrophage and astrocyte activation, as well as fibronectin and laminin deposition, defining glial scar boundaries [52–54]. The pathway of transcription factor Signal Transducer and Activator of Transcription 3 (STAT3) is also crucial, by guiding astrocytes proliferation, for establishing glial scar borders that isolate infiltrating cells into the lesion [55,56]. These events are essential for wound repair, but also interfere with axonal regrowth. A physical barrier is formed by connective tissue deposition and reactive gliosis, affecting cellular migration [57–59]. Neurite outgrowth is also inhibited by some chemical components, such as chondroitin sulfate proteoglycans (CSPGs) [60–62]. It is crucial to note that the scar plays a complex role in SCI. Some groups have shown that glial scars can lower inflammation and toxic species sequestration by repairing the BSCB [51,63] and it was also shown that without the glial scar, the inflammatory status could be an equivalent barrier [51]. Recently, Popovich lab demonstrated that the response to injury by astrocytes is coordinated by microglia and the depletion of microglia leads to an increase of the cystic cavity and worsen functional recovery [64].

The global picture of pathophysiological events after SCI can be seen in **Figure I.3**.

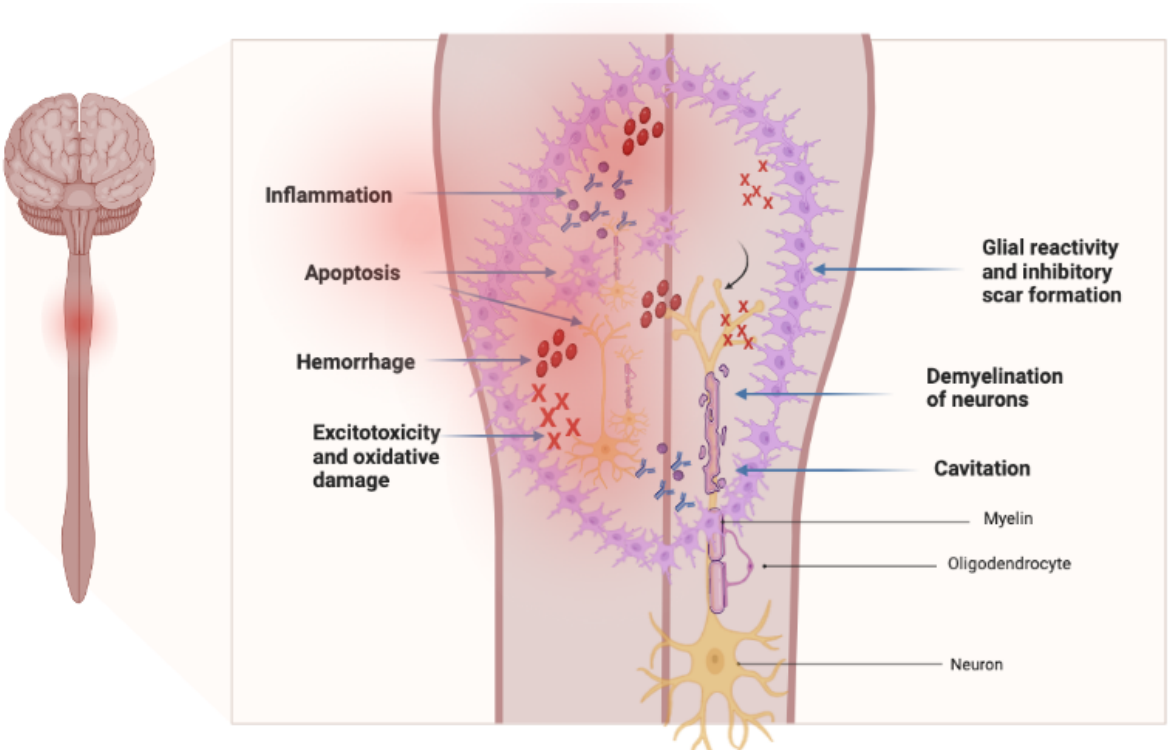


Figure I.3. Representation of an injured spinal cord with the respective secondary pathophysiological events occurring after injury.

Chronic Phase

Upon secondary injury, the chronic phase begins and lasts for the lifetime of the person who has sustained a SCI. During this phase, the lesion stabilizes, which involves the maturation of scar tissue, cysts and/or cavities. [65]. Wallerian degeneration of damaged axons continues, and the removal of severed axons and their cell bodies may take years [66–68]. The lesion itself is characterized by cystic cavitation and myelomalacia. The neurological deficits can be considered stable, and the lesion fully matured after a period of 1–2 years post-injury [69].

Accordingly, SCI pathophysiology is characterized by multiple mechanisms that interact in ways that are still not completely understood, while limiting recovery as well as enhancing it at the same time. This ambivalence is obvious, for example, in the role of astrocytes. These cells restore ionic homeostasis and the BSCB and limit immune cell infiltration. At the same time, astrocytic scars are also responsible for severely restricting axon regeneration and diminishing functional recovery. It is essential to note that every injury has a particular cause and a specific injury in order to gain a deeper understanding of the pathophysiology of human SCI and then focus on therapeutic solutions that are more multidirectional in answering this question.

2. Current clinical management

Despite substantial investment in preclinical research aimed at better understanding the pathophysiology of acute SCI and developing targeted therapies to improve outcomes from SCI, very few have reached clinical practice. Current clinical approaches toward SCI treatment involve aggressive intensive care measures, early surgical decompression and stabilization, followed by blood pressure augmentation to reduce secondary injury [70–72].

The evidence for the neurologic benefit of early surgical decompression has led to the recognition that the injured spinal cord should be decompressed with urgency. A prospective multicenter study [73] conducted by Wilson et al. in 2012 compared patients who received early surgery (< 24 hours after SCI) to those who received delayed surgery (≥ 24 hours after SCI). The AIS motor score did not differ for primary outcomes; however, after adjusting for preoperative status and neurological level, early surgery led to superior results.

A recent systematic review and meta-analysis evaluated 18 studies assessing the timing of surgery from 1996 to 2012 [74]. Although there was significant heterogeneity in these studies, early surgery showed significantly better total motor score (weighted mean difference [WMD]: 5.94, 95% confidence interval [CI]: 0.74, 11.15), additional neurological improvement (odds ratio [OR]: 2.23, 95% CI 1.35, 3.67), and shorter hospital length of stay (WMD: − 9.98 days, 95% CI − 13.10, − 6.85) than late surgery.

In 2012, the group of Michael G. Fehlings finished a prospective multicenter study: The Surgical Timing in Acute Spinal Cord Injury Study (STASCIS) [75], comparing 6-month surgical outcome after early (< 24 hours) or late (≥ 24 hours) treatment for cervical SCI. A total of 313 patients were enrolled (182 with early surgery, and 131 with late surgery). Patients who underwent early surgery showed a greater likelihood of ≥ 2 improvement in AIS grades (OR 2.57, 95% CI 1.11, 5.97) at 6-month follow-up. This trial may be a landmark study, and perhaps more than any other it assumes responsibility for the shift to earlier surgeries for SCI. However, some limitations have been pointed out. The trial does not refer to whether a power calculation was performed and whether this led to the recruitment of an adequate sample size. Equally, it is not clear that the defined primary outcome and method of analysis were selected a priori. Nevertheless, these results have increased the urgency of SCI early surgery. Additionally, Jug and colleagues [76] conducted a study in 2015 to compare neurological recovery after cervical traumatic spinal cord injury after surgical decompression and instrumented fusion within 8 hours versus 8–24 hours following injury and found that patients who underwent earlier surgery had a considerably better rate of improvement of ≥ 2 AIS grade (45.5% vs. 10%, p = 0.017).

Lately, some groups tried to evaluate the consequence of ultra-early decompression. Burke, John F. et al. [77] grouped patients into ultra-early decompression (within 12 hours of injury), early decompression (within 12 to 24 hours), and late groups (after 24 hours of trauma). The group who received surgery within 12 hours after the presentation had a relative improvement in AIS grade from admission to discharge. In addition, 88.8% of patients with an AIS grade A converted to a higher grade (AIS B or better) in the ultra-early group, compared to 38.4% in the early and late groups ($p = 0.054$).

Additionally, another immediate priority in SCI is to ensure the stabilization and control of the cardiovascular and hemodynamic parameters [78]. During the acute phase of SCI, the pathophysiology is aggravated due to some cardiovascular instability (hypotension, hypoxemia, and pulmonary dysfunction). Even in the absence of hemorrhagic shock, hypotension can ensue after SCI because of the disruption of sympathetic innervation. This is named neurogenic shock and can result in spinal cord hypoperfusion and worsened injury to the spinal cord. Bradycardia is a hallmark of neurogenic shock, but the clinician must maintain a high level of suspicion that other hemodynamic patterns of shock may coexist, especially in the polytrauma patient. Several published reports have suggested that preventing hypotension after SCI and even augmenting mean arterial pressure (MAP) may benefit neurological recovery [78,79]. Consequently, both the 2002 and the 2013 American Association of Neurological Surgeons/ Congress of Neurological Surgeons (AANS/CNS) guidelines recommend hemodynamic support treatment with the vasopressor drug to prevent hypotension and augment MAP to > 85–90 mmHg [70,71,80].

Until very recently, methylprednisolone sodium succinate (MPSS) was a first-line drug treatment for SCI patients [80]. Corticosteroids are now a controversial neuroprotective treatment for acute SCI. Numerous major randomized controlled trials, namely the National Acute Spinal Cord Injury Studies (NASCIS), have assessed the role of methylprednisolone [81–83]. Although primary endpoints were not improved with the use of steroids, the secondary analysis suggested treatment within 8 hours produced an improvement in Functional Independence Measure scores at 1-year post-injury [81–83]. Major apprehensions regarding steroid involve an increased risk of wound infection, pulmonary embolism, gastrointestinal hemorrhage, sepsis, and death, supporting the decision to implement steroid use bears substantial risks. While the AANS/CNS Joint Section guidelines did not recommend steroid use after SCI citing a lack of supportive level I and II evidence as well as a level I–III evidence indicating possible harm [84,85], recommendations from AO-Spine suggested a 24-hours infusion of high dose methylprednisolone may be offered to patients within 8 hours of injury based on a meta-analysis (of three randomized trials and one prospective observational trial) [70] assessing early use of steroids, that showed an improvement in AIS motor scores

(mean difference 3.21, 95%CI 0.10, 6.33, p = 0.04). A combined risk analysis also showed no impact of steroids on risk factors when accounting for all studies. Nevertheless, numerous limitations of this analysis included the low precision of motor scores in the meta-analysis, as well as the likely minimal appreciable functional outcome difference with such a low motor score change. Of note, the AO-Spine guidelines recommend < 48 hours of steroid infusion based on the NASCIS III finding that this dosing more prominently increases the risks of steroids than the benefits.

Other drugs, such as thyrotropin-releasing hormone (TRH), naloxone, and Monosialotetrahexosylganglioside (GM1) have been tested in large multicenter clinical trials [86], however, none have demonstrated strong favorable effects for SCI patients. The lack of convincing clinical therapies to treat SCI conditions emphasizes the importance of shadowing novel therapeutic approaches aimed to improve the quality of life of SCI patients.

3. Innovative Therapeutic Approaches

The lack of efficient treatment for SCI leads to the search for a variety of novel approaches to tackle SCI damaging outcomes. The primary emphasis of this review lies on the combinatorial therapeutic approaches that have been investigated over the past decade. However, we have also provided updates on single-based therapies currently undergoing clinical testing, along with highlighting recently explored therapeutic agents.

3.1. Molecular treatments

Pharmacological therapy focuses on the modulation of a specific secondary event to promote neuroprotection or neuroregeneration of spinal cord tissue to improve motor and sensory function in patients. Overall, drugs are divided into neuroprotective compounds, which aim to limit the damage induced by the secondary pathophysiological events of SCI, and neuroregenerative drugs, which induce neuronal and axonal regrowth. While many compounds have been trialed with promising results, none has yet completed a stage III trial and has been approved for the pharmacological management of SCI.

3.1.1. Ion channel antagonists

The potent physiological effects of calcium blockers are mediated by their pharmacological activity on vascular smooth muscle and not by calcium flux across neurons and glial cells [87]. In experimental models of SCI, nimodipine (a calcium channel blocker) improved spinal blood flow and rescued hypoperfusion in the spinal column, then mitigates the reduction in spinal cord blood flow within the

penumbra of the lesion [88,89]. Interestingly, in vitro studies revealed that, compared to other calcium blockers, nimodipine shows greater effects on cerebral circulation than peripheral circulation [90]. This administration significantly improves neurological recovery after spinal cord trauma or ischemia [91]. Despite this, there are concerns about calcium channel blocker administration in acute spinal cord injury, given that systemic hypotension, due to autoregulation, could be detrimental [92].

The administration of sodium channel blockers remarkably protected the white matter and led to functional recovery [93]. Their neuroprotective effects on the spinal cord are exerted by opposing the increase in Na+ concentration and by blocking axonal Na+/Ca2+ exchangers [94]. For instance, the microinjections of tetrodotoxin, a potent voltage-gated sodium channel blocker, reduce neurological deficits and tissue loss after blunting experimental SCI [93].

Similar neuroprotective properties have been observed with another sodium channel blocker, riluzole, after a clip compression injury to a rat spinal cord [95]. It has been reported that this drug has an important role in protecting against excitotoxicity cell death since it blocks sodium channels in the injured neurons, thereby restricting presynaptic glutamate release [95]. Motor recovery was also observed in SCI animal models, explicitly in Basso, Beattie and Bresnahan (BBB) locomotor scores [96] and on inclined plane testing [97]. Considering non-behavioral results, tissue-sparing improvements [96] and reduced microtubule-associated protein 2 (MAP-2) loss were also described [98]. Riluzole is currently being studied by Fehlings and colleagues, who have already published several preclinical studies and started clinical tests [99,100]. At first, a phase I clinical trial was conducted to evaluate the safety and pharmacokinetics of this drug in acute SCI patients [101]. In this trial, some improved ASIA motor scores were associated with the treatment, and this provide the stimulus for a phase IIb/III double-blinded, placebo-controlled trial, the Riluzole in Acute Spinal Cord Injury Study (RISCIS) [102]. RISCIS patients are randomized into two groups: riluzole or placebo. Researchers focused on a more homogeneous group of cervical SCI patients, which received treatment within 12 hours after injury. The group in the experimental arm received 100 mg of riluzole every 12 hours in the first 24 hours post-injury and then 50 mg twice-a-day for the following 13 days. Changes in ASIA motor scores between baseline and 180 days are the primary outcome measure and then they also evaluate changes in Spinal Cord Independence Measure (SCIMIII), ASIA grade, Graded Redefined Assessment of Strength Sensibility and Prehension (GRASSP), Pain Numeric Rating Scale, Short-Form Health Survey-36 (SF-36) and European Quality of Life Five Dimension (EQ-5D) [102]. Unfortunately, this study was finished early because of the slow enrollment and no results have yet been published. Additionally, riluzole was recently also indicated for the management of patients with amyotrophic lateral sclerosis [103].

Another pharmacological class is blocking potassium channels, like 4-aminopyridine (4-AP). Patients with chronic SCI taking 4-AP demonstrated an increase in axonal regeneration with mild levels of recovery [104], [105]. In certain small clinical trials, chronic SCI patients with incomplete injury have shown improved patient satisfaction, quality of life scores, sensory and motor scores, decreased spasm and increased amplitude following fampiridine administration (a fast potassium channel blocker) [106], [107]. It was also demonstrated that potassium channel blockers enhance axonal transmission between demyelinating nodes and enhances neuronal and neuromuscular transmission in the preserved axons [106]. In addition, this drug has good tolerance and few side effects [105]. Despite these, in another study, by Hayes et al., patients treated with 4-AP exhibit less impact on functional status [107]. Therefore, further research with additional detail is urgently needed to investigate potassium channel blockers' potential benefits in improving axon conduction in chronically injured patients.

A non-specific cation channel blocker, Glibenclamide (GLC) is a sulfonylurea receptor 1-regulated that is normally used to treat diabetes mellitus type 2 by increasing insulin release [108]. It exhibits substantial neuroprotective effects in ischemic hemorrhagic stroke and traumatic brain injury models [109,110] and in SCI it can reduce hemorrhagic necrosis, edema, and inflammation. In animal models, it was found that GLC reduced hemorrhage 24 hours after injury and decreased the size of the lesion at 1-6 weeks [111], [112]. GLC also significantly improves functional recovery following SCI within 6 weeks, but the improvement of bilateral injury (33% reduction in lesion size) was more limited compared with unilateral injury (57% reduction in lesion size) at 6 weeks [111]. The findings indicate that the severity of the damage could have a significant impact on functional recovery. Newly, GLC is being investigated for its potential neuroprotective properties in cervical spinal cord injuries as part of a phase I/II clinical trial (SCING, NCT02524379). Patients initiated an oral drug regimen, which must be started within 8 hours of injury and continued for 72 hours. Unfortunately, the study was terminated early because there were not enough participants to analyze the data.

3.1.2. Cyclooxygenase (COX) inhibitors:

COX inhibitors are a preferred choice for the treatment of SCI in the damaging secondary pathway [113]. Ibuprofen is a common non-steroidal anti-inflammatory and a nonselective COX inhibitor [114] that recently has been found to promote both behavioral and histologic improvements after administration in thoracic contusion SCI animal models [115,116]. In addition to its ability to attenuate inflammation, ibuprofen also reduces Ras homolog family member A (RhoA) activation, which can promote axonal sprouting. Still, some issues raise doubts about the clinical applicability of this treatment. For instance,

in preclinical studies, dosages varied from 60 – 70 mg/kg/day, which exceeds the maximum daily dosage recommendation [117]. Additionally, the therapeutic effect of ibuprofen was not always demonstrated. Researchers have demonstrated that in rats with spinal cord injuries, ibuprofen reduced central hyperexcitability and ameliorated mechanical hyperalgesia, but failed to improve locomotor performance [118].

3.1.3. IL-4 cytokine

It had been shown that macrophages can either display neuroprotective or neurotoxic effects in SCI, depending on their phenotype [119]. Evidence suggests that molecules promoting the polarization of pro-regenerative macrophages, such as IL-4, possess neuroprotective properties and can contribute to SCI recovery [120]. Specifically, delayed administration of IL-4, 48 hours after the injury, has been shown to shift macrophages/microglia towards a regenerative phenotype in a mice model of SCI, contrasting with the acute administration that has no immunomodulatory effect [120]. Using a rat model of SCI, it has also been shown that repeated systemic IL-4 administration (once a week) exerts neuroprotective effects without apparent complications [121]. Systemic administration of IL-4 promoted a substantial elevation in serum levels of the anti-inflammatory cytokine IL-10, leading to the protection of neurons and oligodendrocytes. Despite these benefits, IL-4 and other possible cytokine therapies suffer from some major drawbacks: their short half-life, stability, and possible side effects. The use of bioengineered solutions to avoid the rapid degradation of these molecules in circulation or the synthesis of novel molecules that could work as agonist can be an appealing option to optimize present cytokine-based therapies [122].

3.1.4. Minocycline

Minocycline has the highest penetration into the central nervous system (CNS) through the brain-blood barrier of the tetracycline-class antibiotics, and previous studies have shown it to exhibit significant neuroprotective properties in Huntington's disease and multiple sclerosis [123,124]

Minocycline targets multiple processes responsible for mediating cell death. It can considerably prevent the activation of IL-1β, TNF-α, COX-2, and MMPs [124] and impede the expression of caspase-1 and caspase-3 levels [125], inhibits inducible nitric oxide synthase, minimizing microglial activation [126] and protects neurons from glutamate excitotoxicity at the injured spinal cord tissue area [127]. Moreover, this therapy reduces the lesion size and promotes tissue sparing following acute SCI [123].

A previous mouse model study also demonstrated that minocycline has a significant impact on functional outcomes. The treated mice showed continual and substantial improvements over a 4-week recovery period. Weight support and plantar stepping were consistent, while some mice showed evidence of coordinated forelimbs and hindlimbs [128].

Recently, a research group showed the results of a phase II double-blinded, placebo-controlled clinical trial of minocycline for acute traumatic SCI with 52 patients. The treated group showed better motor recovery after one year compared to the controls, but the results were not statistically significant [129]. They were also capable to find that minocycline was given intravenously within 12 hours and 7 days and achieved steady-state serum concentrations comparable to those seen in animal studies with no significant adverse events. Therefore, the development of a phase-III trial entitled 'Minocycline in Acute Spinal Cord Injury' (MASC - NCT01828203) has been driven by these initial outcomes and was evaluated for seven days using an intravenous administration of minocycline. This treatment was associated with apparent improvement in neurological and functional outcomes when compared to controls [130].

3.1.5. Glutamate receptor antagonists

The mechanism of excitotoxicity has been studied as a target of neuroprotective trials for spinal cord injury. By moderating this secondary event, neurons and oligodendrocytes may be preserved and motor function can be restored.

In experimental studies, antagonists of ionotropic receptors (NMDA and AMPA-kainate receptor) have been studied for treating SCI and histological and biochemical analyses showed neuroprotective effects [131–134]. The drug also exhibits anti-apoptotic, anti-inflammatory, and anti-proliferative effects on astrocytes [134].

As glutamate and its receptor are widely distributed throughout the human CNS, NMDA-targeting systemic clinical therapies and applications have been challenging to avoid possible side effects [135], [136]. Gacyclidine, an NMDA receptor antagonist, has been evaluated in France in a phase-II double-blind, randomized trial of patients with SCI [137]. At the 1-month follow-up, patients presented an improvement in the ASIA scale; however, results at 12 months post-treatment failed to show a long-term benefit effect. White matter glial loss is reduced, and locomotor function can be improved by focal microinjections of NBQX, an AMPA receptor blocker, into the injured spinal cord areas after blunt injury [138]. Unfortunately, this invasive form of receptor is antagonism has not been developed into pharmacological therapies due to several side effects [138].

Magnesium plays an imperative role in neuronal cellular physiology by competing with Ca^{2+} ions and acting as an endogenous Ca^{2+} channel blocker and as a NMDA receptor antagonist [139]. There was a direct association between lower magnesium levels post-injury and secondary injury in both animal models of SCI and traumatic brain injury (TBI) in humans [140], [141]. Still, the effective dosage of magnesium used in rodent SCI models (600 mg/kg) far exceeds the tolerable human dosage [142]. For this reason, formulations of Polyethylene Glycol (PEG) containing magnesium were developed increasing the efficiency of a smaller dose of magnesium [143].

3.1.6. Levetiracetam

Levetiracetam is a new antiepileptic drug that blocks presynaptic neurotransmitter release by binding to synaptic vesicle protein 2A (SV2A) [144–148]. Therefore, modulating excitatory amino acid levels after trauma may be an effective strategy to reduce acute excitotoxicity.

Recently, it has been shown that Levetiracetam modulates glutamate excitability and reduces lipid peroxidation after SCI in rats. More important, Levetiracetam-treated animals presented significant improvements in gross and fine motor function either when administrated in cervical or in thoracic injuries. Levetiracetam treatment increased motor neuron survival, preserved their axons, and reduced the size of the cavity surrounding the lesion [149]. Furthermore, it was seen that Levetiracetam inhibits the expression of perforin in a mouse model of SCI [150]. According to this study, treating animals with Levetiracetam restored BSCB, reduced nerve cell apoptosis in the trauma area, and increased hind limb motor function, resulting in nerve conduction and walking coordination being protected. Levetiracetam has not been clinically tested for SCI yet.

3.1.7. Anti-Nogo-A

It is known that regeneration after CNS injury is limited by two factors: the CNS's inhibitory environment and its limited intrinsic regenerative capacity. As earlier described, this is in part due to myelin-associated neurite growth inhibitors such as Nogo-A. Anti-Nogo-A is a monoclonal antibody that has been extensively tested to promote axonal regeneration after SCI and the efficacy has been demonstrated in rodent models [151–153], as well as primate models of cervical SCI [154,155]. Additionally, it was seen that neutralizing Nogo-A with anti-Nogo-A led to axonal regrowth, compensatory sprouting, and increased motor recovery after sectioning the corticospinal tract of adult rats [153,156,157].

Based on the previous positive pre-clinical results, a non-randomized, open-label phase I clinical trial of humanized anti-Nogo-A antibody (ATI-355; Novartis Pharmaceuticals) was initiated in 2006 [158]. The

drug was delivered in sub-acute SCI and was a well-tolerated response to anti-Nogo-A in the 52 patients with trauma between C5 and T12 levels, suggesting the administration of intrathecal antibodies may be considered in future trials of SCI [158].

Most recently, in Zurich, Zörner et al. initiated a phase II clinical trial (Nogo Inhibition in Spinal Cord Injury – NISCI – NCT03935321) that started to test the efficacy of the anti-Nogo-A antibody treatment after SCI in severely injured paraplegic and tetraplegic patients [159]. This study is still ongoing.

3.1.8. PDE4 inhibitors

Numerous animal model studies in the past decade have demonstrated that pharmacological elevation of cyclic AMP (cAMP) alone, or in combination with other treatments, can promote axonal regeneration after spinal cord injury. Elevation of cAMP via the phosphodiesterase 4 (PDE4) inhibitor, Rolipram, decreases neuronal sensitivity to myelin inhibitors, has a neuron and glial-protective effect, increases growth potential, promotes axonal regeneration, and improves locomotor function after experimental SCI [160–163].

Rolipram was originally developed as a possible antidepressant drug in the early nineties but has since been discontinued from psychiatric use, due to a narrow therapeutic window with significant gastrointestinal side effects [164,165]. More recently, Rolipram underwent a phase II clinical trial for multiple sclerosis (NCT00011375) but so far it has not been clinically tested for SCI.

Recently, our team also explored a PDE4 inhibitor, Roflumilast (Rof). Rof has the same binding site as Rolipram and and has already been approved for human use in chronic obstructive pulmonary disease (COPD) [166]. It was seen that increase the intracellular levels of cAMP [167]. Moreover, 8 weeks post-injury, the animals significantly recovered by achieving occasional weight-supported plantar steps. Histological evaluation showed a significant decrease in cavity size, less reactive microglia, as well as higher axonal regeneration in treated animals. Overall, Rof promotes functional recovery and supports neuroregeneration in a severe thoracic contusion injury model and may be important in SCI treatment [167].

3.1.9. Chondroitinase ABC (ChABC)

CSPGs inhibits axonal regeneration in the injured CNS. ChABC is an enzyme obtained from the bacteria lysate of Proteus vulgaris [168] that has been widely explored in different SCI models [168–171]. ChABC's therapeutic effect after SCI is due to its ability to cleave the inhibitory glycosaminoglycans from the protein core of CSPGs. This removes the inhibition of axonal growth caused by intact CSPGs. Both

local and systemic administration of ChABC showed to increase axonal regeneration and functional recovery after SCI [172,173].

3.2. Cell-based strategies

Cell transplantation therapies are being tested around the globe to treat spinal cord injuries as well as other conditions. Cells are used either to provide the lesion site with relevant CNS populations that will replace dying cells, help recreate the lost connections, or as sources that guarantee trophic support and enhance neural regeneration [174]. A wide range of experimental animal studies has demonstrated that cell-based therapies promote functional recovery after SCI. [175–178]. Since the end of the 19th century, stem cells have been known to differentiate into cells from all three germ layers (pluripotent) [179], making them an attractive source for a variety of applications [180–182].

3.2.1. **Embryonic stem cells** (ESCs)

ESCs are derived from blastocysts and were the first stem cells to be used [183]. Different approaches have been applied to study the ability of ESCs to differentiate into neural and glial cells in vitro, and ESC-based strategies have also been researched in various models of SCI. In one thoracic SCI study, Keirstead et al. transplanted neural stem cells (NSCs) derived from mouse ESCs into a rat-injured spinal cord. A high percentage of transplanted cells survived and differentiated into oligodendrocytes and astrocytes [184].

An FDA-approved phase I clinical trial provided by Geron's company started in 2009. A cohort of patients with complete subacute thoracic SCI was transplanted with pre-differentiated oligodendrocyte precursor cells (OPCs) derived from human ESCs. Unfortunately, the trial was aborted later in 2011 due to financial concerns [185]. Despite this, no safety issues have been reported in patients who have undergone ESC transplants to date. Besides technical issues regarding ESCs therapy, there is a controversial debate regarding human ESCs research since the use of human oocytes and embryos is involved [186]. Therefore, **induced pluripotent stem cells** (iPSCs) have been proposed as an alternative to obtaining stem cells directly from adult tissues for autologous transplantation. Yamanaka's lab developed iPSCs as part of pioneering research in 2006. In this study, four transcription factors were introduced into differentiated adult cells to reverse their phenotype into pluripotent cells [187]. Since iPSCs can be derived directly from adult tissues, they offer an unlimited source of autologous cells without the risk of immune rejection.

In a preclinical study, neural stem progenitor cells (NSPCs) derived from murine and human iPSCs (iPSC-NSPCs) were transplanted into a nonhuman primate model of contusive SCI to investigate their therapeutic potential. As in previous rodent studies, the transplanted cells survived and differentiated into astrocytes, neurons, and oligodendrocytes without forming tumors. Functional recovery was also promoted by the treatment [188]. According to the amount of preclinical evidence demonstrating efficacy and safety, the first clinical trial with iPSC-NSPCs was about to begin in Japan in 2021, where patients with complete subacute SCI were being recruited [189]. This research restarted after the COVID-19 pandemic ended and is still ongoing.

Another cell population with therapeutic potential for SCI research is adult multipotent **neural stem cells** (NSCs). The three main neural cell lineages in the mammalian central nervous system can be generated in culture by these cells [190].

Nine days after grafting human neural stem progenitor cells (NSPCs) into primates with cervical contusion SCI, A. Iwanami et al. showed that cells survived and differentiated into neural lineages. Additionally, the transplanted primates showed a decrease in the lesion cavity and an increase in spontaneous motor activity. [191]. Furthermore, it was seen in mice with traumatic SCI that demyelinated axons were remyelinated after the transplantation of human CNS cells grown in aggregates. These cells also differentiated into neurons that have the skill of synapse formation with host neurons [192]. In response to encouraging results, Stem Cells Inc. Company (Switzerland) began the world's first clinical trial using NSCs to treat spinal cord injury in humans [193]. Phase I/II clinical trial was initiated in 2011 to evaluate the safety and preliminary efficacy of purified fetal **human neural stem cells** (HuCNS-SC) as a treatment for chronic thoracic SCI, including complete and incomplete injuries. The study enrolled twelve patients (seven with complete injuries [ASIA A] and five with incomplete injuries [ASIA B]). This study showed improvements in overall Upper Extremity Motor Score (UEMS) and Graded Redefined Assessment of Strength, Sensibility, and Prehension (GRASSP) strength component at final follow-up were observed in those who received the HuCNS-SC [194]. Unfortunately, this study was premature terminated because the functional recovery observed was below the required clinical efficacy threshold set by the sponsor. More recently, the six-year follow-up data of a phase I/IIa trial involving surgical transplantation of 20 million HuCNS-SC cells into the thoracic cord of AIS A or B patients was published [195]. Cervical cord imaging control showed no tumor formation or malformation of the lesion zone. Additionally, this study demonstrated short- and long-term safety and feasibility of cell transplantation for patients with complete and incomplete SCI [195]. Four out of five ASIA A patients have gained significant sensory function after transplantation. However, a remarkable loss of function was observed in one AIS A subject after

withdrawal of immunosuppression. Despite most of the patients remained stable, these results point to the importance of continuing to develop safe autologous NSCs to be transplanted in SCI patients.

3.2.2. Glial cells

A major glial cell type in the peripheral nervous system, where spontaneous regeneration takes place, is **Schwann cells** (SCs). These cells surround neurons, providing their myelin sheath and several pre-clinical studies have shown that SCs can be used to treat the damaged spinal cord. One of the original studies, from Paino and Bunge, in 1991, showed axonal ingrowth in SCI-rats when SCs were enclosed in collagen rolls, similar to what had been observed previously for peripheral nerve grafts [196]. In a subsequent study, two million SCs were transplanted one week after a moderate midthoracic contusion, resulting in increased spared tissue, myelinated axons, and brainstem and propriospinal axons reaching further distances beyond the graft. The improvement in motor function was modest but statistically significant. [197]. Based on these favorable pre-clinical results, a clinical trial was conducted [198]. Autologous transplants of SCs from the sural nerve in subacute SCI patients were tested by The Miami Project to Cure Paralysis. After transplantation into the lesion epicenter in a total of six subjects, there was no evidence of additional spinal cord damage, mass lesion, or syrinx formation, with no adverse effects up to one-year post-transplantation. According to these findings, SCs transplantation can be safe and further studies must be conducted.

3.2.3. Mesenchymal cells

Multipotent stromal cells, mesenchymal stem cells (MSCs) possess the ability to self-renew and can differentiate into different cell types (including osteoblasts, adipocytes, and chondrocytes).

MSCs are among the most used types of regenerative cells [199]. The first uses of MSCs included transplants of bone marrow [200,201] but MSC-like cells have been identified in numerous other tissue sources, including adipose tissue [202], the umbilical cord [203], skeletal muscles [204], dental tissues [205], synovial fluid [206], palatine tonsils [207], parathyroid gland [208], fallopian tube [209], brain [210], placenta [211], endometrial tissues [212] and uterine cervix [213].

The direct transplantation of MSCs after a SCI has been extensively studied and details information can be founded in these reviews [214]. One approach that has been explored more recently is not the direct injection of MSCs but rather the administration of the paracrine factors these cells produce, known as

secretome. For instance, it was demonstrated that Adipose Stem Cells (ASCs) derived secretome has a neuroprotective effect on cortical neurons [215] and it enhances the metabolic viability and cell density of hippocampal neuronal cultures [216]. Moreover, this secretome presents an anti-inflammatory effect over human monocytes, reducing the production of TNF-a, Nitric Oxide (NO) or Prostaglandin E2 (PGE2) and inducing the production of Transforming Growth Factor (TGF)-B1 and IL-10 by M2 macrophages [217]. Recently, ASCs secretome demonstrated to induce neurite outgrowth from DRG explants, when compared to MSCs isolated from the bone marrow [218]. Interestingly, the expression of neuroregulatory molecules in the secretome of ASCs does not significantly change with cell passaging, not influencing the impact of their secretome in neuronal differentiation and axonal growth [219]. The secretome of ASCs was also able to promote functional recovery when injected into a SCI compression mice model [220]. Thus, future applications of ASC-secreted factors in a SCI context could avoid problems including cell rejection or low cell survival/grafting. More information about the use of MSCs secretome for CNS disorders can be found in the Pinho, AG et al review [221].

3.3. Neuromodulation Technologies

Researchers have unraveled the cellular and molecular events that cause spinal cord injury, allowing the development of pharmacologic agents, biomaterials, and stem-cell therapies. Despite these efforts, spinal cord injuries still lack standard care for regenerating axons and restoring silent axon function. As a result of these challenges, neuromodulation is becoming a more prominent therapeutic approach.

There is a substantial decrease in sustained excitability in the lumbosacral circuitry as a result of severe spinal cord lesions [222]. Therefore, much effort has been expended in the past decade to tune the physiological state of lumbar spinal circuits to a level sufficient to allow stepping and standing, mentioning the electrical stimulations of the muscles [223,224] dorsal roots [225], epidural [226–228] or intraspinal [224,225] electrical spinal cord stimulation, and a combinatorial approach with pharmacological agents that will be explored in the last section of this article.

It is interesting to notice that recent clinical studies have found that epidural electrical stimulation (EES) of the spinal cord can restore volitional movement below the level of SCI [229–231]. These findings have been further sustained by the re-establishment of independent stepping from complete SCI with EES and task-specific training [232,233]. Moreover, using a Brain-Spine Interface (BSI), that consists of fully implanted recording and stimulation systems establishing a direct link between cortical signals and the spinal cord regions involved in the production of walking, it was possible to significantly improve the neurological functional of a patient with incomplete SCI [234]. Although a limited number of studies, the

positive impact of EES on autonomic, cardiac, respiratory, and motor functions further support that EES holds potential as a therapeutic intervention after SCI. A therapeutic approach based on these findings was designed to target spared lumbar circuits and projection neurons with residual fibers below the injury to improve recovery from SCI [224,230,235].

EES is currently one of the most promising therapeutic interventions to promote functional recovery after a SCI, nevertheless, the mechanisms enabling motor control when delivering neuromodulation therapies and improvements with rehabilitation remain highly debated [236].

3.4. Biomaterials

A well-known limitation of the CNS is its limited capacity to regenerate. Besides the inhibitory environment created after damage, no physical matrix is available for neurons and endogenous cells to adhere to. The emergence of tissue engineering and regenerative medicine strategies [237] has allowed scientists to combine fields such as biology, physics, chemistry, and biomaterials science to replace damaged tissues, like the spinal cord. Each biomaterial application requires a specific type of material. This choice is often determined by the physical and mechanical properties of the tissue: 1) hydrogels, which are highly hydrated materials with water molecules and hydrophilic polymer networks, and scaffolds have been developed to accelerate the engraftment, survival, and differentiation of stem cells. The mechanical and physical properties of these materials make them ideal for use in CNS tissue repair [238]; 2) micro and nanoparticles, in most cases, used to deliver drugs [239]; and 3) fibers and conduits, often used to guide axonal growth [240].

Using a postcontusive chronic SCI rat model, Gelain and colleagues developed a neural prosthetic scaffold with guidance channels and transplanted into a cyst cavity[241]. After six months, significant cord reconstruction was detected, as the cysts were restored by newly formed tissue containing neural and stromal cells. Nerve fibers were found within and between the guidance channels. Furthermore, a well-developed vascular network, basal lamina, and myelin were observed. This anatomical and histological framework led to the replacement of large tissue gaps and promoted axonal regeneration and functional recovery.

In 2012, Liu and colleagues studied the potential application of electrospun collagen nanofibers for the treatment of spinal cord injuries [242]. The study revealed that nanofibers influenced astrocyte behavior and guided neurite outgrowth in vitro. In vivo experiments showed promising results in terms of cellular penetration, reduced macrophage invasion over time, and early neural fiber sprouting. The findings highlight the feasibility of fabricating three-dimensional spiral constructs using electrospun collagen fibers

and suggest the potential of these scaffolds for SCI treatment. In the same year, it was demonstrated that a 3D scaffold (a blend of starch with polycaprolactone) used to replace delaminated vertebra, in a T8-T9 spinal hemisection rat model promoted spine stabilization and lead to some improved motor function. The scaffolds stabilized the vertebral column, and concurrently prevented the infiltration of connective tissue into the injury site [243].

Two clinical trials were already conducted employing purely biomaterials (without cells or molecules). THE INSPIRE study (NCT02138110) was an open-label, non-randomized, single-arm, multicenter study to evaluate the safety and probable benefit of the poly(lactic-co-glycolic acid)-b-poly(L-lysine) scaffolds. The trial recruited individuals with thoracic AIS A traumatic spinal cord injury at neurological level T2-T12. Results demonstrated that acute scaffold implantation within the spinal cord appeared to be safe with no long-term neurological problems identified during the 24-month follow-up. Patients remained stable, with minor AIS conversions observed in some patients at 12 months. These results support the safety of this scaffold implantation in this SCI population. After the results of this first trial, a randomized, controlled, single-blind, multicenter clinical study is now ongoing (NCT03762655). The INSPIRE 2 intention is to evaluate the safety and therapeutic benefits of the same scaffold in a similar patient group as compared to standard of care open spine surgery.

Further developments in the physical and chemical manipulation of biomaterials may lead to more axonal regeneration and neural repair. Biomaterial-based therapies are usually performed in combination with other therapeutic strategies, such as cell transplantation and biomolecules. This combinational approach will be further explored below.

3.5. Combinational strategies in SCI treatments

As shown previously, we can have significant results with some single approach for spinal cord injury, but others tend to stop below the expectation and have so far failed. A major reason for therapeutic failures is the inability to maintain an adequate concentration around the lesion site or within the spinal cord [229,230]. Other reasons for the failure can be related to the presence of diverse pathways of the lesion in the SCI. Getting significant functional gains for patients with SCI may require a combinatorial approach due to its multifactorial nature and recently, advances in combinatorial therapies, which address different aspects of spinal cord pathophysiology, offer hope for spinal cord regeneration.

Thus, nowadays, it might be wise to focus on developing novel combinations of approaches that can handle different events (**Figure I.4**). The recent developments in combinational approaches are described below.

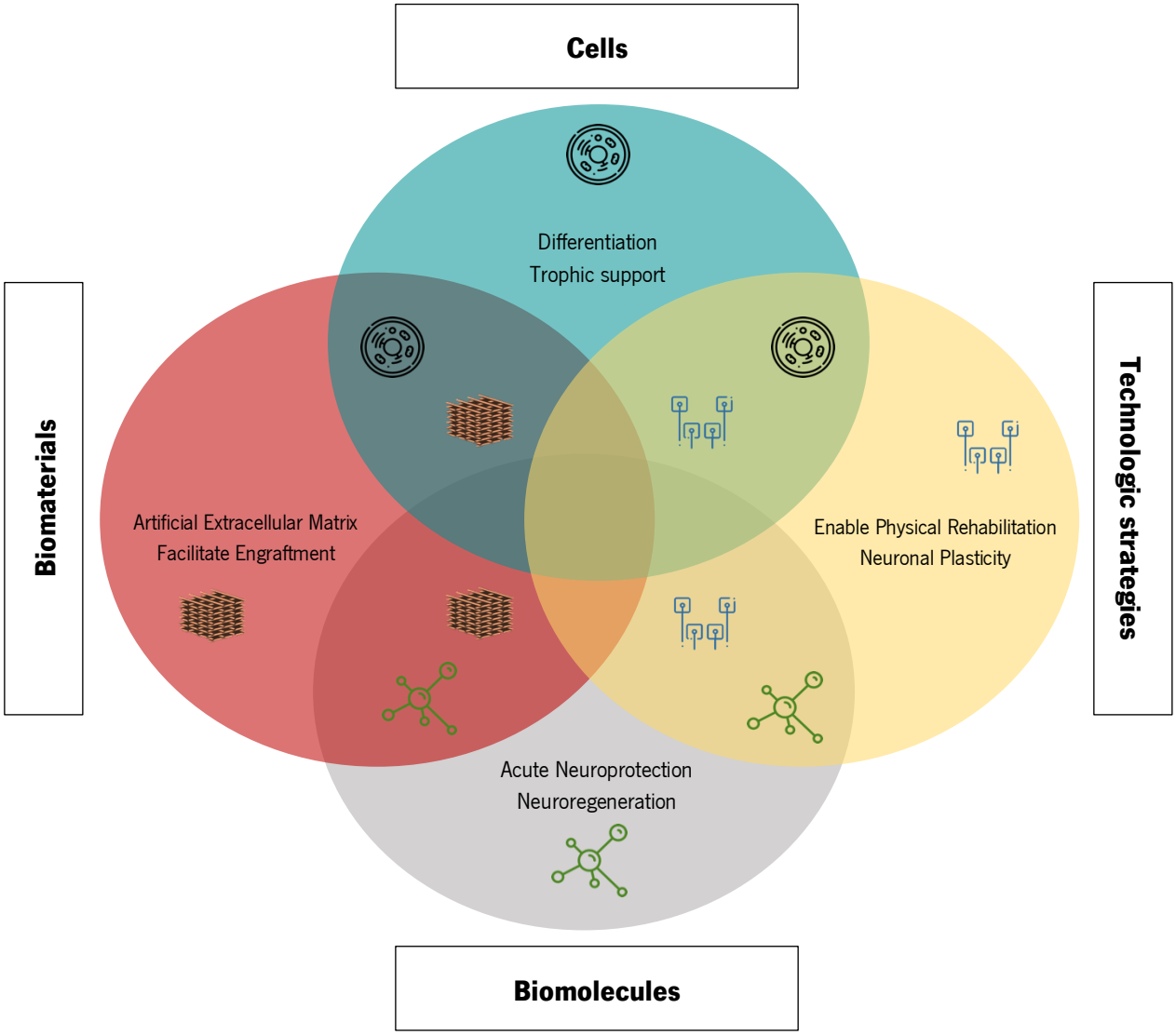


Figure I. 4. *Schematic representation of possible combinatorial treatments against spinal cord injuries. Cells and scaffolds can be used alone or in combination with pharmacological agents and other biomolecules such as growth factors. Technological strategies such as epidural electrical stimulation are being tested in combination with biomolecules or cells.*

3.5.1. Combination of molecular agents

Several pharmacological treatments have already been combined. In 2013, Martin Swab's group tested the **Anti-Nogo-A antibody** and **ChABC** enzyme, two promising treatments that promote functional recovery after SCI, separately and in combination and the last one was more effective. The combination treatment produced greater increases in sprouting and axon regeneration [244]. The **ChABC** also had been combined with other drugs, such as **Neurotrophin-3** (NT-3). A previous study had shown that NT-

3 causes NMDA receptors to switch back to their immature form (expressing the NR2D subunit) and increases motoneuron's plasticity. In a lateral T8 hemisection SCI model, elevating the spinal levels of NT-3, and consequently, the NR2D subunit was combined with ChABC. Rats receiving the full combination group exhibited the most axonal sprouting and behavioral recovery in the BBB locomotor test, and in the case of electrical conductivity across the lesion, it was only restored in rats receiving the full combination. [245,246]. Other group tried to combine **Curcumin** with **Epigallocatechin gallate** (EGCG) in a rat model of acute compressive SCI. Both drugs are well known for its anti-inflammatory role potential and alone or together, they increased axonal sprouting, reduced glial scar formation, and decreased the levels of macrophage inflammatory protein 1-alpha, IL-1B, IL-4 and IL-6 cytokines [247].

Interesting, it also been tried the interaction between **gabapentin** and **NMDA receptor antagonists**, dextromethrophan and MK-801, in relieving neuropathic pain-like actions in rats after SCI. With 15 or 30 mg/kg gabapentin combined with 10 mg/kg of dextromethorphan proportionated a full alleviation of allodynia. MK-801 also augmented the effect of gabapentin in SCI rats [248].

Rilozule and **Magnesium** were also combined with the aim to protect the spinal cord against glutamate excitotoxicity. Riluzole is a sodium channel blocker agent that exerts neuroprotection in SCI mainly through blocking of persistent sodium currents, inhibition of presynaptic glutamate release, as well as increased glutamate uptake. Magnesium acts as an N-methyl-D-aspartate receptor blocker preventing excitotoxicity caused by excessive activation of this glutamate receptor. However, when tested together it was not observed any synergistic or cumulative effect, and Riluzole was the only treatment that were able to achieve functional and histological improvements [249].

Due to the pivotal role neuronal apoptosis plays in secondary cellular events in SCI, a group investigated the ability of co-administration of the **poly(ADP-ribose)-polymerase 1 (PARP-1) inhibitor, 3-aminobenzamide** (3-AB) and **caspase-3 inhibitor** z-DEVD-fmk to attenuate apoptosis in a rat SCI model [250] and the results indicate that combination therapy is useful for neuronal function recovery in rats with SCI.

In 2020, a group from China combined **melatonin** with **methylprednisolone**. Inhibition of axonal degeneration and reduction of nerve damage can be achieved with melatonin [251–253] and this group tried to combine it with a lesser dosage of methylprednisolone to ameliorate the side effects without a loss of effectiveness. They saw that melatonin could synergize with methylprednisolone to improve acute SCI through the PI3K-AKT1 pathway with fewer adverse reactions [254]. **Methylprednisolone** also was combined with Granulocyte colony-stimulating factor (**G-CSF**.) G-CSF is a glycoprotein which works as growth-stimulating factor for hematopoietic progenitor cells. This combination promoted functional and

histological improvements superior to those achieved by either of these drugs alone when treating spinal cord contusion injuries in rats. Thus, the two drugs together had a synergistic effect [255].

A combination of **ceftriaxone** with **methylprednisolone** was also done in a randomized, triple-blind clinical trial, where sixty individuals with acute traumatic SCI to evaluate whether the combination therapy is superior to methylprednisolone alone. Results showed that the combination is responsible for improving inflammation and functional outcomes 6 months after the intervention [256].

The combined action of **methylprednisolone** with recombinant human **erythropoietin** (rhEPO) was also tested in a pilot double-blinded randomized controlled trial with acute cervical SCI patients. They were randomly assigned to either methylprednisolone alone or EPO plus methylprednisolone, but the group failed to prove that the combination therapy compared to methylprednisolone alone improves neurological functions [257]. In another study, but using SCI rats, **EPO** was combined with **IL-6**. Using EPO after acute SCI promoted functional recovery. The combination of EPO and IL-6 revealed benefits, but with inferior results compared to those of isolated EPO; furthermore, the isolated use of IL-6 resulted in no benefit [258].

Another combinational approach was the use of a monoclonal antibody, anti-High Mobility Group Box-1 (**anti-HMGB1 mAb**) with epothilone B (**Epo B**). HMGB1 is a damage-associated molecular pattern (DAMP) involved in BSCB disruption and inflammatory response playing an important role in the secondary damage following CNS injuries and Epo B is an anticancer drug that can contribute to increase microtubule stability and promote axon regeneration. While Epo B alone failed to increase functional recovery, these drugs together increased hindlimb movement compared with anti-HMGB1 mAb alone. According to these results, anti-HMGB1 mAb alone decreased the extent of the lesion and increased the number of neurons surviving around the lesion, while Epo B facilitated axon outgrowth only in conjunction with anti-HMGB1 mAb, indicating that Epo B's therapeutic effect requires anti-HMGB1 mAb-dependent tissue preservation [259].

From another perspective and to alleviation of chronic pain after SCI, it was investigated the simultaneous intrathecal administration of **Gamma-aminobutyric acid (GABA) agonist** and **endomorphin-1**. The inhibitory GABA system plays an important role in reducing neuropathic pain. Additionally, endomorphins has been shown to reduce chronic pain caused by nervous system damage. So, this combination was tested for reduction of neuropathic pain in a rat model of SCI and it was seen that it can significantly increase the pain threshold compared to injection of endomorphin-1 or muscimol alone. It also increased the expression of $\alpha 2$ subunits of GABA receptors, and NR1 subunits of NMDA receptors in the spinal cord [260].

In 2021, the combined effect of three Neurotrophins was studied to examine whether the combination applied after 90 or 120 minutes of the injury could attenuate cord pathology. Brain-derived neurotrophic factor (**BDNF)**, Ciliary Neurotrophic Factor (**CNTF**) and glial cell line-derived neurotrophic factor (**GDNF**) were topically administrated over the injured spinal cord and significantly reduced the BSCB breakdown, edema formation, cell injury and overexpression of enzyme hemeoxygenase-2 (HO-2) by downregulation of carbon monoxide (CO) production [261].

