Universidad Católica de Valencia San Vicente Mártir

TFG

TRABAJO FIN DE GRADO

GRADO EN BIOTECNOLOGÍA

Role of Neuregulin-1 type III overexpression in the Corticospinal tract after Spinal cord injury.

Lucía Domínguez Gámbez
Tutor/a: Victoria Moreno Manzano
Cotutor/a: María Pedraza Boti
Curso académico: 2022 - 2023

PRINCIPE FELIPE
CENTRO DE INVESTIGACION

Facultad de Veterinaria y Ciencias Experimentales
Universidad Católica de Valencia San Vicente Mártir

INDEX

FIGURE LIST

TABLE LIST

ACRONYMS

ADAM 17	A disintegrin and metalloprotease domain 17
ARIs	Axon regeneration inhibitors
AVMA	American Veterinary Medical Association
BRAF	Rapidly accelerated fibrosarcoma kinase B-type
CNS	Central nervous system
CRD	Cystein-rich domain
CREB	cAMP response element binding protein
CSPGs	Chondroitin sulfate proteoglycans
CST	Corticospinal tract
EGF	Epidermal growth factor
EM	Electron microscopy
GFP	Green fluorescent protein
GGF	Glial growth factor
GSK3β	Glycogen synthase kinase 3β
HS	Horse serum
IF	Immnufluorescence
IFA	Immunofluorescence assay
LME	Lesión de la médula espinal
M1	Primary motor cortex
MAG	Myelin associated glycoproteins
MAIs	Myelin-associated inhibitors
MBP	Myelin basic protein
NRG1	Neuregulin-1
NRG1-I	Neuregulin-1 type I
NRG1-II	Neuregulin-1 type II
NRG1-III	Neuregulin-1 type III
NRGs	Neuregulins
OCT	Optimum Cutting Temperature
OPCs	Oligodendrocyte precursor cells
PAP	PTEN antagonist peptides
PBS	Phosphate-buffered saline
PCAF	p300/CBP-associated factor
PFA	Paraformaldehyde
PNS	Peripheral nervous system

PTEN	Phosphatase and Tensin Homolog
RAGs	Regeneration-associated genes
RFP	Red fluorescent protein
RGM	Repulsive guidance molecule
RhoA	Ras homolog gene family member A
ROCK2	Rho associated coiled-coil containing protein kinase 2
ROIs	Regions of interest
SCI	Spinal cord injury
SEM	Standard error of mean
Sema3A	Semaphorin 3A
SNC	Sistema nervioso central
STAT 3	Signal transducer and activator of transcription 3
T8	8th Thoracic vertebra
TACE	Tumor necrosis factor-α-converting enzyme
TMc	C-terminal transmembrane domain
TMn	N-terminal transmembrane domain

ABSTRACT

Spinal cord injury (SCI) is a neurological and pathological state that leads to devastating consequences on major motor, sensory and autonomic functions of affected patients. The main reasons for failure on SCI neural recovery are its dynamic and complex pathophysiology together with its intrinsic limitations on neuronal regeneration of adult central nervous system (CNS). To provide recovery solutions, many molecules implied on axonal regeneration, preservation and neurite outgrowth had been studied on the last years. This study was focused on evaluating the potential role of Neuregulin-1 type III (NRG1-III) overexpression in the corticospinal tract (CST), in a rat adult model of SCI.

To accomplish the study, NRG1-III and the green fluorescent protein (GFP) were specifically transfected into the cerebral motor cortex of adult female rats, so the CST could be anterogradely traced, while the contralateral side was traced only with the red fluorescent protein (RFP), being used as the control condition. Two weeks after tracing the CST, a SCI was performed by doing a dorsal hemisection. Immunohistological analyses were carried out four weeks after the injury.

Study's data showed that axonal projections from NRG1-III- overexpressed neurons showed larger distance to the lesion area, more GAP43 expression, less sprouting and more retraction bulbs, indicating that NRG1-III induced less axonal preservation and/or regeneration response added to higher axonal retraction and degeneration. Moreover, NRG1-III overexpressed neurons had a low density of adjacent oligodendrocytes in comparison with control condition, whereas no differences were found on Myelin basic protein (MBP) expression. Thus, the extrinsic role on CST myelination or demyelination was unclear, as more accurate analyses are required.

Overall, the work performed in this project suggested a detrimental intrinsic or extrinsic effect of NRG1-III overexpression, however, further analyses and studies are needed to accurately determine the actual role of NRG1-III overexpression in CST after SCI.

KEYWORDS

Spinal cord injury, Neuregulin-1, Corticospinal tract, Axonal regeneration, Axonal degeneration, Sprouting, Myelination and Demyelination.

RESUMEN

La lesión de la médula espinal (LME) es un estado patológico con graves consecuencias en las principales funciones motoras, sensoriales y autonómicas. La difícil recuperación tras una LME está determinada por su compleja fisiopatología y la limitada capacidad de regeneración neuronal que presenta el sistema nervioso central (SNC) adulto. En los últimos años, se han estudiado diversas moléculas implicadas en regeneración y preservación axonal, y crecimiento de neuritas, con el fin de mejorar la recuperación neuronal tras la lesión. El objetivo principal de este estudio fue evaluar el posible papel de la sobreexpresión de Neuregulina-1 tipo III (NRG1-III) en el tracto corticoespinal, en un modelo de LME en ratas adultas.

Para llevar a cabo el estudio, dos semanas antes de la lesión se trazó el tracto corticoespinal en sentido anterógrado; transfectando la NRG1-III y la proteína fluorescente verde (GFP) en la corteza motora del hemisferio izquierdo. Mientras que el lado contralateral, se trazó únicamente con la proteína fluorescente roja (RFP) y fue tratado como condición control. Cuatro semanas después de la lesión se realizaron los análisis inmunohistológicos.

Los datos del estudio mostraron que las proyecciones axonales de las neuronas con sobreexpresión de NRG1-III presentaron mayor distancia al área de la lesión, mayor expresión de GAP43, menor brotación axonal y mayor número de bulbos de retracción, indicando que la NRG1-III indujo una respuesta de preservación y/o regeneración axonal menor, añadida a una mayor retracción y degeneración de los axones. Por otro lado, los oligodendrocitos adyacentes a las neuronas con sobreexpresión de NRG1-III mostraron menor densidad que la población control, sin embargo, no se encontraron diferencias en la expresión de la proteína básica de mielina (MBP).

En conclusión, el trabajo realizado en este proyecto sugirió que la sobreexpresión de NRG1-III tuvo efectos intrínsecos o extrínsecos perjudiciales. No obstante, se necesitan más análisis y estudios para poder determinar con precisión el papel de la sobreexpresión de NRG1-III en el tracto corticoespinal tras una LME.

PALABRAS CLAVE

Lesión de la médula espinal, Neurregulina-1, Tracto corticoespinal, Regeneración axonal, Degeneración axonal, Brotación axonal, Mielinización y Desmielinización.

INTRODUCTION

1. SPINAL CORD

The nervous system is responsible for perception, behavior, memory, and all voluntary movements. It is divided into central nervous system (CNS) which includes brain and spinal cord, and the peripheral nervous system (PNS) composed of the rest of cranial nerves, spinal nerves, and the autonomic nervous system (Farley et al., 2014).

Spinal cord carries the sensory information from the PNS to the brain and transmit the motor responses from the brain to the motor neurons. It is contained in the thecal sac, which lies in the vertebral canal of the vertebral column (Nieuwenhuys et al., 2008), anchored to the dura by the denticulate