Other combinations had been tried with steroids like, for example, **TRH**, **GM-1 ganglioside,** or **naloxone**. Compared with single-treatment regimens, these combination therapies may have some benefits. However, it must be noted that the beneficial effects of this treatment were not observed in all forms of SCI patients, as in severe SCI patients [262,263].

3.5.2. Combination of cells and molecular agents

Several authors tested the effect of combining cells with pharmacological agents in order to better integrate the cells into the neural tissue or to increase their survival upon transplantation. Synergistic effects can be seen in the combination of **ChABC** with neural **NSPCs**. By digesting CSPGs, ChABC mitigated CSPG's inhibitory effect on NSPCs migration and stimulated neurite outgrowth after transplantation [264].

Combined with **hUCM-SCs**, **Taxol**, an anti-cancer drug that inhibited scar formation and enhanced axonal lengthening, was found to improve functional recovery following spinal cord injury through the enhancement of anti-inflammatory, anti-astrogliosis, anti-apoptotic, and axonal preservation effects [265].

In 2018, **hUCM-SCs** were also combined with **Quercitin**. This drug is capable of reducing the inflammatory microenvironment after SCI. A number of effects were observed following this conjugation, including neurological functional recovery, axonal preservation, macrophage polarization, reduction of cystic cavity size, and a reduction in proinflammatory cytokines. Several anti-inflammatory cytokines were also increased, including IL-4, IL-10, and TGF-1 [266].

In another study, **methylprednisolone** combined with amniotic membrane mesenchymal stem cells (**AM-MSC**) reduced secondary damage and enhanced functional recovery. When methylprednisolone (50 mg/kg) was combined with delayed AM-MSC transplantation, myeloperoxidase activity and proinflammatory cytokines were significantly reduced, as well as cell apoptosis. Furthermore, this combination also increased the levels of the anti-inflammatory cytokines and the survival rate of AM-MSCs in the injury site [267].

Another group investigated the integration of **SCs** with a hormonal compound, **17β-estradiol**. 17β-estradiol protects different cell types and reduces tissue damage and the combination with **SCs** showed to decrease neurological and behavioral deficits, protected neurons and oligodendrocytes, and significantly decreased the number of Iba-1+ (microglia) and GFAP+ cells (astrocyte) in rats with SCI [268].

It was also found that combining **OECs** and **Minocycline** reduced levels of IL-1β, TNF-α, caspase-3, oxidative stress, astrogliosis, and cavitation while improving axonal regeneration and functional recovery [269]. **OECs** were additionally combined with **ChABC** in a rat SCI model. The group postulated that transplantation of canine OECs genetically modified by lentiviral transduction to express ChABC would offer new delivery system of ChABC and potentiate a synergistic therapy. In this study, a modest functional improvement was observed in the combinational transplanted animals, who recovered greater forepaw reaching accuracy (Whishaw testing) and a normal gait when compared to the OECs transplanted group or controls [270].

Satoshi Nori et al. investigated for the treatment of chronic SCI in an immunodeficient rat model reprogrammed human NPCs toward an oligodendrogenic fate (oNPCs) with sustained delivery of ChABC in an innovative release strategy in a crosslinked methylcellulose biomaterial. This combinatorial improved long-term survival of oNPCs around the lesion center, facilitated oligodendrocyte differentiation and the remyelination of the spared axons by engrafted oNPCs and improved neurobehavioral recovery [271].

The combination of **HuCNS-SC** and lithium chloride (**Li**) was done to see if it improves cell survival, proliferation, and differentiation in a rat spinal contusion model compared with the individual treatments. Both HuCNS-SC + Li and the Li groups revealed significant improvement in locomotor scores and motor evoked potential (MEP) more than HuCNS-SC alone [272].

In 2020, it was seen that **NSCs** loaded with **tetrahedral framework nucleic acid** had similar neuroprotective effects. The combined outcome of this approach resulted in a significant increase in motor function recovery and tissue regeneration in SCI lesion sites [273]. Other group evaluated the effect of Epidermal Neural Crest Stem Cells (**EPI-NCSCs**) combined with **astaxanthin**, a powerful antioxidant, on damage induced by SCI. Based on their results, astaxanthin + EPI-NCSCs had better effects on behavioral recovery, motor neuron protection, and less demyelination compared with both treatments alone [274].

NSCs were also tested in conjunction with **teramethylpyrazine** (TMP). This combination improved functional outcome in rats with SCI and offered spinal cord protection by increasing BDNF and decreasing

Nogo-A levels, as well as restricting the JAK2/STAT3 signal transduction pathway, proposing that TMP could be a useful agent in NSCs transplantation in the treatment of SCI [275].

It was also evaluated, in a contusive SCI rat model, the combination of **IPSC-NSCs** and **MSCs** with a pH-responsive polyacetal-curcumin nanoconjugate (**PA-C**) [276]. This allows curcumin to be released on a sustained basis. The **PA-C** delivery intrathecal with stem cell transplantation failed to show significant improvements in locomotor but had significantly smaller scars and induced the preservation of β-III Tubulin-positive axons. **MSCs** were also combined with platelet-rich plasma (**PRP**), which is a biological product derived from peripheral blood with a variety of growth factors. The results showed that this co-treatment improved axon regeneration, decreased cell apoptosis and improved locomotor function and acted synergistically in the SCI [277].

Z. Zeraatpisheh et al., in 2022, found that co-administration of **FTY720** (an immunomodulatory drug) with **NSPCs**, seven days after a SCI, could enhance functional recovery and transplanted cells' survival and migration [278].

In biological processes such as migration, proliferation, and cell survival, signaling molecules, such as growth factors, seem to play an essential role [279] and cellular transplantation can be further enhanced through the application of these neurotrophins. Thus, some groups test this combination and showed that the use of **MSCs** along with either nerve growth factor **NGF** [280] or **bFGF** [281] can promote axonal regeneration, cell differentiation, and functional recovery following SCI incidence in adult rats. One group from Korea, tried to potentiate the utility of adipose-derived mesenchymal stem cells (**Ad-MSCs**) by transfecting them with **BDNF** and heme oxygenase-1 (**HO-1**), through a lentivirus, to create BDNF overexpressed Ad-MSCs (BDNF-MSCs), and HO-1 overexpressed Ad-MSCs (HO-1-MSCs) [282]. They injected BDNF-MSCs and HO-1-MSCs as a combination group, in SCI dogs, and compared to which one injected individually. The combination group showed an increase in neuroregeneration and a significant improvement in hindlimb functions than in other groups.

Ana Alastrue-Agudo and her group tried to combine the transplantation of ependymal stem/progenitor cells of the spinal cord after injury (**epSPCi**) with **FM19G11** pharmacological treatment (an inhibitor of hypoxia inducible factor). This combination improved some locomotor functions compared to the control group but did not significantly improve the BBB scores in comparison with the individual treatments [283].

Focusing on different aspects and to ameliorate spasticity, which is a frequent complication of SCI with no ideal treatment, Wei-Ming Sun et al combined **sural nerve grafting** with an infusion of acidic FGF (**aFGF**) in a monkey thoracic hemisection model. The combined treatment markedly reduced the

spasticity in leg muscle tone, patella tendon reflex, and fanning of toes, and may be seen as a complementary approach in decrease spasticity in patients with SCI [284].

3.5.3. Neural Tissue Engineering

Tissue engineering is a fast-evolving discipline that pursues to repair, replace or regenerate tissues or organs by translating fundamental knowledge in physics, chemistry and biology into effective materials, or devices and clinical approaches [285]. It evolved from biomaterials development and is the process of creating functional tissues by combining scaffolds, cells, and biologically active molecules. Using tissue engineering, damaged tissues or whole organs can be restored, maintained, or improved.

3.5.3.1. Combination of cells and biomaterials

The inhibitory circumstances derived from SCI undermine cell survival. In order to resolve this issue, biomaterials have been proposed to protect the cells upon transplantation and also to modulate their behavior [286–288]. Biomaterial can fulfill multiple functions for SCI transplantation strategies: (A) specific three-dimensional microarchitectures can be designed for cell seeding and axonal growth in a directed linear pattern, facilitating axonal growth across the lesion [289–291] (B) serves as a physical matrix for cell adhesion and thereby enhancing survival and retention of grafted cells at the lesion site [292–295]; and affect host cell migration (e.g., SCs and astrocytes) [296]; (C) influence the behavior of grafted cells and differentiation [297]; and (D) control the release of encapsulated bio-active molecules [298].

Multiples studies had shown that embedding cells into both natural and synthetic materials led to improvements in cell survival, axonal regeneration, and functional recovery in pre-clinical SCI models (**Table I.1**), and a recent systematic meta-analysis from Michael G. Fehlings laboratory demonstrated the superiority of a combined application of scaffolds and MSCs versus separate applications of each one, in terms of improvements in motor function, during interventions in the acute phase of SCI in animal studies [299]. Of note, one pre-clinical study combined **MSCs** transplant and a bioengineered nanofiber-hydrogel composite (**NHC**) and did not find a significant effect of NHC on MSC transplant survival, and hind limb function was similar across all groups [300].

In 2013, it was seen that the use of nerve guidance scaffolds (templated agarose scaffolds), filled with genetically engineered bone marrow stromal cells for the secretion of **BDNF** could imply improvements in the growth of axons as well as motor functions even during severe SCI [301].

In 2015, a work by Zhang *et al.* employed **chitosan** scaffolds to create a permissive microenvironment for improved survival of human dental pulp stem cells (**DPSCs**), as well as enhanced neural differentiation. The study showed that chitosan scaffolds enhanced cell viability and promoted neural differentiation of DPSCs in vitro. The DPSCs incubated with chitosan scaffolds also exhibited augmented secretion of neurotrophic factors, such as GDNF, BDNF, b-NGF, and NT-3. The Wnt/β-catenin signaling pathway was found to play a key role in the neural differentiation of DPSCs in conjunction with chitosan scaffolds. Furthermore, transplantation of DPSCs along with chitosan scaffolds into an animal model of SCI resulted in significant recovery of hind limb locomotor functions. The findings suggest that chitosan scaffolds provide a supportive microenvironment for DPSC survival and neural differentiation [302].

In another study, a **3D gelatin** sponge combined with genetically engineered **BM-MSCs** and **Schwann cells** was transplanted into the transected spinal cord of rats and denoted a substantial improvement of hindlimb function and cortical motor evoked potential [303]. Furthermore, in 2019, it was described that the loading of differentiated human endometrial stem cells/Schwann cells (**hEnSCs**) on **PCL/gelatin** scaffolds and transplantation into hemisected SCI rats leads to improve both sensory and motor functions [304].

Human embryonic stem cells (**hESCs**) derived-NSCs wrapped in **hyaluronic acid** hydrogel and injected into the lesion site were found to produce differentiation into oligodendrocytes and further improvement of behavioral recovery [305]. Also, important to mention, combined applications of NSCs with collagen [306], Matrigel [307], and collagen/silk fibroin bio-printed [308] scaffolds have been shown to promote spinal cord repair.

Another therapeutic strategy tested for SCI repair is the application of **3D silk** fibrous scaffold joined with dermal fibroblast-reprogrammed neurons, which is shown to improve functional recovery [309]. A study had found that the combination of **decellularized spinal cord** scaffolds with **OECs**, leading to improvements in cell proliferation and axon regeneration [310] or the combined application of polyvinylidene fluoride trifluoroethylene (**PVDF-TrFE**) conduits with **SCs**, causing enhancements in axonal growth and formation of blood vessels following transection [311]. Moreover, the combination of activated **SCs** and **BM-MSCs** through a multichannel PLGA scaffold and injection into fully transected spinal cords were shown to improve functional recovery [312].

Even though **NPCs** transplantation has been found to be beneficial, its low survival and differentiation rates pose several significant limitations. One group from Spain tried a new approach of using a combination of **electric and magnetic stimulation** to induce proliferation and further neuronal differentiation [313]. Thus, they used both **wireless electrical and magnetic stimulation** to

condition in vitro **NPCs** seeded on alignments of **poly(lactic acid) nanofibrous scaffolds** prior transplantation. A continuous stimulation pattern and an intermittent stimulation pattern were compared. As a result of continuous stimulation, NPCs proliferate and differentiate into oligodendrocytic and neuronal lineages preferentially. In contrast, intermittent stimulation patterns did not affect NPCs proliferation with detrimental effects on neural differentiation [313].

Eduardo D. Gomes *et al.* investigated the therapeutic potential of a hydrogel made from **Gellan Gum** (GG) modified with Gly-Arg-Gly-Asp-Ser (GRGDS) peptide, combined with **ASCs** and **OECs**, for cervical SCI in rats. The study found that five weeks post-injury, the group receiving the combinatorial therapy of hydrogel and cells showed increased inspiratory bursting of the affected hemidiaphragm compared to untreated animals, particularly at the ventral portion of the muscle. Additionally, this group showed a significant reduction in mechanical allodynia, as assessed by Von Frey testing, indicating a potential improvement in sensory function. No differences were observed in forelimb motor function or markers for axonal regrowth, astrogliosis, and inflammatory cells [314].

Table I.1. *Pre-clinical combinatorial approaches for SCI repair: cells and biomaterials (from 2013 to 2023).*

Model/Injury	Cells	Biomaterial	Outcome	Reference
Rat/Contusion	MSCs	Nanofiber-hydrogel composite (NHC)	Did not find a significant effect of NHC on MSC transplant survival, and hind limb function was similar across all groups	[300]
Rat/Compression T2	Adult rat SVZ-derived NSPCs	PDGF-A-conjugated HAMC	Enhanced sparing of host oligodendrocytes and neurons, increased fine motor (horizontal ladder) changes but not gross motor skills (BBB)	[298]
Rat/Hemisection T13-L2	Neonatal NPCs	4 mm porous collagen	Increased neuronal differentiation, increased in	[315]

		scaffold + neutralizing antibody of EGFR	BBB score and angle on inclined plane	
Rat/Transection T9-10	co-culture SC-NT-3 and BMSC-TrkC	2 mm 3D gelatin sponge scaffold	Enhanced axonal growth throughout the biomaterial, synaptic association of these cells with serotonergic neurons and some CST axons, upregulation of c-Fos in the grafted as well as host lumbar spinal cord cells and improvement of BBB score	[303]
Rat/Transection T3	BMSCs	2 mm multi-component fiber bundled agarose scaffolds	Increased growth of raphespinal, reticulospinal tracts and sensory fibers into but not exiting scaffold	[301]
Rat/ Hemisection T9	BMSCs	3 mm honeycomb collagen scaffold	Rats implanted with HC scaffolds containing BMSCs displayed better motor and sensory recovery than those implanted with HC scaffolds only	[316]
Rat/Contusion T9	Human DPSCs	Chitosan scaffolds	Higher cell viability and neural differentiation in the DPSC/chitosan-scaffold group and also a marked recovery of hind limb locomotor functions.	[302]
Rat/Hemisection T9	co-hEnSC	PCL/gelatin nanofibrous sc	The growth of neuronal cells was confirmed, and the recovery of sensory and motor	[304]

			functions was also confirmed in this group.	
Rat/ Contusion T10-11	hESC	HA-based hydrogel	Encapsulation of (hESC-NS) in HA-based hydrogel can increased differentiation these cells into oligodendrocytes and improved locomotor function.	[305]
Rat/Excision T8-9	hUC-MSCs	collagen scaffold [(NeuroRegen Scaffold (NRS)]	NRS and hUC-MSCs promoted locomotion in rats and improved cortical motor- and somatosensory-evoked potentials	[317]
Rat/Compression T10-12	NSCs	porous collagen scaffolds	Enhancing NSC neuronal differentiation and functional integration in vivo, enabling robust axonal elongation, and reducing astrogliosis.	[306]
Rat/ Contusion T10	NSCs	Matrigel scaffold	SCI rats transplanted with NSCs in Matrigel showed improved behavioral recovery and neuronal and reactive astrocyte marker expression levels compared to PBS- or Matrigel-transplanted rats.	[307]
Rat/ Transection T10	NSCs	Print 3D collagen/silk fibroin scaffold	3D-CF + NSCs group with increased amplitude in motor evoked potential tests. The filling of injury cavity was the best and glial scarring was reduced in the same group.	[308]

Rat/ Transection T3	NPCs	Print 3D biomimetic hydrogel scaffolds	Injured host axons regenerate into 3D biomimetic scaffolds and synapse onto NPCs implanted into the device and that implanted NPCs in turn extend axons out of the scaffold and into the host spinal cord below the injury	[318]
Rat/Complete transection T9	hbNSPCs and hscNSPCs	Aligned collagen sponge scaffold (ACSS)	Compared with the hbNSPC–ACSS, the hscNSPC–ACSS effectively promoted long-term cell survival and neuronal differentiation and reduce inflammation and glial scar formation. The transplanted hscNSPC–ACSS improved recovery of locomotor functions.	[319]
Rat/Transection T8	Adult rat SCs	4 mm PAN/PVC and fluid or pre-gelled Matrigel	Greater growth of virally traced brainstem-derived axons into and up to the caudal interface, formed synaptic junctions with the help of newly formed supportive astrocytic protrusions	[320]
Rat/Hemisection C5	Adult GFP-SCs	2 mm alginate-based anisotropic capillary hydrogel	Serotonergic growth through and caudal to the biomaterial up to the 1 mm SC injection site	[321]

Rat/ Transection T8	SCs	piezoelectric polyvinylidene fluoride trifluoroethylene (PVDF-TrFE)	Caused enhancements in axonal growth and formation of blood vessels following transection	[311]
Rat/Transection T9-10	Activated SCs and/or rat BM-MSCs	3 mm PLGA scaffold	Both exhibited significant recovery of nerve function as shown by the BBB score and electrophysiological test results.	[312]
Rat/Hemisection T13	OEC	2 mm collagen-based multi-channel 3D matrices	No improvement in CatWalk gait analysis or alleviation of allodynia	[322]
Rat/Transection T9-10	OECs	2 mm PLGA scaffolds	Increased BBB score and crossing of inclined plane, increased axonal preservation, decreased astrogliosis	[323]
Rat/Hemisection C2	ASCs and OECs	Gellan Gum (GG)-based hydrogel	Beneficial effects on both diaphragmatic recovery and sensory function	[324]
Rat/ Transection T9-10	OECs	Spinal cord decellularized (DC) scaffolds	Better axon regeneration promoting protein expression than the SCI model and improve the proliferation and distribution of astrocytes at the site of injury.	[310]
Mouse/ Contusion T9	NS/PCs	FTY720-incorporated PLGA nanoparticles	Improvement in neurological functions and minimized tissue damage. Increased the survival of transplanted cells and promoted their differentiation towards more oligodendrocytic phenotype	[325]
Rat/Transection T9-10	MSCs	Microhydrogel fibers	Significantly improved electrophysiological expression and re-established limb motor function	[326]

For all the combinations of biomaterials and cells mentioned above, approximately 80% reported functional and histological recovery, while 12% showed only histological improvement with no functional recovery and 8% had no improvements at all.

Based in these previous positive results, several clinical trials combining cells with scaffolds were initiated (**Table I.2**).

Table I.2. *Current clinical trials on scaffolds with cells for SCI treatment. Adverse event (AE); Functional Independence Measure (FIM); Motor Evoked Potential (MEP); Magnetic resonance imaging (MRI), Olfactory nerve fibroblasts (ONFs); Somatosensory Evoked Potential (SSEP).*

ClinicalTrials.gov identifier	Stem cells	Biomaterial	SCI	ASIA Scale	Nº Patients	Timing for Transplantation	Primary Outcomes
NCT03933072 [327]	Autologous OECs, ONFs	Collagen scaffold	Complete SCI between C5-T10	ASIA A	2	> 8 months	ASIA Berg Balance scale Deep sensation Vibration Joint position sense
NCT02688049 [328]	MSCs, NSCs	NeuroRegen Scaffold	Complete SCI between C5-T12	ASIA A	30	Chronic SCI	ASIA SSEP MEP
NCT02688062 [329]	BMMCs	NeuroRegen Scaffold	Complete hsSCI – thoracic	ASIA A	22	Chronic SCI	ASIA SSEP/MEP FIM MRI Bladder/Bowel Function Safety/Tolerability/AE
NCT02510365 [330]	Autologous BMMCs	NeuroRegen Scaffolds	Complete SCI – thoracic	ASIA A	7	< 4 months	ASIA SSEP MEP

3.5.3.2. Combination of biomaterials and molecular agents

Nowadays, there is a preference for localized delivery over systemic delivery since the outcomes are more efficient in terms of reducing detrimental side effects [331]. It is important to note that systemic delivery of an agent commonly requires high doses of the drug, which increases the risk of negative side effects [332]. However, controlled drug delivery greatly reduces the risk of these side effects. The most common way to deliver drugs locally is to use hydrogels, fibers, microparticles, and nanoparticles, either implanted or injected, or a combination thereof. For instance, the difference between systemic and localized/controlled delivery of **methylprednisolone** shown that only the delivery using nanoparticles of Poly(lactide-co-glycolide) (**PLGA**) embedded in agarose hydrogels reduced lesion volume and downregulate pro-inflammatory proteins [332]. Similarly, PLGA/F-127 gels can also be used to locally deliver **hirudin**, a thrombin inhibitor, with an improved motor function and a reduction of activated macrophages in patients with spinal cord injuries [333].

Susana R. Cerqueira et al. investigates the potential of dendrimer **nanoparticles** loaded with **methylprednisolone** as a therapeutic approach for SCI. The authors report that these nanoparticles can effectively penetrate the blood-brain barrier and reduce microglial activation, which is an important factor in SCI pathology. The study also demonstrates that the dendrimer nanoparticles are biocompatible and have a low toxicity profile, indicating their potential as a safe therapeutic option [334].

PEG formulations containing **magnesium** were developed to increase the effectiveness of tolerable magnesium dose [143]. SCI rats with this combination demonstrated enhanced tissue sparing and functional recovery [142].

In 2013, a work from China found that sustained delivery of dibutyryl cyclic adenosine monophosphate (**dbcAMP**), an analog of cAMP, loaded in poly(propylene carbonate) (**PPC**) fibers, into the spinal cord, can promote functional recovery, axonal regenerative sprouting, stimulate axonal outgrowth and reduce astrogliosis after spinal cord hemisection [335]. Furthermore, in 2014, Simonetta Papa et al. showed that after SCI, To-Pro3-loaded poly(methyl methacrylate) (**PMMA**) nanoparticles embedded in **agarose** carbomer hydrogel could be a promising drug carrier for the selective administration in activated microglia/macrophages and thus potentially able to counteract relevant secondary inflammatory events in SCI [336].

In another work was shown that **Cabazitaxel** [a microtubule inhibitor micelles (Cab-M)] embedded in a **P10R5-LA** copolymer hydrogel reduces scar formation and axon inhibitory molecules. Additionally, this hydrogel helped sustain the Cab-M release [337]. Another group combined **Cetuximab** with **Taxol co-modified collagen** scaffolds and showed significantly increased neural regeneration. Furthermore, scaffold transplantation decreased the deposition of scar-related inhibitors within the lesion in a rat severe SCI model [338].

In a different approach, a group in 2021 tried to implant **Taxol**-modified linear ordered **collagen** scaffold (LOCS) after a scar tissue removal surgery (one or two surgeries) in canine SCI model. They saw that Taxol-LOCS combination induces axonal regeneration, neurogenesis, and electrophysiological and functional recovery after one scar tissue removal surgery. The researchers also discovered that one scar tissue removal was associated with more activation of endogenous NSCs in the injured rather than the second procedure [339].

Bighinati et al. developed an implantable polymeric delivery system and implanted a biomaterial (electrospun poly (l-lactic acid) [**PLLA**]) loaded with **ibuprofen** and **triiodothyronine** (T3) to reduce inflammation in a rat model of T8 contusion SCI. PLLA + ibuprofen + T3 improved endogenous regeneration and functional locomotion by reducing glutamate release at 24 hours and eight days post-

injury compared to PLLA-implanted rats. The treatment also reduced the lesion volume at sixty days post-injury.

Serpin is an inhibitor of serine protease and an immunomodulatory biologic drug, which can decrease inflammation and protect neurons for SCI therapy. In animal models of SCI, **chitosan**, and stable **collagen** hydrogel combined to produce a highly biodegradable material with low antigenicity and good biocompatibility. Thus, serpin delivered by chitosan-collagen hydrogel (CCH) was found to maintain the therapeutic effect and promote functional recovery in SCI rats [340].

Recently, Zahra Nazemi and colleagues showed another form of a co-delivery system. In this method, **paclitaxel (PTX)** is encapsulated in **PLGA** microspheres. The encapsulated product was injected into a **minocycline**-loaded hydrogel and inserted into the lesion site. As a result, inflammatory cell activity and neuronal regeneration decreased, as well as fibrotic scar size [341] and the co-delivered system also avoids the toxicity of PTX on peripheral nerve tissue [342].

Another study combined small molecules known for increasing neuronal differentiation of rat spinal cord NSCs, such as **LDN193189** (a potent inhibitor of the bone morphogenetic pathway, inhibiting ALK1, ALK2, ALK3, and ALK6), **SB-431542** (potent and selective inhibitor of TGF-β type I), **CHIR99021** (potent glycogen synthase kinase (GSK) 3 inhibitor) and **P7C3-A20** (an aminopropyl carbazole that elevates cellular nicotinamide adenine dinucleotide), loaded in a collagen hydrogel. The authors showed that this combination induced neurogenesis and inhibited astrogliogenesis of endogenous NSCs in the lesion site [343].

Biomaterials had also been combined with growth factors and extracellular vesicles (EVs) (**Table I.3**). As both growth factors and EVs have low stability, short half-lives, and rapid inactivation by enzymes, biomaterials can prolong and sustain its delivery, thereby increasing overall effectiveness [344].

According to one study from 2012, **PLGA** microspheres can be used to deliver Sonic Hedgehog (**Shh**) [345]. Shh is a multifunctional growth factor that has a critical role in spinal cord formation, acting as an axon guidance molecule and promoting the formation of motor neurons and oligodendrocytes from ventral cord progenitor cells. Sustained Shh delivery by PLGA microspheres led to nerve fibers' sprouting, oligodendrocytes' proliferation, and decreased gliosis [345].

Fibroblast growth factor 2 (FGF2; important role in the regulation of cell survival, cell division, cell differentiation, and cell migration [346]) and **NT-3** (promotes the survival and differentiation of existing neurons and the growth and differentiation of new neurons [347]) combined with **PLGA** nanoparticles also demonstrated prolonged, but localized release when dispersed in **hyaluronan-methylcellulose** hydrogels [348,349]. Another study tested the sustained delivery of vascular endothelial growth factor

(VEGF), angiopoietin-1 (**Ang-1**), basic Fibroblast Growth Factor (**bFGF**) using **PLGA** microspheres. Results demonstrated increased miR-210 expression, angiogenesis, suppressed Eph-A3, higher recruiting of neural precursors, and an increased spared white matter [350].

The gelatin nanostructured lipid carriers (**GNLs**) were also used to prolong the nerve growth factor (**NGF**) release and to enhance neuroprotection and recovery after SCI by downregulating the endoplasmic reticulum stress-induced apoptosis proteins. [351]. The **poly (propylene fumarate)-collagen scaffold** coated with **NT-3** also demonstrated to promote the regeneration of neural tissues, leading to functional recovery [352]. Furthermore, a recent study by Liming Li and his team used **exosomes derived from hMSCs** immobilized in peptide-modified adhesive hydrogel (**Exo-pGel**) to treat injured nerve tissues. This combination licit significant nerve recovery and urinary tissue preservation by effectively mitigating inflammation and oxidation [353]

Table I.3. *Pre-Clinical Combinatorial Approaches for SCI repair: Biomaterials and small molecules.*

Model	Molecules	Biomaterial	Outcome	Reference
Mice/Contusion T9-10	Shh	PLGA microspheres	Sprouting of nerve fibers, the proliferation of oligodendrocytes, the decrease of gliosis and behavioral recovery after SCI	[345]
Rat/Compression T2	FGF2	PLGA nanoparticles	Achieved higher blood vessel density in the dorsal horns, due to either greater angiogenesis near the epicenter of the injury or vasoprotection acutely after spinal cord injury.	[348]
Rat/Compression T1-2	NT-3	PLGA nanoparticles	Significant axon growth with no effect on the astroglial response to injury and functional recovery in comparison with vehicle and injury controls.	[349]

Rat/Contusion T9	VEGF, Ang-1, and bFGF	PLGA microspheres	Stimulate neoangiogenesis and neurogenesis, accelerating recovery of neurologic function.	[350]
Rat/Compression T9	NGF	GNLs	Enhanced the neuroprotection effect in SCI rats compared with NGF alone. This combination has higher monodispersity, half-life and stability.	[351]
Rat/ Transection T9	NT-3	Poly (propylene fumarate)-collagen scaffold	Improves the inhibitory microenvironment, facilitates axonal and neuronal regeneration, survival of various types of functional neurons and remyelination and synapse formation of regenerated axons.	[352]
Rat/ Transection T9-10	Exosomes derived from hMSCs	Peptide-modified adhesive hydrogel	Significant nerve recovery and urinary tissue preservation by effectively mitigating inflammation and oxidation.	[353]
Rat/transection T2	BDNF	Hydrogel - HA and MC	Enhancing axonal growth and promoting neural stem cell formation	[354]
Rat/Compression T9	HGF	Gelatin-FA hydrogel	HGF combined with gelatin-FA hydrogel promoted endogenous repair and recovery more effectively than HGF with hydrogel.	[355]

For all the combinations of biomaterials and small molecules mentioned above, approximately 78% reported functional and histological recovery and 22% showed histological recovery with no functional improvements.

3.5.3.3. Combination of biomaterials, cells and biomolecules

In 2020, Roxana Rodríguez-Barrera et al. tried a different approach and combined immunization with neural derived peptides (**INDP**), specifically with A9187–99 peptide, scar removal [(**SR**); scar was removed by performing a single incision with a double-bladed scalpel leaving a space of 2 mm in length] and the use of and a fibrin glue matrix (FGM) with MSCs (**FGM-MSCs**). The combination (SR + INDP + FGM-MSCs) modifies the non-permissive microenvironment post SCI, but it was not capable of inducing a proper axonal regeneration or neurogenesis when compared to the treatment with INDP alone. When compared to the rest of the groups, INDP alone significantly increased motor recovery, anti-inflammatory cytokines, regeneration-associated molecules, axonal regeneration, and neurogenesis [356].

To reach an approach that can give neuroprotection and promote neuroregeneration, another group combined **INDP**, inhibition of glial scar formation (dipyridyl: **DPY**), as well as the use of biocompatible matrix (**FGM**) impregnated with **bone marrow MSCs**. The combination therapy (DPY+ INDP + FG + MSCs) was the best strategy to promote motor and sensory function recovery. A significant increase in axonal density and tissue preservation was also observed in the combination therapy group [357].

Satoshi Nori et al., in 2018, describes a study that evaluated the therapeutic potential of combining human oligodendrogenic neural progenitor cells (**oNPCs**) with sustained delivery of **ChABC** using an innovative affinity release strategy in a **crosslinked methylcellulose hydrogel** for the treatment of chronic SCI in a thoracic compression rat model. The researchers transplanted oNPCs into rats with chronic SCI and administered ChABC to enhance the survival and differentiation of the transplanted cells [358]. The study found that the combination therapy improved motor function in the rats, as well as increased myelin thickness and decreased the formation of glial scars. The findings of this study highlight the potential of using stem cell-based therapies and ChABC for the treatment of chronic SCI [358].

In another work, it was hypothesized that human dental stem cells of the apical papilla (**SCAP**) combined with pharmacologically active microcarriers (**PAMs**) releasing **BDNF** would recuperate rat locomotor function by immunomodulation and neuroprotection. SCAP BDNF-PAMs were administered in a rat contusion model of SCI. SCAP BDNF-PAMs enhanced rat BBB scoring, decreased the expression of inducible nitric oxide synthase (iNOS) and increased the expression of βIII tubulin, Growth Associated Protein 43 (GAP 43), and 5-hydroxytryptamine (5-HT) [359].

3.5.3.4. Combination of Neuromodulation Technologies with molecules or cells.

It was hypothesized that combinations of **electrical stimulation with pharmacological or/and cell transplantation** may cooperate synergistically to activate and functionally remodel spinal locomotor circuits, possibly allowing a coordinated and context-dependent function of the paralyzed hindlimbs of rats after a complete SCI.

In 2009, Courtine G lab tested a combination of 5-HT2A and 5-HT1A/7 **serotonin agonists** and **EES** at two positions distal to the lesion and they found that this combination allows rats with SCI to produce full weight-bearing bipedal treadmill locomotion that is almost undifferentiable from voluntary stepping before the injury. Additionally, the group demonstrated that sensory input allows adaptive motor patterns to develop without supraspinal influences [360]. Other groups tested similar combinations with similarly encouraging results [361–363].

In a different perspective, others have tried to see the effects of combination of **transcranial magnetic stimulation (TMS)** with cells, specifically **BMSC** transplantation, or a **Raf inhibitor (RafI)** on spinal cord injury in rats [364]. Based on their findings, combination therapy reduced spinal cord lesions and neuronal apoptosis caused by SCI, increased GAP43, NGF and BDNF expression levels, downregulated GFAP expression levels, and inhibited Raf/MEK/ERK signaling pathway activation in SCI model rats compared to monotherapy.

Another multi-combinational approach has been done by Eleni Sinopoulou et al., with cervical contused rats receiving intraspinal injections of a neuroplasticity-promoting mediator, lentiviral-**ChABC**, and 11 weeks of **cortical EES** (3 hours daily, 5 days/week) and behavioral **rehabilitation** (15 minutes daily, 5 days/week). With this combination treatment, muscle activity and upper limb dexterity significantly increased compared to the single treatment or no treatment [365].

In order to bypass an extrinsic factor that inhibit axon elongation, repulsive guidance molecule-a (RGMa), that is upregulated around spinal cord lesion sites, Hajime Yamanaka et al. used a combinational approach with an antibody **anti-RGMa** to promotes axon sprouting, regeneration, and motor recovery after SCI and a repetitive transcranial magnetic stimulation (**rTMS**). Dexterous hand movements were restored more effectively with the combination of antibody and rTMS [366].

Other neuromodulatory technology that is being used is photobiomodulation. A non-invasive light-driven intervention, or low-level laser/light therapy (LLLT) [367], that uses red and near-infrared (NIR) light to stimulate healing processes, reduce pain, and reduce inflammation in several tissues, including the nervous system [368–374]. The mechanism is not fully understood, however, the absorption of light by cytochrome C oxidase accelerates electron reactions in mammalian mitochondria. Additionally, it increases energy metabolism and ATP production and prevents NO-induced cell death [375].

Kentaro Yamane et al. [370] conjugated human umbilical cord mesenchymal stem cells **(hUCMSCs)** and **photobiomodulation** with a laser in rat SCI model. In this study, the animals were distributed into SCI, hUCMSCs, laser and combination treatments (hUCMSCs + Laser) groups. They saw that the combined treatment significantly reduced secondary damage and promoted functional recovery compared to either monotherapy [376]. The **photobiomodulation** therapy was also combined with **ChABC** to decrease neuropathic pain, a frequent complication after SCI. With this attempt, there was a reduction in allodynia and thermal hyperalgesia with photobiomodulation, ChABC, and the combination, but it did not reduce mechanical hyperalgesia [377]. Evidence has suggested that **photobiomodulation** and human adipose-derived stem cells (**hADSCs**) have neuroprotective effects after SCI. The combined effect of the hADSCs and laser therapy significantly improved motor function and alleviated hyperalgesia and allodynia associated with SCI [378].

Conclusions

Spinal cord regeneration is a complex physiological process, and no optimal treatment has yet been developed for fully repairing the spinal cord.

There have been several studies in recent years showing the effects of various treatment options on SCI. Despite this, these results are difficult to summarize because of the dispersed research and the complexity of the involved processes. Moreover, some factors might have been overlooked when designing the studies. For instance, cell type and injection site might be influenced by several factors, including the patient's age and lesion size. Yet, most studies generally transplant the same number of cells to all patients. Therefore, clinical trials lack an accurate view of cell use and novel therapeutic approaches are still needed to provide surgeons with user-friendly and efficient protocols. To achieve common goals, physicians and basic scientists must cooperate.

Another problem with most clinical trials involving SCI is the lack of a comparable control group. Possibly, this is because patients refused to be included in the control group. To overcome this problem, it will be important to involve more patients in the studies to better summarize the results. This may be possible with the cooperation between several hospitals and research centers.

Several approaches such as biomolecules, stem cells, and 3D scaffolds are being explored to regenerate the spinal cord. Despite the evidence suggesting that these strategies can be useful for spinal cord reconstruction, they do not appear to adequately improve clinical outcomes. So, the idea of combining these strategies emerge as a more effective solution than using them alone. Theoretically, combinatorial

strategies have the highest chance of creating an environment that mimics a healthy spinal cord's niche with potentially better results. For the injured spinal cord to be restored structurally and functionally, a combination of neuroprotective and neuroregenerative agents may need to be used. In our analysis, most of the works reported functional and/or histological improvements when using a combinatorial approach. However, it is important to be aware of the publication bias, when only positive results are published, it can create a skewed view of the scientific evidence and lead to incorrect conclusions. Publication bias can lead to wasted resources and missed opportunities to learn from unsuccessful experiments. Moreover, combinatorial trials also own many challenges. The combination may cause toxic effects or block the effects of one or more therapeutic agents. For all studies (pre-clinical or clinical trials), a large number of animals/individuals are needed since controls should be included for all groups and combinations. Moreover, different time-points of administration, dosages or routes of administration can produce different results. So, identifying the most effective combinatorial therapies for SCI is complex, time consuming and expensive, but it might be crucial to find a successful clinical treatment.

References

1. James, S. L., Bannick, M. S., Montjoy-Venning, W. C., Lucchesi, L. R., Dandona, L., Dandona, R., Hawley, C., Hay, S. I., Jakovljevic, M., Khalil, I., Krohn, K. J., Mokdad, A. H., Naghavi, M., Nichols, E., Reiner, R. C., Smith, M., Feigin, V. L., Vos, T., Murray, C. J. L., ... Zaman, S. B. (2019). Global, regional, and national burden of traumatic brain injury and spinal cord injury, 1990-2016: A systematic analysis for the Global Burden of Disease Study 2016. The Lancet Neurology, 18(1), 56–87. https://doi.org/10.1016/S1474-4422(18)30415-0
2. Ackery, A., Tator, C., & Krassioukov, A. (2004). A Global Perspective on Spinal Cord Injury Epidemiology. Journal of Neurotrauma, 21(10), 1355–1370. https://doi.org/10.1089/NEU.2004.21.1355
3. Singh, A., Tetreault, L., Kalsi-Ryan, S., Nouri, A., & Fehlings, M. G. (2014). Global prevalence and incidence of traumatic spinal cord injury. Clinical Epidemiology, 6, 309–331. https://doi.org/10.2147/CLEP.S68889
4. University of Alabama at Birmingham. (2021). Spinal Cord Injury Facts and Figures at a Glance. National Spinal Cord Injury Statistical Center.
5. Sadowsky, C., Volshteyn, O., Schultz, L., & McDonald, J. W. (2002). Spinal cord injury. Disability and Rehabilitation, 24(13), 680–687. https://doi.org/10.1080/09638280110110640
6. Wilson, J. R., Cadotte, D. W., & Fehlings, M. G. (2012). Clinical predictors of neurological outcome, functional status, and survival after traumatic spinal cord injury: a systematic review. Journal of Neurosurgery: Spine, 17(Suppl1), 11–26. https://doi.org/10.3171/2012.4.AOSPINE1245
7. Hachem, L. D., Ahuja, C. S., & Fehlings, M. G. (2017). Assessment and management of acute spinal cord injury: From point of injury to rehabilitation. The Journal of Spinal Cord Medicine, 40(6), 665–675. https://doi.org/10.1080/10790268.2017.1329076
8. Middleton, J. W., Dayton, A., Walsh, J., Rutkowski, S. B., Leong, G., & Duong, S. (2012). Life expectancy after spinal cord injury: a 50-year study. Spinal Cord 2012 50:11, 50(11), 803–811. https://doi.org/10.1038/sc.2012.55
9. Shavelle, R. M., Paculdo, D. R., Tran, L. M., Strauss, D. J., Brooks, J. C., & De Vivo, M. J. (2015). Mobility, continence, and life expectancy in persons with ASIA impairment scale Grade D spinal cord injuries. American Journal of Physical Medicine and Rehabilitation, 94(3), 180–191. https://doi.org/10.1097/PHM.0000000000000140
10. Oyinbo C. A. (2011). Secondary injury mechanisms in traumatic spinal cord injury: a nugget of this multiply cascade - PubMed. Acta Neurobiologiae Experimentalis, 71(2), 281–299.
11. Dumont, Randall J.; Okonkwo, David O.; Verma, Subodh; Hurlbert, R. John; Boulos, Paul T.; Ellegala, Dilantha B.; Dumont, A. S. (2001). Acute Spinal Cord Injury, Part I: Pathophysiologic Mechanism... : Clinical Neuropharmacology. Clinical Neuropharmacology, 24(5), 254–264.
12. L H Sekhon M G Fehlings. (2001). Epidemiology, Demographics, and Pathophysiology of Acute Spi... : Spine. Spine, 26(24S), S2–S12.
13. Tator, C. H., & Fehlings, M. G. (1991). Review of the secondary injury theory of acute spinal cord trauma with emphasis on vascular mechanisms. Journal of Neurosurgery, 75(1), 15–26. https://doi.org/10.3171/JNS.1991.75.1.0015
14. Rowland, J. W., Hawryluk, G. W. J., Kwon, B., & Fehlings, M. G. (2008). Current status of acute spinal cord injury pathophysiology and emerging therapies: promise on the horizon. Neurosurgical Focus, 25(5). https://doi.org/10.3171/FOC.2008.25.11.E2
15. Fehlings, M. G., Smith, J. S., Kopjar, B., Arnold, P. M., Yoon, S. T., Vaccaro, A. R., Brodke, D. S., Janssen, M. E., Chapman, J. R., Sasso, R. C., Woodard, E. J., Banco, R. J., Massicotte, E. M., Dekutoski, M. B., Gokaslan, Z. L., Bono, C. M., & Shaffrey, C. I. (2012). Perioperative and delayed

complications associated with the surgical treatment of cervical spondylotic myelopathy based on 302 patients from the AOSpine North America Cervical Spondylotic Myelopathy Study. Journal of Neurosurgery. Spine, 16(5), 425–432. https://doi.org/10.3171/2012.1.SPINE11467

16. Choo, A. M. Te, Liu, J., Liu, Z., Dvorak, M., Tetzlaff, W., & Oxland, T. R. (2009). Modeling spinal cord contusion, dislocation, and distraction: Characterization of vertebral clamps, injury severities, and node of Ranvier deformations. Journal of Neuroscience Methods, 181(1), 6–17. https://doi.org/10.1016/J.JNEUMETH.2009.04.007
17. Figley, S. A., Khosravi, R., Legasto, J. M., Tseng, Y. F., & Fehlings, M. G. (2014). Characterization of Vascular Disruption and Blood–Spinal Cord Barrier Permeability following Traumatic Spinal Cord Injury. Journal of Neurotrauma, 31(6), 541. https://doi.org/10.1089/NEU.2013.3034
18. Hall, E. D., & Springer, J. E. (2004). Neuroprotection and acute spinal cord injury: A reappraisal. NeuroRX 2004 1:1, 1(1), 80–100. https://doi.org/10.1602/NEURORX.1.1.80
19. Alizadeh, A., Dyck, S. M., & Karimi-Abdolrezaee, S. (2019). Traumatic spinal cord injury: An overview of pathophysiology, models and acute injury mechanisms. Frontiers in Neurology, 10, 282. https://doi.org/10.3389/FNEUR.2019.00282/BIBTEX
20. Tator, C. H., & Fehlings, M. G. (1991). Review of the secondary injury theory of acute spinal cord trauma with emphasis on vascular mechanisms. Journal of Neurosurgery, 75(1), 15–26. https://doi.org/10.3171/JNS.1991.75.1.0015
21. Anwar, M. A., Al Shehabi, T. S., & Eid, A. H. (2016). Inflammogenesis of secondary spinal cord injury. Frontiers in Cellular Neuroscience, 10(APR), 98. https://doi.org/10.3389/FNCEL.2016.00098/BIBTEX
22. Mautes, A. E., Weinzierl, M. R., Donovan, F., & Noble, L. J. (2000). Vascular Events After Spinal Cord Injury: Contribution to Secondary Pathogenesis. Physical Therapy, 80(7), 673–687. https://doi.org/10.1093/PTJ/80.7.673
23. Cohen, D. M., Patel, C. B., Ahobila-Vajjula, P., Sundberg, L. M., Chacko, T., Liu, S. J., & Narayana, P. A. (2009). Blood–spinal cord barrier permeability in experimental spinal cord injury: dynamic contrast-enhanced MRI. NMR in Biomedicine, 22(3), 332–341. https://doi.org/10.1002/NBM.1343
24. Stys, P. K. (1998). Anoxic and ischemic injury of myelinated axons in CNS white matter: From mechanistic concepts to therapeutics. Journal of Cerebral Blood Flow and Metabolism, 18(1), 2–25.https://doi.org/10.1097/00004647-19980100000002/ASSET/IMAGES/LARGE/10.1097_00004647-199801000-00002-FIG1.JPEG
25. Philip V Peplow, Svetlana A Dambinova, Thomas A Gennarelli, B. M. (2017). Acute Brain Impairment: Scientific Discoveries and Translational Research. In Royal Society of Chemistry.
26. Hall, E. D., Wang, J. A., Bosken, J. M., & Singh, I. N. (2016). Lipid peroxidation in brain or spinal cord mitochondria after injury. Journal of Bioenergetics and Biomembranes, 48(2), 169–174. https://doi.org/10.1007/S10863-015-9600-5/FIGURES/1
27. Nouvelot, I. J. E. P. D. D. A. G. J. M. M. A. (1995). Modulation of mouse cerebral Na+,K(+)-ATPase activity by oxygen free radicals - PubMed. Neuroreport, 7(1), 333–337.
28. Hall, E. D., & Braughler, J. M. (1982). Effects of intravenous methylprednisolone on spinal cord lipid peroxidation and Na+ + K+)-ATPase activity. Dose-response analysis during 1st hour after contusion injury in the cat. Journal of Neurosurgery, 57(2), 247–253. https://doi.org/10.3171/JNS.1982.57.2.0247
29. Quadri, S. A., Farooqui, M., Ikram, A., Zafar, A., Khan, M. A., Suriya, S. S., Claus, C. F., Fiani, B., Rahman, M., Ramachandran, A., Armstrong, I. I. T., Taqi, M. A., & Mortazavi, M. M. (2018). Recent update on basic mechanisms of spinal cord injury. Neurosurgical Review 2018 43:2, 43(2), 425–441. https://doi.org/10.1007/S10143-018-1008-3

30. Villegas, R., Martinez, N. W., Lillo, J., Pihan, P., Hernandez, D., Twiss, J. L., & Court, F. A. (2014). Calcium Release from Intra-Axonal Endoplasmic Reticulum Leads to Axon Degeneration through Mitochondrial Dysfunction. Journal of Neuroscience, 34(21), 7179–7189. https://doi.org/10.1523/JNEUROSCI.4784-13.2014
31. Xu, G. Y., Liu, S., Hughes, M. G., & McAdoo, D. J. (2008). Glutamate-induced losses of oligodendrocytes and neurons and activation of caspase-3 in the rat spinal cord. Neuroscience, 153(4), 1034–1047. https://doi.org/10.1016/J.NEUROSCIENCE.2008.02.065
32. Tator, C. H., Hashimoto, R., Raich, A., Norvell, D., Fehlings, M. G., Harrop, J. S., Guest, J., Aarabi, B., & Grossman, R. G. (2012). Translational potential of preclinical trials of neuroprotection through pharmacotherapy for spinal cord injury. Journal of Neurosurgery: Spine, 17(Suppl1), 157–229. https://doi.org/10.3171/2012.5.AOSPINE12116
33. Fehlings, M. G., & Nguyen, D. H. (2010). Immunoglobulin G: A potential treatment to attenuate neuroinflammation following spinal cord injury. Journal of Clinical Immunology, 30(SUPPL. 1), 109–112. https://doi.org/10.1007/S10875-010-9404-7/FIGURES/1
34. Donnelly, D. J., & Popovich, P. G. (2008). Inflammation and its role in neuroprotection, axonal regeneration and functional recovery after spinal cord injury. Experimental Neurology, 209(2), 378–388. https://doi.org/10.1016/J.EXPNEUROL.2007.06.009
35. Phillip G. Popovich, Ping Wei, B. T. S. (1998). Cellular inflammatory response after spinal cord injury in sprague-dawley and lewis rats - Popovich - 1997 - Journal of Comparative Neurology - Wiley Online Library. The Journal of Comparative Neurology.
36. Hellenbrand, D. J., Quinn, C. M., Piper, Z. J., Morehouse, C. N., Fixel, J. A., & Hanna, A. S. (2021). Inflammation after spinal cord injury: a review of the critical timeline of signaling cues and cellular infiltration. Journal of Neuroinflammation, 18(1), 1–16. https://doi.org/10.1186/S12974-021-02337-2/FIGURES/5
37. Fleming, J. C., Norenberg, M. D., Ramsay, D. A., Dekaban, G. A., Marcillo, A. E., Saenz, A. D., Pasquale-Styles, M., Dietrich, W. D., & Weaver, L. C. (2006). The cellular inflammatory response in human spinal cords after injury. Brain, 129(12), 3249–3269. https://doi.org/10.1093/BRAIN/AWL296
38. Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., & Freeman, B. A. (1990). Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proceedings of the National Academy of Sciences, 87(4), 1620–1624. https://doi.org/10.1073/PNAS.87.4.1620
39. P.G. Popovich, B.T. Stokes, & C.C. Whitacre. (1996). Concept of autoimmunity following spinal cord injury: Possible roles for T lymphocytes in the traumatized central nervous system - Popovich - 1996 - Journal of Neuroscience Research - Wiley Online Library. Journal of Neuroscience Research.
40. Howe, C. L., Ure, D., Adelson, J. D., LaFrance-Corey, R., Johnson, A., & Rodriguez, M. (2007). CD8+ T cells directed against a viral peptide contribute to loss of motor function by disrupting axonal transport in a viral model of fulminant demyelination. Journal of Neuroimmunology, 188(1–2), 13–21. https://doi.org/10.1016/j.jneuroim.2007.04.005
41. Noble, L. J., Donovan, F., Igarashi, T., Goussev, S., & Werb, Z. (2002). Matrix metalloproteinases limit functional recovery after spinal cord injury by modulation of early vascular events. The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 22(17), 7526–7535. https://doi.org/10.1523/JNEUROSCI.22-17-07526.2002
42. Taoka, Y., Okajima, K., Uchiba, M., Murakami, K., Kushimoto, S., Johno, M., Naruo, M., Okabe, H., & Takatsuki, K. (1997). Role of neutrophils in spinal cord injury in the rat. Neuroscience, 79(4), 1177–1182. https://doi.org/10.1016/S0306-4522(97)00011-0
43. Ditor, D. S., Bao, F., Chen, Y., Dekaban, G. A., & Weaver, L. C. (2006). A therapeutic time window for anti-CD 11d monoclonal antibody treatment yielding reduced secondary tissue damage and

enhanced behavioral recovery following severe spinal cord injury. Journal of Neurosurgery. Spine, 5(4), 343–352. https://doi.org/10.3171/SPI.2006.5.4.343
44. Hauben, E., Agranov, E., Gothilf, A., Nero, U., Cohen, A., Smirnov, I., Steinman, L., & Schwartz, M. (2001). Posttraumatic therapeutic vaccination with modified myelin self-antigen prevents complete paralysis while avoiding autoimmune disease. The Journal of Clinical Investigation, 108(4), 591–599. https://doi.org/10.1172/JCI12837
45. Schori, H., Shechter, R., Shachar, I., & Schwartz, M. (2007). Genetic manipulation of CD74 in mouse strains of different backgrounds can result in opposite responses to central nervous system injury. Journal of Immunology (Baltimore, Md. : 1950), 178(1), 163–171. https://doi.org/10.4049/JIMMUNOL.178.1.163
46. N K Prasad, G Papoff, A Zeuner, E Bonnin, M D Kazatchkine, G Ruberti, & S V Kaveri. (1998). Therapeutic preparations of normal polyspecific IgG (IVIg) induce apoptosis in human lymphocytes and monocytes: a novel mechanism of action of IVIg involving the Fas apoptotic pathway - PubMed. Journal of Immunology, 161(7), 3781–3790.
47. Monteiro, S., Pinho, A. G., Macieira, M., Serre-Miranda, C., Cibrão, J. R., Lima, R., Soares-Cunha, C., Vasconcelos, N. L., Lentilhas-Graça, J., Duarte-Silva, S., Miranda, A., Correia-Neves, M., Salgado, A. J., & Silva, N. A. (2020). Splenic sympathetic signaling contributes to acute neutrophil infiltration of the injured spinal cord. Journal of Neuroinflammation, 17(1), 1–14. https://doi.org/10.1186/S12974-020-01945-8/FIGURES/5
48. Beattie, M. S., Li, Q., & Bresnahan, J. C. (2000). Cell death and plasticity after experimental spinal cord injury. Progress in Brain Research, 128, 9–21. https://doi.org/10.1016/S0079-6123(00)28003-5
49. Sheri L. Shuman, Jacqueline C. Bresnahan, & Michael S. Beattie. (1998). Apoptosis of microglia and oligodendrocytes after spinal cord contusion in rats - Shuman - 1997 - Journal of Neuroscience Research - Wiley Online Library. Journal of Neuroscience Research, 50(5), 798–808.
50. Zhang, Y., Yang, S., Liu, C., Han, X., Gu, X., & Zhou, S. (2021). Deciphering glial scar after spinal cord injury. Burns & Trauma, 9. https://doi.org/10.1093/BURNST/TKAB035
51. Anderson, M. A., Burda, J. E., Ren, Y., Ao, Y., O'Shea, T. M., Kawaguchi, R., Coppola, G., Khakh, B. S., Deming, T. J., & Sofroniew, M. V. (2016). Astrocyte scar formation aids central nervous system axon regeneration. Nature 2016 532:7598, 532(7598), 195–200. https://doi.org/10.1038/nature17623
52. Kimura-Kuroda, J., Teng, X., Komuta, Y., Yoshioka, N., Sango, K., Kawamura, K., Raisman, G., & Kawano, H. (2010). An in vitro model of the inhibition of axon growth in the lesion scar formed after central nervous system injury. Molecular and Cellular Neuroscience, 43(2), 177–187. https://doi.org/10.1016/J.MCN.2009.10.008
53. Logan, A., Berry, M., Gonzalez, A. M., Frautschy, S. A., Sporn, M. B., & Baird, A. (1994). Effects of Transforming Growth Factor β1, on Scar Production in the Injured Central Nervous System of the Rat. European Journal of Neuroscience, 6(3), 355–363. https://doi.org/10.1111/J.1460-9568.1994.TB00278.X
54. East, E., Golding, J. P., & Phillips, J. B. (2009). A versatile 3D culture model facilitates monitoring of astrocytes undergoing reactive gliosis. Journal of Tissue Engineering and Regenerative Medicine, 3(8), 634. https://doi.org/10.1002/TERM.209
55. Renault-Mihara, F., Mukaino, M., Shinozaki, M., Kumamaru, H., Kawase, S., Baudoux, M., Ishibashi, T., Kawabata, S., Nishiyama, Y., Sugai, K., Yasutake, K., Okada, S., Nakamura, M., & Okano, H. (2017). Regulation of RhoA by STAT3 coordinates glial scar formation. Journal of Cell Biology, 216(8), 2533–2550. https://doi.org/10.1083/JCB.201610102/VIDEO-3
56. Wanner, I. B., Anderson, M. A., Song, B., Levine, J., Fernandez, A., Gray-Thompson, Z., Ao, Y., & Sofroniew, M. V. (2013). Glial Scar Borders Are Formed by Newly Proliferated, Elongated Astrocytes

That Interact to Corral Inflammatory and Fibrotic Cells via STAT3-Dependent Mechanisms after Spinal Cord Injury. Journal of Neuroscience, 33(31), 12870–12886. https://doi.org/10.1523/JNEUROSCI.2121-13.2013
57. Bott, K., Upton, Z., Schrobback, K., Ehrbar, M., Hubbell, J. A., Lutolf, M. P., & Rizzi, S. C. (2010). The effect of matrix characteristics on fibroblast proliferation in 3D gels. Biomaterials, 31(32), 8454–8464. https://doi.org/10.1016/J.BIOMATERIALS.2010.07.046
58. Harris, G. M., Madigan, N. N., Lancaster, K. Z., Enquist, L. W., Windebank, A. J., Schwartz, J., & Schwarzbauer, J. E. (2017). Nerve Guidance by a Decellularized Fibroblast Extracellular Matrix. Matrix Biology, 60–61, 176–189. https://doi.org/10.1016/J.MATBIO.2016.08.011
59. Franze, K., Janmey, P. A., & Guck, J. (2013). Mechanics in Neuronal Development and Repair. Https://Doi.Org/10.1146/Annurev-Bioeng-071811-150045, 15, 227–251. https://doi.org/10.1146/ANNUREV-BIOENG-071811-150045
60. Jones, L. L., Margolis, R. U., & Tuszynski, M. H. (2003). The chondroitin sulfate proteoglycans neurocan, brevican, phosphacan, and versican are differentially regulated following spinal cord injury. Experimental Neurology, 182(2), 399–411. https://doi.org/10.1016/S0014-4886(03)00087-6
61. Bradbury, E. J., & Carter, L. M. (2011). Manipulating the glial scar: Chondroitinase ABC as a therapy for spinal cord injury. Brain Research Bulletin, 84(4–5), 306–316. https://doi.org/10.1016/J.BRAINRESBULL.2010.06.015
62. Jones, L. L., Yamaguchi, Y., Stallcup, W. B., & Tuszynski, M. H. (2002). NG2 Is a Major Chondroitin Sulfate Proteoglycan Produced after Spinal Cord Injury and Is Expressed by Macrophages and Oligodendrocyte Progenitors. Journal of Neuroscience, 22(7), 2792–2803. https://doi.org/10.1523/JNEUROSCI.22-07-02792.2002
63. Faulkner, J. R., Herrmann, J. E., Woo, M. J., Tansey, K. E., Doan, N. B., & Sofroniew, M. V. (2004). Reactive astrocytes protect tissue and preserve function after spinal cord injury. The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 24(9), 2143–2155. https://doi.org/10.1523/JNEUROSCI.3547-03.2004
64. Brennan, F. H., Li, Y., Wang, C., Ma, A., Guo, Q., Li, Y., Pukos, N., Campbell, W. A., Witcher, K. G., Guan, Z., Kigerl, K. A., Hall, J. C. E., Godbout, J. P., Fischer, A. J., McTigue, D. M., He, Z., Ma, Q., & Popovich, P. G. (2022). Microglia coordinate cellular interactions during spinal cord repair in mice. Nature Communications 2022 13:1, 13(1), 1–20. https://doi.org/10.1038/s41467-022-31797-0
65. Rowland, J. W., Hawryluk, G. W. J., Kwon, B., & Fehlings, M. G. (2008). Current status of acute spinal cord injury pathophysiology and emerging therapies: promise on the horizon. Neurosurgical Focus, 25(5), E2. https://doi.org/10.3171/FOC.2008.25.11.E2
66. Beattie, M. S., Hermann, G. E., Rogers, R. C., & Bresnahan, J. C. (2002). Chapter 4 Cell death in models of spinal cord injury. Progress in Brain Research, 137, 37–47. https://doi.org/10.1016/S0079-6123(02)37006-7
67. Coleman, M. P., & Perry, V. H. (2002). Axon pathology in neurological disease: A neglected therapeutic target. Trends in Neurosciences, 25(10), 532–537. https://doi.org/10.1016/S0166-2236(02)02255-5
68. Ehlers, M. D. (2004). Deconstructing the axon: Wallerian degeneration and the ubiquitin-proteasome system. Trends in Neurosciences, 27(1), 3–6. https://doi.org/10.1016/j.tins.2003.10.015
69. McDonald, J. W., Becker, D., Sadowsky, C. L., Jane, J. A., Conturo, T. E., & Schultz, L. M. (2002). Late recovery following spinal cord injury: Case report and review of the literature. Journal of Neurosurgery: Spine, 97(2), 252–265. https://doi.org/10.3171/SPI.2002.97.2.0252
70. Fehlings, M. G., Tetreault, L. A., Wilson, J. R., Kwon, B. K., Burns, A. S., Martin, A. R., Hawryluk, G., & Harrop, J. S. (2017). A Clinical Practice Guideline for the Management of Acute Spinal Cord Injury:

Introduction, Rationale, and Scope. Global Spine Journal, 7(3_supplement), 84S-94S. https://doi.org/10.1177/2192568217703387
71. Hadley, M. N., & Walters, B. C. (2013). Introduction to the guidelines for the management of acute cervical Spine and spinal cord injuries. Neurosurgery, 72(SUPPL.2), 5–16. https://doi.org/10.1227/NEU.0B013E3182773549
72. Ahuja, C. S., Badhiwala, J. H., & Fehlings, M. G. (2020). "Time is spine": the importance of early intervention for traumatic spinal cord injury. Spinal Cord 2020 58:9, 58(9), 1037–1039. https://doi.org/10.1038/s41393-020-0477-8
73. Wilson, J. R., Singh, A., Craven, C., Verrier, M. C., Drew, B., Ahn, H., Ford, M., & Fehlings, M. G. (2012). Early versus late surgery for traumatic spinal cord injury: the results of a prospective Canadian cohort study. Spinal Cord 2012 50:11, 50(11), 840–843. https://doi.org/10.1038/sc.2012.59
74. Van Middendorp, J. J., Hosman, A. J. F., & Doi, S. A. R. (2013). The Effects of the Timing of Spinal Surgery after Traumatic Spinal Cord Injury: A Systematic Review and Meta-Analysis. Journal of Neurotrauma, 30(21), 1781–1794. https://doi.org/10.1089/NEU.2013.2932
75. Fehlings, M. G., Vaccaro, A., Wilson, J. R., Singh, A., Cadotte, D. W., Harrop, J. S., Aarabi, B., Shaffrey, C., Dvorak, M., Fisher, C., Arnold, P., Massicotte, E. M., Lewis, S., & Rampersaud, R. (2012). Early versus Delayed Decompression for Traumatic Cervical Spinal Cord Injury: Results of the Surgical Timing in Acute Spinal Cord Injury Study (STASCIS). PLOS ONE, 7(2), e32037. https://doi.org/10.1371/JOURNAL.PONE.0032037
76. Jug, M., Kejžar, N., Vesel, M., Al Mawed, S., Dobravec, M., Herman, S., & Bajrović, F. F. (2015). Neurological Recovery after Traumatic Cervical Spinal Cord Injury Is Superior if Surgical Decompression and Instrumented Fusion Are Performed within 8 Hours versus 8 to 24 Hours after Injury: A Single Center Experience. Journal of Neurotrauma, 32(18), 1385–1392. https://doi.org/10.1089/NEU.2014.3767
77. Burke, J. F., Yue, J. K., Ngwenya, L. B., Winkler, E. A., Talbott, J. F., Pan, J. Z., Ferguson, A. R., Beattie, M. S., Bresnahan, J. C., Haefeli, J., Whetstone, W. D., Suen, C. G., Huang, M. C., Manley, G. T., Tarapore, P. E., & Dhall, S. S. (2019). Ultra-Early (<12 Hours) Surgery Correlates with Higher Rate of American Spinal Injury Association Impairment Scale Conversion after Cervical Spinal Cord Injury. Neurosurgery, 85(2), 199–203. https://doi.org/10.1093/NEUROS/NYY537
78. Levi, Lion M.D.; Wolf, Aizik M.D.; Belzberg, H. M. D. (1993). Hemodynamic Parameters in Patients with Acute Cervical Cord... : Neurosurgery. Neurosurgery, 1007–1017.
79. Vale, F. L., Burns, J., Jackson, A. B., & Hadley, M. N. (1997). Combined medical and surgical treatment after acute spinal cord injury: results of a prospective pilot study to assess the merits of aggressive medical resuscitation and blood pressure management. Journal of Neurosurgery, 87(2), 239–246. https://doi.org/10.3171/JNS.1997.87.2.0239
80. Ryken, T. C., Hurlbert, R. J., Hadley, M. N., Aarabi, B., Dhall, S. S., Gelb, D. E., Rozzelle, C. J., Theodore, N., & Walters, B. C. (2013). The acute cardiopulmonary management of patients with cervical spinal cord injuries. Neurosurgery, 72(SUPPL.2), 84–92. https://doi.org/10.1227/NEU.0B013E318276EE16
81. Bracken, M. B., Shepard, M. J., Holford, T. R., Leo-Summers, L., Aldrich, E. F., Fazl, M., Fehlings, M., Herr, D. L., Hitchon, P. W., Marshall, L. F., Nockels, R. P., Pascale, V., Perot, P. L., Piepmeier, J., Sonntag, V. K. H., Wagner, F., Wilberger, J. E., Winn, H. R., & Young, W. (1997). Administration of Methylprednisolone for 24 or 48 Hours or Tirilazad Mesylate for 48 Hours in the Treatment of Acute Spinal Cord Injury: Results of the Third National Acute Spinal Cord Injury Randomized Controlled Trial. JAMA, 277(20), 1597–1604. https://doi.org/10.1001/JAMA.1997.03540440031029

82. Bracken, M. B., Shepard, M. J., Hellenbrand, K. G., Collins, W. F., Leo, L. S., Freeman, D. F., Wagner, F. C., Flamm, E. S., Eisenberg, H. M., Goodman, J. H., Perot, P. L., Green, B. A., Grossman, R. G., Meagher, J. N., Young, W., Fischer, B., Clifton, G. L., Hunt, W. E., & Rifkinson, N. (1985). Methylprednisolone and neurological function 1 year after spinal cord injury: Results of the National Acute Spinal Cord Injury Study. Journal of Neurosurgery, 63(5), 704–713. https://doi.org/10.3171/JNS.1985.63.5.0704
83. Bracken, M. B., Shepard, M. J., Collins, W. F., Holford, T. R., Young, W., Baskin, D. S., Eisenberg, H. M., Flamm, E., Leo-Summers, L., Maroon, J., Marshall, L. F., Perot, P. L. Jr., Piepmeier, J., Sonntag, V. K. H., Wagner, F. C., Wilberger, J. E., & Winn, H. R. (2010). A Randomized, Controlled Trial of Methylprednisolone or Naloxone in the Treatment of Acute Spinal-Cord Injury. The New England Journal of Medicine, 322(20), 1405–1411. https://doi.org/10.1056/NEJM199005173222001
84. Walters, B. C., Hadley, M. N., Hurlbert, R. J., Aarabi, B., Dhall, S. S., Gelb, D. E., Harrigan, M. R., Rozelle, C. J., Ryken, T. C., & Theodore, N. (2013). Guidelines for the management of acute cervical spine and spinal cord injuries: 2013 update. Neurosurgery, 60(SUPPL. 1), 82–91. https://doi.org/10.1227/01.NEU.0000430319.32247.7F
85. Hurlbert, R. J., Hadley, M. N., Walters, B. C., Aarabi, B., Dhall, S. S., Gelb, D. E., Rozzelle, C. J., Ryken, T. C., & Theodore, N. (2015). Pharmacological therapy for acute spinal cord injury. Neurosurgery, 76, S71–S83. https://doi.org/10.1227/01.NEU.0000462080.04196.F7
86. Hawryluk, G. W. J., Rowland, J., Kwon, B. K., & Fehlings, M. G. (2008). Protection and repair of the injured spinal cord: a review of completed, ongoing, and planned clinical trials for acute spinal cord injury: A review. Neurosurgical Focus, 25(5), E14. https://doi.org/10.3171/FOC.2008.25.11.E14
87. Kusuyama, K., Tachibana, T., Yamanaka, H., Okubo, M., Yoshiya, S., & Noguchi, K. (2018). Upregulation of calcium channel alpha-2-delta-1 subunit in dorsal horn contributes to spinal cord injury-induced tactile allodynia. Spine Journal, 18(6), 1062–1069. https://doi.org/10.1016/j.spinee.2018.01.010
88. Faden, A. I., Jacobs, T. P., & Smith, M. T. (1984). Evaluation of the calcium channel antagonist nimodipine in experimental spinal cord ischemia. Journal of Neurosurgery, 60(4), 796–799. https://doi.org/10.3171/JNS.1984.60.4.0796
89. Fehlings, M. G., Tator, C. H., & Linden, R. D. (1989). The effect of nimodipine and dextran on axonal function and blood flow following experimental spinal cord injury. Journal of Neurosurgery, 71(3), 403–416. https://doi.org/10.3171/JNS.1989.71.3.0403
90. Langley, M., & Sorkin, E. (1989). Nimodipine. Drugs, 669–669.
91. Winkler, T., Sharma, H. S., Stålberg, E., Badgaiyan, R. D., Gordh, T., & Westman, J. (2003). An L-type calcium channel blocker, nimodipine influences trauma induced spinal cord conduction and axonal injury in the rat. Acta Neurochirurgica, Supplementum, 86, 425–432. https://doi.org/10.1007/978-3-7091-0651-8_88/COVER
92. Agrawal, S. K., Nashmi, R., & Fehlings, M. G. (2000). Role of L- and N-type calcium channels in the pathophysiology of traumatic spinal cord white matter injury. Neuroscience, 99(1), 179–188. https://doi.org/10.1016/S0306-4522(00)00165-2
93. Rosenberg, L. J., Teng, Y. D., & Wrathall, J. R. (1999). Effects of the Sodium Channel Blocker Tetrodotoxin on Acute White Matter Pathology After Experimental Contusive Spinal Cord Injury. Journal of Neuroscience, 19(14), 6122–6133. https://doi.org/10.1523/JNEUROSCI.19-14-06122.1999
94. Schwartz, G., & Fehlings, M. G. (2002). Secondary injury mechanisms of spinal cord trauma: a novel therapeutic approach for the management of secondary pathophysiology with the sodium channel blocker riluzole. Progress in Brain Research, 137, 177–190. https://doi.org/10.1016/S0079-6123(02)37016-X

95. Caglar, Y. S., Demirel, A., Dogan, I., Huseynov, R., Eroglu, U., Ozgural, O., Cansiz, C., Bahadir, B., Kilinc, M. C., & Al-Beyati, E. S. M. (2018). Effect of Riluzole on Spinal Cord Regeneration with Hemisection Method Before Injury. World Neurosurgery, 114, e247–e253. https://doi.org/10.1016/J.WNEU.2018.02.171
96. Vasconcelos, N. L., Gomes, E. D., Oliveira, E. P., Silva, C. J., Lima, R., Sousa, N., Salgado, A. J., & Silva, N. A. (2016). Combining neuroprotective agents: effect of riluzole and magnesium in a rat model of thoracic spinal cord injury. Spine Journal, 16(8), 1015–1024. https://doi.org/10.1016/j.spinee.2016.04.013
97. Guyot, M. C., Palfi, S., Stutzmann, J. M., Mazière, M., Hantraye, P., & Brouillet, E. (1997). Riluzole protects from motor deficits and striatal degeneration produced by systemic 3-nitropropionic acid intoxication in rats. Neuroscience, 81(1), 141–149. https://doi.org/10.1016/S0306-4522(97)00192-9
98. Lang-Lazdunski, L., Heurteaux, C., Mignon, A., Mantz, J., Widmann, C., Desmonts, J. M., & Lazdunski, M. (2000). Ischemic spinal cord injury induced by aortic cross-clamping: prevention by riluzole. European Journal of Cardio-Thoracic Surgery, 18(2), 174–181. https://doi.org/10.1016/S1010-7940(00)00430-9
99. Schwartz, G., & Fehlings, M. G. (2001). Evaluation of the neuroprotective effects of sodium channel blockers after spinal cord injury: improved behavioral and neuroanatomical recovery with riluzole. Journal of Neurosurgery: Spine, 94(2), 245–256. https://doi.org/10.3171/SPI.2001.94.2.0245
100. Wu, Y., Satkunendrarajah, K., Teng, Y., Chow, D. S. L., Buttigieg, J., & Fehlings, M. G. (2013). Delayed post-injury administration of riluzole is neuroprotective in a preclinical rodent model of cervical spinal cord injury. Journal of Neurotrauma, 30(6), 441–452. https://doi.org/10.1089/NEU.2012.2622/ASSET/IMAGES/LARGE/FIGURE8.JPEG
101. Grossman, R. G., Fehlings, M. G., Frankowski, R. F., Burau, K. D., Chow, D. S. L., Tator, C., Teng, A., Toups, E. G., Harrop, J. S., Aarabi, B., Shaffrey, C. I., Johnson, M. M., Harkema, S. J., Boakye, M., Guest, J. D., & Wilson, J. R. (2014). A prospective, multicenter, Phase i matched-comparison group trial of safety, pharmacokinetics, and preliminary efficacy of riluzole in patients with traumatic spinal cord injury. Journal of Neurotrauma, 31(3), 239–255. https://doi.org/10.1089/NEU.2013.2969/ASSET/IMAGES/LARGE/FIGURE5.JPEG
102. Fehlings, M. G., Nakashima, H., Nagoshi, N., Chow, D. S. L., Grossman, R. G., & Kopjar, B. (2016). Rationale, design and critical end points for the Riluzole in Acute Spinal Cord Injury Study (RISCIS): a randomized, double-blinded, placebo-controlled parallel multi-center trial. Spinal Cord, 54(1), 8. https://doi.org/10.1038/SC.2015.95
103. Bellingham, M. C. (2011). A Review of the Neural Mechanisms of Action and Clinical Efficiency of Riluzole in Treating Amyotrophic Lateral Sclerosis: What have we Learned in the Last Decade? CNS Neuroscience & Therapeutics, 17(1), 4–31. https://doi.org/10.1111/J.1755-5949.2009.00116.X
104. Grijalva, I., García-Pérez, A., Díaz, J., Aguilar, S., Mino, D., Santiago-Rodríguez, E., Guizar-Sahagún, G., Castañeda-Hernández, G., Maldonado-Julián, H., & Madrazo, I. (2010). High Doses of 4-Aminopyridine Improve Functionality in Chronic Complete Spinal Cord Injury Patients with MRI Evidence of Cord Continuity. Archives of Medical Research, 41(7), 567–575. https://doi.org/10.1016/J.ARCMED.2010.10.001
105. Page, J. C., Park, J., Chen, Z., Cao, P., & Shi, R. (2018). Parallel Evaluation of Two Potassium Channel Blockers in Restoring Conduction in Mechanical Spinal Cord Injury in Rat. Journal of Neurotrauma, 35(9), 1057–1068. https://doi.org/10.1089/NEU.2017.5297
106. Darlington, C. (2000). Fampridine Acorda Therapeutics. Current Opinion in Investigational Drugs (London, England : 2000), 1(3), 375–379.

107. Hayes, K. C. (2011). Impact of extended-release dalfampridine on walking ability in patients with multiple sclerosis. Neuropsychiatric Disease and Treatment, 7(1), 229–239. https://doi.org/10.2147/NDT.S10469
108. Proks, P., Reimann, F., Green, N., Gribble, F., & Ashcroft, F. (2002). Sulfonylurea Stimulation of Insulin Secretion. Diabetes, 51(suppl_3), S368–S376. https://doi.org/10.2337/DIABETES.51.2007.S368
109. Kurland, D. B., Tosun, C., Pampori, A., Karimy, J. K., Caffes, N. M., Gerzanich, V., & Simard, J. M. (2013). Glibenclamide for the Treatment of Acute CNS Injury. Pharmaceuticals 2013, Vol. 6, Pages 1287-1303, 6(10), 1287–1303. https://doi.org/10.3390/PH6101287
110. Caffes, N., Kurland, D. B., Gerzanich, V., & Marc Simard, J. (2015). Glibenclamide for the Treatment of Ischemic and Hemorrhagic Stroke. International Journal of Molecular Sciences 2015, Vol. 16, Pages 4973-4984, 16(3), 4973–4984. https://doi.org/10.3390/IJMS16034973
111. Simard, J. M., Popovich, P. G., Tsymbalyuk, O., & Gerzanich, V. (2012). Spinal cord injury with unilateral versus bilateral primary hemorrhage — Effects of glibenclamide. Experimental Neurology, 233(2), 829–835. https://doi.org/10.1016/J.EXPNEUROL.2011.11.048
112. Simard, J. M., Woo, S. K., Schwartzbauer, G. T., & Gerzanich, V. (2012). Sulfonylurea receptor 1 in central nervous system injury: A focused review. Journal of Cerebral Blood Flow and Metabolism, 32(9), 1699–1717. https://doi.org/10.1038/JCBFM.2012.91/ASSET/IMAGES/LARGE/10.1038_JCBFM.2012.91-FIG1.JPEG
113. Chen, Y., Wang, B., & Zhao, H. (2018). Thymoquinone reduces spinal cord injury by inhibiting inflammatory response, oxidative stress and apoptosis via PPAR-γ and PI3K/Akt pathways. Experimental and Therapeutic Medicine, 15(6), 4987–4994. https://doi.org/10.3892/ETM.2018.6072/HTML
114. Praveen Rao, P. N., & Knaus, E. E. (2008). Evolution of Nonsteroidal Anti-Inflammatory Drugs (NSAIDs): Cyclooxygenase (COX) Inhibition and Beyond. Journal of Pharmacy & Pharmaceutical Sciences, 11(2), 81s–110s. https://doi.org/10.18433/J3T886
115. Fu, Q., Hue, J., & Li, S. (2007). Nonsteroidal Anti-Inflammatory Drugs Promote Axon Regeneration via RhoA Inhibition. Journal of Neuroscience, 27(15), 4154–4164. https://doi.org/10.1523/JNEUROSCI.4353-06.2007
116. Wang, X., Budel, S., Baughman, K., Gould, G., Song, K. H., & Strittmatter, S. M. (2009). Ibuprofen Enhances Recovery from Spinal Cord Injury by Limiting Tissue Loss and Stimulating Axonal Growth. Journal of Neurotrauma, 26(1), 81–95. https://doi.org/10.1089/NEU.2007.0464
117. Kwon, B. K., Okon, E., Hillyer, J., Mann, C., Baptiste, D., Weaver, L. C., Fehlings, M. G., & Tetzlaff, W. (2011). A Systematic Review of Non-Invasive Pharmacologic Neuroprotective Treatments for Acute Spinal Cord Injury. Journal of Neurotrauma, 28(8), 1545–1588. https://doi.org/10.1089/NEU.2009.1149
118. Redondo-Castro, E., & Navarro, X. (2014). Chronic ibuprofen administration reduces neuropathic pain but does not exert neuroprotection after spinal cord injury in adult rats. Experimental Neurology, 252, 95–103. https://doi.org/10.1016/J.EXPNEUROL.2013.11.008
119. Amo-Aparicio, J., Garcia-Garcia, J., Francos-Quijorna, I., Urpi, A., Esteve-Codina, A., Gut, M., Quintana, A., & Lopez-Vales, R. (2021). Interleukin-4 and interleukin-13 induce different metabolic profiles in microglia and macrophages that relate with divergent outcomes after spinal cord injury. Theranostics, 11(20), 9805–9820. https://doi.org/10.7150/THNO.65203
120. Francos-Quijorna, I., Amo-Aparicio, J., Martinez-Muriana, A., & López-Vales, R. (2016). IL-4 drives microglia and macrophages toward a phenotype conducive for tissue repair and functional recovery after spinal cord injury. Glia, 64(12), 2079–2092. https://doi.org/10.1002/GLIA.23041

121. Lima, R., Monteiro, S., Lopes, J. P., Barradas, P., Vasconcelos, N. L., Gomes, E. D., Assunção-Silva, R. C., Teixeira, F. G., Morais, M., Sousa, N., Salgado, A. J., & Silva, N. A. (2017). Systemic Interleukin-4 Administration after Spinal Cord Injury Modulates Inflammation and Promotes Neuroprotection. Pharmaceuticals 2017, Vol. 10, Page 83, 10(4), 83. https://doi.org/10.3390/PH10040083
122. Monteiro, S., Salgado, A. J., & Silva, N. A. (2018). Immunomodulation as a neuroprotective strategy after spinal cord injury. Neural Regeneration Research, 13(3), 423–424. https://doi.org/10.4103/1673-5374.228722
123. Squair, J. W., Ruiz, I., Phillips, A. A., Zheng, M. M. Z., Sarafis, Z. K., Sachdeva, R., Gopaul, R., Liu, J., Tetzlaff, W., West, C. R., & Krassioukov, A. V. (2018). Minocycline Reduces the Severity of Autonomic Dysreflexia after Experimental Spinal Cord Injury. Journal of Neurotrauma, 35(24), 2861–2871. https://doi.org/10.1089/NEU.2018.5703
124. zhang, H., Xiang, Z., Duan, X., Jiang, J. li, Xing, Y. M., Zhu, C., Song, Q., & Yu, Q. R. (2019). Antitumor and anti-inflammatory effects of oligosaccharides from Cistanche deserticola extract on spinal cord injury. International Journal of Biological Macromolecules, 124, 360–367. https://doi.org/10.1016/J.IJBIOMAC.2018.11.132
125. Elmore, S. (2007). Apoptosis: A Review of Programmed Cell Death. Toxicologic Pathology, 35(4), 495. https://doi.org/10.1080/01926230701320337
126. Scholz, R., Sobotka, M., Caramoy, A., Stempfl, T., Moehle, C., & Langmann, T. (2015). Minocycline counter-regulates pro-inflammatory microglia responses in the retina and protects from degeneration. Journal of Neuroinflammation, 12(1), 1–14. https://doi.org/10.1186/S12974-015-0431-4/FIGURES/7
127. Tikka, T. M., & Koistinaho, J. E. (2001). Minocycline Provides Neuroprotection Against N-Methyl-d-aspartate Neurotoxicity by Inhibiting Microglia. The Journal of Immunology, 166(12), 7527–7533. https://doi.org/10.4049/JIMMUNOL.166.12.7527
128. Wells, J. E. A., Hurlbert, R. J., Fehlings, M. G., & Yong, V. W. (2003). Neuroprotection by minocycline facilitates significant recovery from spinal cord injury in mice. Brain, 126(7), 1628–1637. https://doi.org/10.1093/BRAIN/AWG178
129. Casha, S., Zygun, D., McGowan, M. D., Bains, I., Yong, V. W., & John Hurlbert, R. (2012). Results of a phase II placebo-controlled randomized trial of minocycline in acute spinal cord injury. Brain, 135(4), 1224–1236. https://doi.org/10.1093/BRAIN/AWS072
130. Casha, S., Zygun, D., McGowan, M. D., Bains, I., Yong, V. W., & John Hurlbert, R. (2013). Minocycline in Acute Spinal Cord Injury (MASC) - Full Text View - ClinicalTrials.gov. ClinicalTrials. Gov. https://clinicaltrials.gov/ct2/show/NCT01828203
131. Wrathall, J. R., Teng, Y. D., & Choiniere, D. (1996). Amelioration of Functional Deficits from Spinal Cord Trauma with Systemically Administered NBQX, an Antagonist of Non-N-methyl-D-aspartate receptors. Experimental Neurology, 137(1), 119–126. https://doi.org/10.1006/EXNR.1996.0012
132. Wrathall, J. R., Teng, Y. D., & Marriott, R. (1997). Delayed Antagonism of AMPA/Kainate Receptors Reduces Long-Term Functional Deficits Resulting from Spinal Cord Trauma. Experimental Neurology, 145(2), 565–573. https://doi.org/10.1006/EXNR.1997.6506
133. Ha, K. Y., Kim, Y. H., Rhyu, K. W., & Kwon, S. E. (2008). Pregabalin as a neuroprotector after spinal cord injury in rats. European Spine Journal, 17(6), 864–872. https://doi.org/10.1007/S00586-008-0653-6/FIGURES/5
134. Ha, K. Y., Carragee, E., Cheng, I., Kwon, S. E., & Kim, Y. H. (2011). Pregabalin as a Neuroprotector after Spinal Cord Injury in Rats: Biochemical Analysis and Effect on Glial Cells. Journal of Korean Medical Science, 26(3), 404–411. https://doi.org/10.3346/JKMS.2011.26.3.404
135. Wang, H. J., Cahoon, R., Cahoon, E. B., Zheng, H., Patel, K. P., & Zucker, I. H. (2015). Glutamatergic receptor dysfunction in spinal cord contributes to the exaggerated exercise pressor reflex in heart

failure. American Journal of Physiology - Heart and Circulatory Physiology, 308(5), H447–H455. https://doi.org/10.1152/AJPHEART.00735.2014/ASSET/IMAGES/LARGE/ZH400515139800008.JPEG
136. Cai, J., Sun, Y., Yin, Z., Wang, D., Shi, K., Fu, Y., Cao, X., & Ge, Y. (2018). Analysis of FK506-mediated functional recovery and neuroprotection in a rat model of spinal cord injury indicates that EGF is modulated in astrocytes. Experimental and Therapeutic Medicine, 16(2), 501–510. https://doi.org/10.3892/ETM.2018.6283/HTML
137. Kim, Y. H., Ha, K. Y., & Kim, S. Il. (2017). Spinal Cord Injury and Related Clinical Trials. Clinics in Orthopedic Surgery, 9(1), 1–9. https://doi.org/10.4055/CIOS.2017.9.1.1
138. Mori, E., Ueta, T., Maeda, T., Ideta, R., Yugue, I., Kawano, O., & Shiba, K. (2018). Sequential neurological improvements after conservative treatment in patients with complete motor paralysis caused by cervical spinal cord injury without bone and disc injury. Journal of Neurosurgery: Spine, 29(1), 1–9. https://doi.org/10.3171/2017.10.SPINE17844
139. Nowak, L., Bregestovski, P., Ascher, P., Herbet, A., & Prochiantz, A. (1984). Magnesium gates glutamate-activated channels in mouse central neurones. Nature 1984 307:5950, 307(5950), 462–465. https://doi.org/10.1038/307462a0
140. Lemke, M., Demediuk, P., McIntosh, T. K., Vink, R., & Faden, A. I. (1987). Alterations in tissue Mg++, Na+ and spinal cord edema following impact trauma in rats. Biochemical and Biophysical Research Communications, 147(3), 1170–1175. https://doi.org/10.1016/S0006-291X(87)80192-4
141. Cernak, I., Savic, V. J., Kotur, J., Prokic, V., Veljovic, M., & Grbovic, D. (2009). Characterization of Plasma Magnesium Concentration and Oxidative Stress Following Graded Traumatic Brain Injury in Humans. Journal of Neurotrauma, 17(1), 53–68. https://doi.org/10.1089/NEU.2000.17.53
142. Kaptanoglu, E., Beskonakli, E., Solaroglu, I., Kilinc, A., & Taskin, Y. (2003). Magnesium sulfate treatment in experimental spinal cord injury: Emphasis on vascular changes and early clinical results. Neurosurgical Review, 26(4), 283–287. https://doi.org/10.1007/S10143-003-0272-Y/TABLES/2
143. Kwon, B. K., Roy, J., Lee, J. H. T., Okon, E., Zhang, H., Marx, J. C., & Kindy, M. S. (2009). Magnesium Chloride in a Polyethylene Glycol Formulation as a Neuroprotective Therapy for Acute Spinal Cord Injury: Preclinical Refinement and Optimization. Journal of Neurotrauma, 26(8), 1379–1393. https://doi.org/10.1089/NEU.2009.0884
144. Lynch, B. A., Lambeng, N., Nocka, K., Kensel-Hammes, P., Bajjalieh, S. M., Matagne, A., & Fuks, B. (2004). The synaptic vesicle is the protein SV2A is the binding site for the antiepileptic drug levetiracetam. Proceedings of the National Academy of Sciences of the United States of America, 101(26), 9861–9866. https://doi.org/10.1073/PNAS.0308208101/ASSET/329D471F-1001-41CF-89A6-C93AD65511F9/ASSETS/GRAPHIC/ZPQ0260452550006.JPEG
145. Lambeng, N., Gillard, M., Vertongen, P., Fuks, B., & Chatelain, P. (2005). Characterization of [3H]ucb 30889 binding to synaptic vesicle protein 2A in the rat spinal cord. European Journal of Pharmacology, 520(1–3), 70–76. https://doi.org/10.1016/J.EJPHAR.2005.07.029
146. Yang, X. F., Weisenfeld, A., & Rothman, S. M. (2007). Prolonged Exposure to Levetiracetam Reveals a Presynaptic Effect on Neurotransmission. Epilepsia, 48(10), 1861–1869. https://doi.org/10.1111/J.1528-1167.2006.01132.X
147. Crowder, K. M., Gunther, J. M., Jones, T. A., Hale, B. D., Zhang, H. Z., Peterson, M. R., Scheller, R. H., Chavkin, C., & Bajjalieh, S. M. (1999). Abnormal neurotransmission in mice lacking synaptic vesicle protein 2A (SV2A). Proceedings of the National Academy of Sciences of the United States of America, 96(26), 15268–15273. https://doi.org/10.1073/PNAS.96.26.15268/ASSET/BD2F4684-78F1-4660-8F50-FC224C500ACB/ASSETS/GRAPHIC/PQ2694549006.JPEG

148. Lima, R., Monteiro, A., Salgado, A. J., Monteiro, S., & Silva, N. A. (2022). Pathophysiology and Therapeutic Approaches for Spinal Cord Injury. International Journal of Molecular Sciences 2022, Vol. 23, Page 13833, 23(22), 13833. https://doi.org/10.3390/IJMS232213833
149. Lima, R., Gomes, E. D., Cibrão, J. R., Rocha, L. A., Assunção-Silva, R. C., Rodrigues, C. S., Neves-Carvalho, A., Monteiro, S., Salgado, A. J., & Silva, N. A. (2021). Levetiracetam treatment leads to functional recovery after thoracic or cervical injuries of the spinal cord. Npj Regenerative Medicine 2021 6:1, 6(1), 1–10. https://doi.org/10.1038/s41536-021-00121-7
150. Xia, H., Huang, J., Liu, J., Zhang, H., Chen, D., Zhang, W., Zhou, X., Wei, Z., & Liu, Z. (2022). Levetiracetam Attenuates the Spinal Cord Injury Induced by Acute Trauma via Suppressing the Expression of Perforin. Computational and Mathematical Methods in Medicine, 2022. https://doi.org/10.1155/2022/7218666
151. Schnell, L., & Schwab, M. E. (1990). Axonal regeneration in the rat spinal cord produced by an antibody against myelin-associated neurite growth inhibitors. Nature 1990 343:6255, 343(6255), 269–272. https://doi.org/10.1038/343269a0
152. Merkler, D., Metz, G. A. S., Raineteau, O., Dietz, V., Schwab, M. E., & Fouad, K. (2001). Locomotor Recovery in Spinal Cord-Injured Rats Treated with an Antibody Neutralizing the Myelin-Associated Neurite Growth Inhibitor Nogo-A. Journal of Neuroscience, 21(10), 3665–3673. https://doi.org/10.1523/JNEUROSCI.21-10-03665.2001
153. Bregman, B. S., Kunkel-Bagden, E., Schnell, L., Dai, H. N., Gao, D., & Schwab, M. E. (1995). Recovery from spinal cord injury mediated by antibodies to neurite growth inhibitors. Nature 1995 378:6556, 378(6556), 498–501. https://doi.org/10.1038/378498a0
154. Freund, P., Schmidlin, E., Wannier, T., Bloch, J., Mir, A., Schwab, M. E., & Rouiller, E. M. (2006). Nogo-A–specific antibody treatment enhances sprouting and functional recovery after cervical lesion in adult primates. Nature Medicine 2006 12:7, 12(7), 790–792. https://doi.org/10.1038/nm1436
155. Freund, P., Wannier, T., Schmidlin, E., Bloch, J., Mir, A., Schwab, M. E., & Rouiller, E. M. (2007). Anti-Nogo-A antibody treatment enhances sprouting of corticospinal axons rostral to a unilateral cervical spinal cord lesion in adult macaque monkey. Journal of Comparative Neurology, 502(4), 644–659. https://doi.org/10.1002/CNE.21321
156. Liebscher, T., Schnell, L., Schnell, D., Scholl, J., Schneider, R., Gullo, M., Fouad, K., Mir, A., Rausch, M., Kindler, D., Hamers, F. P. T., & Schwab, M. E. (2005). Nogo-A antibody improves regeneration and locomotion of spinal cord–injured rats. Annals of Neurology, 58(5), 706–719. https://doi.org/10.1002/ANA.20627
157. Schwab, M. E. (2004). Nogo and axon regeneration. Current Opinion in Neurobiology, 14(1), 118–124. https://doi.org/10.1016/j.conb.2004.01.004
158. Kucher, K., Johns, D., Maier, D., Abel, R., Badke, A., Baron, H., Thietje, R., Casha, S., Meindl, R., Gomez-Mancilla, B., Pfister, C., Rupp, R., Weidner, N., Mir, A., Schwab, M. E., & Curt, A. (2018). First-in-man intrathecal application of neurite growth-promoting anti-nogo- a antibodies in acute spinal cord injury. Neurorehabilitation and Neural Repair, 32(6–7), 578–589. https://doi.org/10.1177/1545968318776371/ASSET/IMAGES/LARGE/10.1177_1545968318776371-FIG1.JPEG
159. Zörner, B., & Schwab, M. E. (2010). Anti-Nogo on the go: from animal models to a clinical trial. Annals of the New York Academy of Sciences, 1198(SUPPL. 1), E22–E34. https://doi.org/10.1111/J.1749-6632.2010.05566.X
160. Nikulina, E., Tidwell, J. L., Dai, H. N., Bregman, B. S., & Filbin, M. T. (2004). The phosphodiesterase inhibitor rolipram delivered after a spinal cord lesion promotes axonal regeneration and functional recovery. Proceedings of the National Academy of Sciences of the United States of America,

101(23), 8786–8790. https://doi.org/10.1073/PNAS.0402595101/ASSET/07733D1F-F0B4-4401-9203-5C0635C63266/ASSETS/GRAPHIC/ZPQ0240450730005.JPEG
161. Pearse, D. D., Pereira, F. C., Marcillo, A. E., Bates, M. L., Berrocal, Y. A., Filbin, M. T., & Bunge, M. B. (2004). cAMP and Schwann cells promote axonal growth and functional recovery after spinal cord injury. Nature Medicine 2004 10:6, 10(6), 610–616. https://doi.org/10.1038/nm1056
162. Gao, Y., Deng, K., Hou, J., Bryson, J. B., Barco, A., Nikulina, E., Spencer, T., Mellado, W., Kandel, E. R., & Filbin, M. T. (2004). Activated CREB is sufficient to overcome inhibitors in myelin and promote spinal axon regeneration in vivo. Neuron, 44(4), 609–621. https://doi.org/10.1016/j.neuron.2004.10.030
163. Beaumont, E., Whitaker, C. M., Burke, D. A., Hetman, M., & Onifer, S. M. (2009). Effects of rolipram on adult rat oligodendrocytes and functional recovery after contusive cervical spinal cord injury. Neuroscience, 163(4), 985–990. https://doi.org/10.1016/J.NEUROSCIENCE.2009.07.039
164. Heckman, P. R. A., Roig Kuhn, F., Raven, F., Bolsius, Y. G., Prickaerts, J., Meerlo, P., & Havekes, R. (2020). Phosphodiesterase inhibitors roflumilast and vardenafil prevent sleep deprivation-induced deficits in spatial pattern separation. Synapse, 74(6), e22150. https://doi.org/10.1002/SYN.22150
165. Bielekova, B., Richert, N., Howard, T., Packer, A. N., Blevins, G., Ohayon, J., McFarland, H. F., Stürzebecher, C. S., & Martin, R. (2009). Treatment with the phosphodiesterase type-4 inhibitor rolipram fails to inhibit blood—brain barrier disruption in multiple sclerosis. Multiple Sclerosis Journal, 15(10), 1206–1214. https://doi.org/10.1177/1352458509345903
166. Garnock-Jones, K. P. (2015). Roflumilast: A Review in COPD. Drugs 2015 75:14, 75(14), 1645–1656. https://doi.org/10.1007/S40265-015-0463-1
167. Sousa, C. S., Lima, R., Cibrão, J. R., Gomes, E. D., Fernandes, L. S., Pinho, T. S., Silva, D., Campos, J., Salgado, A. J., & Silva, N. A. (2023). Pre-Clinical Assessment of Roflumilast Therapy in a Thoracic Model of Spinal Cord Injury. Pharmaceutics 2023, Vol. 15, Page 1556, 15(5), 1556. https://doi.org/10.3390/PHARMACEUTICS15051556
168. Prabhakar, V., Capila, I., Bosques, C. J., Pojasek, K., & Sasisekharan, R. (2005). Chondroitinase ABC I from Proteus vulgaris: cloning, recombinant expression and active site identification. Biochemical Journal, 386(1), 103–112. https://doi.org/10.1042/BJ20041222
169. Tester, N. J., & Howland, D. R. (2008). Chondroitinase ABC improves basic and skilled locomotion in spinal cord injured cats. Experimental Neurology, 209(2), 483–496. https://doi.org/10.1016/J.EXPNEUROL.2007.07.019
170. García-Alías, G., Barkhuysen, S., Buckle, M., & Fawcett, J. W. (2009). Chondroitinase ABC treatment opens a window of opportunity for task-specific rehabilitation. Nature Neuroscience 2009 12:9, 12(9), 1145–1151. https://doi.org/10.1038/nn.2377
171. Bradbury, E. J., Moon, L. D. F., Popat, R. J., King, V. R., Bennett, G. S., Patel, P. N., Fawcett, J. W., & McMahon, S. B. (2002). Chondroitinase ABC promotes functional recovery after spinal cord injury. Nature 2002 416:6881, 416(6881), 636–640. https://doi.org/10.1038/416636a
172. Barritt, A. W., Davies, M., Marchand, F., Hartley, R., Grist, J., Yip, P., McMahon, S. B., & Bradbury, E. J. (2006). Chondroitinase ABC Promotes Sprouting of Intact and Injured Spinal Systems after Spinal Cord Injury. Journal of Neuroscience, 26(42), 10856–10867. https://doi.org/10.1523/JNEUROSCI.2980-06.2006
173. Shields, L. B. E., Zhang, Y. P., Burke, D. A., Gray, R., & Shields, C. B. (2008). Benefit of chondroitinase ABC on sensory axon regeneration in a laceration model of spinal cord injury in the rat. Surgical Neurology, 69(6), 568–577. https://doi.org/10.1016/J.SURNEU.2008.02.009
174. Gomes, E. D., Rocha, L. A., Assunção-Silva, R. C., Lima, R., Silva, N. A., & Salgado, A. J. (2020). Cell therapies for spinal cord injury regeneration. Spinal Cord Injury (SCI) Repair Strategies, 157–186. https://doi.org/10.1016/B978-0-08-102807-0.00009-0

175. Syková, E., & Jendelová, P. (2006). Magnetic Resonance Tracking of Transplanted Stem Cells in Rat Brain and Spinal Cord. Neurodegenerative Diseases, 3(1–2), 62–67. https://doi.org/10.1159/000092095
176. Urdzíková, L., Jendelová, P., Glogarová, K., Burian, M., Hájek, M., & Syková, E. (2006). Transplantation of Bone Marrow Stem Cells as well as Mobilization by Granulocyte-Colony Stimulating Factor Promotes Recovery after Spinal Cord Injury in Rats. Journal of Neurotrauma, 23(9), 1379–1391. https://doi.org/10.1089/NEU.2006.23.1379
177. Rossi, S. L., & Keirstead, H. S. (2009). Stem cells and spinal cord regeneration. Current Opinion in Biotechnology, 20(5), 552–562. https://doi.org/10.1016/J.COPBIO.2009.09.008
178. Willerth, S. M., & Sakiyama-Elbert, S. E. (2008). Cell therapy for spinal cord regeneration. Advanced Drug Delivery Reviews, 60(2), 263–276. https://doi.org/10.1016/J.ADDR.2007.08.028
179. Bianco, P., Robey, P. G., & Simmons, P. J. (2008). Mesenchymal Stem Cells: Revisiting History, Concepts, and Assays. Cell Stem Cell, 2(4), 313–319. https://doi.org/10.1016/j.stem.2008.03.002
180. Wobus, A. M., Holzhausen, H., Jäkel, P., & Schöneich, J. (1984). Characterization of a pluripotent stem cell line derived from a mouse embryo. Experimental Cell Research, 152(1), 212–219. https://doi.org/10.1016/0014-4827(84)90246-5
181. Polak, J. M., & Bishop, A. E. (2006). Stem Cells and Tissue Engineering: Past, Present, and Future. Annals of the New York Academy of Sciences, 1068(1), 352–366. https://doi.org/10.1196/ANNALS.1346.001
182. Elisseeff, J. H. (2004). Embryonic stem cells: Potential for more impact. Trends in Biotechnology, 22(4), 155–156. https://doi.org/10.1016/j.tibtech.2004.02.006
183. Ying, Q. L., Stavridis, M., Griffiths, D., Li, M., & Smith, A. (2003). Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. Nature Biotechnology 2003 21:2, 21(2), 183–186. https://doi.org/10.1038/nbt780
184. Keirstead, H. S., Nistor, G., Bernal, G., Totoiu, M., Cloutier, F., Sharp, K., & Steward, O. (2005). Human Embryonic Stem Cell-Derived Oligodendrocyte Progenitor Cell Transplants Remyelinate and Restore Locomotion after Spinal Cord Injury. Journal of Neuroscience, 25(19), 4694–4705. https://doi.org/10.1523/JNEUROSCI.0311-05.2005
185. Lukovic, D., Stojkovic, M., Moreno-Manzano, V., Bhattacharya, S. S., & Erceg, S. (2013). Perspectives and Future Directions of Human Pluripotent Stem Cell-Based Therapies: Lessons from Geron's Clinical Trial for Spinal Cord Injury. Stem Cells and Development, 23(1), 1–4. https://doi.org/10.1089/SCD.2013.0266
186. Sleeboom-Faulkner, M. (2008). Debates on Human Embryonic Stem Cell Research in Japan: Minority Voices and their Political Amplifiers. Science as Culture, 17(1), 85–97. https://doi.org/10.1080/09505430801915455
187. Takahashi, K., & Yamanaka, S. (2006). Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. Cell, 126(4), 663–676. https://doi.org/10.1016/j.cell.2006.07.024
188. Kobayashi, Y., Okada, Y., Itakura, G., Iwai, H., Nishimura, S., Yasuda, A., Nori, S., Hikishima, K., Konomi, T., Fujiyoshi, K., Tsuji, O., Toyama, Y., Yamanaka, S., Nakamura, M., & Okano, H. (2012). Pre-Evaluated Safe Human iPSC-Derived Neural Stem Cells Promote Functional Recovery after Spinal Cord Injury in Common Marmoset without Tumorigenicity. PLOS ONE, 7(12), e52787. https://doi.org/10.1371/JOURNAL.PONE.0052787
189. Sugai, K., Sumida, M., Shofuda, T., Yamaguchi, R., Tamura, T., Kohzuki, T., Abe, T., Shibata, R., Kamata, Y., Ito, S., Okubo, T., Tsuji, O., Nori, S., Nagoshi, N., Yamanaka, S., Kawamata, S., Kanemura, Y., Nakamura, M., & Okano, H. (2021). First-in-human clinical trial of transplantation of

iPSC-derived NS/PCs in subacute complete spinal cord injury: Study protocol. Regenerative Therapy, 18, 321–333. https://doi.org/10.1016/J.RETH.2021.08.005
190. Weiss, S., Dunne, C., Hewson, J., Wohl, C., Wheatley, M., Peterson, A. C., & Reynolds, B. A. (1996). Multipotent CNS Stem Cells Are Present in the Adult Mammalian Spinal Cord and Ventricular Neuroaxis. Journal of Neuroscience, 16(23), 7599–7609. https://doi.org/10.1523/JNEUROSCI.16-23-07599.1996
191. Iwanami, A., Yamane, J., Katoh, H., Nakamura, M., Momoshima, S., Ishii, H., Tanioka, Y., Tamaoki, N., Nomura, T., Toyama, Y., & Okano, H. (2005). Establishment of graded spinal cord injury model in a nonhuman primate: The common marmoset. Journal of Neuroscience Research, 80(2), 172–181. https://doi.org/10.1002/JNR.20435
192. Cummings, B. J., Uchida, N., Tamaki, S. J., Salazar, D. L., Hooshmand, M., Summers, R., Gage, F. H., & Anderson, A. J. (2005). Human neural stem cells differentiate and promote locomotor recovery in spinal cord-injured mice. Proceedings of the National Academy of Sciences of the United States of America, 102(39), 14069–14074. https://doi.org/10.1073/PNAS.0507063102/SUPPL_FILE/07063FIG9.JPG
193. Levi, A. D., Okonkwo, D. O., Park, P., Jenkins, A. L., Kurpad, S. N., Parr, A. M., Ganju, A., Aarabi, B., Kim, D., Casha, S., Fehlings, M. G., Harrop, J. S., Anderson, K. D., Gage, A., Hsieh, J., Huhn, S., Curt, A., & Guzman, R. (2018). Emerging safety of intramedullary transplantation of human neural stem cells in chronic cervical and thoracic spinal cord injury. Clinical Neurosurgery, 82(4), 562–575. https://doi.org/10.1093/NEUROS/NYX250
194. Levi, A. D., Anderson, K. D., Okonkwo, D. O., Park, P., Bryce, T. N., Kurpad, S. N., Aarabi, B., Hsieh, J., & Gant, K. (2019). Clinical Outcomes from a Multi-Center Study of Human Neural Stem Cell Transplantation in Chronic Cervical Spinal Cord Injury. Journal of Neurotrauma, 36(6), 891–902. https://doi.org/10.1089/NEU.2018.5843/ASSET/IMAGES/LARGE/FIGURE5.JPEG
195. Curt, A., Hsieh, J., Schubert, M., Hupp, M., Friedl, S., Freund, P., Huber, E., Pfyffer, D., Sutter, R., Jutzeler, C., Wüthrich, R. P., Min, K., Casha, S., Fehlings, M. G., & Guzman, R. (2020). The Damaged Spinal Cord Is a Suitable Target for Stem Cell Transplantation. Neurorehabilitation and Neural Repair, 34(8), 758–768. https://doi.org/10.1177/1545968320935815
196. Paino, C. L., & Bunge, M. B. (1991). Induction of axon growth into schwann cell implants grafted into lesioned adult rat spinal cord. Experimental Neurology, 114(2), 254–257. https://doi.org/10.1016/0014-4886(91)90043-C
197. Takami, T., Oudega, M., Bates, M. L., Wood, P. M., Kleitman, N., & Bunge, M. B. (2002). Schwann Cell But Not Olfactory Ensheathing Glia Transplants Improve Hindlimb Locomotor Performance in the Moderately Contused Adult Rat Thoracic Spinal Cord. Journal of Neuroscience, 22(15), 6670–6681. https://doi.org/10.1523/JNEUROSCI.22-15-06670.2002
198. Anderson, K. D., Guest, J. D., Dietrich, W. D., Bartlett Bunge, M., Curiel, R., Dididze, M., Green, B. A., Khan, A., Pearse, D. D., Saraf-Lavi, E., Widerström-Noga, E., Wood, P., & Levi, A. D. (2017). Safety of Autologous Human Schwann Cell Transplantation in Subacute Thoracic Spinal Cord Injury. Journal of Neurotrauma, 34(21), 2950–2963. https://doi.org/10.1089/NEU.2016.4895
199. Murray, I. R., & Péault, B. (2015). Q&A: Mesenchymal stem cells - where do they come from and is it important? BMC Biology, 13(1), 1–6. https://doi.org/10.1186/S12915-015-0212-7/FIGURES/1
200. Tavassoli, M., & Crosby, W. H. (1968). Transplantation of Marrow to Extramedullary Sites. Science, 161(3836), 54–56. https://doi.org/10.1126/SCIENCE.161.3836.54
201. Friedenstein, A. J., Chailakhjan, R. K., & Lalykina, K. S. (1970). THE DEVELOPMENT OF FIBROBLAST COLONIES IN MONOLAYER CULTURES OF GUINEA-PIG BONE MARROW AND SPLEEN CELLS. Cell Proliferation, 3(4), 393–403. https://doi.org/10.1111/J.1365-2184.1970.TB00347.X

202. Zuk, P. A., Zhu, M., Ashjian, P., De Ugarte, D. A., Huang, J. I., Mizuno, H., Alfonso, Z. C., Fraser, J. K., Benhaim, P., & Hedrick, M. H. (2002). Human adipose tissue is a source of multipotent stem cells. Molecular Biology of the Cell, 13(12), 4279–4295. https://doi.org/10.1091/MBC.E02-02-0105/ASSET/IMAGES/LARGE/MK1222051007.JPEG
203. Lee, O. K., Kuo, T. K., Chen, W. M., Lee, K. Der, Hsieh, S. L., & Chen, T. H. (2004). Isolation of multipotent mesenchymal stem cells from umbilical cord blood. Blood, 103(5), 1669–1675. https://doi.org/10.1182/BLOOD-2003-05-1670
204. Jackson, W. M., Nesti, L. J., & Tuan, R. S. (2010). Potential therapeutic applications of muscle-derived mesenchymal stem and progenitor cells. Expert Opinion on Biological Therapy, 10(4), 505–517. https://doi.org/10.1517/14712591003610606
205. Huang, G. T. J., Gronthos, S., & Shi, S. (2009). Mesenchymal Stem Cells Derived from Dental Tissues vs. Those from Other Sources: Their Biology and Role in Regenerative Medicine. Journal of Dental Research, 88(9), 792–806. https://doi.org/10.1177/0022034509340867
206. Jones, E. A., English, A., Henshaw, K., Kinsey, S. E., Markham, A. F., Emery, P., & McGonagle, D. (2004). Enumeration and Phenotypic Characterization of Synovial Fluid Multipotential Mesenchymal Progenitor Cells in Inflammatory and Degenerative Arthritis. Arthritis and Rheumatism, 50(3), 817–827. https://doi.org/10.1002/ART.20203
207. Janjanin, S., Djouad, F., Shanti, R. M., Baksh, D., Gollapudi, K., Prgomet, D., Rackwitz, L., Joshi, A. S., & Tuan, R. S. (2008). Human palatine tonsil: A new potential tissue source of multipotent mesenchymal progenitor cells. Arthritis Research and Therapy, 10(4), 1–12. https://doi.org/10.1186/AR2459/FIGURES/5
208. Shih, Y. R. V., Kuo, T. K., Yang, A. H., Lee, O. K., & Lee, C. H. (2009). Isolation and characterization of stem cells from the human parathyroid gland. Cell Proliferation, 42(4), 461–470. https://doi.org/10.1111/J.1365-2184.2009.00614.X
209. Jazedje, T., Perin, P. M., Czeresnia, C. E., Maluf, M., Halpern, S., Secco, M., Bueno, D. F., Vieira, N. M., Zucconi, E., & Zatz, M. (2009). Human fallopian tube: A new source of multipotent adult mesenchymal stem cells discarded in surgical procedures. Journal of Translational Medicine, 7(1), 1–10. https://doi.org/10.1186/1479-5876-7-46/FIGURES/4
210. Paul, G., Özen, I., Christophersen, N. S., Reinbothe, T., Bengzon, J., Visse, E., Jansson, K., Dannaeus, K., Henriques-Oliveira, C., Roybon, L., Anisimov, S. V., Renström, E., Svensson, M., Haegerstrand, A., & Brundin, P. (2012). The Adult Human Brain Harbors Multipotent Perivascular Mesenchymal Stem Cells. PLOS ONE, 7(4), e35577. https://doi.org/10.1371/JOURNAL.PONE.0035577
211. Fukuchi, Y., Nakajima, H., Sugiyama, D., Hirose, I., Kitamura, T., & Tsuji, K. (2004). Human Placenta-Derived Cells Have Mesenchymal Stem/Progenitor Cell Potential. Stem Cells, 22(5), 649–658. https://doi.org/10.1634/STEMCELLS.22-5-649
212. Schwab, K. E., Hutchinson, P., & Gargett, C. E. (2008). Identification of surface markers for prospective isolation of human endometrial stromal colony-forming cells. Human Reproduction, 23(4), 934–943. https://doi.org/10.1093/HUMREP/DEN051
213. Eiró, N., Sendon-Lago, J., Seoane, S., Bermúdez, M. A., Lamelas, M. L., Garcia-Caballero, T., Schneider, J., Perez-Fernandez, R., & Vizoso, F. J. (2014). Potential therapeutic effect of the secretome from human uterine cervical stem cells against both cancer and stromal cells compared with adipose tissue stem cells. Oncotarget, 5(21), 10692–10708. https://doi.org/10.18632/ONCOTARGET.2530
214. Cofano, F., Boido, M., Monticelli, M., Zenga, F., Ducati, A., Vercelli, A., & Garbossa, D. (2019). Mesenchymal Stem Cells for Spinal Cord Injury: Current Options, Limitations, and Future of Cell Therapy. International Journal of Molecular Sciences, 20(11). https://doi.org/10.3390/IJMS20112698

215. Szekiova, E., Slovinska, L., Blasko, J., Plsikova, J., & Cizkova, D. (2018). The neuroprotective effect of rat adipose tissue-derived mesenchymal stem cell-conditioned medium on cortical neurons using an in vitro model of SCI inflammation. Neurological Research, 40(4), 258–267. https://doi.org/10.1080/01616412.2018.1432266
216. Ribeiro, C. A., Fraga, J. S., Grãos, M., Neves, N. M., Reis, R. L., Gimble, J. M., Sousa, N., & Salgado, A. J. (2012). The secretome of stem cells isolated from the adipose tissue and Wharton jelly acts differently on central nervous system derived cell populations. Stem Cell Research and Therapy, 3(3), 1–7. https://doi.org/10.1186/SCRT109/TABLES/1
217. Guillén, M. I., Platas, J., Pérez del Caz, M. D., Mirabet, V., & Alcaraz, M. J. (2018). Paracrine anti-inflammatory effects of adipose tissue-derived mesenchymal stem cells in human monocytes. Frontiers in Physiology, 9(MAY), 661. https://doi.org/10.3389/FPHYS.2018.00661/BIBTEX
218. Assunção-Silva, R. C., Mendes-Pinheiro, B., Patrício, P., Behie, L. A., Teixeira, F. G., Pinto, L., & Salgado, A. J. (2018). Exploiting the impact of the secretome of MSCs isolated from different tissue sources on neuronal differentiation and axonal growth. Biochimie, 155, 83–91. https://doi.org/10.1016/J.BIOCHI.2018.07.026
219. Serra, S. C., Costa, J. C., Assunção-Silva, R. C., Teixeira, F. G., Silva, N. A., Anjo, S. I., Manadas, B., Gimble, J. M., Behie, L. A., & Salgado, A. J. (2018). Influence of passage number on the impact of the secretome of adipose tissue stem cells on neural survival, neurodifferentiation and axonal growth. Biochimie, 155, 119–128. https://doi.org/10.1016/J.BIOCHI.2018.09.012
220. Pinho, A. G., Cibrão, J. R., Lima, R., Gomes, E. D., Serra, S. C., Lentilhas-Graça, J., Ribeiro, C., Lanceros-Mendez, S., Teixeira, F. G., Monteiro, S., Silva, N. A., & Salgado, A. J. (2022). Immunomodulatory and regenerative effects of the full and fractioned adipose tissue derived stem cells secretome in spinal cord injury. Experimental Neurology, 351. https://doi.org/10.1016/J.EXPNEUROL.2022.113989
221. Pinho, A. G., Cibrão, J. R., Silva, N. A., Monteiro, S., & Salgado, A. J. (2020). Cell Secretome: Basic Insights and Therapeutic Opportunities for CNS Disorders. Pharmaceuticals 2020, Vol. 13, Page 31, 13(2), 31. https://doi.org/10.3390/PH13020031
222. Harkema, S. J. (2008). Plasticity of interneuronal networks of the functionally isolated human spinal cord. Brain Research Reviews, 57(1), 255–264. https://doi.org/10.1016/J.BRAINRESREV.2007.07.012
223. Mushahwar, V. K., & Horch, K. W. (2000). Muscle recruitment through electrical stimulation of the lumbo-sacral spinal cord. IEEE Transactions on Rehabilitation Engineering, 8(1), 22–29. https://doi.org/10.1109/86.830945
224. Mushahwar, V. K., Jacobs, P. L., Normann, R. A., Triolo, R. J., & Kleitman, N. (2007). New functional electrical stimulation approaches to standing and walking. Journal of Neural Engineering, 4(3), S181. https://doi.org/10.1088/1741-2560/4/3/S05
225. Barthélemy, D., Leblond, H., & Rossignol, S. (2007). Characteristics and mechanisms of locomotion induced by intraspinal microstimulation and dorsal root stimulation in spinal cats. Journal of Neurophysiology, 97(3), 1986–2000. https://doi.org/10.1152/JN.00818.2006/ASSET/IMAGES/LARGE/Z9K0030780730010.JPEG
226. Lavrov, I., Courtine, G., Dy, C. J., Van Den Brand, R., Fong, A. J., Gerasimenko, Y., Zhong, H., Roy, R. R., & Edgerton, V. R. (2008). Facilitation of Stepping with Epidural Stimulation in Spinal Rats: Role of Sensory Input. Journal of Neuroscience, 28(31), 7774–7780. https://doi.org/10.1523/JNEUROSCI.1069-08.2008
227. Lavrov, I., Dy, C. J., Fong, A. J., Gerasimenko, Y., Courtine, G., Zhong, H., Roy, R. R., & Edgerton, V. R. (2008). Epidural Stimulation Induced Modulation of Spinal Locomotor Networks in Adult Spinal Rats. Journal of Neuroscience, 28(23), 6022–6029. https://doi.org/10.1523/JNEUROSCI.0080-08.2008

228. Musienko, P. E., Zelenin, P. V., Orlovsky, G. N., & Deliagina, T. G. (2010). Facilitation of postural limb reflexes with epidural stimulation in spinal rabbits. Journal of Neurophysiology, 103(2), 1080–1092. https://doi.org/10.1152/JN.00575.2009/ASSET/IMAGES/LARGE/Z9K0021099430010.JPEG
229. Harkema, S., Gerasimenko, Y., Hodes, J., Burdick, J., Angeli, C., Chen, Y., Ferreira, C., Willhite, A., Rejc, E., Grossman, R. G., & Edgerton, V. R. (2011). Effect of epidural stimulation of the lumbosacral spinal cord on voluntary movement, standing, and assisted stepping after motor complete paraplegia: A case study. The Lancet, 377(9781), 1938–1947. https://doi.org/10.1016/S0140-6736(11)60547-3
230. Angeli, C. A., Edgerton, V. R., Gerasimenko, Y. P., & Harkema, S. J. (2014). Altering spinal cord excitability enables voluntary movements after chronic complete paralysis in humans. Brain, 137(5), 1394–1409. https://doi.org/10.1093/BRAIN/AWU038
231. Angeli, C. A., Boakye, M., Morton, R. A., Vogt, J., Benton, K., Chen, Y., Ferreira, C. K., & Harkema, S. J. (2018). Recovery of Over-Ground Walking after Chronic Motor Complete Spinal Cord Injury. New England Journal of Medicine, 379(13), 1244–1250. https://doi.org/10.1056/NEJMOA1803588/SUPPL_FILE/NEJMOA1803588_DISCLOSURES.PDF
232. Wagner, F. B., Mignardot, J. B., Le Goff-Mignardot, C. G., Demesmaeker, R., Komi, S., Capogrosso, M., Rowald, A., Seáñez, I., Caban, M., Pirondini, E., Vat, M., McCracken, L. A., Heimgartner, R., Fodor, I., Watrin, A., Seguin, P., Paoles, E., Van Den Keybus, K., Eberle, G., … Courtine, G. (2018). Targeted neurotechnology restores walking in humans with spinal cord injury. Nature 2018 563:7729, 563(7729), 65–71. https://doi.org/10.1038/s41586-018-0649-2
233. Gill, M. L., Grahn, P. J., Calvert, J. S., Linde, M. B., Lavrov, I. A., Strommen, J. A., Beck, L. A., Sayenko, D. G., Van Straaten, M. G., Drubach, D. I., Veith, D. D., Thoreson, A. R., Lopez, C., Gerasimenko, Y. P., Edgerton, V. R., Lee, K. H., & Zhao, K. D. (2018). Neuromodulation of lumbosacral spinal networks enables independent stepping after complete paraplegia. Nature Medicine 2018 24:11, 24(11), 1677–1682. https://doi.org/10.1038/s41591-018-0175-7
234. Lorach, H., Galvez, A., Spagnolo, V., Martel, F., Karakas, S., Intering, N., Vat, M., Faivre, O., Harte, C., Komi, S., Ravier, J., Collin, T., Coquoz, L., Sakr, I., Baaklini, E., Hernandez-Charpak, S. D., Dumont, G., Buschman, R., Buse, N., … Courtine, G. (2023). Walking naturally after spinal cord injury using a brain–spine interface. Nature 2023 618:7963, 618(7963), 126–133. https://doi.org/10.1038/s41586-023-06094-5
235. Courtine, G., Gerasimenko, Y., Van Den Brand, R., Yew, A., Musienko, P., Zhong, H., Song, B., Ao, Y., Ichiyama, R. M., Lavrov, I., Roy, R. R., Sofroniew, M. V., & Edgerton, V. R. (2009). Transformation of nonfunctional spinal circuits into functional states after the loss of brain input. Nature Neuroscience 2009 12:10, 12(10), 1333–1342. https://doi.org/10.1038/nn.2401
236. Wernig, A. (2015). No dawn yet of a new age in spinal cord rehabilitation. Brain, 138(7), e362–e362. https://doi.org/10.1093/BRAIN/AWU371
237. Langer, R. (2007). Editorial: Tissue Engineering: Perspectives, Challenges, and Future Directions. Tissue Engineering, 13(1), 1–2. https://doi.org/10.1089/TEN.2006.0219
238. Gomes, E. D., Mendes, S. S., Leite-Almeida, H., Gimble, J. M., Tam, R. Y., Shoichet, M. S., Sousa, N., Silva, N. A., & Salgado, A. J. (2016). Combination of a peptide-modified gellan gum hydrogel with cell therapy in a lumbar spinal cord injury animal model. Biomaterials, 105, 38–51. https://doi.org/10.1016/J.BIOMATERIALS.2016.07.019
239. Saraiva, C., Paiva, J., Santos, T., Ferreira, L., & Bernardino, L. (2016). MicroRNA-124 loaded nanoparticles enhance brain repair in Parkinson's disease. Journal of Controlled Release, 235, 291–305. https://doi.org/10.1016/J.JCONREL.2016.06.005

240. Schaub, N. J., Johnson, C. D., Cooper, B., & Gilbert, R. J. (2016). Electrospun Fibers for Spinal Cord Injury Research and Regeneration. Https://Home.Liebertpub.Com/Neu, 33(15), 1405–1415. https://doi.org/10.1089/NEU.2015.4165
241. Gelain, F., Panseri, S., Antonini, S., Cunha, C., Donega, M., Lowery, J., Taraballi, F., Cerri, G., Montagna, M., Baldissera, F., & Vescovi, A. (2011). Transplantation of nanostructured composite scaffolds results in the regeneration of chronically injured spinal cords. ACS Nano, 5(1), 227–236. https://doi.org/10.1021/NN102461W/SUPPL_FILE/NN102461W_SI_002.PDF
242. Liu, T., Houle, J. D., Xu, J., Chan, B. P., & Chew, S. Y. (2012). Nanofibrous Collagen Nerve Conduits for Spinal Cord Repair. Tissue Engineering Part A, 18(9–10), 1057–1066. https://doi.org/10.1089/TEN.TEA.2011.0430
243. Silva, N. A., Sousa, R. A., Fraga, J. S., Fontes, M., Leite-Almeida, H., Cerqueira, R., Almeida, A., Sousa, N., Reis, R. L., & Salgado, A. J. (2012). Benefits of Spine Stabilization with Biodegradable Scaffolds in Spinal Cord Injured Rats. Tissue Engineering Part C: Methods, 19(2), 101–108. https://doi.org/10.1089/TEN.TEC.2012.0264
244. Zhao, R. R., Andrews, M. R., Wang, D., Warren, P., Gullo, M., Schnell, L., Schwab, M. E., & Fawcett, J. W. (2013). Combination treatment with anti-Nogo-A and chondroitinase ABC is more effective than single treatments at enhancing functional recovery after spinal cord injury. European Journal of Neuroscience, 38(6), 2946–2961. https://doi.org/10.1111/EJN.12276
245. García-Alías, G., Petrosyan, H. A., Schnell, L., Horner, P. J., Bowers, W. J., Mendell, L. M., Fawcett, J. W., & Arvanian, V. L. (2011). Chondroitinase ABC Combined with Neurotrophin NT-3 Secretion and NR2D Expression Promotes Axonal Plasticity and Functional Recovery in Rats with Lateral Hemisection of the Spinal Cord. Journal of Neuroscience, 31(49), 17788–17799. https://doi.org/10.1523/JNEUROSCI.4308-11.2011
246. García-Alías, G., & Fawcett, J. W. (2012). Training and anti-CSPG combination therapy for spinal cord injury. Experimental Neurology, 235(1), 26–32. https://doi.org/10.1016/J.EXPNEUROL.2011.09.009
247. Ruzicka, J., Urdzikova, L. M., Svobodova, B., Amin, A. G., Karova, K., Dubisova, J., Zaviskova, K., Kubinova, S., Schmidt, M., Jhanwar-Uniyal, M., & Jendelova, P. (2018). Does combined therapy of curcumin and epigallocatechin gallate have a synergistic neuroprotective effect against spinal cord injury? Neural Regeneration Research, 13(1), 119–127. https://doi.org/10.4103/1673-5374.224379
248. Shi, T., Hao, J. X., Wiesenfeld-Hallin, Z., & Xu, X. J. (2018). Gabapentin and NMDA receptor antagonists interacts synergistically to alleviate allodynia in two rat models of neuropathic pain. Scandinavian Journal of Pain, 18(4), 687–693. https://doi.org/10.1515/SJPAIN-2018-0083/MACHINEREADABLECITATION/RIS
249. Vasconcelos, N. L., Gomes, E. D., Oliveira, E. P., Silva, C. J., Lima, R., Sousa, N., Salgado, A. J., & Silva, N. A. (2016). Combining neuroprotective agents: effect of riluzole and magnesium in a rat model of thoracic spinal cord injury. Spine Journal, 16(8), 1015–1024. https://doi.org/10.1016/j.spinee.2016.04.013
250. Zhao, W., Li, H., Hou, Y., Jin, Y., & Zhang, L. (2019). Combined Administration of Poly-ADP-Ribose Polymerase-1 and Caspase-3 Inhibitors Alleviates Neuronal Apoptosis After Spinal Cord Injury in Rats. World Neurosurgery, 127, e346–e352. https://doi.org/10.1016/J.WNEU.2019.03.116
251. Uyanikgil, Y., Cavusoglu, T., Kilic, K. D., Yigitturk, G., Celik, S., Tubbs, R. S., & Turgut, M. (2017). Useful effects of melatonin in peripheral nerve injury and development of the nervous system. Journal of Brachial Plexus and Peripheral Nerve Injury, 12(1), e1–e6. https://doi.org/10.1055/S-0036-1597838/ID/JR1600009-47

252. Zhang, Y., Zhang, W. X., Zhang, Y. J., Liu, Y. D., Liu, Z. J., Wu, Q. C., Guan, Y., & Chen, X. M. (2018). Melatonin for the treatment of spinal cord injury. Neural Regeneration Research, 13(10), 1685–1692. https://doi.org/10.4103/1673-5374.238603
253. Naseem, M., & Parvez, S. (2014). Role of melatonin in traumatic brain injury and spinal cord injury. Scientific World Journal, 2014. https://doi.org/10.1155/2014/586270
254. Bi, J., Sun, P., Feng, E., Shen, J., Chen, C., Tan, H., Li, Z., & Lin, Y. (2021). Melatonin Synergizes With Methylprednisolone to Ameliorate Acute Spinal Cord Injury. Frontiers in Pharmacology, 12. https://doi.org/10.3389/FPHAR.2021.723913
255. Jacobsen Teixeira, W. G., Cristante, A. F., Marcon, R. M., Bispo, G., Ferreira, R., & Pessoa de Barros-Filho, T. E. (2018). Granulocyte Colony-Stimulating Factor Combined with Methylprednisolone Improves Functional Outcomes in Rats with Experimental Acute Spinal Cord Injury. Clinics, 73, 1. https://doi.org/10.6061/CLINICS/2018/E235
256. Mirzaei, F., Meshkini, A., Habibi, B., Salehpour, F., Rafei, E., Fathi, W., Alavi, S. H. N., Majdi, A., Aghasan, S. R., & Alavi, S. A. N. (2020). Ceftriaxone Plus Methylprednisolone Combination Therapy Versus Methylprednisolone Monotherapy in Patients With Acute Spinal Cord Injury: A Randomized, Triple-Blind Clinical Trial. International Journal of Spine Surgery, 14(5), 706. https://doi.org/10.14444/7102
257. Ganjeifar, B., Rezaee, H., Keykhosravi, E., Tavallaii, A., Bahadorkhan, G., Nakhaei, M., & Abouei Mehrizi, M. A. (2021). The effect of combination therapy with erythropoietin and methylprednisolone in patients with traumatic cervical spinal cord injury: a pilot randomized controlled trial. Spinal Cord 2021 59:3, 59(3), 347–353. https://doi.org/10.1038/s41393-020-00604-2
258. de Barros, A. G. C., Cristante, A. F., Dos Santos, G. B., Natalino, R. J. M., Ferreira, R. J. R., & de Barros-Filho, T. E. P. (2019). Evaluation of the effects of erythropoietin and interleukin-6 in rats submitted to acute spinal cord injury. Clinics (Sao Paulo, Brazil), 74. https://doi.org/10.6061/CLINICS/2019/E674
259. Zhu, Y., Uezono, N., Yasui, T., Nakajo, M., Nagai, T., Wang, D., Nishibori, M., & Nakashima, K. (2021). Combinatrial treatment of anti-High Mobility Group Box-1 monoclonal antibody and epothilone B improves functional recovery after spinal cord contusion injury. Neuroscience Research, 172, 13–25. https://doi.org/10.1016/J.NEURES.2021.04.002
260. Hosseini, M., Karami, Z., Yousefifard, M., Janzadeh, A., Zamani, E., & Nasirinezhad, F. (2020). Simultaneous intrathecal injection of muscimol and endomorphin-1 alleviates neuropathic pain in rat model of spinal cord injury. Brain and Behavior, 10(5), e01576. https://doi.org/10.1002/BRB3.1576
261. Sharma, A., Feng, L., Muresanu, D. F., Huang, H., Menon, P. K., Sahib, S., Ryan Tian, Z., Lafuente, J. V., Buzoianu, A. D., Castellani, R. J., Nozari, A., Wiklund, L., & Sharma, H. S. (2021). Topical application of CNTF, GDNF and BDNF in combination attenuates blood-spinal cord barrier permeability, edema formation, hemeoxygenase-2 upregulation, and cord pathology. Progress in Brain Research, 266, 357–376. https://doi.org/10.1016/BS.PBR.2021.06.013
262. Baptiste, D. C., & Fehlings, M. G. (2007). Update on the treatment of spinal cord injury. Progress in Brain Research, 161, 217–233. https://doi.org/10.1016/S0079-6123(06)61015-7
263. Winkler, T., Sharma, H. S., Stålberg, E., & Westman, J. (1998). Spinal cord bioelectrical activity, edema and cell injury following a focal trauma to the rat spinal cord. An experimental study using pharmacological and morphological approaches. Spinal Cord Monitoring, 283–363. https://doi.org/10.1007/978-3-7091-6464-8_13
264. Ikegami, T., Nakamura, M., Yamane, J., Katoh, H., Okada, S., Iwanami, A., Watanabe, K., Ishii, K., Kato, F., Fujita, H., Takahashi, T., Okano, H. J., Toyama, Y., & Okano, H. (2005). Chondroitinase ABC combined with neural stem/progenitor cell transplantation enhances graft cell migration and outgrowth of growth-associated protein-43-positive fibers after rat spinal cord injury. European

Journal of Neuroscience, 22(12), 3036–3046. https://doi.org/10.1111/J.1460-9568.2005.04492.X
265. Zhilai, Z., Hui, Z., Anmin, J., Shaoxiong, M., Bo, Y., & Yinhai, C. (2012). A combination of taxol infusion and human umbilical cord mesenchymal stem cells transplantation for the treatment of rat spinal cord injury. Brain Research, 1481, 79–89. https://doi.org/10.1016/J.BRAINRES.2012.08.051
266. Wang, X., Wang, Y. Y., Zhang, L. L., Li, G. T., & Zhang, H. T. (2018). Combinatory effect of mesenchymal stromal cells transplantation and quercetin after spinal cord injury in rat. European Review for Medical and Pharmacological Sciences, 22(9), 2876–2887. https://doi.org/10.26355/EURREV_201805_14990
267. Gao, S., Ding, J., Xiao, H. J., Li, Z. Q., Chen, Y., Zhou, X. S., Wang, J. E., Wu, J., & Shi, W. Z. (2014). Anti-inflammatory and anti-apoptotic effect of combined treatment with methylprednisolone and amniotic membrane mesenchymal stem cells after spinal cord injury in rats. Neurochemical Research, 39(8), 1544–1552. https://doi.org/10.1007/S11064-014-1344-9/FIGURES/4
268. Namjoo, Z., Moradi, F., Aryanpour, R., Piryaei, A., Joghataei, M. T., Abbasi, Y., Hosseini, A., Hassanzadeh, S., Taklimie, F. R., Beyer, C., & Zendedel, A. (2018). Combined effects of rat Schwann cells and 17β-estradiol in a spinal cord injury model. Metabolic Brain Disease, 33(4), 1229–1242. https://doi.org/10.1007/S11011-018-0220-8/FIGURES/6
269. Pourkhodadad, S., Oryan, S., Kaka, G., & Sadraie, S. H. (2019). Neuroprotective Effects of Combined Treatment with Minocycline and Olfactory Ensheathing Cells Transplantation against Inflammation and Oxidative Stress after Spinal Cord Injury. Cell Journal (Yakhteh), 21(2), 220–228. https://doi.org/10.22074/CELLJ.2019.6126
270. Prager, J., Ito, D., Carwardine, D. R., Jiju, P., Chari, D. M., Granger, N., & Wong, L. F. (2021). Delivery of chondroitinase by canine mucosal olfactory ensheathing cells alongside rehabilitation enhances recovery after spinal cord injury. Experimental Neurology, 340, 113660. https://doi.org/10.1016/J.EXPNEUROL.2021.113660
271. Nori, S., Khazaei, M., Ahuja, C. S., Yokota, K., Ahlfors, J. E., Liu, Y., Wang, J., Shibata, S., Chio, J., Hettiaratchi, M. H., Führmann, T., Shoichet, M. S., & Fehlings, M. G. (2018). Human Oligodendrogenic Neural Progenitor Cells Delivered with Chondroitinase ABC Facilitate Functional Repair of Chronic Spinal Cord Injury. Stem Cell Reports, 11(6), 1433–1448. https://doi.org/10.1016/j.stemcr.2018.10.017
272. Mohammadshirazi, A., Sadrosadat, H., Jaberi, R., Zareikheirabadi, M., Mirsadeghi, S., Naghdabadi, Z., Ghaneezabadi, M., Fardmanesh, M., Baharvand, H., & Kiani, S. (2019). Combinational therapy of lithium and human neural stem cells in rat spinal cord contusion model. Journal of Cellular Physiology, 234(11), 20742–20754. https://doi.org/10.1002/JCP.28680
273. Ma, W., Zhan, Y., Zhang, Y., Xie, X., Mao, C., & Lin, Y. (2020). Enhanced Neural Regeneration with a Concomitant Treatment of Framework Nucleic Acid and Stem Cells in Spinal Cord Injury. ACS Applied Materials and Interfaces, 12(2), 2095–2106. https://doi.org/10.1021/ACSAMI.9B19079/ASSET/IMAGES/MEDIUM/AM9B19079_0001.GIF
274. Mohaghegh Shalmani, L., Valian, N., Pournajaf, S., Abbaszadeh, F., Dargahi, L., & Jorjani, M. (2020). Combination therapy with astaxanthin and epidermal neural crest stem cells improves motor impairments and activates mitochondrial biogenesis in a rat model of spinal cord injury. Mitochondrion, 52, 125–134. https://doi.org/10.1016/J.MITO.2020.03.002
275. Zhang, H., Huang, Z., Guo, M., Meng, L., Piao, M., Zhang, M., & Yu, H. (2021). Effect of combination therapy with neural stem cell transplantation and teramethylpyrazine in rats following acute spinal cord injury. Neuroreport, 32(16), 1311–1319. https://doi.org/10.1097/WNR.0000000000001725

276. Bonilla, P., Hernandez, J., Giraldo, E., González-Pérez, M. A., Alastrue-Agudo, A., Elkhenany, H., Vicent, M. J., Navarro, X., Edel, M., & Moreno-Manzano, V. (2021). Human-induced neural and mesenchymal stem cell therapy combined with a curcumin nanoconjugate as a spinal cord injury treatment. International Journal of Molecular Sciences, 22(11), 5966. https://doi.org/10.3390/IJMS22115966/S1
277. Salarinia, R., Hosseini, M., Mohamadi, Y., Ghorbani, A., Alamdari, D. H., Mafinezhad, A., & Sadeghnia, H. (2020). Combined use of platelet-rich plasma and adipose tissue-derived mesenchymal stem cells shows a synergistic effect in experimental spinal cord injury. Journal of Chemical Neuroanatomy, 110, 101870. https://doi.org/10.1016/J.JCHEMNEU.2020.101870
278. Zeraatpisheh, Z., Shamsi, F., Sarkoohi, P., Torabi, S., Alipour, H., & Aligholi, H. (2022). Effects of FTY720 on Neural Cell Behavior in Two and Three-Dimensional Culture and in Compression Spinal Cord Injury. Cellular and Molecular Bioengineering, 15(4), 331–340. https://doi.org/10.1007/S12195-022-00724-0
279. Orazizadeh, M., Rashidi, I., Saremi, J., & Latifi, M. (2009). Focal adhesion kinase (FAK) involvement in human endometrial remodeling during the menstrual cycle. Iranian Biomedical Journal, 13(2), 95–101.
280. Huang, F., Wang, J., & Chen, A. (2006). Effects of co-grafts mesenchymal stem cells and nerve growth factor suspension in the repair of spinal cord injury. Journal of Huazhong University of Science and Technology. [Medical Sciences], 26(2), 206–210. https://doi.org/10.1007/BF02895817/METRICS
281. Kim, K. N., Oh, S. H., Lee, K. H., & Yoon, D. H. (2006). Effect of human mesenchymal stem cell transplantation combined with growth factor infusion in the repair of injured spinal cord. Acta Neurochirurgica, Supplementum, 99(99), 133–136. https://doi.org/10.1007/978-3-211-35205-2_25/COVER
282. Khan, I. U., Yoon, Y., Kim, A., Jo, K. R., Choi, K. U., Jung, T., Kim, N., Son, Y. S., Kim, W. H., & Kweon, O. K. (2018). Improved Healing after the Co-Transplantation of HO-1 and BDNF Overexpressed Mesenchymal Stem Cells in the Subacute Spinal Cord Injury of Dogs. Cell Transplantation, 27(7), 1140–1153. https://doi.org/10.1177/0963689718779766/ASSET/IMAGES/LARGE/10.1177_0963689718779766-FIG2.JPEG
283. Alastrue-Agudo, A., Rodriguez-Jimenez, F. J., Mocholi, E. L., De Giorgio, F., Erceg, S., & Moreno-Manzano, V. (2018). FM19G11 and Ependymal Progenitor/Stem Cell Combinatory Treatment Enhances Neuronal Preservation and Oligodendrogenesis after Severe Spinal Cord Injury. International Journal of Molecular Sciences 2018, Vol. 19, Page 200, 19(1), 200. https://doi.org/10.3390/IJMS19010200
284. Sun, W. M., Ma, C. L., Xu, J., & He, J. P. (2021). Reduction in post-spinal cord injury spasticity by combination of peripheral nerve grafting and acidic fibroblast growth factor infusion in monkeys. Journal of International Medical Research, 49(6). https://doi.org/10.1177/03000605211022294/ASSET/IMAGES/LARGE/10.1177_03000605211022294-FIG2.JPEG
285. Di Silvio, L. (2007). Bone tissue engineering and biomineralization. Tissue Engineering Using Ceramics and Polymers, 319–331. https://doi.org/10.1533/9781845693817.2.319
286. Narimanpour, Z., Nazm Bojnordi, M., Somayeh, E. B., Elham, V., Jamileh, S., & Ghasemi, H. H. (2022). Silk Nanofibrous Electrospun Scaffold Amplifies Proliferation and Stemness Profile of Mouse Spermatogonial Stem Cells. Regenerative Engineering and Translational Medicine, 8(1), 86–93. https://doi.org/10.1007/S40883-020-00189-5/FIGURES/7
287. Rezaei, N., Bojnordi, M. N., & Ghasemi Hamidabadi, H. (2020). Differentiation of bone marrow stromal stem cells seeded on silk scaffold to mature oligodendrocyte using cerebrospinal fluid.

Journal of Chemical Neuroanatomy, 106, 101790. https://doi.org/10.1016/J.JCHEMNEU.2020.101790
288. Fathi, A., Khanmohammadi, M., Goodarzi, A., Foroutani, L., Mobarakeh, Z. T., Saremi, J., Arabpour, Z., & Ai, J. (2020). Fabrication of chitosan-polyvinyl alcohol and silk electrospun fiber seeded with differentiated keratinocyte for skin tissue regeneration in animal wound model. Journal of Biological Engineering, 14(1), 1–14. https://doi.org/10.1186/S13036-020-00249-Y/TABLES/3
289. Gros, T., Sakamoto, J. S., Blesch, A., Havton, L. A., & Tuszynski, M. H. (2010). Regeneration of long-tract axons through sites of spinal cord injury using templated agarose scaffolds. Biomaterials, 31(26), 6719–6729. https://doi.org/10.1016/J.BIOMATERIALS.2010.04.035
290. Günther, M. I., Günther, M., Schneiders, M., Rupp, R., & Blesch, A. (2015). AngleJ: A new tool for the automated measurement of neurite growth orientation in tissue sections. Journal of Neuroscience Methods, 251, 143–150. https://doi.org/10.1016/J.JNEUMETH.2015.05.021
291. Onuma-Ukegawa, M., Bhatt, K., Hirai, T., Kaburagi, H., Sotome, S., Wakabayashi, Y., Ichinose, S., Shinomiya, K., Okawa, A., & Enomoto, M. (2015). Bone Marrow Stromal Cells Combined With a Honeycomb Collagen Sponge Facilitate Neurite Elongation In Vitro and Neural Restoration in the Hemisected Rat Spinal Cord. Cell Transplantation, 24(7), 1283–1297. https://doi.org/10.3727/096368914X682134
292. Hurtado, A., Moon, L. D. F., Maquet, V., Blits, B., Jérôme, R., & Oudega, M. (2006). Poly (d,l-lactic acid) macroporous guidance scaffolds seeded with Schwann cells genetically modified to secrete a bi-functional neurotrophin implanted in the completely transected adult rat thoracic spinal cord. Biomaterials, 27(3), 430–442. https://doi.org/10.1016/J.BIOMATERIALS.2005.07.014
293. Olson, H. E., Rooney, G. E., Gross, L., Nesbitt, J. J., Galvin, K. E., Knight, A., Chen, B., Yaszemski, M. J., & Windebank, A. J. (2009). Neural Stem Cell– and Schwann Cell–Loaded Biodegradable Polymer Scaffolds Support Axonal Regeneration in the Transected Spinal Cord. Tissue Engineering Part A, 15(7), 1797–1805. https://doi.org/10.1089/TEN.TEA.2008.0364
294. Bozkurt, G., Mothe, A. J., Zahir, T., Kim, H., Shoichet, M. S., & Tator, C. H. (2010). Chitosan channels containing spinal cord-derived stem/progenitor cells for repair of subacute spinal cord injury in the rat. Neurosurgery, 67(6), 1733–1744. https://doi.org/10.1227/NEU.0B013E3181F9AF35
295. Park, S. S., Lee, Y. J., Lee, S. H., Lee, D., Choi, K., Kim, W. H., Kweon, O. K., & Han, H. J. (2012). Functional recovery after spinal cord injury in dogs treated with a combination of Matrigel and neural-induced adipose-derived mesenchymal Stem cells. Cytotherapy, 14(5), 584–597. https://doi.org/10.3109/14653249.2012.658913
296. Suzuki, H., Kanchiku, T., Imajo, Y., Yoshida, Y., Nishida, N., Gondo, T., Yoshii, S., & Taguchi, T. (2015). Artificial collagen-filament scaffold promotes axon regeneration and long tract reconstruction in a rat model of spinal cord transection. Medical Molecular Morphology, 48(4), 214–224. https://doi.org/10.1007/S00795-015-0104-5/FIGURES/7
297. Mekhail, M., Almazan, G., & Tabrizian, M. (2015). Purine-crosslinked injectable chitosan sponges promote oligodendrocyte progenitor cells' attachment and differentiation. Biomaterials Science, 3(2), 279–287. https://doi.org/10.1039/C4BM00215F
298. Mothe, A. J., Tam, R. Y., Zahir, T., Tator, C. H., & Shoichet, M. S. (2013). Repair of the injured spinal cord by transplantation of neural stem cells in a hyaluronan-based hydrogel. Biomaterials, 34(15), 3775–3783. https://doi.org/10.1016/J.BIOMATERIALS.2013.02.002
299. Yousefifard, M., Maleki, S. N., Askarian-Amiri, S., Vaccaro, A. R., Chapman, J. R., Fehlings, M. G., Hosseini, M., & Rahimi-Movaghar, V. (2019). A combination of mesenchymal stem cells and scaffolds promotes motor functional recovery in spinal cord injury: a systematic review and meta-analysis. Journal of Neurosurgery: Spine, 32(2), 269–284. https://doi.org/10.3171/2019.8.SPINE19201

300. Haggerty, A. E., Maldonado-Lasunción, I., Nitobe, Y., Yamane, K., Marlow, M. M., You, H., Zhang, C., Cho, B., Li, X., Reddy, S., Mao, H. Q., & Oudega, M. (2022). The Effects of the Combination of Mesenchymal Stromal Cells and Nanofiber-Hydrogel Composite on Repair of the Contused Spinal Cord. Cells 2022, Vol. 11, Page 1137, 11(7), 1137. https://doi.org/10.3390/CELLS11071137
301. Gao, M., Lu, P., Bednark, B., Lynam, D., Conner, J. M., Sakamoto, J., & Tuszynski, M. H. (2013). Templated agarose scaffolds for the support of motor axon regeneration into sites of complete spinal cord transection. Biomaterials, 34(5), 1529–1536. https://doi.org/10.1016/J.BIOMATERIALS.2012.10.070
302. Zhang, J., Lu, X., Feng, G., Gu, Z., Sun, Y., Bao, G., Xu, G., Lu, Y., Chen, J., Xu, L., Feng, X., & Cui, Z. (2016). Chitosan scaffolds induce human dental pulp stem cells to neural differentiation: potential roles for spinal cord injury therapy. Cell and Tissue Research, 366(1), 129–142. https://doi.org/10.1007/S00441-016-2402-1/FIGURES/6
303. Zeng, X., Qiu, X. C., Ma, Y. H., Duan, J. J., Chen, Y. F., Gu, H. Y., Wang, J. M., Ling, E. A., Wu, J. L., Wu, W., & Zeng, Y. S. (2015). Integration of donor mesenchymal stem cell-derived neuron-like cells into host neural network after rat spinal cord transection. Biomaterials, 53, 184–201. https://doi.org/10.1016/J.BIOMATERIALS.2015.02.073
304. Babaloo, H., Ebrahimi-Barough, S., Derakhshan, M. A., Yazdankhah, M., Lotfibakhshaiesh, N., Soleimani, M., Joghataei, M. T., & Ai, J. (2019). PCL/gelatin nanofibrous scaffolds with human endometrial stem cells/Schwann cells facilitate axon regeneration in spinal cord injury. Journal of Cellular Physiology, 234(7), 11060–11069. https://doi.org/10.1002/JCP.27936
305. Zarei-Kheirabadi, M., Sadrosadat, H., Mohammadshirazi, A., Jaberi, R., Sorouri, F., Khayyatan, F., & Kiani, S. (2020). Human embryonic stem cell-derived neural stem cells encapsulated in hyaluronic acid promotes regeneration in a contusion spinal cord injured rat. International Journal of Biological Macromolecules, 148, 1118–1129. https://doi.org/10.1016/J.IJBIOMAC.2020.01.219
306. Kourgiantaki, A., Tzeranis, D. S., Karali, K., Georgelou, K., Bampoula, E., Psilodimitrakopoulos, S., Yannas, I. V., Stratakis, E., Sidiropoulou, K., Charalampopoulos, I., & Gravanis, A. (2020). Neural stem cell delivery via porous collagen scaffolds promotes neuronal differentiation and locomotion recovery in spinal cord injury. Npj Regenerative Medicine 2020 5:1, 5(1), 1–14. https://doi.org/10.1038/s41536-020-0097-0
307. Wang, J., Chu, R., Ni, N., & Nan, G. (2020). The effect of Matrigel as scaffold material for neural stem cell transplantation for treating spinal cord injury. Scientific Reports 2020 10:1, 10(1), 1–11. https://doi.org/10.1038/s41598-020-59148-3
308. Jiang, J. P., Liu, X. Y., Zhao, F., Zhu, X., Li, X. Y., Niu, X. G., Yao, Z. T., Dai, C., Xu, H. Y., Ma, K., Chen, X. Y., & Zhang, S. (2020). Three-dimensional bioprinting collagen/silk fibroin scaffold combined with neural stem cells promotes nerve regeneration after spinal cord injury. Neural Regeneration Research, 15(5), 959–968. https://doi.org/10.4103/1673-5374.268974
309. Koffler, J., Zhu, W., Qu, X., Platoshyn, O., Dulin, J. N., Brock, J., Graham, L., Lu, P., Sakamoto, J., Marsala, M., Chen, S., & Tuszynski, M. H. (2019). Biomimetic 3D-printed scaffolds for spinal cord injury repair. Nature Medicine 2019 25:2, 25(2), 263–269. https://doi.org/10.1038/s41591-018-0296-z
310. Yu, F., Li, P., Du, S., Lui, K. H. W., Lin, Y., Chen, L., Ren, Q., Wang, J., Mei, J., Xiao, J., & Zhu, J. (2021). Olfactory ensheathing cells seeded decellularized scaffold promotes axonal regeneration in spinal cord injury rats. Journal of Biomedical Materials Research Part A, 109(5), 779–787. https://doi.org/10.1002/JBM.A.37066
311. Lee, Y. S., Wu, S., Arinzeh, T. L., & Bunge, M. B. (2017). Enhanced noradrenergic axon regeneration into schwann cell-filled PVDF-TrFE conduits after complete spinal cord transection. Biotechnology and Bioengineering, 114(2), 444–456. https://doi.org/10.1002/BIT.26088

312. Yang, E. Z., Zhang, G. W., Xu, J. G., Chen, S., Wang, H., Cao, L. L., Liang, B., & Lian, X. F. (2017). Multichannel polymer scaffold seeded with activated Schwann cells and bone mesenchymal stem cells improves axonal regeneration and functional recovery after rat spinal cord injury. Acta Pharmacologica Sinica 2017 38:5, 38(5), 623–637. https://doi.org/10.1038/aps.2017.11
313. Cuenca-Ortolá, I., Martínez-Rojas, B., Moreno-Manzano, V., García Castelló, M., Monleón Pradas, M., Martínez-Ramos, C., & Más Estellés, J. (2022). A Strategy for Magnetic and Electric Stimulation to Enhance Proliferation and Differentiation of NPCs Seeded over PLA Electrospun Membranes. Biomedicines 2022, Vol. 10, Page 2736, 10(11), 2736. https://doi.org/10.3390/BIOMEDICINES10112736
314. Gomes, E. D., Ghosh, B., Lima, R., Goulão, M., Moreira-Gomes, T., Martins-Macedo, J., Urban, M. W., Wright, M. C., Gimble, J. M., Sousa, N., Silva, N. A., Lepore, A. C., & Salgado, A. J. (2020). Combination of a Gellan Gum-Based Hydrogel With Cell Therapy for the Treatment of Cervical Spinal Cord Injury. Frontiers in Bioengineering and Biotechnology, 8, 984. https://doi.org/10.3389/FBIOE.2020.00984/BIBTEX
315. Ma, F., Li, X., Li, X., Xiao, Z., Han, J., Chen, L., Xiao, H., Ma, F., Hou, X., Sun, J., Ding, W., Zhao, Y., Chen, B., & Dai, J. (2013). Promotion of neuronal differentiation of neural progenitor cells by using EGFR antibody functionalized collagen scaffolds for spinal cord injury repair. Biomaterials. https://doi.org/10.1016/j.biomaterials.2013.03.062
316. Onuma-Ukegawa, M., Bhatt, K., Hirai, T., Kaburagi, H., Sotome, S., Wakabayashi, Y., Ichinose, S., Shinomiya, K., Okawa, A., & Enomoto, M. (2015). Bone marrow stromal cells combined with a honeycomb collagen sponge facilitate neurite elongation in vitro and neural restoration in the hemisected rat spinal cord. Cell Transplantation, 24(7), 1283–1297. https://doi.org/10.3727/096368914X682134/ASSET/IMAGES/LARGE/10.3727_096368914X682134-FIG6.JPEG
317. Wang, N., Xiao, Z., Zhao, Y., Wang, B., Li, X., Li, J., & Dai, J. (2018). Collagen scaffold combined with human umbilical cord-derived mesenchymal stem cells promote functional recovery after scar resection in rats with chronic spinal cord injury. Journal of Tissue Engineering and Regenerative Medicine, 12(2), e1154–e1163. https://doi.org/10.1002/TERM.2450
318. Koffler, J., Zhu, W., Qu, X., Platoshyn, O., Dulin, J. N., Brock, J., Graham, L., Lu, P., Sakamoto, J., Marsala, M., Chen, S., & Tuszynski, M. H. (2019). Biomimetic 3D-printed scaffolds for spinal cord injury repair. Nature Medicine 2019 25:2, 25(2), 263–269. https://doi.org/10.1038/s41591-018-0296-z
319. Zou, Y., Ma, D., Shen, H., Zhao, Y., Xu, B., Fan, Y., Sun, Z., Chen, B., Xue, W., Shi, Y., Xiao, Z., Gu, R., & Dai, J. (2020). Aligned collagen scaffold combination with human spinal cord-derived neural stem cells to improve spinal cord injury repair. Biomaterials Science, 8(18), 5145–5156. https://doi.org/10.1039/D0BM00431F
320. Williams, R. R., Henao, M., Pearse, D. D., & Bunge, M. B. (2015). Permissive Schwann cell graft/spinal cord interfaces for axon regeneration. Cell Transplantation, 24(1), 115–131. https://doi.org/10.3727/096368913X674657
321. Liu, S., Sandner, B., Schackel, T., Nicholson, L. S., Chtarto, A., Tenenbaum, L., Puttagunta, R., Müller, R., Weidner, N., & Blesch, A. (2017). Regulated viral BDNF delivery in combination with Schwann cells promotes axonal regeneration through capillary alginate hydrogels after spinal cord injury. Acta Biomaterialia, 60, 167–180. https://doi.org/10.1016/J.ACTBIO.2017.07.024
322. Deumens, R., Van Gorp, S. F. J., Bozkurt, A., Beckmann, C., Führmann, T., Montzka, K., Tolba, R., Kobayashi, E., Heschel, I., Weis, J., & Brook, G. A. (2013). Motor outcome and allodynia are largely unaffected by novel olfactory ensheathing cell grafts to repair low-thoracic lesion gaps in the adult rat spinal cord. Behavioural Brain Research, 237(1), 185–189. https://doi.org/10.1016/J.BBR.2012.09.036

323. Wang, C., Sun, C., Hu, Z., Huo, X., Yang, Y., Liu, X., Botchway, B. O. A., Davies, H., & Fang, M. (2018). Improved Neural Regeneration with Olfactory Ensheathing Cell Inoculated PLGA Scaffolds in Spinal Cord Injury Adult Rats. Neurosignals, 25(1), 1–14. https://doi.org/10.1159/000471828
324. Gomes, E. D., Ghosh, B., Lima, R., Goulão, M., Moreira-Gomes, T., Martins-Macedo, J., Urban, M. W., Wright, M. C., Gimble, J. M., Sousa, N., Silva, N. A., Lepore, A. C., & Salgado, A. J. (2020). Combination of a Gellan Gum-Based Hydrogel With Cell Therapy for the Treatment of Cervical Spinal Cord Injury. Frontiers in Bioengineering and Biotechnology, 8, 984. https://doi.org/10.3389/FBIOE.2020.00984/BIBTEX
325. Zeraatpisheh, Z., Mirzaei, E., Nami, M., Alipour, H., Mahdavipour, M., Sarkoohi, P., Torabi, S., Azari, H., & Aligholi, H. (2021). Local delivery of fingolimod through PLGA nanoparticles and PuraMatrix-embedded neural precursor cells promote motor function recovery and tissue repair in spinal cord injury. European Journal of Neuroscience, 54(4), 5620–5637. https://doi.org/10.1111/EJN.15391
326. Yao, S., He, F., Cao, Z., Sun, Z., Chen, Y., Zhao, H., Yu, X., Wang, X., Yang, Y., Rosei, F., & Wang, L. N. (2020). Mesenchymal Stem Cell-Laden Hydrogel Microfibers for Promoting Nerve Fiber Regeneration in Long-Distance Spinal Cord Transection Injury. ACS Biomaterials Science and Engineering, 6(2), 1165–1175. https://doi.org/10.1021/ACSBIOMATERIALS.9B01557/ASSET/IMAGES/MEDIUM/AB9B01557_0008.GIF
327. Autologous Bulbar Olfactory Ensheathing Cells and Nerve Grafts for Treatment of Patients With Spinal Cord Transection - Full Text View - ClinicalTrials.gov. (n.d.). Retrieved July 10, 2023, from https://classic.clinicaltrials.gov/ct2/show/NCT03933072
328. NeuroRegen ScaffoldTM With Stem Cells for Chronic Spinal Cord Injury Repair - Full Text View - ClinicalTrials.gov. (n.d.). Retrieved December 10, 2023, from https://classic.clinicaltrials.gov/ct2/show/NCT02352077
329. Functional Neural Regeneration Collagen Scaffold Transplantation in Acute Spinal Cord Injury Patients - No Study Results Posted - ClinicalTrials.gov. (n.d.). Retrieved March 8, 2023, from https://clinicaltrials.gov/ct2/show/results/NCT02510365
330. Chen, W., Zhang, Y., Yang, S., Sun, J., Qiu, H., Hu, X., Niu, X., Xiao, Z., Zhao, Y., Zhou, Y., Dai, J., & Chu, T. (2020). NeuroRegen Scaffolds Combined with Autologous Bone Marrow Mononuclear Cells for the Repair of Acute Complete Spinal Cord Injury: A 3-Year Clinical Study. Cell Transplantation, 29. https://doi.org/10.1177/0963689720950637/ASSET/IMAGES/LARGE/10.1177_0963689720950637-FIG2.JPEG
331. Zustiak, S. P., Sheth, S., & Imaninezhad, M. (2020). Pharmacological therapies and factors delivery for spinal cord injury regeneration. Spinal Cord Injury (SCI) Repair Strategies, 223–248. https://doi.org/10.1016/B978-0-08-102807-0.00012-0
332. Chvatal, S. A., Kim, Y. T., Bratt-Leal, A. M., Lee, H., & Bellamkonda, R. V. (2008). Spatial distribution and acute anti-inflammatory effects of Methylprednisolone after sustained local delivery to the contused spinal cord. Biomaterials, 29(12), 1967. https://doi.org/10.1016/J.BIOMATERIALS.2008.01.002
333. Sellers, D. L., Kim, T. H., Mount, C. W., Pun, S. H., & Horner, P. J. (2014). Poly(lactic-co-glycolic) acid microspheres encapsulated in Pluronic F-127 prolong hirudin delivery and improve functional recovery from a demyelination lesion. Biomaterials, 35(31), 8895–8902. https://doi.org/10.1016/J.BIOMATERIALS.2014.06.051
334. Cerqueira, S. R., Oliveira, J. M., Silva, N. A., Leite-Almeida, H., Ribeiro-Samy, S., Almeida, A., Mano, J. F., Sousa, N., Salgado, A. J., & Reis, R. L. (2013). Microglia Response and In Vivo Therapeutic

Potential of Methylprednisolone-Loaded Dendrimer Nanoparticles in Spinal Cord Injury. Small, 9(5), 738–749. https://doi.org/10.1002/SMLL.201201888

335. Xia, T., Ni, S., Li, X., Yao, J., Qi, H., Fan, X., & Wang, J. (2013). Sustained delivery of dbcAMP by poly(propylene carbonate) micron fibers promotes axonal regenerative sprouting and functional recovery after spinal cord hemisection. Brain Research, 1538, 41–50. https://doi.org/10.1016/J.BRAINRES.2013.09.027
336. Papa, S., Ferrari, R., De Paola, M., Rossi, F., Mariani, A., Caron, I., Sammali, E., Peviani, M., Dell'Oro, V., Colombo, C., Morbidelli, M., Forloni, G., Perale, G., Moscatelli, D., & Veglianese, P. (2014). Polymeric nanoparticle system to target activated microglia/macrophages in spinal cord injury. Journal of Controlled Release, 174(1), 15–26. https://doi.org/10.1016/J.JCONREL.2013.11.001
337. Liu, S., Schackel, T., Weidner, N., & Puttagunta, R. (2018). Biomaterial-Supported Cell Transplantation Treatments for Spinal Cord Injury: Challenges and Perspectives. Frontiers in Cellular Neuroscience, 11. https://doi.org/10.3389/FNCEL.2017.00430
338. Fan, C., Li, X., Zhao, Y., Xiao, Z., Xue, W., Sun, J., Li, X., Zhuang, Y., Chen, Y., & Dai, J. (2018). Cetuximab and Taxol co-modified collagen scaffolds show combination effects for the repair of acute spinal cord injury. Biomaterials Science, 6(7), 1723–1734. https://doi.org/10.1039/C8BM00363G
339. Yin, W., Xue, W., Zhu, H., Shen, H., Xiao, Z., Wu, S., Zhao, Y., Cao, Y., Tan, J., Li, J., Liu, W., Wang, L., Meng, L., Chen, B., Zhao, M., Jiang, X., Li, X., Ren, C., & Dai, J. (2021). Scar tissue removal-activated endogenous neural stem cells aid Taxol-modified collagen scaffolds in repairing chronic long-distance transected spinal cord injury. Biomaterials Science, 9(13), 4778–4792. https://doi.org/10.1039/D1BM00449B
340. Kwiecien, J. M., Zhang, L., Yaron, J. R., Schutz, L. N., Kwiecien-Delaney, C. J., Awo, E. A., Burgin, M., Dabrowski, W., & Lucas, A. R. (2020). Local Serpin Treatment via Chitosan-Collagen Hydrogel after Spinal Cord Injury Reduces Tissue Damage and Improves Neurologic Function. Journal of Clinical Medicine, 9(4). https://doi.org/10.3390/JCM9041221
341. Nazemi, Z., Nourbakhsh, M. S., Kiani, S., Heydari, Y., Ashtiani, M. K., Daemi, H., & Baharvand, H. (2020). Co-delivery of minocycline and paclitaxel from injectable hydrogel for treatment of spinal cord injury. Journal of Controlled Release : Official Journal of the Controlled Release Society, 321, 145–158. https://doi.org/10.1016/J.JCONREL.2020.02.009
342. Nazemi, Z., Nourbakhsh, M. S., Kiani, S., Heydari, Y., Ashtiani, M. K., Daemi, H., & Baharvand, H. (2020). Co-delivery of minocycline and paclitaxel from injectable hydrogel for treatment of spinal cord injury. Journal of Controlled Release, 321, 145–158. https://doi.org/10.1016/J.JCONREL.2020.02.009
343. Yang, Y., Fan, Y., Zhang, H., Zhang, Q., Zhao, Y., Xiao, Z., Liu, W., Chen, B., Gao, L., Sun, Z., Xue, X., Shu, M., & Dai, J. (2021). Small molecules combined with collagen hydrogel direct neurogenesis and migration of neural stem cells after spinal cord injury. Biomaterials, 269, 120479. https://doi.org/10.1016/J.BIOMATERIALS.2020.120479
344. Wang, Z., Wang, Z., Lu, W. W., Zhen, W., Yang, D., & Peng, S. (2017). Novel biomaterial strategies for controlled growth factor delivery for biomedical applications. NPG Asia Materials 2017 9:10, 9(10), e435–e435. https://doi.org/10.1038/am.2017.171
345. Lowry, N., Goderie, S. K., Lederman, P., Charniga, C., Gooch, M. R., Gracey, K. D., Banerjee, A., Punyani, S., Silver, J., Kane, R. S., Stern, J. H., & Temple, S. (2012). The effect of long-term release of Shh from implanted biodegradable microspheres on recovery from spinal cord injury in mice. Biomaterials, 33(10), 2892–2901. https://doi.org/10.1016/J.BIOMATERIALS.2011.12.048

346. Ornitz, D. M., Xu, J., Colvin, J. S., McEwen, D. G., MacArthur, C. A., Coulier, F., Gao, G., & Goldfarb, M. (1996). Receptor specificity of the fibroblast growth factor family. Journal of Biological Chemistry, 271(25), 15292–15297. https://doi.org/10.1074/JBC.271.25.15292
347. Kakizawa, S. (2021). Neurotrophin-3. Handbook of Hormones: Comparative Endocrinology for Basic and Clinical Research, 483–485. https://doi.org/10.1016/B978-0-12-820649-2.00123-6
348. Kang, C. E., Baumann, M. D., Tator, C. H., & Shoichet, M. S. (2013). Localized and Sustained Delivery of Fibroblast Growth Factor-2 from a Nanoparticle-Hydrogel Composite for Treatment of Spinal Cord Injury. Cells Tissues Organs, 197(1), 55–63. https://doi.org/10.1159/000339589
349. Elliott Donaghue, I., Tator, C. H., & Shoichet, M. S. (2014). Sustained delivery of bioactive neurotrophin-3 to the injured spinal cord. Biomaterials Science, 3(1), 65–72. https://doi.org/10.1039/C4BM00311J
350. Yu, S., Yao, S., Wen, Y., Wang, Y., Wang, H., & Xu, Q. (2016). Angiogenic microspheres promote neural regeneration and motor function recovery after spinal cord injury in rats. Scientific Reports 2016 6:1, 6(1), 1–13. https://doi.org/10.1038/srep33428
351. Zhu, S. P., Wang, Z. G., Zhao, Y. Z., Wu, J., Shi, H. X., Ye, L. B., Wu, F. Z., Cheng, Y., Zhang, H. Y., He, S., Wei, X., Fu, X. B., Li, X. K., Xu, H. Z., & Xiao, J. (2016). Gelatin Nanostructured Lipid Carriers Incorporating Nerve Growth Factor Inhibit Endoplasmic Reticulum Stress-Induced Apoptosis and Improve Recovery in Spinal Cord Injury. Molecular Neurobiology, 53(7), 4375–4386. https://doi.org/10.1007/S12035-015-9372-2/FIGURES/6
352. Chen, X., Zhao, Y., Li, X., Xiao, Z., Yao, Y., Chu, Y., Farkas, B., Romano, I., Brandi, F., & Dai, J. (2018). Functional Multichannel Poly(Propylene Fumarate)-Collagen Scaffold with Collagen-Binding Neurotrophic Factor 3 Promotes Neural Regeneration After Transected Spinal Cord Injury. Advanced Healthcare Materials, 7(14), 1800315. https://doi.org/10.1002/ADHM.201800315
353. Li, L., Li, L., Zhang, Y., Mu, J., Chen, J., Zhang, C., Cao, H., Gao, J., & Gao, J. (2020). Transplantation of Human Mesenchymal Stem-Cell-Derived Exosomes Immobilized in an Adhesive Hydrogel for Effective Treatment of Spinal Cord Injury. Nano Letters, 20(6), 4298–4305. https://doi.org/10.1021/ACS.NANOLETT.0C00929/SUPPL_FILE/NL0C00929_SI_006.MP4
354. Khaing, Z. Z., Agrawal, N. K., Park, J. H., Xin, S., Plumton, G. C., Lee, K. H., Huang, Y. J., Niemerski, A. L., Schmidt, C. E., & Grau, J. W. (2016). Localized and sustained release of brain-derived neurotrophic factor from injectable hydrogel/microparticle composites fosters spinal learning after spinal cord injury. Journal of Materials Chemistry B, 4(47), 7560–7571. https://doi.org/10.1039/C6TB01602B
355. Yamane, K., Mazaki, T., Shiozaki, Y., Yoshida, A., Shinohara, K., Nakamura, M., Yoshida, Y., Zhou, D., Kitajima, T., Tanaka, M., Ito, Y., Ozaki, T., & Matsukawa, A. (2018). Collagen-Binding Hepatocyte Growth Factor (HGF) alone or with a Gelatin- furfurylamine Hydrogel Enhances Functional Recovery in Mice after Spinal Cord Injury. Scientific Reports 2018 8:1, 8(1), 1–12. https://doi.org/10.1038/s41598-018-19316-y
356. Rodríguez-Barrera, R., Flores-Romero, A., Buzoianu-Anguiano, V., Garcia, E., Soria-Zavala, K., Incontri-Abraham, D., Garibay-López, M., Juárez-Vignon Whaley, J. J., & Ibarra, A. (2020). Use of a Combination Strategy to Improve Morphological and Functional Recovery in Rats With Chronic Spinal Cord Injury. Frontiers in Neurology, 11, 189. https://doi.org/10.3389/FNEUR.2020.00189/BIBTEX
357. García, E., Rodríguez-Barrera, R., Buzoianu-Anguiano, V., Flores-Romero, A., Malagón-Axotla, E., Guerrero-Godinez, M., De La Cruz-Castillo, E., Castillo-Carvajal, L., Rivas-Gonzalez, M., Santiago-Tovar, P., Morales, I., Borlongan, C., & Ibarra, A. (2019). Use of a combination strategy to improve neuroprotection and neuroregeneration in a rat model of acute spinal cord injury. Neural Regeneration Research, 14(6), 1060–1068. https://doi.org/10.4103/1673-5374.250627

358. Nori, S., Khazaei, M., Ahuja, C. S., Yokota, K., Ahlfors, J. E., Liu, Y., Wang, J., Shibata, S., Chio, J., Hettiaratchi, M. H., Führmann, T., Shoichet, M. S., & Fehlings, M. G. (2018). Human Oligodendrogenic Neural Progenitor Cells Delivered with Chondroitinase ABC Facilitate Functional Repair of Chronic Spinal Cord Injury. Stem Cell Reports, 11(6), 1433. https://doi.org/10.1016/J.STEMCR.2018.10.017
359. Kandalam, S., De Berdt, P., Ucakar, B., Vanvarenberg, K., Bouzin, C., Gratpain, V., Diogenes, A., Montero-Menei, C. N., & des Rieux, A. (2020). Human dental stem cells of the apical papilla associated to BDNF-loaded pharmacologically active microcarriers (PAMs) enhance locomotor function after spinal cord injury. International Journal of Pharmaceutics, 587, 119685. https://doi.org/10.1016/J.IJPHARM.2020.119685
360. Courtine, G., Gerasimenko, Y., Van Den Brand, R., Yew, A., Musienko, P., Zhong, H., Song, B., Ao, Y., Ichiyama, R. M., Lavrov, I., Roy, R. R., Sofroniew, M. V., & Edgerton, V. R. (2009). Transformation of nonfunctional spinal circuits into functional states after the loss of brain input. Nature Neuroscience 2009 12:10, 12(10), 1333–1342. https://doi.org/10.1038/nn.2401
361. Gerasimenko, Y. P., Ichiyama, R. M., Lavrov, I. A., Courtine, G., Cai, L., Zhong, H., Roy, R. R., & Edgerton, V. R. (2007). Epidural spinal cord stimulation plus quipazine administration enable stepping in complete spinal adult rats. Journal of Neurophysiology, 98(5), 2525–2536. https://doi.org/10.1152/JN.00836.2007/ASSET/IMAGES/LARGE/Z9K0110785250010.JPEG
362. Landry, E. S., Lapointe, N. P., Rouillard, C., Levesque, D., Hedlund, P. B., & Guertin, P. A. (2006). Contribution of spinal 5-HT1A and 5-HT7 receptors to locomotor-like movement induced by 8-OH-DPAT in spinal cord-transected mice. European Journal of Neuroscience, 24(2), 535–546. https://doi.org/10.1111/J.1460-9568.2006.04917.X
363. Yao, Q., Guan, J., Ma, L., Cheng, L., Duan, F., Xu, F., Zhao, W., Duan, W., Wu, H., Chen, Z., & Jian, F. (2021). Wireless Epidural Electrical Stimulation in Combination With Serotonin Agonists Improves Intraspinal Metabolism in Spinal Cord Injury Rats. Neuromodulation, 24(3), 416–426. https://doi.org/10.1111/ner.13344
364. FENG, S., WANG, S., SUN, S., SU, H., & ZHANG, L. (2021). Effects of combination treatment with transcranial magnetic stimulation and bone marrow mesenchymal stem cell transplantation or raf inhibition on spinal cord injury in rats. Molecular Medicine Reports, 23(4), 1–10. https://doi.org/10.3892/MMR.2021.11934/HTML
365. Sinopoulou, E., Spejo, A. B., Roopnarine, N., Burnside, E. R., Bartus, K., De Winter, F., McMahon, S. B., & Bradbury, E. J. (2022). Chronic muscle recordings reveal recovery of forelimb function in spinal injured female rats after cortical epidural stimulation combined with rehabilitation and chondroitinase ABC. Journal of Neuroscience Research, 100(11), 2055–2076. https://doi.org/10.1002/JNR.25111
366. Yamanaka, H., Takata, Y., Nakagawa, H., Isosaka-Yamanaka, T., Yamashita, T., & Takada, M. (2021). An enhanced therapeutic effect of repetitive transcranial magnetic stimulation combined with antibody treatment in a primate model of spinal cord injury. PLOS ONE, 16(6), e0252023. https://doi.org/10.1371/JOURNAL.PONE.0252023
367. Cardoso, F. dos S., Salehpour, F., Coimbra, N. C., Gonzalez-Lima, F., & Gomes da Silva, S. (2022). Photobiomodulation for the treatment of neuroinflammation: A systematic review of controlled laboratory animal studies. Frontiers in Neuroscience, 16, 1610. https://doi.org/10.3389/FNINS.2022.1006031/BIBTEX
368. Rojas, J. C., & Gonzalez-Lima, F. (2013). Neurological and psychological applications of transcranial lasers and LEDs. Biochemical Pharmacology, 86(4), 447–457. https://doi.org/10.1016/J.BCP.2013.06.012
369. Rojas, J. C., & Gonzalez-Lima, F. (2011). Low-level light therapy of the eye and brain. Eye and Brain, 3, 49–67. https://doi.org/10.2147/EB.S21391

370. De Almeida, P., Lopes-Martins, R. Á. B., Tomazoni, S. S., Albuquerque-Pontes, G. M., Santos, L. A., Vanin, A. A., Frigo, L., Vieira, R. P., Albertini, R., De Tarso Camillo De Carvalho, P., & Leal-Junior, E. C. P. (2013). Low-Level Laser Therapy and Sodium Diclofenac in Acute Inflammatory Response Induced by Skeletal Muscle Trauma: Effects in Muscle Morphology and mRNA Gene Expression of Inflammatory Markers. Photochemistry and Photobiology, 89(2), 501–507. https://doi.org/10.1111/J.1751-1097.2012.01232.X
371. Arany, P. R. (2016). Craniofacial Wound Healing with Photobiomodulation Therapy. Http://Dx.Doi.Org/10.1177/0022034516648939, 95(9), 977–984. https://doi.org/10.1177/0022034516648939
372. Hamblin, M. R., & Hamblin, M. R. (2017). Mechanisms and applications of the anti-inflammatory effects of photobiomodulation. AIMS Biophysics 2017 3:337, 4(3), 337–361. https://doi.org/10.3934/BIOPHY.2017.3.337
373. Cardoso, F. dos S., Gonzalez-Lima, F., & Gomes da Silva, S. (2021). Photobiomodulation for the aging brain. Ageing Research Reviews, 70, 101415. https://doi.org/10.1016/J.ARR.2021.101415
374. Cardoso, F. dos S., de Souza Oliveira Tavares, C., Araujo, B. H. S., Mansur, F., Lopes-Martins, R. Á. B., & Gomes da Silva, S. (2022). Improved Spatial Memory And Neuroinflammatory Profile Changes in Aged Rats Submitted to Photobiomodulation Therapy. Cellular and Molecular Neurobiology, 42(6), 1875–1886. https://doi.org/10.1007/S10571-021-01069-4/METRICS
375. Hassan, M. P., Abdollahifar, M. A., Aliaghaei, A., Tabeie, F., Vafaei-Nezhad, S., Norouzian, M., & Abbaszadeh, H. A. (2021). Photobiomodulation therapy improved functional recovery and overexpression of interleukins-10 after contusion spinal cord injury in rats. Journal of Chemical Neuroanatomy, 117, 102010. https://doi.org/10.1016/J.JCHEMNEU.2021.102010
376. Chen, H., Wang, Y., Tu, W., Wang, H., Yin, H., Sha, H., & Li, Y. (2021). Effects of photobiomodulation combined with MSCs transplantation on the repair of spinal cord injury in rat. Journal of Cellular Physiology, 236(2), 921–930. https://doi.org/10.1002/JCP.29902
377. Janzadeh, A., Sarveazad, A., Hamblin, M. R., Teheripak, G., Kookli, K., & Nasirinezhad, F. (2020). The effect of chondroitinase ABC and photobiomodulation therapy on neuropathic pain after spinal cord injury in adult male rats. Physiology & Behavior, 227, 113141. https://doi.org/10.1016/J.PHYSBEH.2020.113141
378. Sarveazad, A., Janzadeh, A., Taheripak, G., Dameni, S., Yousefifard, M., & Nasirinezhad, F. (2019). Co-administration of human adipose-derived stem cells and low-level laser to alleviate neuropathic pain after experimental spinal cord injury. Stem Cell Research and Therapy, 10(1), 1–15. https://doi.org/10.1186/S13287-019-1269-Y/FIGURES/7

Chapter II

Pre-clinical assessment

of roflumilast therapy in a thoracic model of spinal cord injury.

The results presented in this chapter were published as an original article in an international peer reviewed journal: Sousa, C.S.; Lima, R.; Cibrão, J.R.; Gomes, E.D.; Fernandes, L.S.; Pinho, T.S.; Silva, D.; Campos, J.; Salgado, A.J.; Silva, N.A. Pre-Clinical Assessment of Roflumilast Therapy in a Thoracic Model of Spinal Cord Injury. Pharmaceutics. 2023; 15(5):1556.

Pre-Clinical Assessment of Roflumilast Therapy in a Thoracic Model of Spinal Cord Injury

Carla S. Sousa [1,2,3,†], Rui Lima [1,2,†], Jorge R. Cibrão [1,2], Eduardo D. Gomes [1,2], Luís S. Fernandes [1,2], Tiffany S. Pinho [1,2], Deolinda Silva [1,2], Jonas Campos [1,2], António J. Salgado [1,2] and Nuno A. Silva [1,2,*]

1 Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, 4710-057 Braga, Portugal

2 ICVS/3B's Associate Lab, PT Government Associated Lab, 4805-017 Guimarães, Portugal

3 Department of Neurosurgery, Hospital Garcia de Orta, 2805-267 Almada, Portugal

* Correspondence: nunosilva@med.uminho.pt; Tel.: +351-253604875

† These authors contributed equally to this work.

Abstract

The failure of axons to regenerate after a spinal cord injury (SCI) remains one of the greatest challenges in neuroscience. The initial mechanical trauma is followed by a secondary injury cascade, creating a hostile microenvironment, which not only is not permissive to regeneration but also leads to further damage. One of the most promising approaches for promoting axonal regeneration is to maintain the levels of cyclic adenosine monophosphate (cAMP), specifically by a phosphodiesterase-4 (PDE4) inhibitor expressed in neural tissues. Therefore, in our study, we evaluated the therapeutic effect of an FDA-approved PDE4 inhibitor, Roflumilast (Rof), in a thoracic contusion rat model. Results indicate that the treatment was effective in promoting functional recovery. Rof-treated animals showed improvements in both gross and fine motor function. Eight weeks post-injury, the animals significantly recovered by achieving occasional weight-supported plantar steps. Histological assessment revealed a significant decrease in cavity size, less reactive microglia, as well as higher axonal regeneration in treated animals. Molecular analysis revealed that IL-10 and IL-13 levels, as well as VEGF, were increased in the serum of Rof-treated animals. Overall, Roflumilast promotes functional recovery and supports neuroregeneration in a severe thoracic contusion injury model and may be important in SCI treatment.

Keywords: Roflumilast; spinal cord injury; motor recovery; rat model

1. Introduction

Spinal cord injury (SCI) has a dramatic impact on motor, sensory and autonomic functions [1]. Currently, SCI is managed only through surgery decompression and maintaining the mean arterial blood pressure [2,3]. The use of steroids as a standard of care is currently highly controversial [4]. As a result, it is crucial to develop novel therapeutic strategies to repair spinal cord injuries. SCI is characterized by a complex pathophysiology. Initial mechanical trauma is followed by damaging secondary injury events that lead to further injury, creating an adverse microenvironment for regeneration [5,6]. A major discovery in this field has been the identification of inhibitory proteins in CNS myelin. In 1988, Schwab and colleagues provided the first direct evidence that CNS myelin contains proteins that inhibit neurite outgrowth in vitro [7]. Nogo-A, myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp) are among these proteins. They have no sequence similarity or structural homology, yet surprisingly they all bind to a common receptor complex to mediate inhibition. These inhibitors are responsible for the Rho-mediated activation of downstream effectors, such as Rho-associated kinase (ROCK), which then induces actin polymerization, leading to growth cone collapse and the inhibition of neurite outgrowth [8,9]. MAG, Nogo and OMgp signal through a common pathway, so inhibiting protein kinase C PKC, Rho and ROCK is logical to promote axonal regeneration. However, it is also possible to manipulate neurons at the molecular level so that they no longer respond to myelin inhibitors. The approach is based on the observation that MAG initially permits neurite outgrowth and only becomes inhibitory beyond a certain developmental time point. In 2001, Cai et al. demonstrated a direct correlation between neuronal adenosine monophosphate (cAMP) levels and the inhibition of neurite outgrowth by MAG and myelin. The levels of endogenous cAMP are high in P1 dorsal root ganglion (DRG) neurons, and substantial neurite outgrowth is observed despite the presence of MAG and myelin; however, at P3–4, there is an abrupt decrease in neuronal cAMP, which coincides with myelin inhibition [10]. These findings raise the possibility that pharmacological agents could be used to elevate cAMP levels in neurons, thereby increasing their ability to regenerate. Many promising treatments have resulted from the identification of cAMP as a modulator of axonal regeneration [11], some of which use phosphodiesterase-4 (PDE4) inhibitors [11]. However, of all the mediators that have been tested to date, possibly the most significant finding was that acute administration of Rolipram alone significantly improved axonal integrity and functional outcome post-SCI [12–14]. Unfortunately, Rolipram has been abandoned for human use because of its side effects [10,15]. However, this does not undermine the therapeutic potential of second-generation PDE4 inhibitors. For instance, it was demonstrated that Roflumilast (Rof) has the same binding

site as Rolipram and can increase the intracellular levels of cAMP. Additionally, Rof presents excellent pharmacokinetic properties, such as rapid absorption and distribution, linear kinetics, good bioavailability and low toxicity [16], and has already been approved for human use in chronic obstructive pulmonary disease (COPD) [17]. Moreover, it was demonstrated that Rof was able to protect the neural tissue after cerebral ischemia in rats by modulating the immune response. Roflumilast administered intraperitoneally decreases the expression of pro-inflammatory cytokines and protects the neurons in the brain [18]. Additionally, Rof is also able to change the microglia phenotype from a pro-inflammatory status to a pro-regenerative one, both in vitro and in vivo [19]. Taking into account all the above-mentioned properties, herein we aimed to study the therapeutic action of Roflumilast in a severe thoracic SCI rat model.

2. Materials and Methods

2.1. Spinal Cord Cultures

To evaluate if Roflumilast was able to induce specific cAMP-signaling markers, we employed an in vitro culture system composed of mixed cells of spinal cord isolated from Wistar Han pups within 5 days (P5). Spinal cords were isolated by hydraulic extrusion according to [20]. Cells were kept for 7 days in NeurobasalTM medium with 2% B27, 1% Pen/Strep, 1% L-Glutamine and Glial-derived neurotrophic factor (GDNF) at 10 ng/mL. Cells were then exposed for 48 h to Roflumilast at 20 uM in NeurobasalTM medium with 1% Pen/Strep. Controls had exposure to the same media without Roflumilast. Cells were fixed in cold 4% PFA for 10 min, then triple-immunolabeled for b3 tubulin, cAMP protein kinase (cAMPKA) and the cAMP response element binding protein (CREB). Four wells for each group were automatically imaged, focusing on neuronal enriched regions of interest (ROIs). Acquisition settings such as exposure time and detector gain were kept constant throughout groups. A total of 40 photomicrographs per condition(10/well) at 20× magnification were taken on an inverted widefield microscrope (Olympus IX81, Tokyo, Japan). Image analysis was performed in FIJI with an automatic macro that used the b3 tubulin-labeled neuronal cell bodies to segment, the cytoplasmic cAMPKA and the nuclear CREB. Measurements of the area of each marker of interest were taken and normalized to the number of positive b3 tubulin-labeled cells.

2.2. Organotypic Cultures

To assess the effect of Roflumilast on axonal regeneration, we used organotypic cultures of spinal cord slices (350 µm) from the thoracic region of Wistar Han pups within 6 days (P6). The spinal cords were dissected under aseptic conditions and placed on cold HBSS containing 1% Pen/Strep. Then, 350 µm slices were obtained using a tissue chopper. Preserved spinal cord slices were selected under magnification. The slices were then divided into two groups with or without 20 nM of Rof in NeurobasalTM medium with 2% B27, 1% Pen/Strep, 1% L-Glutamine and 6 mg/mL of glucose (medium was changed every 2 days for both groups). Finally, slices were fixed and immunolabeled for neurofilament following the protocol described below. The area occupied by the axons in each spinal cord slice explant was calculated using the ImageJ (NIH) plugin Neurite-J. Our analysis was focused only on the neurofilament that was detected outside the spinal cord slice, measuring the newly growing axons instead of the total area of neurofilament because there were some minor differences in the area of the spinal cord slice alone. Briefly, the image was automatically translated to eight bits, and a binary mask was created with the aid of the "Analysis Particles" function, which enables the correct segmentation of axonal structures based on an intensity-threshold image coupled with morphological parameters such as structure size and circularity. The mask generated can then be added as input to the Neurite-J plugin.

2.3. ELISA Assay

For the protein extraction, samples were prepared in RIPA buffer (Tris-HCl 20 mM; NaCl 150 mM; EDTA 5 mM; 0.1% SDS). All samples were tested in duplicate. We used an ELISA kit for cAMP (Enzo Complete cAMP ELISA Kit). After the wells were filled with the respective samples, adequate controls and anti-cAMP antibodies, the plate was sealed and incubated for 2 h at room temperature with orbital agitation (500 rpm). Following incubation, the plate was washed, and cAMP conjugate and pNpp substrate were added to the wells to initiate the colorimetric reaction, which took 1 h at room temperature. The reaction was finished with the addition of ELISA Stop solution (HRP), and the optical densities were read at 405 nm using a spectrophotometer. cAMP concentration was inferred based on a four-parameter logistic calibration curve.

2.4. In Vivo Study Design

The objective of this study was to evaluate the therapeutic potential of Rof treatment after SCI. We assessed the effects of Rof treatment in a rat model, evaluating functional recovery and histological and

molecular modifications. The number of animals in this study was determined using G*power software (12 animals per group). Fifteen animals per group were randomly treated with Roflumilast or saline, and all data were collected blind to the treatment group. All procedures were carried out in accordance with EU directive 2010/63/EU and were approved by the ethical committee in life and health sciences (Ref: DGAV 022405, SECVS116/2016, University of Minho, Braga, Portugal).

2.5. Spinal Cord Injury Model and Treatment

A total of forty-two Wistar Han female rats (8–10 weeks old, weighing 210–260 g) were used in this study (30 rats for the pre-clinical trial and 12 for cAMP quantification). Animals were kept under standard laboratory conditions (12 h light/12 h dark cycles, 22 ◦C, relative humidity of 55%, ad libitum access to standard food and water) and housed in pairs. Animal handling was performed 3 days prior to surgery. The T8 contusion model was induced, as previously described [21,22]. General anesthesia was induced by an intraperitoneal (i.p.) injection of a ketamine (100 mg/mL, Imalgene/Merial) and medetomidine hydrochloride (1 mg/mL, Dormitor/Pfizer) mixture at a volume ratio of 1.5:1. Once anesthetized, animals received subcutaneous injections of the analgesic butorphanol (10 mg/mL, Butomidor/Richter Pharma AG) and the antibiotic enrofloxacin (5 mg/mL, Baytril/Bayer). The fur was shaved from the surgical site and the skin was disinfected with ethanol 70% and chlorohexidine. Surgical procedures were performed under sterile conditions. The animals were placed in a prone position and a dorsal midline incision was made at the level of the thoracic spine (T5–T12). The paravertebral muscles were retracted, and the spinous process of T5 was identified by observing a prominent vessel that was placed between the fourth and the fifth thoracic processes. Using the back edge of the scalpel blade, the spinous processes were counted from T5 until T8, and the latter was marked with a small cut. Then, the spinous process and laminar arc of T8 was removed, and the spinal cord was exposed. The dura was left intact. A weight drop trauma model was used, which consisted of dropping a 10 g weight rod from a 10 cm height onto the exposed spinal cord. The rod was guided through a stabilized tube positioned perpendicular to the center of the spinal cord. After the trauma, the muscles were sutured with Vicryl sutures (Johnson and Johnson®, New Brunswick, NJ, USA), and the incision closed with surgical staples (Fine Science Tools®, North Vancouver, BC, Canada). Anesthesia was inverted using atipamezole (5 mg/mL, Antisedan/Pfizer®, New York, NY, USA). One-hour post-injury, the animals were randomly divided into two experimental groups, the Roflumilast treatment group and the control that only received vehicle (saline). The post-operative care for all rats included butorphanol (Richter Pharma AG®, Wels, Austria)

administration twice daily for a five-day period, and vitamins (Duphalyte, Pfizer®), saline and enrofloxacin (Bayer®, Leverkusen, Germany) twice daily for seven days. The bladders were manually expressed twice a day until animals recovered spontaneous voiding. A weekly body weight measurement was taken as a measure of general health. If a weight loss over 10% of body weight was detected, a high-calorie oral supplement (Nutri-Cal®, Bondurant, IA, USA) was administered daily. For Rof or saline administration, we inserted an intrathecal 28 G catheter 7 days before inflicting the contusion. Under general anesthesia, a dorsal midline incision was made. The spinous process and laminar arc of T13 were removed, the dura was gently opened, and the catheter was introduced up to the level of T8 and then retracted to T9 (1 level below the level of contusion). The laminectomy previously performed at T8 exposed the spinal cord and was used as a window of observation to confirm that the tip of the catheter was rightly placed. At the skin, in the exit point, the catheter was fixed subcutaneously in one point, and the skin was closed with Vicryl sutures (Johnson and Johnson®), leaving approximately 1 cm of catheter length outside. In order to prevent the rats from reaching the catheters, the exit point was placed near the back of their heads. For repeated administrations, the catheter tip was heated to close its extremity each time after an injection. Before a new treatment administration, the catheter was opened by cutting approximately 1 mm off its extremity. Animals were functionally evaluated (Basso, Beattie and Bresnahan (BBB) scale) on day 2 post-procedure and were excluded if any motor disability was detected. Rof and saline were administered locally through the intrathecal catheter (100 μg/kg) on days 2, 4 and 6 post-injury and then weekly by intraperitoneal injection (1 mg/kg) until the eighth week. The selected dosage of Roflumilast was taken into account based on previously published work, namely, it was demonstrated that 1 mg/kg of Roflumilast administrated intraperitoneally had superior neuroprotective effects than 0.5 mg/kg in the cerebral ischemia injury model [18]. The methodological scheme and temporal line for the in vivo study can be found on **Figure II.1**.

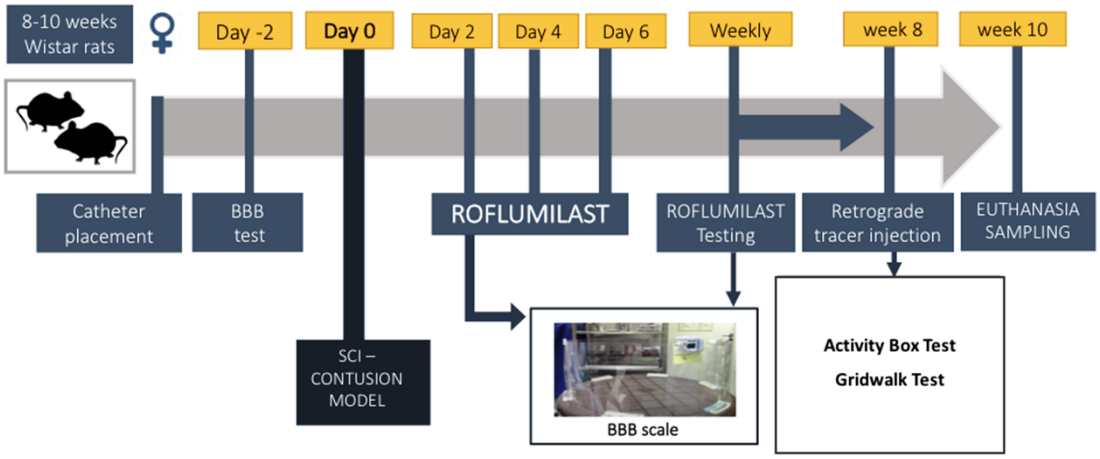


Figure II.1. *Methodological scheme and temporal line for the in vivo study design.*

2.6. Behavioral Assessment

The Basso, Beattie and Bresnahan (BBB) locomotor rating scale was used to evaluate functional recovery, as previously described [23]. All behavioral tests were performed blind on the treatment groups. The BBB test was executed two days post-injury and weekly thereafter for a 7-week period. A BBB score of 0 indicates no movement of the hindlimbs. From 1 to 8, a BBB score indicates joint movement but no weight support. A BBB score from 9 to 20 indicates the ability to support weight and use the limb for locomotion but with some degree of abnormality. A BBB score of 21 corresponds to locomotion in a normal rat. Activity Box Test (ABT) measure distance traveled, velocity and number of rearing movements of animals to assess exploratory behavior. The test was conducted in an open arena (43.2 × 43.2 cm) with transparent acrylic walls (MedAssociates Inc., Fairfax, VT, USA). Animals started the test at the center of the arena and were allowed 5 min to explore it. Data were collected using activity monitoring software. Velocity was used as a measure of locomotor activity [24,25]. Gridwalk test (GWT) assesses deficits in descending motor control by examining the ability of the rat to navigate across a 1.3 m long wire mesh walkway [26]. A training session was performed before surgery, in which groups of four animals freely explored the grid for 10 min. The test consisted of recording each rat's performance in a 3 min trial, where the animals freely explored the grid. For each 3 min trial, we selected 30 s based on continuous movements of the animal on the grid and then determined the number of errors committed by the rat. The Gridwalk test was performed 8 weeks post-injury.

2.7. Fluoro-Gold–Retrograde Axonal Tracer

Retrograde tracing explores neuronal retrograde axonal transport and is commonly used to elucidate the relationship between neuronal structure and function across the nervous system. Fluoro-Gold (hidroxistilbamidine) is a fluorescent and retrogradely transported anatomical tracer, which is commonly used to label neurons in the brain and spinal cord for light microscopic fluorescence microscopy [25] and was used to evaluate neuroregeneration. A 0.3 µL injection of Fluoro-Gold was injected with a rate of 0.1 uL/min using a glass micropipette at the level of T13 two weeks before the sacrifice (8 weeks post-injury). The injection on the ventral horns (coordinates: X = ±0.4; Y = T13; Z = −2.5) was performed bilaterally and guided by a stereotaxic apparatus (Stoelting 51730D, Kiel, WI, USA). Neuronal cellular bodies were quantified by calculating the ratio of positive neurons, labeled rostral and caudal, to the injury site (approximately 1 cm from epicenter of the lesion).

2.8. Serum Cytokine and VEGF Mediator Analysis

Blood was collected from the tail vein 1-, 4- and 8-weeks post-injury and allowed to clot for 30 min before centrifugation (10 min at 10,000× g). Then, serum was collected and frozen at −80 ◦C. An enzyme-linked immunosorbent assay for IL-4, IL-5, IL-13, IL-10, IL-1a, IL-1b, IL-2, IL-6, IL-12p, IL-17, IL-18, IFNg, TNFα and VEGF detection (Millipore, Burlington, MA, USA) was used, and the assay was performed as previously described [28]. Samples were analyzed using a MAGPIX Luminex xMAP® (Austin, TX, USA) instrument. Values for IL-4, IL-5, IL-1a, IL-1b, IL-2, IL-6, IL-12p, IL-17, IL-18, IFNg and TNFα were below the limit of detection and, for this reason, were not analyzed in the results section.

2.9. Animals Sacrifice and Spinal Cords collection:

We deep anesthetized animals ten weeks after injury with intraperitoneal sodium pentobarbital (200 mg/mL, Eutasil/Ceva Sante Animale) and trans-cardially perfused with cold 0.9% saline, followed by 300 mL of 4% paraformaldehyde (PFA) in 1× phosphatebuffered saline (PBS). After rough dissection of the vertebral column and spinal cord, tissues were fixed in a 4% PFA solution for 24 h (4 ◦C). The spinal cord was then dissected from the vertebral column and immersed in a cryoprotectant solution (30% sucrose) for 48 h at 4 ◦C. Afterwards, a 2 cm length of spinalcord tissues, centered on the lesion, was submerged in optimal cutting temperature (OCT) embedding medium, frozen on dry ice and stored at −20 ◦C. For Fluoro-Gold analysis, we also isolated 1 cm of both rostral and caudal segments immediately next to the 2 cm epicenter of spinal cord tissues. Longitudinal sections of 20 µm thickness were obtained using a

cryostat (Leica CM1900, LeicaBiosystems, Wetzlar, Germany) and thaw-mounted onto charged microscope slides (Superfrost Plus, Thermo Scientific, Waltham, MA, USA). Fluoro-Gold staining was analyzed using transversal sections. All histological procedures and evaluations were performed in a blind manner to the treatment groups.

2.9. Immunohistochemistry Protocol

For immunofluorescence staining, slices were washed with PBS, permeabilized with 0.2% Triton X-100 for 10 min, and blocked with 5% fetal calf serum in 0.2% Triton X-100 for 30 min. Then, the following primary antibodies were incubated overnight at room temperature (RT): rabbit anti-GFAP for astrocytes (1:200; Dako, Singapore), rabbit anti-IBA1 for microglia and macrophages (1:1000; Pharmingen, San Diego, CA, USA), chicken anti-B3 tubulin for Neurons (1:100; Millipore, Burlington, MA, USA), mouse anti-CREB (1:100; Santa Cruz, Santa Cruz, CA, USA) and rabbit anti-cAMPKA (1:1000; Abcam, Cambridge, UK). The following day, primary antibodies were then probed (3 h incubation) with the appropriate Alexa 594- or Alexa 488-conjugated secondary antibodies (1:1000; Invitrogen). Sections were counterstained with DAPI for 30 min (1:1000; Sigma) and mounted with ImmuMount® (Thermo Scientific). Between steps, five washes with PBS (1×) were performed. For all immunofluorescence procedures, appropriate negative controls were obtained by omitting the relevant primary antibodies. Images were acquired using a confocal point-scanning microscope (Olympus FV1000). All images were analyzed using ImageJ and FIJI software.

2.10. Immunofluorescence Analysis

Spinal cord immunostaining was analyzed by collecting photomicrographs of entire longitudinal slices with the cavity in the center. After obtaining micrographs through confocal microscopy, the photos were opened using the Image J software. The immunofluorescence quantification in each photomicrograph was assessed by the positive staining area for IBA1 and the negative staining area (cavity) for GFAP. For IBA1+, we measured the percent of reactive microglia (ameboid microglia), which was calculated by dividing the area of ameboid microglia by the total area of the spinal cord slice. For GFAP, the data plotted in the graphs represent the mean area of the cavity per section. For the spared tissue analysis, the preserved tissue area present in the injury epicenter was automatically segmented based on automatic intensity thresholding and normalized to the total epicenter area. The analysis of the GAP-43 regenerating fibers was conducted on whole-slice mosaic images. Rectangular ROIs were drawn every 200 μm from

the epicenter in both directions of the rostro–caudal axis. GAP-43 labeling area was quantified after intensity thresholding, and the value was normalized by the area of the spinal cord within each ROI.

2.11. Statistical Analysis

Statistical analysis was performed using the GraphPad Prism 8.00 software. The normality of the data was evaluated using Kolmogorov–Smirnov normality test. Data from the BBB test was assessed using two-way ANOVA test. Differences between groups were compared using the post hoc Bonferroni test. Activity box test, Gridwalk test, immunofluorescence and cytokine concentration data were analyzed using Student's t-test or Mann–Whitney according to normality test. Statistical significance was defined as $p < 0.05$ (95% confidence level). Data are shown as mean +/− standard error (SEM).

3. Results

3.1. Roflumilast Increases Nuclear CREB Labeling in Spinal Cord Neurons In Vitro

To understand if Roflumilast could mediate cAMP-driven plasticity responses, we treated spinal cord neurons derived from Wistar Han pups at (P5) after 7 days in culture (**Figure II.2A**). Using immunofluorescence, we assessed the expression of (cAMPKA) and (CREB) after 48 h of Roflumilast treatment and respective control (**Figure II.2B** and **II.2C**). The quantification of nuclear CREB and cytoplasmic cAMPKA demonstrated an increase in nuclear CREB translocation marked by an enhanced neuronal nuclear CREB staining area (8.767 ± 0.7375 vs. 5.942 ± 0.8331), while cAMPKA levels were unchanged when compared to controls (**Figure II.2D)** and **II.2E)**, $p = 0.0441$).

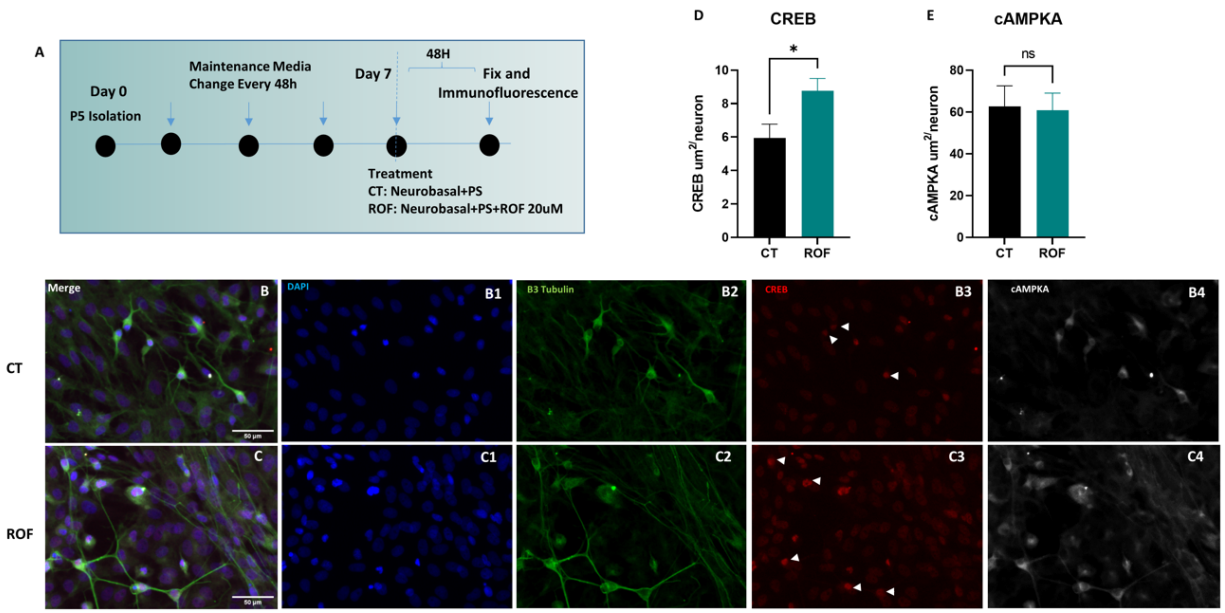


Figure II.2. *The effect of Roflumilast on cAMP-driven responses in spinal cord neurons.* ***A)*** *Experimental outline.* ***B)***, ***C)*** *Representative photomicrographs of controls and Roflumilast-treated cells, respectively.* ***D)*** *Neuronal nuclear CREB area quantification. Unpaired t-test, 6df, p = 0.0441. Controls n = 4, Roflumilast n = 4.* ***E)*** *Neuronal cytoplasmic cAMPKA area quantification. * p < 0.05; ns = non-significant.*

3.2. Roflumilast Administration Promotes Axonal Growth In Vitro

We used an organotypic culture of spinal cord slices (350 µm) from the thoracic region of Wistar Han pups within 6 days (P6) to assess the effect of Roflumilast on axonal regeneration. The cultures were incubated with basal medium + 20 nM Roflumilast or basal medium alone. The spinal cord slices cultured with Roflumilast showed higher levels of neurofilament expression (**Figure II.3A** and **II.3B**), as measured by the total area of positive staining (735,600 ± 12,862 µm²), indicating a superior axonal regeneration in this group when compared to controls (512,635 ± 62,363 µm²). These results showed that Rof promotes axonal regeneration in spinal cord neurons (**Figure II.3C**; p = 0.008).

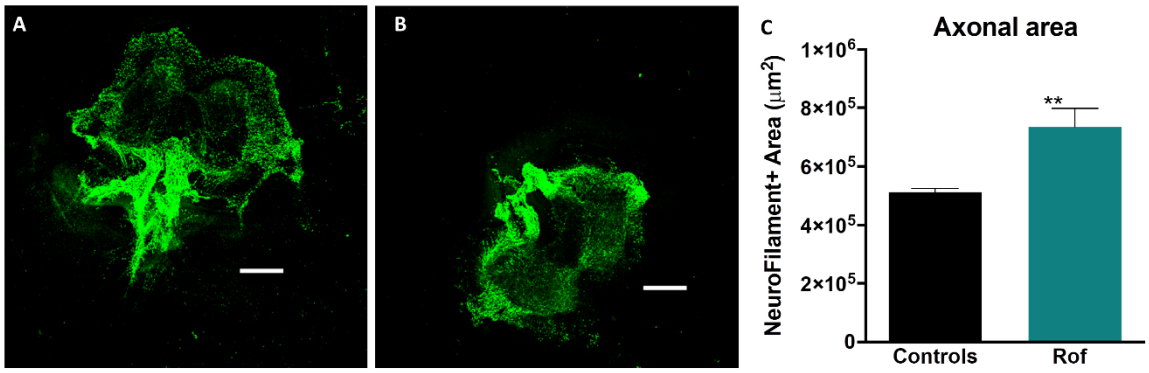


Figure II.3. *The effect of Roflumilast on axonal regeneration in vitro.* ***A)*** *Neurofilament + staining for cultures with Neurobasal + Roflumilast.* ***B)*** *Neurofilament + staining for cultures with only Neurobasal.* ***C)*** *Neurofilament + area in organotypic cultures. Unpaired t-test, 11df, p = 0.008. Controls n = 4, Roflumilast n = 5. ** p < 0.01.*

3.3. Roflumilast Treatment Increases Cyclic AMP Concentration In Vivo

ELISA analysis, performed on the spinal cords of injured animals, showed a significantly higher concentration of cAMP in rats treated with Roflumilast than in control animals. cAMP concentration in the Rof group was 3.85-fold higher than that in controls (**Figure II.4A**; p = 0.029), showing that Rof successfully elevated the levels of cAMP in the spinal cord.

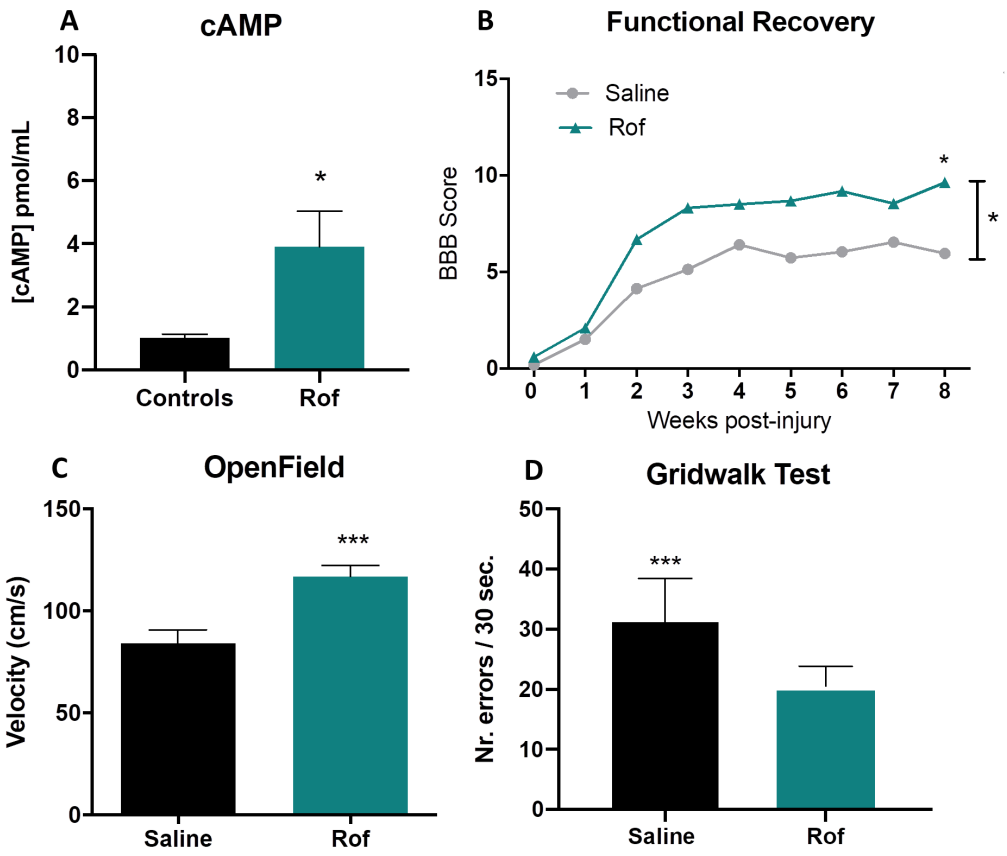


Figure II.4. *Pre-clinical evaluation of Roflumilast using a contusion SCI model.* ***A)*** *In vivo cAMP concentration for Rof-treated animals and controls. Unpaired t-test, 10 df, p = 0.029. Saline n = 6, Roflumilast n = 6.* ***B)*** *Functional recovery after spinal cord injury of Rof-treated animals and controls. Two-way ANOVA F (1, 20) = 4.408, p = 0.0487. Saline n = 11; Roflumilast n = 12.* ***C)*** *Activity box test—8th week. Velocity (cm/s) of Rof-treated animals and controls. Unpaired t-test, 21 df, p = 0.0008. Saline n = 11; Roflumilast n = 12.* ***D)*** *Gridwalk test of Roflumilast-treated animals and controls—8th week. Unpaired t-test, 21 df, p = 0.0001. Saline n = 11; Roflumilast n = 12. * p < 0.05; *** p < 0.001.*

3.4. Roflumilast Treatment Promotes Functional Recovery in a Thoracic Contusion Injury Model

Functional recovery was analyzed using the BBB score, ABT and Gridwalk test. The BBB test was performed weekly and revealed that although both Roflumilast (Rof) and saline-treated animals presented a spontaneous recovery over time, Rof-treated animals presented a significantly better functional recovery at 8 weeks post-injury when compared to saline treatment (Figure 4B; p = 0.048). At week 8, Rof-treated rats presented a mean BBB score of 9.6 ± 3.7, corresponding to the ability to support their own body

weight and to perform occasional plantar steps, while the saline-treated group presented a mean BBB score of 6.0 ± 3.5, which is translated into extensive movement of two joints and a slight movement of the third. Locomotor velocity was evaluated using ABT 8 weeks post-injury. Rof-treated animals presented better locomotor abilities than saline-treated rats. Rof presented a mean velocity of 116.9 ± 5.35 cm/s, while the saline-treated group presented a mean of 84.2 ± 6.50 cm/s velocity (Figure 4C; p = 0.0008). Finally, the Gridwalk test showed that the number of errors per 30 s of continuous locomotion on top of the wire mesh was superior in saline-treated animals, with a mean score of 31.7 ± 2.10 errors per 30 s, when compared to the Rof-treated group, which showed a mean score of 20.5 ± 0.96 errors per 30 s (Figure 4D; p = 0.0001).

3.5. Roflumilast Treatment Induces an Anti-Inflammatory Environment

Blood samples were collected, and the serums were analyzed 1, 4 and 8 weeks post-injury. Cytokine quantification was performed by multiplex analysis. Results revealed that IL-10 and IL-13 were significantly increased in the serum of Roflumilast-treated animals at the first- and fourth-weeks post-injury (**Figure II.5A–D**). The serum concentration of IL-10 in Rof treated rats was 1.6-fold higher than that in saline-treated animals 1-week post-injury (Rof: 0.064 ± 0.002 pg/mL; Saline: 0.039 ± 0.006 pg/mL) and 1.5-fold higher 4 weeks post-injury (Rof: 0.080 ± 0.003 pg/mL; Saline: 0.055 ± 0.007 pg/mL;). The elevation in the serum concentration of IL-13 was also 1.2-fold (Rof: 0.240 ± 0.013 pg/mL; Saline: 0.200 ± 0.007 pg/mL;) and 1.3-fold (Rof: 0.260 ± 0.020 pg/mL; Saline: 0.194 ± 0.046 pg/mL) higher 1 week and 4 weeks post-injury, respectively, in the Rof group. Moreover, the results also demonstrated that VEGF was significantly higher (3.5-folds) at 8 weeks post-injury in the Roftreated group (1.720 ± 0.530 pg/mL) versus the controls (0.490 ± 0.170 pg/mL) (**Figure II.5E**; p = 0.040).

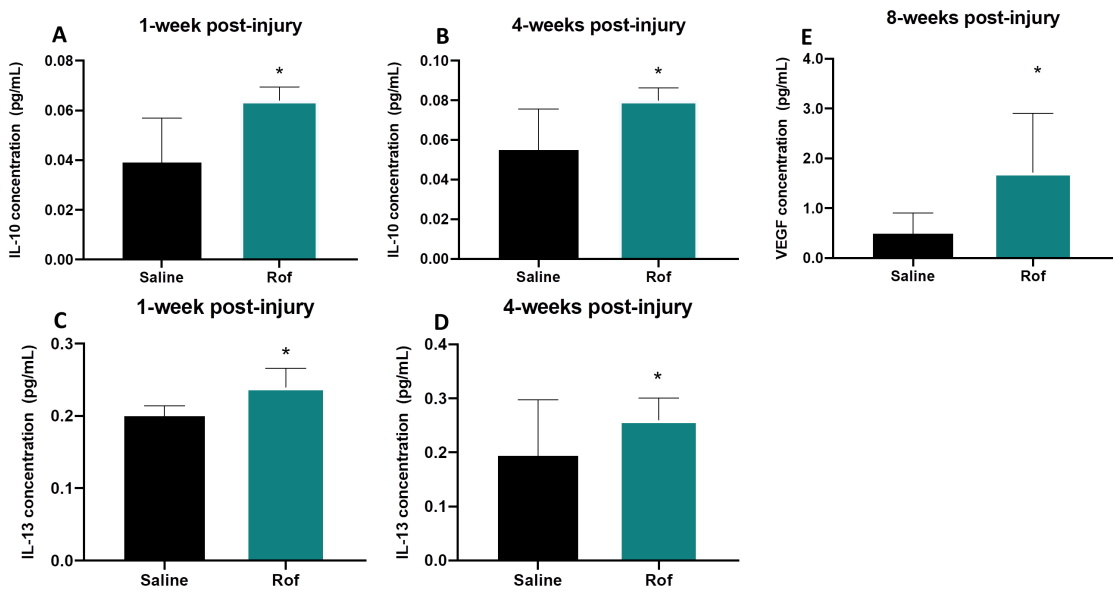


Figure II.5. *Multiplex analysis.* ***A)*** *Serum IL-10 concentration 1-week post-injury. Unpaired t-test, 13 df, p = 0.010. Saline n = 10; Roflumilast n = 5. B) Serum IL-10 concentration 4 weeks post-injury. Unpaired t-test, 14 df, p = 0.013. Saline n = 10; Roflumilast n= 6.* ***C)*** *Serum IL-13 concentration 1-week post-injury. Unpaired t-test, 6 df, p = 0.035. Saline n = 4; Roflumilast n = 4.* ***D)*** *Serum IL-13 concentration 4 weeks post-injury. Unpaired t-test, 7 df, p = 0.027. Saline n = 5; Roflumilast n = 4.* ***E)*** *Serum VEGF concentration 8 weeks post-injury. Unpaired t-test, 9 df, p = 0.040. Saline n = 6; Roflumilast n = 5. * p < 0.05.*

3.6. Roflumilast Treatment Reduces Cavity Size, Increases the Spared Tissue and Reduces Microglia Reactivity

Histological analysis was performed 10 weeks post-injury. Lesion size and spared tissue were quantified using GFAP+ staining (**Figure II.6C** and **II.6D**). An analysis of longitudinal slices of spinal cord tissue revealed that Rof treatment promoted a global reduction of lesion size when compared to the saline-treated group (Rof: 1,286,633 ± 138,341 µm2; Saline: 2,745,543 ± 413,518 µm2. **Figure III.6A**; p = 0.0149) and led to significant increases in the spare tissue (Rof: 33.23 ± 2.14 µm2; Saline: 24.50 ± 1.36 µm2. **Figure II.6B**; p = 0.0036).

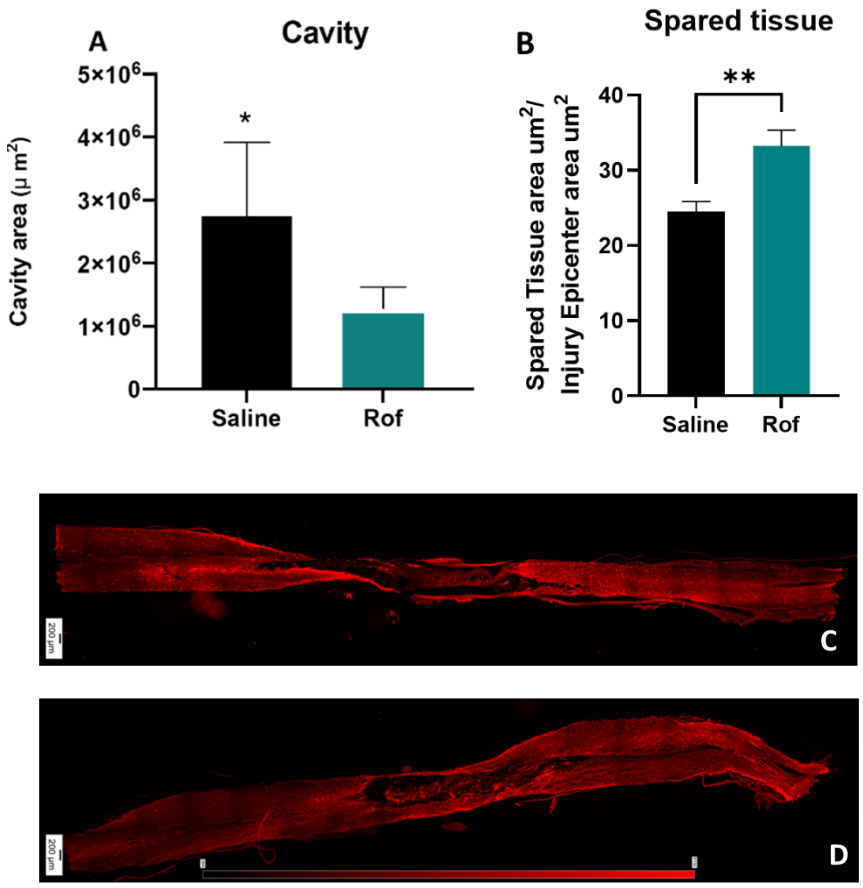


Figure II.6.A) *Cavity Size of Roflumilast-treated animals and controls. Unpaired t-test, 12 df, p = 0.0149. Saline n = 8; Roflumilast n = 6.* ***B)*** *Spared tissue in the injury epicenter of Roflumilast-treated animals and controls. Unpaired t-test, 12 df, p = 0.0036. Saline n = 8; Roflumilast n = 6.* ***C)*** *Longitudinal GFAP+ staining slice of saline-treated group and* ***D)*** *for Rof-treated group. A total of 69 longitudinal slices were analyzed. * p < 0.05; ** p < 0.01.*

In relation to microglia reactivity, we found that the percentage of IBA1 positive cells (**Figure II.7B** and **II.7C**) with amoeboid morphology was significantly lower (12.55 ± 1.97%) in the Rof-treated animals when compared to the controls (37.03 ± 4.41%) (**Figure II.7A**; p = 0.0003).

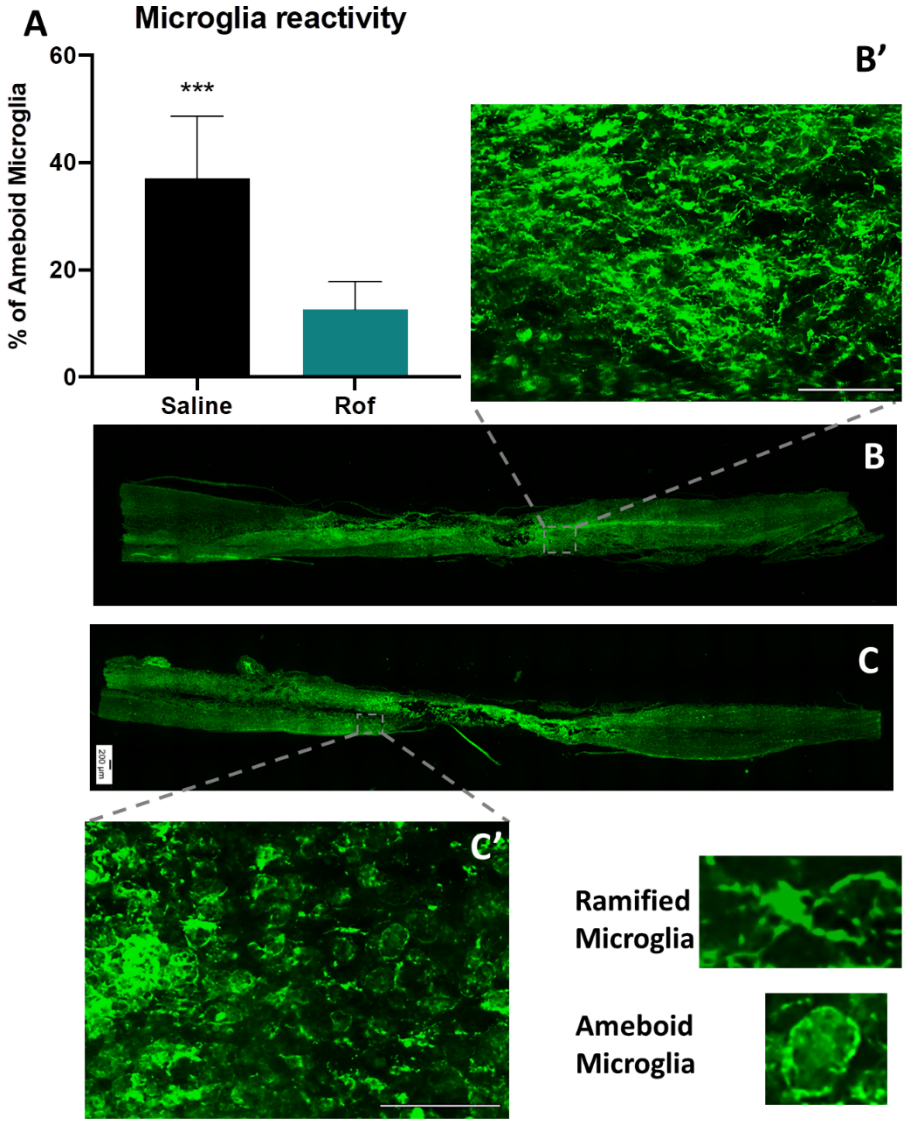


Figure II.7.A) *Microglia staining with IBA1 + to assess the percentage of ameboid microglia in Roflumilast-treated animals and controls. Unpaired t-test, 12 df, p = 0.0003. Saline n = 7; Roflumilast n = 7.* ***B)*** *Longitudinal slice of injured spinal cord, with IBA1+ staining for microglia reactivity in a Rof-treated animal;* ***B')*** *magnification of the dashed square.* ***C)*** *IBA1 + staining in an injured spinal cord of a saline-treated animal;* ***C')*** *magnification of the dashed square. A total of 68 longitudinal slices were analyzed. *** p < 0.001.*

3.7. Roflumilast Treatment Increases Neuronal Regeneration

In order to analyze axonal regeneration in the spinal cord tissue, we performed immunohistochemistry against Growth-Associated Protein 43 (GAP-43). Our analysis demonstrated that animals treated with Roflumilast have a statistically higher level of GAP-43, namely on the rostral side of the spinal cord of injured animals (**Figure II.8**; p = 0.0437).

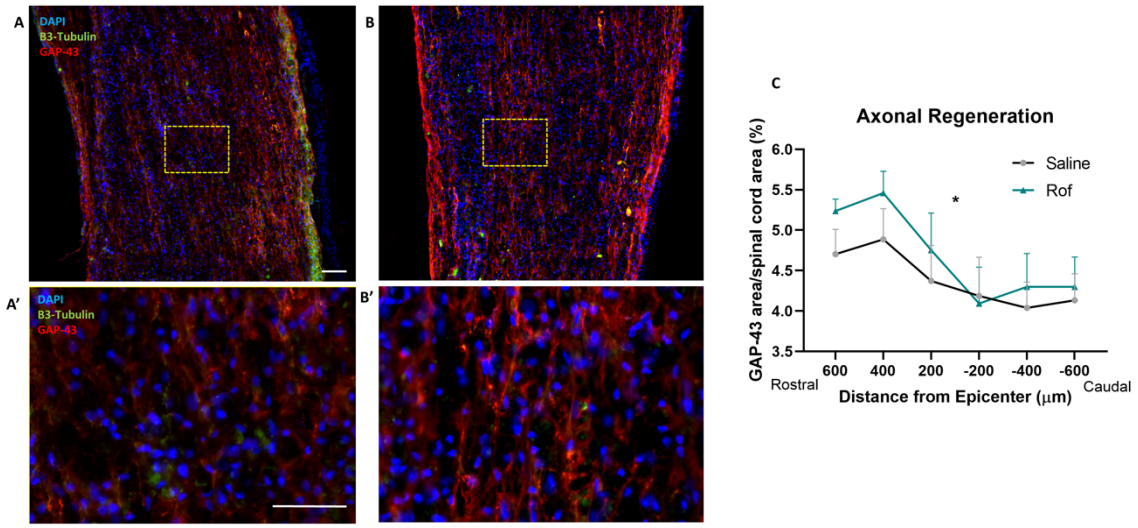


Figure II.8. *Roflumilast increases GAP-43 expression along the rostro–caudal axis of the injured spinal cord.* ***A)*** *and* ***B)*** *Microphotographs of the spinal cord of control and Roflumilast-treated animals.* ***C)*** *Shows the quantification of GAP-43 fibers along the rostro– caudal axis at 200, 400 and 600 μm from the injury epicenter in both directions. Two-way ANOVA F (DFn:1.409, DFd:18.31) = 4.182. Saline n = 7; Roflumilast n = 8. Insets show zoom magnification GAP-43 labeling. Scale Bars: 250 μm at* ***A)*** *and* ***B)*** *and 50 μm at* ***A')*** *and* ***B')****. * p < 0.05.*

Fluoro-Gold, a fluorescent retrograde tracer, was also used to evaluate neuroregeneration. The tracer was stereotaxically injected (bilaterally) on the ventral horns, at T13, eight weeks post-injury. Staining was analyzed in cross-sections of the spinal cord, rostral and caudal, to the injury site (approximately 1 cm from the lesion epicenter). Neuronal cellular bodies were quantified by calculating the ratio of positive neurons, labeled rostral and caudal, to the injury site (FG+ rostral/FG+ caudal). The Fluoro-Gold analysis revealed higher positive staining on Rof-treated animals (0.280 ± 0.036) at the rostral extremity of the thoracic spinal cord when compared to the control group (0.027 ± 0.011), supporting the neuroregenerative effect of Rof treatment (**Figure II.9**; p < 0.0001).

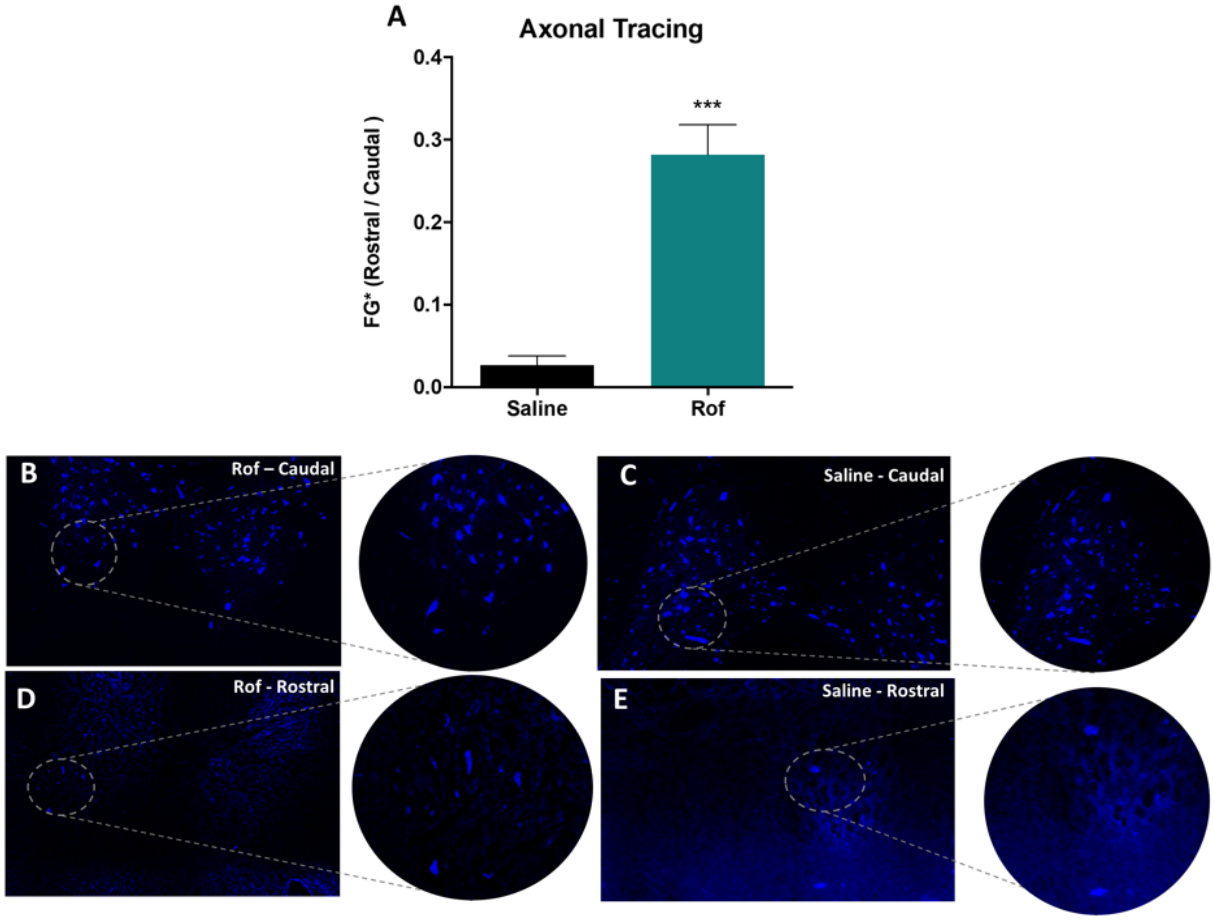


Figure II.9. *Roflumilast promotes axonal regeneration in vivo.* ***A)*** *Fluoro-Gold ratio (Rostral/Caudal) in Roflumilast-treated animals and controls. Unpaired t-test, 12 df, $p < 0.0001$. Saline n = 7; Roflumilast n = 7.* ***B)*** *Fluoro-Gold + staining in a Roflumilast-treated animal caudal to the epicenter.* ***C)*** *Fluoro-Gold + staining caudal to the epicenter in a saline-treated animal.* ***D)*** *Fluoro-Gold + staining in the Roflumilast group rostral to the lesion.* ***E)*** *Fluoro-Gold + staining rostral to the lesion in the saline group. A total of 88 transversal slices were analyzed. *** $p < 0.001$.*

4. Discussion

Despite great advances in knowledge about the processes that regulate axonal regeneration, the failure of axons to properly regenerate after spinal cord injury remains a critical challenge. One of the most important breakthroughs understanding axonal regeneration has been the identification of inhibitory proteins in CNS myelin (such as MAG, Nogo and OMgp) [11,26–29] and the development of strategies that enable axons to overcome myelin inhibition. It has been demonstrated that there is a correlation between neuronal cAMP levels and the inhibition of neurite outgrowth by MAG and myelin. Therefore, when levels of endogenous cAMP are high, substantial neurite outgrowth can be observed, even in the presence of MAG and myelin. However, in case of a decrease, there is the onset of myelin inhibition [10]. These findings suggest that pharmacological agents can be used to elevate cAMP levels in neurons and increase their ability to regenerate. Inhibiting the PDE4 receptor, which is responsible for cAMP hydrolysis, is one of the targets for increasing cAMP levels. PDE4 inhibition increases the levels of cAMP, which activates protein kinase A (PKA) and successively CREB, thereby promoting the transcription of genes associated with synaptic plasticity, axonal growth, and neurogenesis, such as the neurotrophic factor BDNF [15] and Arginase 1 [30]. The beneficial potential of PDE4 inhibition was first tested using Rolipram. The administration of this drug to SCI rats significantly improved axonal integrity and functional outcomes [12–14]. These results demonstrated the therapeutic potential of PDE4 inhibition. However, Rolipram, which was originally developed as an antidepressant drug in the early 1990s, has since been discontinued due to a narrow therapeutic window with significant gastrointestinal side effects as well as nausea and emesis [15,31]. In this sense, it is critical to find alternatives to Rolipram that can be safely tested in persons with SCI. The purpose of this study was to propose Roflumilast as a neuroregenerative drug for treating SCI. Previous studies have demonstrated that Rof, like Rolipram, is also a PDE4 inhibitor that leads to an increase in cAMP [32]. Additionally, Rof presents excellent pharmacokinetic properties, such as rapid absorption and distribution, linear kinetics, significant bioavailability and low toxicity [16], and is approved for human use in COPD [17]. Indeed, Roflumilast is currently the only PDE4 inhibitor approved for the treatment of severe COPD in humans [32]. Taking this into account, we tested the therapeutic potential of Rof in repairing the injured spinal cord. First, to understand whether Rof can promote axonal regeneration, we used organotypic cultures of the spinal cord from the thoracic region. The in vitro results demonstrated that Rof treatment promotes axonal regeneration in spinal cord slices, showing that Roflumilast creates a favorable neuroregenerative environment. We also analyzed axonal regeneration in the spinal cord tissue by performing immunohistochemistry against Growth-Associated Protein 43 (GAP-

43), and the animals treated with Roflumilast have a statistically higher expression of GAP-43 in the rostral side of the spinal cord. GAP-43 is enriched in growth cones and presents itself at high levels during axonal elongation [29], playing a critical role in axonal regeneration [30,31]. As we know, the transected axon is not inherently immobile but is in a latent state of growth, which is suppressed by factors of the local environment. As a result of blocking cAMP hydrolysis, Roflumilast attenuates the effects of inhibitory factors and restores the status of permanent growth inhibitory status. This effect was described for Rolipram [33]. Moreover, elevating neuronal cAMP levels, using db-cAMP or through a conditioning lesion to DRG neurons, has also been demonstrated to promote axonal growth [34,35]. Our results are another example of this mechanism being essential for axonal regeneration. However, we demonstrated this by using a drug that can be safely administered in humans, avoiding the side effects of Rolipram or db-cAMP. To understand whether Rof administration was able to elevate the levels of cAMP in the spinal cord of living animals, we performed molecular analyses and demonstrated that Rof increased the tissue concentration of cAMP in the injured spinal cord when compared to saline-treated animals. The elevation of cAMP caused by Roflumilast can be considered a bypass to the neurotrophin-activated signaling cascade [36] with the consequent activation of pathways such as cAMP/PKA/CREB [36–40] and cAMP/Epac 2 [39], which ultimately leads to axonal regeneration [36–39]. CREB-dependent transcription has been shown to control axon regeneration in both the PNS and CNS [32,34]. On the other hand, the expression of a dominant negative form of CREB has been shown to block axonal growth [36]. Herein, we assessed the pathway cAMP/PKA/CREB by evaluating the expression of (cAMPKA) and (CREB) after 48 h of Roflumilast treatment. We demonstrated an increase in nuclear CREB translocation marked by an enhanced neuronal nuclear CREB staining area in spinal cord neurons, while cAMPKA levels were unchanged when compared to controls. We then tested if the administration of Rof leads to an increase of cAMP in the spinal cord tissue. The increase in cAMP levels in Rof-treated animals was expected considering the mechanism of action of this drug [38,39,41–44]; however, it worked as a confirmation step before testing Rof administration in large animal sets. To test the therapeutic effect, using a contusion injury model at the thoracic level (T8), we observed that Rof treatment promoted locomotor recovery. When provided with Rof, SCI rats were able to achieve weight support and perform occasional plantar steps. In contrast, control animals were only able to perform extensive movements of two joints and a slight movement of a third joint. This improvement in hindlimb motor skills is probably the reason why the treated animals also walked over the ground faster than the non-treated animals. These results suggest that Rof treatment has a beneficial effect on incomplete paraplegia. To explore the impact of Rof's therapeutic effect on the restoration of fine motor skills, we also performed the Gridwalk test. During

this test, the animals were required to place their limbs on a grid wire and walk. Effective achievement of this task demands the activation of more than one neuronal pathway. It involves the coordination of the forelimb and hindlimb, which is mediated by ventrolateral tracts [45,46]; recruits the stepping rhythm, which implies a functioning reticulospinal system; and involves voluntary movement control, which is mainly mediated by the corticospinal tract [47,48]. The results demonstrated that Rof-treated animals recovered fine motor skills to a greater extent than saline-treated animals. Subsequently, we analyzed the long-term effects of Rof treatment at the histological level. Histological analysis of thoracic-injured animals demonstrated that Rof treatment considerably reduced the cavity size and also increased the spare tissue volume, suggesting a sustained protective effect on spinal cord tissue. Moreover, histological analysis revealed that the inflammatory response in spinal cord tissue was significantly reduced with Rof treatment. The persistent and non-resolving immune response after SCI is an important contributor to the secondary damage observed after primary injury. At the initial stages of SCI, the immune response resembles that in non-CNS injured tissue [33]. However, pro-repair immune cells are unable to populate the wounded tissue, and pro-inflammatory cells quickly become the predominant type at the injury site [35]. This immune response is associated with the expression of proinflammatory cytokines, fibrosis, oxidative damage and neurodegeneration, contributing to wound-healing failure [37]. The reduction in ameboid microglia/macrophages is probably due to a direct effect on inflammation by Rof since it has previously been demonstrated that Rof has an anti-inflammatory effect [48,49]. For instance, as seen in the lung, Rof consistently minimizes the release of TNF-α, CCL2, CCL3, CCL4, CCL5 and CXCL9 from human bronchial explants [49]. The accumulation of microglia/macrophages in the lesion epicenter plays a significant role in the inflammatory response during secondary injury. Microglia/macrophages are divided into two main subtypes: the pro-inflammatory subtype, which promotes inflammation and may lead to further tissue damage, and the pro-regenerative subtype, which promotes anti-inflammatory reactions, resolution of inflammation, and tissue repair [50,51]. Elevated intracellular cAMP levels in macrophages exhibit immunosuppressive activity predominantly by antagonizing proinflammatory cytokine production and phagocytosis [52]. Moreover, a previous study has shown that Roflumilast not only significantly decreases the M1/M2 ratio but also reduces the levels of pro-inflammatory molecules in an injured spinal cord [53]. These results led to the hypothesis that cAMP elevation may reduce secondary injury caused by local inflammation. Herein, we also examined the serum cytokines of spinal cord-injured rats, and the analysis revealed that IL-10 and IL-13 levels were increased in the serum of Roflumilast-treated animals at 1- and 4-weeks post-injury. IL-10 is an anti-inflammatory cytokine, and several studies performed over the past few decades have revealed positive effects of IL-10 treatment

post-SCI [54]. IL-10 treatment has been shown to protect against secondary inflammation by downregulating pro-inflammatory cytokines [55–57] and provides trophic support and neuroprotection [58], leading to functional recovery in rat models [59]. IL-13 is also an anti-inflammatory cytokine that has been reported to protect against demyelination and spinal cord injury through immunomodulation. IL-13 suppresses proinflammatory responses and enhances the phagocytic capacity of microglia/macrophages [60]. It was also seen that IL-13 shared many functions with IL-4. Interleukin-4 and IL-13 are signature cytokines of a type II inflammatory response. They are key players in the inflammatory response triggered by either an invading parasite or an allergen. Cellular sources of IL-4 and IL-13 have been studied extensively, and, along with CD4+ T cells, basophils, eosinophils, mast cells and NK+ T cells, appropriately stimulated ILC2 cells have the ability to produce IL-4 and IL-13 [61–63]. Recently, IL-4 has emerged as a promising therapeutic strategy in the context of SCI due to its ability to promote the M2-alternative activation of macrophages [64,65], a phenotype associated with neuroprotection and repair [22,66]. Additionally, our team also showed that IL-4 treatment increased circulating levels of the anti-inflammatory cytokine IL-10 and reduced inflammatory markers in the spinal cord of injured rats [21]. We also observed that VEGF levels were 3.5-fold higher at 8 weeks post-injury in the Roflumilast-treated group ($p = 0.040$). VEGF is a mitogen-activated protein that plays an active role in the formation of new blood vessels and cellular processes, such as proliferation, growth and survival. Importantly, VEGF's role in the nervous system may be related to neurogenesis [67], neuromaturation [68], neuroprotection [68–71] and neuroregeneration [72]. This effect occurs through the VEGF receptor and MAPK/Erk1/2 signal transduction pathways, which mediate growth effects on CNS neurons [69]. As such, VEGF promotes the reorganization of the spinal motor network after SCI [73] and has a protective effect against ischemia [74]. On the other hand, it is important to note that there is a possible link between VEGF to nonspecific axonal sprouting in dorsal horns, dorsal columns, and mechanical allodynia after SCI [75]. Some studies have also shown that treatment with angiogenesis inhibitors suppresses peripheral neuropathy-induced central angiogenesis, neuroinflammation, astrocyte activation and neuropathic pain [75]. Therefore, the role of VEGF must be further evaluated to balance the benefits and constraints. To understand if our treatment promotes axonal regeneration through the lesion site, we used the tracer Fluoro-Gold, which has been demonstrated to undergo retrograde axonal transport. We injected the tracer caudally into the injury site and quantified neurons, labeled rostral, to the lesion. As a result, the tracer needed uptake and traveled through regenerated fibers at the injury site. Using contusion injury allows us to test our therapy in a relevant model; however, it is not the ideal model to test regenerating axons. Full transections models are more appropriate to test axonal regeneration because they fully ablate the

ascending and descending information; however, this model is not clinically relevant. To have confidence in FG results, it is important that all animals were equally injured, and our locomotor analysis 2 days post-injury shows that all animals presented total paralysis of the hind limbs. In this work, we also normalized the number of labeled neurons for each animal. By performing the analysis and calculating the ratio of caudal-labeled neurons with the rostral-labeled neurons, we nullify the interference of the injection in each animal. All animals were injected in the same place of the spinal cord. However, microscopic variabilities in the injection site may have an impact on the amount of tracer uptake and retrograde transportation. Calculating the rostral/caudal FG-labeled neurons ratio shows us that treatment promoted higher axonal regeneration or higher neuronal plasticity. It is important to point out that the same retrograded tracer was used in previous incomplete SCI models [76–79]. Considering this, we showed that Rof-treated animals had higher positive staining in the rostral sections of the thoracic spinal cord compared to the control group. These results support the neuroregenerative effect of Roflumilast.

5. Conclusions

In this study, we demonstrated that Rof is a promising therapeutic drug for acute SCI management. Our results demonstrated the beneficial effects of Rof treatment on both functional recovery and neural regeneration. Considering the therapeutic effect of Rof, the clinical relevance of our findings, the translational potential of this therapeutic strategy and Rof's track record for safety, we believe that Rof has the potential to be applied in SCI clinical practice.

Author Contributions:

Investigation: C.S.S., R.L., J.R.C., E.D.G., T.S.P., D.S., L.S.F. and J.C.; conceptualization: N.A.S.; data curation: C.S.S., R.L. and J.C.; methodology: N.A.S.; validation: C.S.S. and R.L.; formal analysis: C.S.S. and R.L.; writing—original draft preparation: C.S.S.; writing—review and editing: A.J.S., E.D.G. and N.A.S.; project administration: N.A.S.; funding acquisition: A.J.S. and N.A.S. All authors have read and agreed to the published version of the manuscript. Funding: This research was supported by Prémios Santa Casa Neurociências—Prize Melo e Castro for Spinal Cord Injury Research (MC-18-2021) and by national funds, through the Foundation for Science and Technology (FCT)—project PTDC/MED-FAR/29751/2017, project UIDB/50026/2020 and UIDP/50026/2020. Institutional Review Board Statement: The animal study protocol was carried out in accordance with EU directive 2010/63/EU and was approved by the ethical committee in life and health sciences (Ref: DGAV 022405, SECVS116/2016, University of Minho, Braga, Portugal). Informed Consent Statement: Not applicable.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request. Acknowledgments: We would like to acknowledge the financial support by the Foundation for Science and Technology to the following authors: RL (PD/BDE/127836/2016), JRC (SFRH/BD/145860/2019) and NAS (CEECIND/04794/2007). We would like to acknowledge the support of the ICVS Scientific Microscopy Platform, members of the national infrastructure PPBI—Portuguese Platform of Bioimaging (PPBI-POCI-01-0145-FEDER-022122).

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

6. References

1. Monteiro, S.; Pinho, A.G.; Macieira, M.; Serre-Miranda, C.; Cibrão, J.R.; Lima, R.; Soares-Cunha, C.; Vasconcelos, N.L.; LentilhasGraça, J.; Duarte-Silva, S.; et al. Splenic sympathetic signaling contributes to acute neutrophil infiltration of the injured spinal cord. J. Neuroinflamm. 2020, 17, 282.
2. Catapano, J.S.; Hawryluk, G.W.J.; Whetstone, W.; Saigal, R.; Ferguson, A.; Talbott, J.; Bresnahan, J.; Dhall, S.; Pan, J.; Beattie, M.; et al. Higher Mean Arterial Pressure Values Correlate with Neurological Improvement in Patients With Initially Complete Spinal Cord Injuries. World Neurosurg. 2016, 96, 72–79
3. Lee, D.Y.; Park, Y.J.; Song, S.Y.; Hwang, S.C.; Kim, K.T.; Kim, D.H. The importance of early surgical decompression for acute traumatic spinal cord injury. CiOS Clin. Orthop. Surg. 2018, 10, 448–454.
4. Canseco, J.A.; Karamian, B.A.; Bowles, D.R.; Markowitz, M.P.; DiMaria, S.L.; Semenza, N.C.; Leibensperger, M.R.; Smith, M.L.; Vaccaro, A.R. Updated Review: The Steroid Controversy for Management of Spinal Cord Injury. World Neurosurg. 2021, 150, 1–8. [CrossRef] [PubMed]
5. Silva, N.A.; Sousa, N.; Reis, R.L.; Salgado, A.J. From basics to clinical: A comprehensive review on spinal cord injury. Prog. Neurobiol. 2014, 114, 25–57.
6. Fan, B.; Wei, Z.; Yao, X.; Shi, G.; Cheng, X.; Zhou, X.; Zhou, H.; Ning, G.; Kong, X.; Feng, S. Microenvironment Imbalance of Spinal Cord Injury. Cell Transplant. 2018, 27, 853–866.
7. Caroni, P.; Schwab, M.E. Two membrane protein fractions from rat central myelin with inhibitory properties for neurite growth and fibroblast spreading. J. Cell Biol. 1988, 106, 1281–1288.
8. Yamashita, T.; Higuchi, H.; Tohyama, M. The p75 receptor transduces the signal from myelin-associated glycoprotein to Rho. J. Cell Biol. 2002, 157, 565–570.
9. Dergham, P.; Ellezam, B.; Essagian, C.; Avedissian, H.; Lubell, W.D.; McKerracher, L. Rho Signaling Pathway Targeted to Promote Spinal Cord Repair. J. Neurosci. 2002, 22, 6570–6577.
10. Cai, D.; Qiu, J.; Cao, Z.; McAtee, M.; Bregman, B.S.; Filbin, M.T. Neuronal Cyclic AMP Controls the Developmental Loss in Ability of Axons to Regenerate. J. Neurosci. 2001, 21, 4731–4739.
11. Hannila, S.S.; Filbin, M.T. The role of cyclic AMP signaling in promoting axonal regeneration after spinal cord injury. Exp. Neurol. 2008, 209, 321–332.
12. Pearse, D.D.; Pereira, F.C.; Marcillo, A.E.; Bates, M.L.; Berrocal, Y.A.; Filbin, M.T.; Bunge, M.B. cAMP and Schwann cells promote axonal growth and functional recovery after spinal cord injury. Nat. Med. 2004, 10, 610–616.
13. Whitaker, C.M.; Beaumont, E.; Wells, M.J.; Magnuson, D.S.K.; Hetman, M.; Onifer, S.M. Rolipram attenuates acute oligodendrocyte death in the adult rat ventrolateral funiculus following contusive cervical spinal cord injury. Neurosci. Lett. 2008, 438, 200–204.
14. Beaumont, E.; Whitaker, C.M.; Burke, D.A.; Hetman, M.; Onifer, S.M. Effects of Rolipram on Adult Rat Oligodendrocytes and Functional Recovery after Contusive Cervical Spinal Cord Injury. Neuroscience 2009, 163, 985–990.
15. Heckman, P.R.A.; Wouters, C.; Prickaerts, J. Phosphodiesterase Inhibitors as a Target for Cognition Enhancement in Aging and Alzheimer's Disease: A Translational Overview. Curr. Pharm. Des. 2014, 21, 317–331.
16. Huang, J.; Fu, C.X.; Yang, X.Y.; Cui, C.; Yang, S.; Kuang, Y.; Guo, C.X.; Hu, P.; Pei, Q.; Yang, G.P. Pharmacokinetics of single- and multiple-dose roflumilast: An open-label, three-way crossover study in healthy Chinese volunteers. Drug Des. Devel. Ther. 2018, 12, 4047–4057.
17. Garnock-Jones, K.P. Roflumilast: A Review in COPD. Drugs 2015, 75, 1645–1656.
18. Keskin H, Keskin F, Tavaci T, Halici H, Yuksel TN, Ozkaraca M, Bilen A, Halici Z. Neuroprotective effect of roflumilast under cerebral ischaemia/reperfusion injury in juvenile rats through NLRP-mediated inflammatory response inhibition. Clin Exp Parmacol Physiol. 2021, 48, 1103-1110.
19. Vilhena, E. R., Bonato J.M., Schepers, M., Kuneida, J.K.C., Milani, H., Vanmierlo, T., Prickaerts, J., Oliveira, R. M.W. Positive effeccts of Roflumilast on behavior, neuroinflamation, and white matter injury in mice with global cerebral ischemia. Behavioural Pharmacology, 2021, 32, 459 - 471.

20. Lima, R.; Gomes, E.D.; Cibrão, J.R.; Rocha, L.A.; Assunção-Silva, R.C.; Rodrigues, C.S.; Neves-Carvalho, A.; Monteiro, S.; Salgado, A.J.; Silva, N.A. Levetiracetam treatment leads to functional recovery after thoracic or cervical injuries of the spinal cord. npj Regen. Med. 2021, 6, 11.
21. Lima, R.; Monteiro, S.; Lopes, J.P.; Barradas, P.; Vasconcelos, N.L.; Gomes, E.D.; Assunção-Silva, R.C.; Teixeira, F.G.; Morais, M.; Sousa, N.; et al. Systemic Interleukin-4 Administration after Spinal Cord Injury Modulates Inflammation and Promotes Neuroprotection. Pharmaceuticals 2017, 10, 83.
22. Vasconcelos, N.L.; Gomes, E.D.; Oliveira, E.P.; Silva, C.J.; Lima, R.; Sousa, N.; Salgado, A.J.; Silva, N.A. Combining neuroprotective agents: Effect of riluzole and magnesium in a rat model of thoracic spinal cord injury. Spine J. 2016, 16, 1015–1024.
23. Metz, G.A.S.; Merkler, D.; Dietz, V.; Schwab, M.E.; Fouad, K. Efficient testing of motor function in spinal cord injured rats. Brain Res. 2000, 883, 165–177. [CrossRef] [PubMed]
24. Silva, N.A.; Sousa, R.A.; Fraga, J.S.; Fontes, M.; Leite-Almeida, H.; Cerqueira, R.; Almeida, A.; Sousa, N.; Reis, R.L.; Salgado, A. J Benefits of spine stabilization with biodegradable scaffolds in spinal cord injured rats. Tissue Eng. Part C Methods 2013, 19, 101–108.
25. Persson, S.; Havton, L.A. Retrogradely Transported Fluorogold Accumulates in Lysosomes of Neurons and is Detectable Ultrastructurally using Post-embedding Immuno-gold Methods. J. Neurosci. 2009, 184, 42–47.
26. Liu, B.P.; Cafferty, W.B.J.; Budel, S.O.; Strittmatter, S.M. Extracellular regulators of axonal growth in the adult central nervous system. Philos. Trans. R. Soc. Lond. B Biol. Sci. 2006, 361, 1593–1610.
27. Liu, B.P.; Fournier, A.; GrandPré, T.; Strittmatter, S.M. Myelin-associated glycoprotein as a functional ligand for the Nogo-66 receptor. Science 2002, 297, 1190–1193.
28. Schwab, M.E. Nogo and axon regeneration. Curr. Opin. Neurobiol. 2004, 14, 118–124.
29. Filbin, M.T. Myelin-associated inhibitors of axonal regeneration in the adult mammalian. CNS. Nat. Rev. Neurosci. 2003, 4, 703–713.
30. Cai, D.; Deng, K.; Mellado, W.; Lee, J.; Ratan, R.R.; Filbin, M.T. Arginase I and polyamines act downstream from cyclic AMP in overcoming inhibition of axonal growth MAG and myelin in vitro. Neuron 2002, 35, 711–719.
31. Bielekova, B.; Richert, N.; Howard, T.; Packer, A.N.; Blevins, G.; Ohayon, J.; McFarland, H.F.; Stürzebecher, C.S.; Martin, R. Treatment with the phosphodiesterase type-4 inhibitor rolipram fails to inhibit blood-brain barrier disruption in multiple sclerosis. Mult. Scler. J. 2009, 15, 1206–1214.
32. Cortijo, J.; Iranzo, A.; Milara, X.; Mata, M.; Cerdá-Nicolás, M.; Ruiz-Saurí, A.; Tenor, H.; Hatzelmann, A.; Morcillo, E.J. Roflumilast, a phosphodiesterase 4 inhibitor, alleviates bleomycin-induced lung injury. Br. J. Pharmacol. 2009, 156, 534.
33. Nikulina, E.; Tidwell, J.L.; Dai, H.N.; Bregman, B.S.; Filbin, M.T. The phosphodiesterase inhibitor rolipram delivered after a spinal cord lesion promotes axonal regeneration and functional recovery. Proc. Natl. Acad. Sci. USA 2004, 101, 8786–8790.
34. Qiu, J.; Cai, D.; Dai, H.; McAtee, M.; Hoffman, P.N.; Bregman, B.S.; Filbin, M.T. Spinal axon regeneration induced by elevation of cyclic AMP. Neuron 2002, 34, 895–903.
35. Neumann, S.; Bradke, F.; Tessier-Lavigne, M.; Basbaum, A.I. Regeneration of sensory axons within the injured spinal cord induced by intraganglionic cAMP elevation. Neuron 2002, 34, 885–893.
36. Gordon, T. The role of neurotrophic factors in nerve regeneration. Neurosurg. Focus 2009, 26, E3.
37. Cui, Q.; So, K.F. Involvement of cAMP in neuronal survival and axonal regeneration. Anat. Sci. Int. 2004, 79, 209–212.
38. Siddiq, M.M.; Hannila, S.S. Looking downstream: The role of cyclic AMP-regulated genes in axonal regeneration. Front. Mol. Neurosci. 2015, 8, 26.
39. Batty, N.J.; Fenrich, K.K.; Fouad, K. The role of cAMP and its downstream targets in neurite growth in the adult nervous system. Neurosci. Lett. 2016, 652, 56–63.

40. Zhou, G.; Wang, Z.; Han, S.; Chen, X.; Li, Z.; Hu, X.; Li, Y.; Gao, J. Multifaceted Roles of cAMP Signaling in the Repair Process of Spinal Cord Injury and Related Combination Treatments. Front. Mol. Neurosci. 2022, 15, 808510.
41. Fabbri, L.M.; Beghé, B.; Yasothan, U.; Kirkpatrick, P. Roflumilast. Nat. Rev. Drug Discov. 2010, 9, 761–762.
42. Zebda, R.; Paller, A.S. Phosphodiesterase 4 inhibitors. J. Am. Acad. Dermatol. 2018, 78, S43–S52.
43. Pagès, L.; Gavaldà, A.; Lehner, M.d. PDE4 inhibitors: A review of current developments (2005–2009). Expert Opin. Ther. Pat. 2009, 19, 1501–1519.
44. Li, H.; Zuo, J.; Tang, W. Phosphodiesterase-4 inhibitors for the treatment of inflammatory diseases. Front. Pharmacol. 2018, 9, 1048.
45. Brustein, E.; Rossignol, S. Recovery of locomotion after ventral and ventrolateral spinal lesions in the cat. I. Deficits and adaptive mechanisms. J. Neurophysiol. 1998, 80, 1245–1267.
46. Z'Graggen, W.J.; Metz, G.A.S.; Kartje, G.L.; Thallmair, M.; Schwab, M.E. Functional Recovery and Enhanced Corticofugal Plasticity after Unilateral Pyramidal Tract Lesion and Blockade of Myelin-Associated Neurite Growth Inhibitors in Adult Rats. J. Neurosci. 1998, 18, 4744–4757.
47. Muir, G.D.; Whishaw, I.Q. Red nucleus lesions impair overground locomotion in rats: A kinetic analysis. Eur. J. Neurosci. 2000, 12, 1113–1122.
48. Rabe, K.F.; Watz, H.; Baraldo, S.; Pedersen, F.; Biondini, D.; Bagul, N.; Hanauer, G.; Göhring, U.M.; Purkayastha, D.; Román, J.; et al. Anti-inflammatory effects of roflumilast in chronic obstructive pulmonary disease (ROBERT): A 16-week, randomized, placebo-controlled trial. Lancet Respir. Med. 2018, 6, 827–836.
49. Salvator, H.; Buenestado, A.; Brollo, M.; Naline, E.; Victoni, T.; Longchamp, E.; Tenor, H.; Grassin-Delyle, S.; Devillier, P. Clinical Relevance of the Anti-inflammatory Effects of Roflumilast on Human Bronchus: Potentiation by a Long-Acting Beta-2-Agonist. Front. Pharmacol. 2020, 11, 1981.
50. Martinez, F.O.; Helming, L.; Gordon, S. Alternative Activation of Macrophages: An Immunologic Functional Perspective. Annu. Rev. Immunol. 2009, 27, 451–483. [CrossRef]
51. Gensel, J.C.; Zhang, B. Macrophage activation and its role in repair and pathology after spinal cord injury. Brain Res. 2015, 1619, 1–11.
52. Serezani, C.H.; Ballinger, M.N.; Aronoff, D.M.; Peters-Golden, M. Cyclic AMP: Master Regulator of Innate Immune Cell Function. Am. J. Respir. Cell Mol. Biol. 2008, 39, 127.
53. Moradi, K.; Golbakhsh, M.; Haghighi, F.; Afshari, K.; Nikbakhsh, R.; Khavandi, M.M.; Faghani, S.; Badripour, A.; Etemadi, A.; Ashraf-Ganjouei, A.; et al. Inhibition of phosphodiesterase IV enzyme improves locomotor and sensory complications of spinal cord injury via altering microglial activity: Introduction of Roflumilast as an alternative therapy. Int. Immunopharmacol. 2020, 86, 106743.
54. Thompson, C.D.; Zurko, J.C.; Hanna, B.F.; Hellenbrand, D.J.; Hanna, A. The Therapeutic Role of Interleukin-10 after Spinal Cord Injury. J. Neurotrauma 2013, 30, 1311–1324.
55. Florentino, D.F.; Zlotnik, A.; Mosmann, T.R.; Howard, M.; O'garra, A. IL-10 inhibits cytokine production by activated macrophages. J. Immunol. 1991, 147, 3815–3822.
56. Fuchs, A.C.; Granowitz, E.V.; Shapiro, L.; Vannier, E.; Lonnemann, G.; Angel, J.B.; Kennedy, J.S.; Rabson, A.R.; Radwanski, E.; Affrime, M.B.; et al. Clinical, hematologic, and immunologic effects of interleukin-10 in humans. J. Clin. Immunol. 1996, 16, 291–303.
57. Balasingam, V.; Yong, V.W. Attenuation of Astroglial Reactivity by Interleukin-10. J. Neurosci. 1996, 16, 2945–2955.
58. Xin, J.; Wainwright, D.A.; Mesnard, N.A.; Serpe, C.J.; Sanders, V.M.; Jones, K.J. IL-10 within the CNS is necessary for CD4+ T cells to mediate neuroprotection. Brain. Behav. Immun. 2011, 25, 820–829.
59. Zhou, Z.; Peng, X.; Insolera, R.; Fink, D.J.; Mata, M. IL-10 promotes neuronal survival following spinal cord injury. Exp. Neurol. 2009, 220, 183–190.

60. Kolosowska, N.; Keuters, M.H.; Wojciechowski, S.; Keksa-Goldsteine, V.; Laine, M.; Malm, T.; Goldsteins, G.; Koistinaho, J.; Dhungana, H. Peripheral Administration of IL-13 Induces Anti-inflammatory Microglial/Macrophage Responses and Provides Neuroprotection in Ischemic Stroke. Neurotherapeutics 2019, 16, 1304–1319.
61. Moro, K.; Yamada, T.; Tanabe, M.; Takeuchi, T.; Ikawa, T.; Kawamoto, H.; Furusawa, J.I.; Ohtani, M.; Fujii, H.; Koyasu, S. Innate production of TH2 cytokines by adipose tissue-associated c-Kit+Sca-1+ lymphoid cells. Nature 2009, 463, 540–544.
62. Neill, D.R.; Wong, S.H.; Bellosi, A.; Flynn, R.J.; Daly, M.; Langford, T.K.A.; Bucks, C.; Kane, C.M.; Fallon, P.G.; Pannell, R.; et al. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. Nature 2010, 464, 1367.
63. Saenz, S.A.; Siracusa, M.C.; Perrigoue, J.G.; Spencer, S.P.; Urban, J.F.; Tocker, J.E.; Budelsky, A.L.; Kleinschek, M.A.; Kastelein, R.A.; Kambayashi, T.; et al. IL-25 elicits a multi-potent progenitor cell population that promotes Th2 cytokine responses. Nature 2010, 464, 1362.
64. Francos-Quijorna, I.; Amo-Aparicio, J.; Martinez-Muriana, A.; López-Vales, R. IL-4 drives microglia and macrophages toward a phenotype conducive for tissue repair and functional recovery after spinal cord injury. Glia 2016, 64, 2079–2092.
65. Ghosh, M.; Xu, Y.; Pearse, D.D. Cyclic AMP is a key regulator of M1 to M2a phenotypic conversion of microglia in the presence of Th2 cytokines. J. Neuroinflamm. 2016, 13, 9.
66. Monteiro, S.; Salgado, A.J.; Silva, N.A. Immunomodulation as a neuroprotective strategy after spinal cord injury. Neural Regen. Res. 2018, 13, 423.
67. Jin, K.; Zhu, Y.; Sun, Y.; Mao, X.O.; Xie, L.; Greenberg, D.A. Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo. Proc. Natl. Acad. Sci. USA 2002, 99, 11946.
68. Mackenzie, F.; Ruhrberg, C. Diverse roles for VEGF-A in the nervous system. Development 2012, 139, 1371–1380.
69. Khaibullina, A.A.; Rosenstein, J.M.; Krum, J.M. Vascular endothelial growth factor promotes neurite maturation in primary CNS neuronal cultures. Dev. Brain Res. 2004, 148, 59–68.
70. Liu, Y.; Figley, S.; Spratt, S.K.; Lee, G.; Ando, D.; Surosky, R.; Fehlings, M.G. An engineered transcription factor which activates VEGF-A enhances recovery after spinal cord injury. Neurobiol. Dis. 2010, 37, 384–393.
71. Rong, W.; Wang, J.; Liu, X.; Jiang, L.; Wei, F.; Hu, X.; Han, X.; Liu, Z. Naringin treatment improves functional recovery by increasing BDNF and VEGF expression, inhibiting neuronal apoptosis after spinal cord injury. Neurochem. Res. 2012, 37, 1615–1623.
72. Bhuiyan, M.I.H.; Kim, J.C.; Hwang, S.N.; Lee, M.Y.; Kim, S.Y. Ischemic tolerance is associated with VEGF-C and VEGFR-3 signaling in the mouse hippocampus. Neuroscience 2015, 290, 90–102.
73. Guaiquil, V.H.; Pan, Z.; Karagianni, N.; Fukuoka, S.; Alegre, G.; Rosenblatt, M.I. Vegf-B selectively regenerates injured peripheral neurons and restores sensory and trophic functions. Proc. Natl. Acad. Sci. USA 2014, 111, 17272–17277.
74. Han, M.-K.; Kim, M.; Bae, S.Y.; Kang, L.; Han, S.Y.; Lee, Y.-S.; Rha, J.H.; Kim, S.U.; Roh, J.-K. VEGF protects human cerebral hybrid neurons from in vitro ischemia. NeuroReport 2004, 15, 847–850.
75. Wen, Z.H.; Huang, S.Y.; Kuo, H.M.; Chen, C.T.; Chen, N.F.; Chen, W.F.; Tsui, K.H.; Liu, H.T.; Sung, C.S. Fumagillin attenuates spinal angiogenesis, neuroinflammation, and pain in neuropathic rats after chronic constriction injury. Biomedicines 2021, 9, 1187.
76. Yick, L.W.; So, K.F.; Cheung, P.T.; Wu, W.T. Lithium chloride reinforces the regeneration-promoting effect of chondroitinase ABC on rubrospinal neurons after spinal cord injury. J. Neurotrauma 2004, 21, 932–943.
77. McKillop, W.M.; York, E.M.; Rubinger, L.; Liu, T.; Ossowski, N.M.; Xu, K.; Hryciw, T.; Brown, A. Conditional Sox9 ablation improves locomotor recovery after spinal cord injury by increasing reactive sprouting. Exp. Neurol 2016, 283, 1–15.

78. Jakeman, L.B.; Chen, Y.; Lucin, K.M.; McTigue, D.M. Mice lacking L1 cell adhesion molecule have deficits in locomotion and exhibit enhanced corticospinal tract sprouting following mild contusion injury to the spinal cord. Eur. J. Neurosci. 2006, 23, 1997–2011.
79. Baptiste, D.C.; Austin, J.W.; Zhao, W.; Nahirny, A.; Sugita, S.; Fehlings, M.G. Systemic polyethylene glycol promotes neurological recovery and tissue sparing in rats after cervical spinal cord injury. J. Neuropathol. Exp. Neurol. 2009, 68, 661–676.

Chapter III

Roflumilast – Going solo or searching for a friend.

The results presented in this chapter are currently submitted as an original article in an international peer reviewed journal:

Carla S Sousa, et al. From Neuroprotection to Neuroregeneration: Testing Novel Integrated Approaches for Spinal Cord Injury Recovery.

From Neuroprotection to Neuroregeneration: Combining Roflumilast with Levetiracetam or Adipose Stem Cell Secretome for Spinal Cord Injury Recovery.

Carla S. Sousa [1,2,3], Rui Lima [1,2], Eduardo D. Gomes [1,2], Andreia Monteiro [1,2], António J. Salgado [1,2] and Nuno A. Silva [1,2,*]

1 Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, 4710-057 Braga, Portugal

2 ICVS/3B's Associate Lab, PT Government Associated Lab, 4805-017 Guimarães, Portugal

3 Department of Neurosurgery, IPO Porto, 4200-072 Porto, Portugal

* Correspondence: nunosilva@med.uminho.pt; Tel.: +351-253604875

Abstract

The Spinal Cord Injury (SCI) pathophysiology is highly complex, contributing to a poor prognosis, and lack of effective treatments. Previously, we demonstrated that Roflumilast (Rof), leads to functional recovery when tested in a SCI contusion model. However, it is unlikely that Rof treatment on its own could fully restore the injured spinal cord. Therefore, our objective was to scrutinize the synergistic effects of combining the neuro regenerative agent Rof with neuroprotective approaches. Herein we tested two therapies, firstly, Rof combined with Levetiracetam (Lev), and in a second phase, the complementary interplay between Rof and Adipose Stem Cells secretome (Sec). We induced SCI using a weight drop trauma model at the T8 level, and rats were systematically assigned to one of eight groups based on the treatment modality. Functional recovery was assessed according to the Basso, Beattie, and Bresnahan (BBB) scale, Activity Box Test (ABT), Motor Swimming Test (MST), and Von Frey test. Results indicate that the unilateral use of Rof, Lev, or Sec was effective in promoting functional recovery. However, the combination of Rof + Lev or Rof + Sec did not lead to an improvement in functional outcomes when compared to both standalone treatments.

Overall, the combination of Lev + Rof and Rof + Sec did not have a better functional outcome than the single treatments in a severe thoracic contusion injury model. Further studies are needed to find a combinatorial treatment that can lead to superior therapeutic effects with potential clinical application.

Keywords

Phosphodiesterase 4 Inhibitors; Levetiracetam; Secretome; Spinal Cord Injury; Molecular Therapy.

Introduction

Traumatic spinal cord injury (SCI) can result in dramatic neurological disability with a strong social and psychological impact. Primary injury is caused by mechanical forces applied directly to the spinal cord at the time of injury [1]. After that, a cascade of secondary events take place with a progressive process of post-traumatic damage [2], which includes permeability and vascular variations, free radical formation and lipid peroxidation, ionic disruption and glutamate excitotoxicity, dysfunctional inflammatory reaction, apoptosis, and finally glial scarring. The events of the secondary injury create an inhibitory microenvironment that blocks axonal regeneration, greatly contributing to the failure of tissue repair observed after SCI. The identification of inhibitory proteins in central nervous system (CNS) myelin (such as MAG, Nogo, and OMgp) [3–7], greatly contributed to our understanding of the molecular basis of axonal regeneration. For instance, it was discovered a correlation between neuronal cAMP levels and inhibition of neurite outgrowth by myelin debris. When levels of endogenous cAMP were kept high, a significant neurite outgrowth could be observed, even under the presence of myelin [8]. For that reason, increasing the neuronal cAMP levels is known as one of the possible targets to promote axonal regeneration. For example, it has been shown that inhibiting the action of Phosphodiesterase-4 (PDE4), an enzyme responsible for the hydrolysis of cAMP, increases the levels of cAMP, which activates protein kinase A (PKA) and successively CREB, thereby promoting transcription of genes associated with synaptic plasticity, axonal growth, and neurogenesis [9,10]. Recently, our team tested an FDA-approved PDE4 inhibitor, Roflumilast (Rof), as a strategy to promote axonal regeneration and repair after SCI. We observed that Rof was able to increase axonal regeneration in vitro and lead to functional recovery in vivo. These results were accompanied by an increase in the cAMP levels in the spinal cord tissue as well as a significant decrease in the cavity size and less reactive microglia cells [11].

Together with axonal growth inhibition, one of the central pieces of the SCI pathological process is glutamate excitotoxicity. After the SCI, calcium (Ca^{2+}) flux at the synaptic cleft regulates the release of glutamate. As glutamate receptors (NMDA and AMPA) become overactivated, sodium (Na^{+}) and Ca^{2+} influx increases. As a result of this ionic dysregulation, neuronal and glial cells, especially oligodendrocytes and neurons, become vulnerable to cell death [12,13]. Moreover, axonal degeneration is mediated by Ca^{2+} influx from the endoplasmic reticulum (ER) via the IP3 receptor that promotes mitochondrial permeability [14]. Globally, glutamate concentrations increase to neurotoxic levels (excitotoxicity) resulting in axonal demyelination and neuronal cell death [15–17]. Thus, modulating excitatory amino acid levels may be an effective strategy to promote neuroprotection after SCI trauma. Several drugs that modulate excitotoxicity have been tested over the years. Our team has recently shown that Levetiracetam (Lev) had a

neuroprotective role after SCI. Lev is an antiepileptic drug that suppresses presynaptic neurotransmitter release by binding to synaptic vesicle protein SV2A [18–21]. Using a cervical and thoracic rat SCI model, our team demonstrated that Lev treatment promotes significant improvements in both gross and fine motor function, revealing a significant decrease in cavity contusion size, as well as higher neuronal and oligodendrocyte survival [22] .

Another promising therapeutic approach that our team has been testing is the use of the proteins and extracellular vesicles secreted by Mesenchymal Stem Cells, namely the ones derived from adipose tissue (ASCs). Different studies point to the secreted factors and vesicles, known as the secretome, released by these cells as agents for the regenerative effect rather than ASCs differentiation. The secretome can be defined as the factors that are secreted by a cell, tissue, or organism to the extracellular space under defined time and conditions [23]. We have previously reported that the ASC secretome is an inductor of vascularization [24], as well as a promoter of neurite growth, leading to functional recovery when injected in mice with thoracic contusive or full transection SCI [25].

While individual therapies have shown promise in preclinical models, the fact is that they have been unable to demonstrate clinical efficacy when tested in humans. For this reason, it is important to search for complementary treatment strategies that can address diverse aspects of SCI pathology and produce higher levels of regeneration. In this sense, herein we combined the neuroprotective effects of Lev with the neuroregenerative effects of Rof. Moreover, we also searched for synergistic or additive therapeutic effects between the Rof and ASCs secretome.

Materials and methods

This study has two parts, in which Roflumilast was combined with two different therapeutic approaches: Levetiracetam and the secretome derived from ASCs. The assessment focused on functional recovery in a rat model of SCI. A total of 72 animals were subjected to a SCI and randomly treated with the therapeutic agents administered alone or given in combination. Functional recovery was assessed during 8 weeks post-injury. All procedures were carried out by EU directive 2010/63/EU and were approved by the ethical committee in life and health sciences (Ref: DGAV 022405, SECVS116/2016, University of Minho, Braga, Portugal). The ARRIVE guidelines for reporting animal research have been followed [26]. A detailed description of the methods can be found in supplemental materials.

Cell culture and secretome collection

Adipose Stem Cells (ASCs), were collected from human donors, according to the procedure described by Dubois [27]. ASCs in passage 6 (P6) were seeded in α-MEM medium (Invitrogen, USA) supplemented with with 5% human platelet lysate (hPL, Stemmatters, Portugal) and 1% Penicillin-Streptomycin (P/S, Invitrogen USA). ASCs were maintained at 37°C and 5% CO2, with medium changes every 2 or 3 days, until reaching confluency. The secretome was collected as described before by our group [28]. Briefly, P6-P8 hASCs were plated at a density of 4 to 12x103 cells/cm2 and left for 72 hours in a complete α-MEM medium and incubated as previously described. Afterward, the medium was removed, and cells were washed with phosphate-buffered saline (PBS), with no Ca^{2+} and Mg^{2+} (Invitrogen, USA). After washing, Neurobasal A medium (NbA, Invitrogen, USA), supplemented with only 1% kanamycin (Invitrogen, USA), was added to the cells. This media was left for 24 hours at the same incubation parameters. Following this conditioning period, the medium containing the factors/vesicles released by the ASCs – secretome or conditioned media – was collected and centrifuged at 1000g for 10 minutes to pellet cell debris. As a control group we followed the same parameters but without cells in the culture flasks. The secretome or Neurobasal medium only were stored at -80°C, after a snap freeze in liquid nitrogen, until it was required for further experiments.

In vivo study design

The objective of this study was to evaluate the therapeutic efficacy of two distinct treatment combinations, specifically the synergistic effects of Lev and Rof in the first segment, and the complementary interplay between Rof and ASCs secretome (Sec) in the second segment. The assessment focused on the enhancement of functional recovery in a rat model of SCI. In the initial part of the study, a total of 48 animals were allocated into four groups, each consisting of 12 rats. These groups received treatments with Rof alone, Lev alone, a combination of Rof and Lev, or a control group administered with saline. In the latter portion of the study, a total of 24 animals were similarly divided into four groups, with six rats per group. These groups were treated with Rof alone, Sec alone, a combination of Rof and Sec, or Neurobasal alone, serving as the control group. All data collection procedures were conducted in a double-blind manner to minimize any potential bias related to the treatment groups. All procedures were carried out by EU directive 2010/63/EU and were approved by the ethical committee in life and health sciences (Ref: DGAV 022405, SECVS116/2016, University of Minho, Braga, Portugal).

Spinal Cord Injury Model and Treatment

Wistar Han female rats (8 to 10 weeks old, weighing 210–260 g) were used in this study. Animals were kept under standard laboratory conditions (12 hours light: 12 hours dark cycles, 22°C, relative humidity of 55%, ad libitum access to standard food and water), and housed in pairs. Animal handling was carried out 3 days before surgery and then subjected to a severe contusive SCI as previously described [29,30]. Briefly, general anesthesia was induced by intraperitoneal injection (IP) of ketamine (100 mg/mL, Imalgene/Merial, Duluth, GA, USA) and medetomidine hydrochloride (1 mg/mL, Dormitor/Pfizer, New York, NY, USA) mixture, at a volume ratio of 1.5:1. Once anesthetized, animals received subcutaneous injections of the analgesic butorphanol (10 mg/mL, Butomidor/Richter Pharma AG, Wels, Austria), and the antibiotic enrofloxacin (5 mg/mL, Baytril/Bayer, Leverkusen, Germany). The fur was shaved from the surgical site and the skin was disinfected with 70% ethanol and chlorohexidine. Surgical procedures were performed under sterile conditions. The animals were placed in a prone position and a dorsal midline incision was made at the level of the thoracic spine (T5–T12). The paravertebral muscles were retracted and the spinous processes and laminar arc of T8 were removed, exposing the spinal cord. The dura was left intact. A weight drop trauma model was used, which consisted of dropping a 10 g weight rod from a 10 cm height onto the exposed spinal cord. The rod was guided through a stabilized tube that was positioned perpendicularly to the center of the spinal cord. After the trauma, the muscles were sutured with Vicryl suture (Johnson and Johnson, New Brunswick, NJ, USA), and the incision closed with surgical staples (Fine Science Tools, Heidelberg, Germany). Anesthesia was reverted using atipamezole (5 mg/mL, Antisedan/Pfizer, New York, NY, USA).

Post-operative care for all rats included butorphanol (Richter Pharma AG, Wels, Austria) administration twice a day, for five days as well as vitamins (Duphalyte, Pfizer, New York, NY, USA), saline, and enrofloxacin (Bayer, Leverkusen, Germany), twice a day for 7 days. Manual expression of bladders was performed twice a day until animals recovered spontaneous voiding. Body weight was monitored weekly as a parameter of the general health of the animals. If a weight loss over 10% of body weight was detected, a high-calorie oral supplement (Nutri-Cal®) was administered daily.

Combination of Roflumilast and Levetiracetam

For every group, Rof, Lev, Rof + Lev, or Saline administration, an intrathecal catheter was inserted three days before inflicting the contusion. Under general anesthesia, a dorsal midline incision was made. The spinous process and laminar arc of T13 were removed, the dura was gently opened, and the catheter was introduced up to the level of T9. Animals were functionally evaluated [Basso, Beattie, and Bresnahan

(BBB) scale], at day 2 post-procedure, and were excluded if any motor disability was detected. Three days after catheter implantation the SCI was inflicted as described above, then Lev (750 mg/kg) was administered by IP, 4 hours after injury, and treatment with Rof was administered locally through the intrathecal catheter (100 µg/kg) on days 2, 4, and 6 post-injury and then weekly by an IP (1 mg/kg) until the eighth week (**Figure III.1A**). This treatment regimen for Rof, where local and systemic administrations were combined, was chosen due to previous positive results [11] . For the Lev + Rof group, both drugs were administered at the same time points and with the same methods of administration for each drug alone. The control group received a saline solution via the same methods and at the same timings as both drugs, while the Lev group also received a local saline solution at the same time points that the Rof group received its treatment and vice-versa.

Combination of Roflumilast and ASCs secretome

The secretome of ASCs was administered by intravenous injection (IV) at day 0 (30 min post-SCI), 1, 2, and 3 days post-SCI as previously described [31] and treatment with Rof (1 mg/kg) was administered from week 2 and every week until the seventh week by IP injection (**Figure III.1B**). For the Sec + Rof group, we administered the drugs at the same individual time points and method of administration for each drug alone. The control group received the vehicle solution for each specific treatment, the vehicle for Sec was Neurobasal medium and the vehicle for Rof was saline. Animals treated only with Sec receive saline from week 2 and every week until the seventh week by IP injection. Animals treated only with Rof were administered with Neurobasal medium through (IV) at day 0 (30 minutes post-SCI), 1, 2, and 3 days post-SCI.

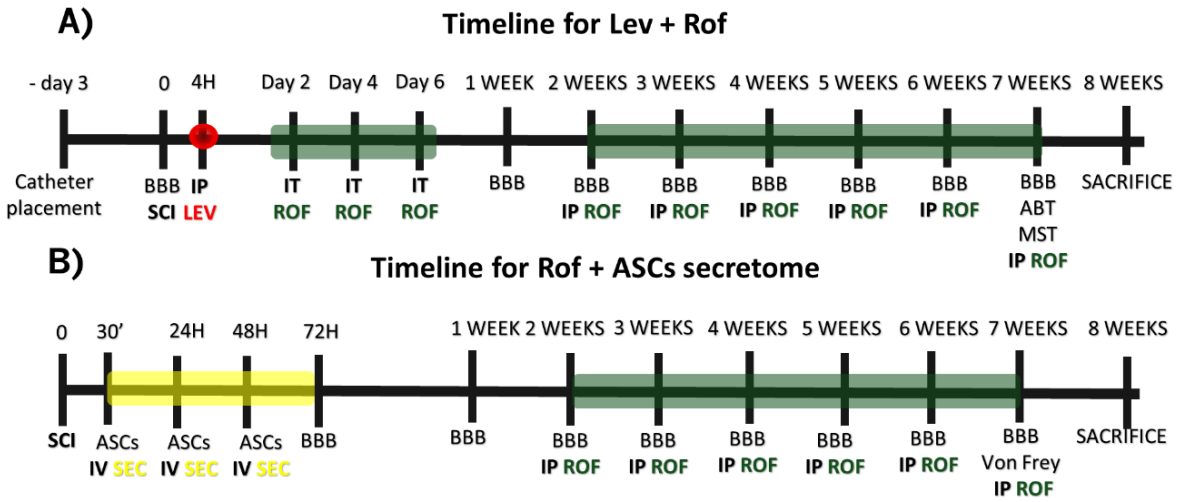


Figure III.1. *Schematic experimental design.* ***A)*** *Therapeutic synergies between Roflumilast (ROF) and Levetiracetam (LEV). Wistar rats underwent intrathecal catheter placement 3 days before spinal cord injury (SCI). LEV was administered by intraperitoneal injection (IP) 4 hours after injury. ROF treatment was administered locally through the intrathecal catheter on days 2, 4, and 6 of pos-injury and then weekly as highlighted in green. Behavioral assessment was performed through Basso, Beattie, and Bresnahan (BBB) scale, Activity Box Test (ABT), Motor Swimming Test (MST) at the timepoints indicated above.* ***B)*** *Therapeutic synergies between Roflumilast (ROF) and secretome of ASCs. Wistar rats undergo secretome administration through intravenous injection 30 minutes after SCI, followed by daily administration until 72 hours post-injury, as highlighted in yellow. Treatment with Rof was administered from week 2 and every week until the seventh week by IP as highlighted in green. Behavioral assessment was performed BBB scale and Von Frey test at the timepoints indicated above.*

Behavioral Assessment

BBB scale

The BBB locomotor rating scale was used to evaluate functional recovery as previously described [22]. The BBB test was executed three days post-injury (dpi) and thereafter weekly for 8 weeks. A BBB score of 0 indicates no hindlimb movement. A BBB score of 1 through 8 indicates joint movement, but no weight support. A BBB score of 9 through 20 indicates an ability to support weight and use the limb for locomotion but with some degree of abnormality. A BBB score of 21 corresponds to the locomotion of a normal rat. All behavioral analyses were executed blindly for the treatment groups.

Activity Box Test (ABT)

This test allows the assessment of exploratory behavior by measuring the total distance traveled, and the velocity of the animals. The test was done in an open arena (43.2 × 43.2 cm) with transparent acrylic walls (Med Associates Inc., Vermont, USA). Animals started the test at the arena's center and were given 5 minutes to explore it. Data was collected using the activity monitor software. Velocity was used as a measure of locomotor activity [32,33].

Motor Swimming Test (MST)

As rats swim, they can perform locomotor movements without fully supporting their body weight since water provides buoyancy. When the animals have been trained, they swim without any apparent

aversions, and stress symptoms do not significantly affect the test. The motor swimming test was performed on a quadrangular pool (water temperature 25°C), where the rat had to reach the platform to get out of the water. As a training period, the animals performed four trials two days before the test. On the day of behavior analysis, all trials were recorded by a video camera. Velocity was assessed by using three trials of each rat. The software Ethovision XT 12 (Noldus, Wageningen, Netherlands) was used to determine the velocity of swimming.

Von Frey Test

The Von Frey test was performed in the seventh week post-injury, as previously described [34]. After 5 minutes of acclimatization to the experimental conditions, animals were placed on an elevated grid. A mechanical allodynia assessment was then conducted using an up-and-down method as described previously [35,36]. A series of Von Frey calibrated monofilaments: 15.0, 8.0, 6.0, 4.0, 2.0, 1.0, 0.6, and 0.4 g (North Coast Medical Inc., USA) was used to probe the sural dermatome of the lesioned hindlimbs.

Using at first the 2.0 g filament, the test would advance upward if no response was elicited (=0) or downward if a brisk withdrawal of the limb was generated (=X) until 6 measurements were obtained around the threshold point [36]. A response did not include movements of the paws associated with locomotion or weight shifting. The 50% response threshold was then calculated using the following formula: $50\%g_threshold=(10Xf+K.\delta)/10000$.

Where Xf = value (in log units) of the final von Frey filament; k = tabular value corresponding to the pattern of positive and negative responses [X and 0 sequences; consult [35]; δ = mean difference (in log units) between stimuli (0.224). If no response was obtained up to maximal force (15.0 g) or conversely, if all filaments elicited a response down to the minimal force (0.4 g), the values 15 and 0.25 were assumed as the 50% withdrawal threshold, respectively.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8.00 software. The normality of the data was evaluated by the Kolmogorov-Smirnov normality tests. When the equal variances criterion was not met, Welch correction was applied. Data from the BBB test was assessed by a two-way ANOVA test. Differences between groups were compared with the post hoc Tukey test. Activity box test, motor swimming test, and the Von Frey were analyzed using the non-parametric Kruskal-Wallis followed by the Dunn's multiple comparisons test. Statistical significance was defined for $p < 0.05$ (95% confidence level). Data are shown as mean +/− standard error (SEM).

Results

Roflumilast and Levetiracetam alone or combined treatments promote functional recovery after SCI.

Four hours post-injury, the animals were administered Lev (750 mg/kg) via IP injection. Subsequently, they received 3 intrathecal injections of Rof (100 ug/kg) at 2, 4, and 6 dpi. Following this, they were administered a weekly IP injection of Roflumilast (1 mg/kg) commencing from the second-week post-injury. The BBB test was performed to evaluate locomotor recovery in an open arena with transparent acrylic walls, 3 dpi and thereafter weekly for 7 weeks. Results revealed that although all groups presented a spontaneous recovery over time, only treated animals showed a significant motor recovery when compared to saline (**Figure III.2.A**; $p = 0.0064$). However, Tukey's multiple comparisons test did not reveal any difference amongst treatments when administered alone or in combination. At 8 weeks post-injury, animals treated with Rof (9.6 ± 1.7), Lev (10.1 ± 1.3), or Lev + Rof (9.8 ± 1.4) were able to perform occasional weight support plantar steps without forelimb hindlimb coordination, while animals treated with saline (5.6 ± 1.1), only achieved extensive movement of two joints and slight movement of the third joint. The locomotor analysis considering two significant indicators of functional recovery, namely, body weight support and coordination, revealed significant differences when analyzed over time between drug-treated animals and the saline-treated group. Only 20% of the animals treated with saline recovered body weight support contrasting with 44% in the Lev ($p=0.0001$), 45% in the Rof ($p<0.0001$), and 62,5% in the Lev+Rof treated animals ($p<0.0001$; **Figure III.2.B**). Concerning coordination, none of the saline-treated animals recovered any level of coordination, while 33% of Lev, 45% of Rof, and 50% of Lev+Rof treated animals recovered at least occasional coordination (**Figure III.2.C**).

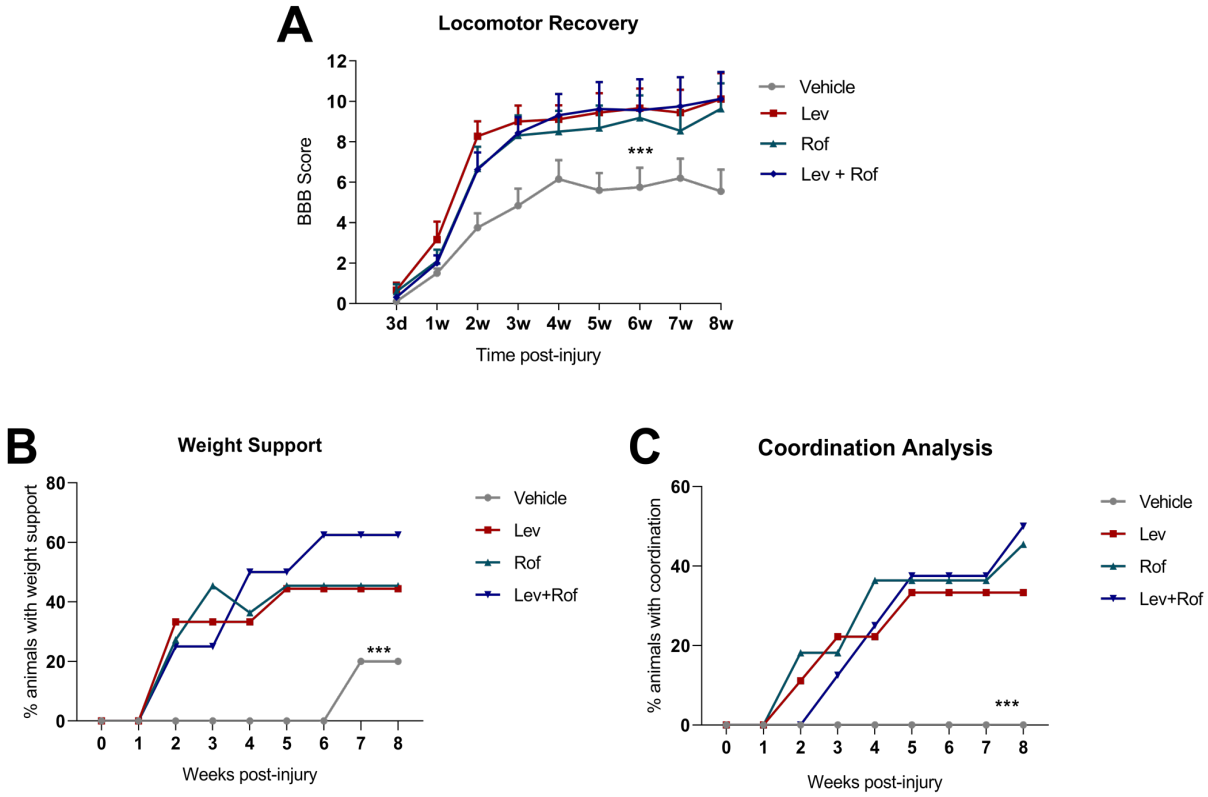


Figure III. 2. *Evaluation of functional recovery after spinal cord injury in animals treated with Rof, Lev, Rof+Lev or vehicle.* ***A)*** *Locomotor recovery analysis demonstrated that only treated animals presented significant motor recovery when compared to vehicle. No differences were observed between the treatments when administered alone or in combination.* ***B)*** *Weight support analysis demonstrated that animals treated with Lev, Rof, and Lev+Rof presented higher recovery body weight support contrasting with the vehicle-treated group.* ***C)*** *Coordination analysis demonstrated that vehicle-treated animals did not recover any level of coordination. All treatment groups presented significant improvement in coordination when compared to controls, however, no statistical differences were observed between treatments. Data was analyzed using 2-way ANOVA p=0.0064 followed by Tukey's multiple comparisons test. Vehicle n = 10; Lev n = 9; Rof n = 11; Lev+Rof n = 8. Values shown as mean ± SEM. ***p<0.001.*

Despite no statistical synergistic or additive effects were detected between the drugs, the animals treated with both drugs presented a higher percentage of recovery in body weight support and coordination.

The activity box test was used to assess the exploratory behavior of the animals by measuring the total distance traveled, and velocity. The test was done in an open arena with transparent acrylic walls. Distance and locomotor velocity were evaluated at 8 weeks post-injury. The animals of all groups roamed

a similar distance with no statistical difference (**Figure III.3.A**, p= 0.0673). However, animals that received Lev, Rof, or a combination of both roamed with significantly more velocity than saline-treated animals (**Figure III.3.B**, p=0.0008). Interestingly, the combination of Lev + Rof had a statistically significant difference when compared to the Lev-treated group (p=0.0206).

The locomotor velocity during swimming was also analyzed. The test was performed on a quadrangular pool, where the rat had to reach a platform to get out of the water. All the trials were recorded by a video camera, after two days of training. In this analysis the Lev-treated animals showed a statistically significant difference when compared to the Saline and Rof groups. These animals presented higher velocity in water (**Figure III.3.C**, p=0.0015), however, there were no differences observed in Lev+Rof treated animals.

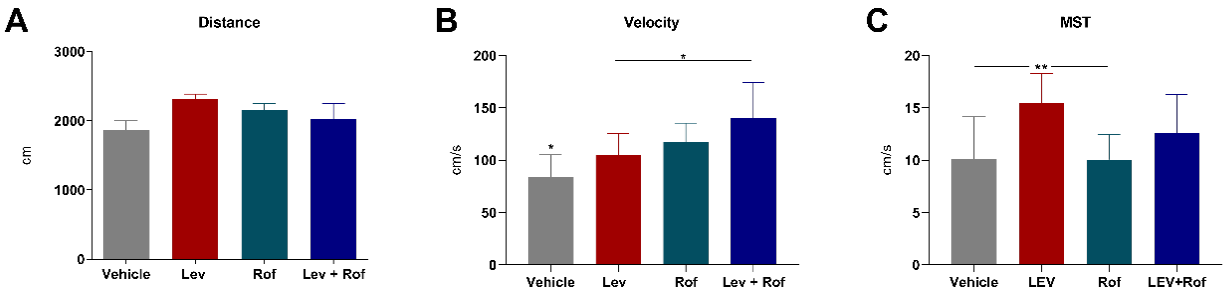


Figure III. 3. *Analysis of exploratory behavior using the Activity Box Test.* ***A)*** *No differences were observed between all groups when analyzing total overground distance.* ***B)*** *Animals treated with our therapeutic agents presented higher velocity than vehicle-treated animals. A synergistic effect between Lev+Rof was observed but only when compared to the Lev-treated group.* ***C)*** *Animals treated with Lev showed a statistically significant difference when compared to the Rof and vehicle-treated groups on the motor swimming test (MST). Values shown as mean ± SEM. *p<0.05; **p<0.01.*

Roflumilast combined with ASCs secretome does not promote functional recovery after SCI

In the first 48 hours post-injury, the animals were administered 3 times with ASCs secretome (500ul/each time) via IV injection. Subsequently, they were administered a weekly IP injection of Rof (1 mg/kg) commencing from the second-week post-injury. The BBB was performed to evaluate locomotor recovery in an open arena, 3 dpi and thereafter weekly for 7 weeks (**Figure III.4.A**). Animals treated with the combination of Rof and Sec had a significantly worse performance than animals treated with Rof (p=0.0335) or Sec alone (p=0.0028). There were no statistical differences between the combinatorial

group and the vehicle-treated animals (p= 0.3900). Moreover, there were no differences between Rof and Sec-treated animals (p= 0.9628).

The locomotor analysis considering two significant indicators of functional recovery - body weight support and coordination - revealed significant differences between groups (**Figure III.4.B** and **4.C**). 50% of the animals treated with the combinatory approach recovery body weight support contrasting with 100% in the Sec (p <0.0001), and 80% in both the Rof (p=0.0002) and vehicle-treated animals (p= 0.0155). Concerning coordination, only 17% of combinatory-treated animals recovered at least occasional coordination, contrasting with 40% in the vehicle-treated group, however, no significant differences were detected between these groups (p= 0.6080). On the other hand, the animals treated with Sec or Rof alone significantly recovered more levels of coordination when compared with either Rof+Sec or vehicle-treated animals. Namely, 50% of Sec (p= 0.0013), and 60% of Rof-treated animals (p= 0.003) recovered at least occasional coordination (p values shown for the analysis against vehicle group). These results indicate the detrimental effects of the combinatorial treatment, however, when administered alone both compounds showed a therapeutic effect.

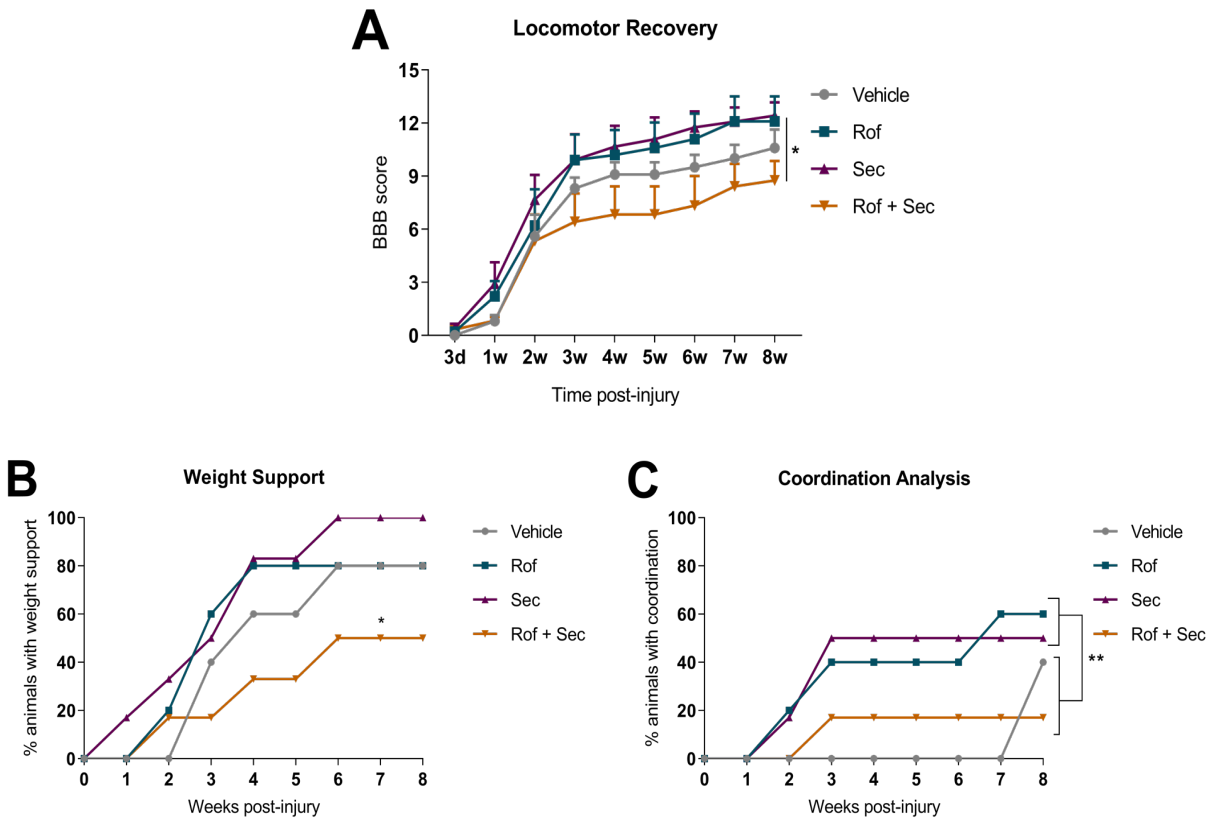


Figure III. 4. *Evaluation of functional recovery after treatment with Rof, Sec, Rof+Sec, or vehicle.* ***A)*** *Locomotor recovery analysis demonstrated that the combination of Rof and Sec leads to a significant worse locomotor recovery when compared to animals treated with Rof or Secretome alone.* ***B)*** *Body weight support analysis revealed statistical differences between treatments with the combinatory therapy showing again detrimental effects.* ***C)*** *Coordination analysis revealed that when treatments are administered alone significant recovery can be achieved when compared with Rof+Sec or vehicle treatment. Differences over time were analyzed using 2-way ANOVA followed by Tukey's multiple comparisons test. Vehicle n=5; Rof n=5; Sec n=6 and Rof+Sec n = 6. Values shown as mean ± SEM. *p<0.05; **p<0.01.*

Treatments administered alone or in combination did not affect mechanical allodynia.

The Von Frey test was performed at the seventh week post-injury to assess mechanical allodynia and hyperalgesia in the animals. This test aims to measure tactile sensitivity and pain responses and involves applying varying degrees of pressure to the paws of SCI animals, using filaments with different force intensities. Results did not reveal any statistical difference between all the treatments administered. Animals from all groups presented a low threshold to mechanical forces, and the treatment with Rof, Sec, or the combination of both did not improve or deteriorate mechanical allodynia in both the left and the right paws (**Figure III.5**).

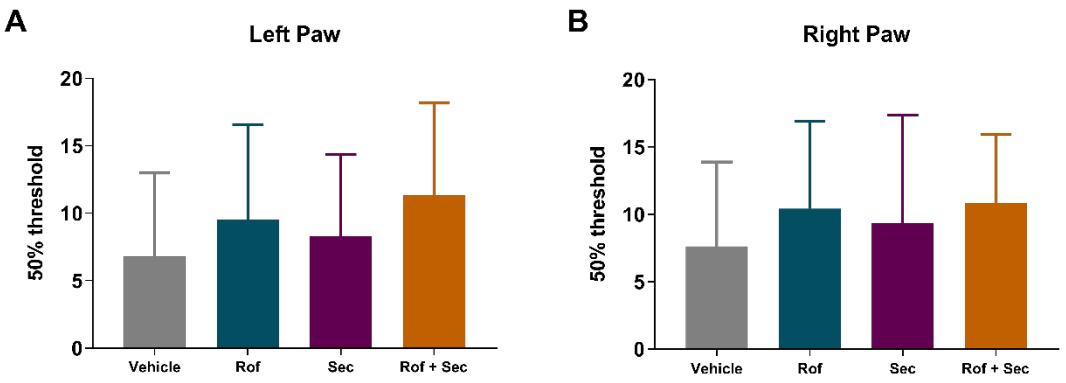


Figure III. 5. *Results from Frey filaments applied both on the* ***A)*** *Left paw and* ***B)*** *Right paw. Statistical analysis did not reveal any differences between groups. Normality analysis using the Kolmogorov-Smirnov test revealed that data does not follow a Gaussian distribution, for this reason, the non-parametric Kruskal-Wallis test was performed followed by the Dunn's multiple comparisons test. Vehicle = 5; Rof = 5; Sec = 6; Rof + Sec = 6. Values shown as mean ± SEM.*

Discussion

Spinal cord regeneration is a complex physiological process, and no optimal treatment has yet been developed to fully repair the spinal cord after injury. There have been numerous studies in recent years showing the effects of several treatment options on SCI. Approaches such as biomolecules, stem cells, and 3D scaffolds are being explored to regenerate the spinal cord [11,37–40]. Despite evidence suggesting that these strategies can be useful for spinal cord reconstruction, the fact is that single-based therapies have not been able to adequately improve clinical outcomes. Significant functional gains for individuals with SCI may require a combinatorial approach that addresses the multifactorial nature of SCI pathophysiology. In this sense, advances in combinatorial therapies, which can address different molecular and cellular secondary events of SCI, offer hope for spinal cord regeneration. Having this in mind, in this study we tested two different combinatorial approaches. Both aimed to promote neuroprotection in the acute phase and neuroregeneration in the chronic phase.

Initially, we aimed to evaluate the potential of a combined treatment approach using Lev and Rof. In our findings, we observed that individually, both Lev and Rof treatment can improve the functional outcome of SCI rats. These data reinforce our previous results where we demonstrated that Lev can protect the injured spinal cord in the acute phase leading to functional and histological recovery [22] , and Rof can promote axonal regeneration in the chronic phase, leading as well to functional improvements[11]. The combined administration of Lev and Rof also significantly improved the motor function of the animals when compared with vehicle-treated animals. However, despite these statistical differences, we did not observe strong synergistic or additive effects resulting from our combination approach. In fact, in the BBB motor score, the animals treated with the agents alone or in combination do not differ. Nonetheless, it's essential to highlight that we observed a higher overground velocity in the group that received the combined treatment when compared with Lev alone, while all treatments were also significantly better than the vehicle group. These may indicate an additive effect and, consequently, this combination may display promise for yielding better functional outcomes when administered together for SCI injuries. Further investigations focusing on various administration regimens, different dosage ratios, and alternative routes of administration are necessary to comprehensively understand whether these two drugs, when combined, might offer a higher degree of recovery compared to when administered individually. It is also important to point out that there was no observable distinction in velocity between the saline group and the Lev+Rof combination group during the swimming task. Moreover, only the Lev-treated animals presented significant improvements in this test. There are several similarities between

swimming and walking, including the phasic relationship between the right and left limb flexion and extension, however, during swimming there are very low levels of both proprioceptive (loading of the limbs) and cutaneous feedback compared to the levels experienced during walking [41]. Cutaneous feedback plays a critical role in hindlimb locomotion, and it was demonstrated that animals exposed to phasic cutaneous feedback during swimming showed improved hindlimb function compared to those without this input [41]. The lack of environmental exploration during swimming, a behavior commonly observed during walking, might also have an impact on the assessment of some animals [42]. These differences between overground walking and swimming could explain our results. Notably, our previous publications indicated that Lev enhanced swimming recovery [22], while the same effect was not seen with Rof treatment [11]. Thus, it is important to direct additional experiments toward comprehending whether Lev distinctly enhances proprioception, particularly through the protection of the spinocerebellar tract and the dorsal columns, which are the two major pathways conveying proprioceptive information.

Given the lack of obvious additive effects between Rof and Lev, in the second part of the study, we aimed to assess whether Rof could potentially exhibit synergistic effects when combined with the secretome of ASCs. In the past years, our team has been investigating the therapeutic potential of the secretome derived from stem cells, particularly those displaying a mesenchymal phenotype, for regenerative applications in the CNS. The use of secretome offers numerous advantages over conventional stem cell transplantation, particularly in terms of manufacturing, storage, and practical application as a readily deployable biologic product. Notably, the secretome can be stored for extended periods without compromising its potency and quality [43,44]. Large-scale production can be achieved under controlled laboratory conditions, and the biological product can be customized to desired cell-specific effects [45]. Importantly, secretome derivatives could circumvent potential challenges linked with cell transplantation, including limitations in cell availability, post-transplantation cell survival, immune compatibility, tumorigenicity, and risk of infection transmission.

Previous research has shown that the secretome of MSCs comprises essential proteins, growth factors, chemokines, and cytokines that facilitate the regenerative processes [46]. Various factors such as Nerve growth factor (NGF), Vascular endothelial growth factor (VEGF), Hepatocyte growth factor (HGF), Insulin-like growth factor 1 (IGF-1), Transforming growth factor-beta 1 (TGF-β1), Interleukin (IL)-10, Glial-derived neurotrophic growth factor (GDNF), basic Fibroblast growth factor (bFGF), Pigment epithelium-derived factor (PEDF), Cadherin 2 (CADH2), Semaphorin 7A (SEM7A), and Glial-derived nexin (GDN) have been identified as contributors to the neurotrophic and immunomodulatory effects of ASCs [47–49]. These factors have been associated with various critical processes such as neurogenesis, neuronal differentiation,

proliferation [47–49], axonal growth, and migration [50–52]. Among the different sources available for isolating MSCs, adipose tissue-derived (ASCs) have emerged as particularly promising for SCI applications. Our previous investigations revealed that ASC secretome significantly promoted the neuronal differentiation of human neural progenitor cells and the axonal growth of dorsal root ganglion explants in vitro [53]. Notably, when compared with secretome derived from other sources like bone marrow or umbilical cord tissue, the ASC-derived secretome demonstrated superiority in these aspects. These outcomes are closely linked to the composition of ASC secretome, containing various well-recognized CNS-related neuroregulatory factors, including PEDF, Semaphorins (SEM), Cadherins (CDH), Interleukin (IL)-6, GDN, Clusterin (CLUS), Decorin (DCN), and Beta-1,4-galactosyltransferase 1 [54]. Moreover, our pre-clinical findings upon injecting the secretome following SCI have consistently shown a notable modulation of the immune response within the spinal cord tissue [55,56].

Considering the well-documented beneficial effects of ASCs and our prior positive outcomes with Rof, herein we aimed to investigate the therapeutic potential of a combination involving the Sec and Rof. Surprisingly, our findings did not unveil any synergistic or additive effects between the two therapeutic agents. Unexpectedly, the combination of Sec and Rof led to detrimental effects on the motor function of the treated animals, both when analyzed on the BBB scale and when assessing the ability of the animals to regain body weight support or coordination. Despite these negative effects from the combined approach, our results reinforce the individual beneficial effects of the therapeutic agents when administered alone, which is consistent with our previous published data [55,56].

Understanding the intricacies of combined treatments is complex. Investigation into the exact reasons for the detrimental effects in this specific combination would need to involve further research, considering various dosages, administration sequences, and the specific physiological response within the context of SCI models. To avoid unintended interaction between the several molecules of the Sec with the Rof, we administered the two therapeutic agents in two different time windows. The Sec was injected up to 48 hours post-injury and then the first injection of Rof happened only two weeks post-injury. In this sense, it is unlikely that a direct interaction between the proteins of the secretome and the Rof could have led to the observed detrimental results. However, we cannot discard some indirect interactions. It is possible that the two therapeutic agents modulate incompatible pathways, ASC secretome and Rof might target antagonistic pathways interfering with the actions of each other, leading to a compromised overall therapeutic response. Moreover, and despite the different time windows of administration used, the sequencing of administration might also play a role. A particular order between the two agents might be essential for their effectiveness, and the combination could have deviated from this optimal pattern.

Based on all our results we discourage the combination of ASCs secretome and Rof for SCI repair.

Even though it is assumed by the scientific community that an effective treatment for SCI may need to rely on combinatorial treatments that target several hallmarks of SCI in an integrated manner, rather than developing an isolated approach, the present work demonstrated that testing combinatorial therapeutic approaches presents numerous challenges. An important challenge is the study of the interactions between therapeutic agents. Combinatorial approaches involve combining agents with different mechanisms of action with potential interactions. Understanding how these treatments interact is complex and may require extensive investigation. Determining the right combinations, doses, and administration schedules is also challenging. Each agent might have distinct pharmacokinetics and dynamics, and determining the most effective and safe dosing regimen will require careful study. Moreover, understanding the individual mechanisms of action and the potential overlap or divergence among multiple therapeutic agents is essential. However, even with this knowledge, it is difficult to predict whether the combined effects of therapeutic agents will be synergistic, additive, or antagonistic. These effects can be unpredictable and may not align with expectations based on the individual actions of the agents. Moreover, the translation to clinical settings will also be a big challenge for combined therapies. Translating single therapies for humans is proving to be complicated, thus doing it for combinatorial therapies will be even more complex. The design of clinical trial will have to consider the statistical power needed to detect treatment effects between single and combined agents, this may reveal to be cost-prohibitive and pose significant logistical challenges. Finally, obtaining regulatory approval for combinatorial therapies can be challenging due to the need for adequate safety and efficacy data for each agent and their combinations, however, it may be facilitated if the therapeutic agents to be combined are already FDA approved, as is the case of Lev and Rof.

The complexity of the SCI and the multifaceted nature of potential treatments will always be an issue, however, this field can also learn valuable lessons from the cancer field. Researchers working on cancer have made significant strides in understanding the challenges and benefits of combining different drugs for more effective treatments as well as on how to overcome some of the obstacles described above. Indeed, many researchers in the SCI field have been focused on finding the best combined treatment. In 2013, Martin Swab's group tested the combination of two promising agents, the Anti-Nogo-A antibody and the ChABC enzyme. The combination treatment was more effective, leading to greater levels of neuronal sprouting and axon regeneration [57]. Another combination tested was the methylprednisolone and the Granulocyte colony-stimulating factor (G-CSF). This combination promoted functional and histological improvements superior to those achieved by either of these drugs alone, demonstrating a

synergistic effect (Teixeira et al., 2018). Recently, Michael Fehling's lab published a systematic meta-analysis that demonstrated that a combined application of scaffolds and MSCs is more effective than scaffolds and MSCs alone in improving motor function following SCI in animal models when used in the acute phase of injury [58]. Finally, Rilozule and Magnesium were also combined to protect the spinal cord against glutamate excitotoxicity. However, when tested together it was not observed any synergistic or cumulative effect, Riluzole was the only treatment that was able to achieve functional and histological improvements [29].

This study stands out for showing that the combination did not produce synergistic effects. It is crucial to consider publication bias, which tends to favor the publication of positive results. This bias can create an imbalanced perception of scientific evidence, potentially leading to incorrect conclusions. Publication bias may result in wasted resources and overlook valuable lessons from unsuccessful experiments. Therefore, we emphasize the significance of publishing data, such as the findings presented in this manuscript.

Conclusions

In conclusion, our study evaluated combinations of Rof with two adjuvant compounds, Lev and the secretome from ASCs, aiming to improve functional recovery after SCI. While the individual treatments with Rof and Lev separately showed efficacy in promoting functional recovery in an SCI rat model, the combined treatment of Rof + Lev did not lead to synergistic effects. However, the combination of Rof + Lev was still significantly better than the vehicle treatment. Contrarily, the combination of Rof with ASCs secretome did not just yield improved functional recovery compared to treatments administered individually but led to detrimental effects. These findings suggest that the combinations tested did not produce superior functional outcomes compared to the standalone treatments. Further investigations are warranted to identify more effective combinatorial treatments with potential clinical applications for SCI.

Acknowledgments

This work has been funded by National funds, through the Foundation for Science and Technology (FCT) – project UIDB/50026/2020, and UIDP/50026/2020. Financial support was also provided by Prémios Santa Casa Neurociências – Prize Melo e Castro for Spinal Cord Injury (MC-18-2021) and by Wings for Life Spinal Cord Research Foundation (WFL-PT-14/23).

References

1. Choo, A. M. Te, Liu, J., Liu, Z., Dvorak, M., Tetzlaff, W., & Oxland, T. R. (2009). Modeling spinal cord contusion, dislocation, and distraction: Characterization of vertebral clamps, injury severities, and node of Ranvier deformations. Journal of Neuroscience Methods, 181(1), 6–17. https://doi.org/10.1016/J.JNEUMETH.2009.04.007
2. Alizadeh, A., Dyck, S. M., & Karimi-Abdolrezaee, S. (2019). Traumatic spinal cord injury: An overview of pathophysiology, models and acute injury mechanisms. Frontiers in Neurology, 10, 282. https://doi.org/10.3389/FNEUR.2019.00282/BIBTEX
3. Liu, B. P., Fournier, A., GrandPré, T., & Strittmatter, S. M. (2002). Myelin-associated glycoprotein as a functional ligand for the Nogo-66 receptor. Science (New York, N.Y.), 297(5584), 1190–1193. https://doi.org/10.1126/SCIENCE.1073031
4. Filbin, M. T. (2003). Myelin-associated inhibitors of axonal regeneration in the adult mammalian CNS. Nature Reviews. Neuroscience, 4(9), 703–713. https://doi.org/10.1038/NRN1195
5. Schwab, M. E. (2004). Nogo and axon regeneration. Current Opinion in Neurobiology, 14(1), 118–124. https://doi.org/10.1016/J.CONB.2004.01.004
6. Liu, B. P., Cafferty, W. B. J., Budel, S. O., & Strittmatter, S. M. (2006). Extracellular regulators of axonal growth in the adult central nervous system. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences, 361(1473), 1593–1610. https://doi.org/10.1098/RSTB.2006.1891
7. Hannila, S. S., & Filbin, M. T. (2008). The role of cyclic AMP signaling in promoting axonal regeneration after spinal cord injury. Experimental Neurology, 209(2), 321–332. https://doi.org/10.1016/j.expneurol.2007.06.020
8. Cai, D., Qiu, J., Cao, Z., McAtee, M., Bregman, B. S., & Filbin, M. T. (2001). Neuronal Cyclic AMP Controls the Developmental Loss in Ability of Axons to Regenerate. Journal of Neuroscience, 21(13), 4731–4739. https://doi.org/10.1523/JNEUROSCI.21-13-04731.2001
9. Cai, D., Deng, K., Mellado, W., Lee, J., Ratan, R. R., & Filbin, M. T. (2002). Arginase I and Polyamines Act Downstream from Cyclic AMP in Overcoming Inhibition of Axonal Growth MAG and Myelin In Vitro. Neuron, 35(4), 711–719. https://doi.org/10.1016/S0896-6273(02)00826-7
10. Heckman, P. R. A., Wouters, C., & Prickaerts, J. (2014). Phosphodiesterase Inhibitors as a Target for Cognition Enhancement in Aging and Alzheimer's Disease: A Translational Overview. Current Pharmaceutical Design, 21(3), 317–331. https://doi.org/10.2174/13816128206661408261146O1
11. Sousa, C. S., Lima, R., Cibrão, J. R., Gomes, E. D., Fernandes, L. S., Pinho, T. S., Silva, D., Campos, J., Salgado, A. J., & Silva, N. A. (2023). Pre-Clinical Assessment of Roflumilast Therapy in a Thoracic Model of Spinal Cord Injury. Pharmaceutics 2023, Vol. 15, Page 1556, 15(5), 1556. https://doi.org/10.3390/PHARMACEUTICS15051556
12. Philip V Peplow, Svetlana A Dambinova, Thomas A Gennarelli, B. M. (2017). Acute Brain Impairment: Scientific Discoveries and Translational Research. In Royal Society of Chemistry. https://books.google.com/books?hl=pt-PT&lr=&id=sbWrDwAAQBAJ&oi=fnd&pg=PA71&dq=Martinez,+B.,+Acute+Brain+Impairment:+Scientific+Discoveries+and+Translational+Research.+Vol.+62.+2017:+Royal+Society+of+Chemistry.&ots=ATTy0cGZOu&sig=BILXZQgPY3pT3ImQIEmhcZ1yHi
13. Quadri, S. A., Farooqui, M., Ikram, A., Zafar, A., Khan, M. A., Suriya, S. S., Claus, C. F., Fiani, B., Rahman, M., Ramachandran, A., Armstrong, I. I. T., Taqi, M. A., & Mortazavi, M. M. (2018). Recent update on basic mechanisms of spinal cord injury. Neurosurgical Review 2018 43:2, 43(2), 425–441. https://doi.org/10.1007/S10143-018-1008-3

14. Villegas, R., Martinez, N. W., Lillo, J., Pihan, P., Hernandez, D., Twiss, J. L., & Court, F. A. (2014). Calcium Release from Intra-Axonal Endoplasmic Reticulum Leads to Axon Degeneration through Mitochondrial Dysfunction. Journal of Neuroscience, 34(21), 7179–7189. https://doi.org/10.1523/JNEUROSCI.4784-13.2014
15. Park, E., Velumian, A. A., & Fehlings, M. G. (2004). The Role of Excitotoxicity in Secondary Mechanisms of Spinal Cord Injury: A Review with an Emphasis on the Implications for White Matter Degeneration. Https://Home.Liebertpub.Com/Neu, 21(6), 754–774. https://doi.org/10.1089/0897715041269641
16. Xu, G. Y., Liu, S., Hughes, M. G., & McAdoo, D. J. (2008). Glutamate-induced losses of oligodendrocytes and neurons and activation of caspase-3 in the rat spinal cord. Neuroscience, 153(4), 1034–1047. https://doi.org/10.1016/J.NEUROSCIENCE.2008.02.065
17. Tator, C. H., Hashimoto, R., Raich, A., Norvell, D., Fehlings, M. G., Harrop, J. S., Guest, J., Aarabi, B., & Grossman, R. G. (2012). Translational potential of preclinical trials of neuroprotection through pharmacotherapy for spinal cord injury. Journal of Neurosurgery: Spine, 17(Suppl1), 157–229. https://doi.org/10.3171/2012.5.AOSPINE12116
18. Crowder, K. M., Gunther, J. M., Jones, T. A., Hale, B. D., Zhang, H. Z., Peterson, M. R., Scheller, R. H., Chavkin, C., & Bajjalieh, S. M. (1999). Abnormal neurotransmission in mice lacking synaptic vesicle protein 2A (SV2A). Proceedings of the National Academy of Sciences of the United States of America, 96(26), 15268–15273. https://doi.org/10.1073/PNAS.96.26.15268/ASSET/BD2F4684-78F1-4660-8F50-FC224C500ACB/ASSETS/GRAPHIC/PQ2694549006.JPEG
19. Lynch, B. A., Lambeng, N., Nocka, K., Kensel-Hammes, P., Bajjalieh, S. M., Matagne, A., & Fuks, B. (2004). The synaptic vesicle is the protein SV2A is the binding site for the antiepileptic drug levetiracetam. Proceedings of the National Academy of Sciences of the United States of America, 101(26), 9861–9866. https://doi.org/10.1073/PNAS.0308208101/ASSET/329D471F-1001-41CF-89A6-C93AD65511F9/ASSETS/GRAPHIC/ZPQ0260452550006.JPEG
20. Lambeng, N., Gillard, M., Vertongen, P., Fuks, B., & Chatelain, P. (2005). Characterization of [3H]ucb 30889 binding to synaptic vesicle protein 2A in the rat spinal cord. European Journal of Pharmacology, 520(1–3), 70–76. https://doi.org/10.1016/J.EJPHAR.2005.07.029
21. Yang, X. F., Weisenfeld, A., & Rothman, S. M. (2007). Prolonged Exposure to Levetiracetam Reveals a Presynaptic Effect on Neurotransmission. Epilepsia, 48(10), 1861–1869. https://doi.org/10.1111/J.1528-1167.2006.01132.X
22. Lima, R., Gomes, E. D., Cibrão, J. R., Rocha, L. A., Assunção-Silva, R. C., Rodrigues, C. S., Neves-Carvalho, A., Monteiro, S., Salgado, A. J., & Silva, N. A. (2021). Levetiracetam treatment leads to functional recovery after thoracic or cervical injuries of the spinal cord. Npj Regenerative Medicine 2021 6:1, 6(1), 1–10. https://doi.org/10.1038/s41536-021-00121-7
23. Pinho, A. G., Cibrão, J. R., Silva, N. A., Monteiro, S., & Salgado, A. J. (2020). Cell Secretome: Basic Insights and Therapeutic Opportunities for CNS Disorders. Pharmaceuticals 2020, Vol. 13, Page 31, 13(2), 31. https://doi.org/10.3390/PH13020031
24. Rocha, L. A., Gomes, E. D., Afonso, J. L., Granja, S., Baltazar, F., Silva, N. A., Shoichet, M. S., Sousa, R. A., Learmonth, D. A., & Salgado, A. J. (2020). In vitro Evaluation of ASCs and HUVECs Co-cultures in 3D Biodegradable Hydrogels on Neurite Outgrowth and Vascular Organization. Frontiers in Cell and Developmental Biology, 8, 543061. https://doi.org/10.3389/FCELL.2020.00489/BIBTEX
25. Pinho, A. G., Cibrão, J. R., Lima, R., Gomes, E. D., Serra, S. C., Lentilhas-Graça, J., Ribeiro, C., Lanceros-Mendez, S., Teixeira, F. G., Monteiro, S., Silva, N. A., & Salgado, A. J. (2022). Immunomodulatory and regenerative effects of the full and fractioned adipose tissue derived stem cells secretome in spinal cord injury. Experimental Neurology, 351, 113989. https://doi.org/10.1016/J.EXPNEUROL.2022.113989

26. du Sert, N. P., Ahluwalia, A., Alam, S., Avey, M. T., Baker, M., Browne, W. J., Clark, A., Cuthill, I. C., Dirnagl, U., Emerson, M., Garner, P., Holgate, S. T., Howells, D. W., Hurst, V., Karp, N. A., Lazic, S. E., Lidster, K., MacCallum, C. J., Macleod, M., … Würbel, H. (2020). Reporting animal research: Explanation and elaboration for the ARRIVE guidelines 2.0. PLoS Biology, 18(7). https://doi.org/10.1371/JOURNAL.PBIO.3000411
27. Dubois, S. G., Floyd, E. Z., Zvonic, S., Kilroy, G., Wu, X., Carling, S., Halvorsen, Y. D. C., Ravussin, E., & Gimble, J. M. (2008). Isolation of human adipose-derived stem cells from biopsies and liposuction specimens. Methods in Molecular Biology, 449, 69–79. https://doi.org/10.1007/978-1-60327-169-1_5/COVER
28. Serra, S. C., Costa, J. C., Assunção-Silva, R. C., Teixeira, F. G., Silva, N. A., Anjo, S. I., Manadas, B., Gimble, J. M., Behie, L. A., & Salgado, A. J. (2018). Influence of passage number on the impact of the secretome of adipose tissue stem cells on neural survival, neurodifferentiation and axonal growth. Biochimie, 155, 119–128. https://doi.org/10.1016/J.BIOCHI.2018.09.012
29. Vasconcelos, N. L., Gomes, E. D., Oliveira, E. P., Silva, C. J., Lima, R., Sousa, N., Salgado, A. J., & Silva, N. A. (2016). Combining neuroprotective agents: effect of riluzole and magnesium in a rat model of thoracic spinal cord injury. Spine Journal, 16(8), 1015–1024. https://doi.org/10.1016/j.spinee.2016.04.013
30. Lima, R., Monteiro, S., Lopes, J. P., Barradas, P., Vasconcelos, N. L., Gomes, E. D., Assunção-Silva, R. C., Teixeira, F. G., Morais, M., Sousa, N., Salgado, A. J., & Silva, N. A. (2017). Systemic Interleukin-4 Administration after Spinal Cord Injury Modulates Inflammation and Promotes Neuroprotection. Pharmaceuticals 2017, Vol. 10, Page 83, 10(4), 83. https://doi.org/10.3390/PH10040083
31. Pinho, A. G., Cibrão, J. R., Lima, R., Gomes, E. D., Serra, S. C., Lentilhas-Graça, J., Ribeiro, C., Lanceros-Mendez, S., Teixeira, F. G., Monteiro, S., Silva, N. A., & Salgado, A. J. (2022). Immunomodulatory and regenerative effects of the full and fractioned adipose tissue derived stem cells secretome in spinal cord injury. Experimental Neurology, 351, 113989. https://doi.org/10.1016/J.EXPNEUROL.2022.113989
32. Silva, N. A., Sousa, R. A., Fraga, J. S., Fontes, M., Leite-Almeida, H., Cerqueira, R., Almeida, A., Sousa, N., Reis, R. L., & Salgado, A. J. (2013). Benefits of spine stabilization with biodegradable scaffolds in spinal cord injured rats. Tissue Engineering - Part C: Methods, 19(2), 101–108. https://doi.org/10.1089/ten.tec.2012.0264
33. Gomes, E. D., Mendes, S. S., Assunção-Silva, R. C., Teixeira, F. G., Pires, A. O., Anjo, S. I., Manadas, B., Leite-Almeida, H., Gimble, J. M., Sousa, N., Lepore, A. C., Silva, N. A., & Salgado, A. J. (2018). Co-Transplantation of Adipose Tissue-Derived Stromal Cells and Olfactory Ensheathing Cells for Spinal Cord Injury Repair. Stem Cells (Dayton, Ohio), 36(5), 696–708. https://doi.org/10.1002/STEM.2785
34. Guimarães, M. R., Soares, A. R., Cunha, A. M., Esteves, M., Borges, S., Magalhães, R., Moreira, P. S., Rodrigues, A. J., Sousa, N., Almeida, A., & Leite-Almeida, H. (2019). Evidence for lack of direct causality between pain and affective disturbances in a rat peripheral neuropathy model. Genes, Brain and Behavior, 18(6), e12542. https://doi.org/10.1111/GBB.12542
35. Chaplan, S. R., Bach, F. W., Pogrel, J. W., Chung, J. M., & Yaksh, T. L. (1994). Quantitative assessment of tactile allodynia in the rat paw. Journal of Neuroscience Methods, 53(1), 55–63. https://doi.org/10.1016/0165-0270(94)90144-9
36. Sotiropoulos, I., Lopes, A. T., Pinto, V., Lopes, S., Carlos, S., Duarte-Silva, S., Neves-Carvalho, A., Pinto-Ribeiro, F., Pinheiro, S., Fernandes, R., Almeida, A., Sousa, N., & Leite-Almeida, H. (2014). Selective impact of Tau loss on nociceptive primary afferents and pain sensation. Experimental Neurology, 261, 486–493. https://doi.org/10.1016/J.EXPNEUROL.2014.07.008

37. Oliveira, E., Assunção-Silva, R. C., Ziv-Polat, O., Gomes, E. D., Teixeira, F. G., Silva, N. A., Shahar, A., & Salgado, A. J. (2017). Influence of Different ECM-Like Hydrogels on Neurite Outgrowth Induced by Adipose Tissue-Derived Stem Cells. Stem Cells International, 2017. https://doi.org/10.1155/2017/6319129
38. Monteiro, S., Salgado, A. J., & Silva, N. A. (2018). Immunomodulation as a neuroprotective strategy after spinal cord injury. Neural Regeneration Research, 13(3), 423. https://doi.org/10.4103/1673-5374.228722
39. Gomes, E. D., Ghosh, B., Lima, R., Goulão, M., Moreira-Gomes, T., Martins-Macedo, J., Urban, M. W., Wright, M. C., Gimble, J. M., Sousa, N., Silva, N. A., Lepore, A. C., & Salgado, A. J. (2020). Combination of a Gellan Gum-Based Hydrogel With Cell Therapy for the Treatment of Cervical Spinal Cord Injury. Frontiers in Bioengineering and Biotechnology, 8, 528244. https://doi.org/10.3389/FBIOE.2020.00984/BIBTEX
40. Rocha, L. A., Silva, D., Barata-Antunes, S., Cavaleiro, H., Gomes, E. D., Silva, N. A., & Salgado, A. J. (2020). Cell and Tissue Instructive Materials for Central Nervous System Repair. Advanced Functional Materials, 30(44), 1909083. https://doi.org/10.1002/ADFM.201909083
41. Muir, G. D., & Steeves, J. D. (1995). Phasic cutaneous input facilitates locomotor recovery after incomplete spinal injury in the chick. Journal of Neurophysiology, 74(1), 358–368. https://doi.org/10.1152/JN.1995.74.1.358
42. Xu, N., Åkesson, E., Holmberg, L., & Sundström, E. (2015). A sensitive and reliable test instrument to assess swimming in rats with spinal cord injury. Behavioural Brain Research, 291, 172–183. https://doi.org/10.1016/J.BBR.2015.05.004
43. Bermudez, M. A., Sendon-Lago, J., Seoane, S., Eiro, N., Gonzalez, F., Saa, J., Vizoso, F., & Perez-Fernandez, R. (2016). Anti-inflammatory effect of conditioned medium from human uterine cervical stem cells in uveitis. Experimental Eye Research, 149, 84–92. https://doi.org/10.1016/J.EXER.2016.06.022
44. Vizoso, F. J., Eiro, N., Cid, S., Schneider, J., & Perez-Fernandez, R. (2017). Mesenchymal Stem Cell Secretome: Toward Cell-Free Therapeutic Strategies in Regenerative Medicine. International Journal of Molecular Sciences 2017, Vol. 18, Page 1852, 18(9), 1852. https://doi.org/10.3390/IJMS18091852
45. McKee, C., & Chaudhry, G. R. (2017). Advances and challenges in stem cell culture. Colloids and Surfaces B: Biointerfaces, 159, 62–77. https://doi.org/10.1016/J.COLSURFB.2017.07.051
46. Paul, G., & Anisimov, S. V. (2013). The secretome of mesenchymal stem cells: Potential implications for neuroregeneration. Biochimie, 95(12), 2246–2256. https://doi.org/10.1016/J.BIOCHI.2013.07.013
47. Steele, F. R., Chader, G. J., Johnson, L. V., & Tombran-Tink, J. (1993). Pigment epithelium-derived factor: neurotrophic activity and identification as a member of the serine protease inhibitor gene family. Proceedings of the National Academy of Sciences, 90(4), 1526–1530. https://doi.org/10.1073/PNAS.90.4.1526
48. Su, H., Wang, L., Huang, W., Qin, D., Cai, J., Yao, X., Feng, C., Li, Z., Wang, Y., So, K. F., Pan, G., Wu, W., & Pei, D. (2013). Immediate expression of Cdh2 is essential for efficient neural differentiation of mouse induced pluripotent stem cells. Stem Cell Research, 10(3), 338–348. https://doi.org/10.1016/J.SCR.2013.01.003
49. Ashwini, A., Naganur, S. S., Smitha, B., Sheshadri, P., Prasanna, J., & Kumar, A. (2018). Cyclosporine A-Mediated IL-6 Expression Promotes Neural Induction in Pluripotent Stem Cells. Molecular Neurobiology, 55(5), 4267–4279. https://doi.org/10.1007/S12035-017-0633-0/FIGURES/11
50. Pasterkamp, R. J., Peschon, J. J., Spriggs, M. K., & Kolodkin, A. L. (2003). Semaphorin 7A promotes axon outgrowth through integrins and MAPKs. Nature 2003 424:6947, 424(6947), 398–405. https://doi.org/10.1038/nature01790

51. Scott, G. A., McClelland, L. A., & Fricke, A. F. (2008). Semaphorin 7a promotes spreading and dendricity in human melanocytes through β1-integrins. Journal of Investigative Dermatology, 128(1), 151–161. https://doi.org/10.1038/sj.jid.5700974
52. Yang, G., & Tang, W. Y. (2017). Resistance of interleukin-6 to the extracellular inhibitory environment promotes axonal regeneration and functional recovery following spinal cord injury. International Journal of Molecular Medicine, 39(2), 437–445. https://doi.org/10.3892/IJMM.2017.2848
53. Assunção-Silva, R. C., Mendes-Pinheiro, B., Patrício, P., Behie, L. A., Teixeira, F. G., Pinto, L., & Salgado, A. J. (2018). Exploiting the impact of the secretome of MSCs isolated from different tissue sources on neuronal differentiation and axonal growth. Biochimie, 155, 83–91. https://doi.org/10.1016/J.BIOCHI.2018.07.026
54. Pires, A. O., Mendes-Pinheiro, B., Teixeira, F. G., Anjo, S. I., Ribeiro-Samy, S., Gomes, E. D., Serra, S. C., Silva, N. A., Manadas, B., Sousa, N., & Salgado, A. J. (2016). Unveiling the Differences of Secretome of Human Bone Marrow Mesenchymal Stem Cells, Adipose Tissue-Derived Stem Cells, and Human Umbilical Cord Perivascular Cells: A Proteomic Analysis. Https://Home.Liebertpub.Com/Scd, 25(14), 1073–1083. https://doi.org/10.1089/SCD.2016.0048
55. Assunção Silva, R. C., Pinto, L., & Salgado, A. J. (2022). Cell transplantation and secretome based approaches in spinal cord injury regenerative medicine. Medicinal Research Reviews, 42(2), 850–896. https://doi.org/10.1002/MED.21865
56. Pinho, A. G., Cibrão, J. R., Lima, R., Gomes, E. D., Serra, S. C., Lentilhas-Graça, J., Ribeiro, C., Lanceros-Mendez, S., Teixeira, F. G., Monteiro, S., Silva, N. A., & Salgado, A. J. (2022). Immunomodulatory and regenerative effects of the full and fractioned adipose tissue derived stem cells secretome in spinal cord injury. Experimental Neurology, 351, 113989. https://doi.org/10.1016/J.EXPNEUROL.2022.113989
57. Zhao, R. R., Andrews, M. R., Wang, D., Warren, P., Gullo, M., Schnell, L., Schwab, M. E., & Fawcett, J. W. (2013). Combination treatment with anti-Nogo-A and chondroitinase ABC is more effective than single treatments at enhancing functional recovery after spinal cord injury. European Journal of Neuroscience, 38(6), 2946–2961. https://doi.org/10.1111/EJN.12276
58. Yousefifard, M., Maleki, S. N., Askarian-Amiri, S., Vaccaro, A. R., Chapman, J. R., Fehlings, M. G., Hosseini, M., & Rahimi-Movaghar, V. (2019). A combination of mesenchymal stem cells and scaffolds promotes motor functional recovery in spinal cord injury: a systematic review and meta-analysis. Journal of Neurosurgery: Spine, 32(2), 269–284. https://doi.org/10.3171/2019.8.SPINE19201

Chapter IV

Discussion and Conclusions

Discussion and Conclusions

Researchers continue in their quest for a therapeutic approach capable of effectively restore the spinal cord function after injury. The intricate pathophysiology of spinal cord injury (SCI) coupled with the limited regeneration capacity of the Central Nervous System (CNS), presents a multifaceted challenge in the pursuit for a successful treatment [1–3].

A variety of approaches have been tested to achieve a relevant therapeutic impact. The most explored therapeutic approaches for SCI include molecular therapies [4], biomaterials [5], neurorehabilitation techniques, and cell transplantation [6]. Molecular approaches target various entities, aiming to either protect the neural tissue during the acute phase of injury or to enhance its intrinsic regenerative ability during the chronic phase [7]. Biomaterials facilitate regeneration by providing physical support, chemical and guidance cues, as well as by facilitating cellular or/and drug delivery into the tissue [8]. Novel neuronal rehabilitation approaches use electrical stimulation to activate remaining fibers below the lesion sites enabling physical training [9]. Finally, cell transplantation involves repopulating the spinal cord with new cells capable of substitute the tissue lost or capable of secrete molecules that can protect or regenerate the nervous tissue [6,10]. In general, molecular therapies are the most extensively explored strategies in the field due to their versatile range of targets that can address different aspects of SCI pathology as well as their more feasibility to be translated into clinical trials compared to other approaches.

In this thesis, we evaluated the therapeutic potential of different approaches that target distinct pathophysiologic processes of the SCI. The introductory Chapter provides an overview of the SCI pathophysiology, current clinical practices, and therapeutic strategies with a special focus given on combinational therapies. In Chapter II, the dysfunctional inflammatory response and inhibitors of axonal regeneration were target using the PDE4 inhibitor, Roflumilast (Rof). In Chapter III, we explored the combination of Rof with the anti-epileptic drug Levetiracetam (Lev), which target glutamate excitotoxicity. Moreover, in this chapter we also combine Rof with ASCs secretome, which target multiple pathways, as such neuroinflammatory or neuroregenerative ones.

The first evaluation involved the therapeutic potential of Food and Drug Administration (FDA)-approved Phosphodiesterase-4 (PDE4) inhibitor, Rof. We tested the therapeutic potential of Rof in repairing the injured spinal cord. First, to understand whether Rof can promote axonal regeneration, we used organotypic cultures of the spinal cord from the thoracic region. Results indicate that the treatment was effective in promoting functional recovery, as evidenced by improvements in both gross and fine motor

function. Eight weeks post-injury, the animals significantly recovered by achieving occasional weight-supported plantar steps. Histological assessment revealed a significant decrease in cavity size, less reactive microglia, as well as higher axonal regeneration in treated animals. Moreover, molecular analysis revealed that Rof has an anti-inflammatory effect, with increased levels of anti-inflammatory cytokines (IL-10 and IL-13), as well as VEGF.

Overall, Rof demonstrated efficacy in promoting functional recovery and supporting neuroregeneration in a severe thoracic contusion injury model, suggesting its significance in SCI treatment.

In this thesis we also aimed to identify adjuvant compounds that could elicit synergetic or additive effects when combined with Rof. We explored two distinctive combinatorial approaches, both aimed at promoting neuroprotection in the acute phase and neuroregeneration in the chronic phase. Initially, we sought to evaluate the potential of combining Rof with Lev, both compounds are already FDA approved and used in clinical practice. Using a T8 contusion rat model, both treatments when administered independently demonstrated effectiveness in promoting functional recovery. However, the combination of Rof + Lev did not lead to an improvement in functional outcomes when compared to both standalone treatments.

Given the lack of obvious additive effects between Rof and Lev, the second part of the study aimed to assess whether Rof could potentially exhibit synergistic effects when combined with the secretome of Adipose-Derived Mesenchymal Stem Cells (Ad-MSCs). In the past years, our team has been investigating the therapeutic potential of the secretome derived from stem cells, particularly those displaying a mesenchymal phenotype, for regenerative applications in the CNS. Taking these results into account, we tested therapeutic potential of combining Rof with secretome obtained from Ad-MSCs using a T8 contusion rat model. Despite these efforts, our findings did not unveil any synergistic or additive effects between the two therapeutic agents. Unexpectedly, the combination of Sec and Rof led to detrimental effects on the motor function of the treated animals, both when analyzed on the Basso, Beattie and Bresnahan (BBB) scale and when assessing the ability of the animals to regain body weight support or coordination. Results revealed that treatment only led to functional improvements when administered alone. Based on all our results, we discourage the combination of Ad-MSCs secretome and Rof for SCI repair.

Overall, the combination of Lev + Rof and Rof + Secretome did not yield better functional outcome than the standalone treatments, however, both experiments reinforce our previous results demonstrating that Lev and Rof alone have a high therapeutic potential and more research should be pursued to demonstrate

their clinical promise. Concerning combinatorial treatment, further studies are imperative to identify approaches that can deliver superior therapeutic effects with clear synergistic effects.

Spinal cord regeneration is a complex physiological process, and no optimal treatment has yet been developed for fully repairing the spinal cord. Several approaches such as biomolecules, stem cells, and 3D scaffolds are being explored to regenerate the spinal cord. Despite the evidence suggesting that these strategies can be useful for spinal cord reconstruction, they do not appear to adequately improve clinical outcomes. So, the idea of combining these strategies emerge as more effective solution than using them alone.

In theory, combinatorial strategies present the greatest likelihood of creating a stimulating environment for tissue regeneration, potentially yielding superior results. To achieve both structural and functional restoration of the injured spinal cord, a combination of neuroprotective and neuroregenerative agents may be necessary. Many studies have reported functional and/or histological improvements when employing a combinatorial approach[11,12] . However, it is crucial to be mindful of publication bias, as selectively reporting positive results can distort the scientific evidence and lead to inaccurate conclusions. Despite the scientific community's assumption that an effective treatment for SCI may necessitate combinatorial approaches targeting various hallmarks of its pathophysiology in an integrated manner rather than pursuing isolated methods, this thesis reveals that testing combinatorial therapeutic approaches presents numerous challenges. A key challenge lies in studying the interactions between therapeutic agents. Combinatorial approaches entail combining agents with different mechanisms of action, introducing potential interactions that are complex and may require extensive investigation. Determining optimal combinations, doses, and administration schedules is challenging and may yield different results with varied options. Each agent may exhibit distinct pharmacokinetics and dynamics, necessitating careful study to identify the most effective and safe dosing regimen. Furthermore, understanding the individual mechanisms of action and potential overlaps or divergences among multiple therapeutic agents is essential. Even with this knowledge, predicting whether the combined effects will be synergistic, additive, or antagonistic remains challenging. The combination may lead to toxic effects or impede the effects of one or more therapeutic agents. Logistically, it is also challenging, conducting combinatorial studies, whether pre-clinical or clinical trials, requires a substantial number of animals/individuals, as controls must be included for all groups and combinations. So, identifying the most effective combinatorial therapies for SCI is complex, time-consuming, and expensive. Nevertheless, it may be crucial for finding a successful clinical treatment. Further investigations are warranted to pinpoint more effective combinatorial treatments with potential clinical applications for SCI.

Finally, and having into account the complexity of the involved processes, it is also important to better design the clinical studies. Many clinical trials involving SCI is the lack of a comparable control group, often due to patient reluctance to be included in such groups. To overcome this problem, it will be important to involve more patients in the studies, this may be possible with the cooperation between several hospitals and research centers. Another issue is the communication gap between surgeons and basic scientists, which poses a significant challenge in the development of user-friendly and efficient protocols for treating SCI. Often, the vital insights gained from basic scientific research on spinal cord regeneration, neuroprotection, and other therapeutic strategies do not seamlessly translate into practical protocols readily applicable in surgical settings. Surgeons, tasked with implementing these treatments, may encounter difficulties in understanding and incorporating complex scientific findings into their procedures. The disconnect hinders the seamless integration of cutting-edge research into clinical practice, emphasizing the need for enhanced collaboration and communication between the two disciplines. Bridging this gap holds the potential to facilitate the translation of scientific advancements into more accessible and effective protocols for the benefit of SCI patients.

References

1. Anjum, A., Yazid, M. D., Daud, M. F., Idris, J., Hwei Ng, A. M., Naicker, A. S., Rashidah Ismail, O. H., Kumar, R. K. A., & Lokanathan, Y. (2020). Spinal Cord Injury: Pathophysiology, Multimolecular Interactions, and Underlying Recovery Mechanisms. International Journal of Molecular Sciences 2020, Vol. 21, Page 7533, 21(20), 7533. https://doi.org/10.3390/IJMS21207533
2. Silva, N. A., Sousa, N., Reis, R. L., & Salgado, A. J. (2014). From basics to clinical: A comprehensive review on spinal cord injury. Progress in Neurobiology, 114, 25–57. https://doi.org/10.1016/J.PNEUROBIO.2013.11.002
3. Lima, R., Monteiro, A., Salgado, A. J., Monteiro, S., & Silva, N. A. (2022). Pathophysiology and Therapeutic Approaches for Spinal Cord Injury. International Journal of Molecular Sciences 2022, Vol. 23, Page 13833, 23(22), 13833. https://doi.org/10.3390/IJMS232213833
4. De Almeida, F. M., Marques, S. A., Dos Santos, A. C. R., Prins, C. A., Dos Santos Cardoso, F. S., Dos Santos Heringer, L., Mendonça, H. R., & Martinez, A. M. B. (2023). Molecular approaches for spinal cord injury treatment. Neural Regeneration Research, 18(1), 23–30. https://doi.org/10.4103/1673-5374.344830
5. Assunção-Silva, R. C., Gomes, E. D., Sousa, N., Silva, N. A., & Salgado, A. J. (2015). Hydrogels and Cell Based Therapies in Spinal Cord Injury Regeneration. In Stem Cells International. https://doi.org/10.1155/2015/948040
6. Gomes, E. D., Rocha, L. A., Assunção-Silva, R. C., Lima, R., Silva, N. A., & Salgado, A. J. (2020). Cell therapies for spinal cord injury regeneration. Spinal Cord Injury (SCI) Repair Strategies, 157–186. https://doi.org/10.1016/B978-0-08-102807-0.00009-0
7. Kim, H. N., McCrea, M. R., & Li, S. (2023). Advances in molecular therapies for targeting pathophysiology in spinal cord injury. Expert Opinion on Therapeutic Targets, 27(3), 171–187. https://doi.org/10.1080/14728222.2023.2194532
8. Feng, C., Deng, L., Yong, Y. Y., Wu, J. M., Qin, D. L., Yu, L., Zhou, X. G., & Wu, A. G. (2023). The Application of Biomaterials in Spinal Cord Injury. International Journal of Molecular Sciences 2023, Vol. 24, Page 816, 24(1), 816. https://doi.org/10.3390/IJMS24010816
9. Mushahwar, V. K., Jacobs, P. L., Normann, R. A., Triolo, R. J., & Kleitman, N. (2007). New functional electrical stimulation approaches to standing and walking. Journal of Neural Engineering, 4(3), S181. https://doi.org/10.1088/1741-2560/4/3/S05
10. Li, J., & Lepski, G. (2013). Cell transplantation for spinal cord injury: A systematic review. BioMed Research International, 2013. https://doi.org/10.1155/2013/786475
11. Griffin, J. M., & Bradke, F. (2020). Therapeutic repair for spinal cord injury: combinatory approaches to address a multifaceted problem. EMBO Molecular Medicine, 12(3). https://doi.org/10.15252/EMMM.201911505/ASSET/61BA2EEB-C1F7-4146-BD86-1265FC55A60F/ASSETS/GRAPHIC/EMMM201911505-FIG-0002-M.PNG
12. Gomes, E., Silva, N., & Salgado, A. (2019). Combinatorial therapies for spinal cord injury: Strategies to induce regeneration. Neural Regeneration Research, 14(1), 69–71. https://doi.org/10.4103/1673-5374.243705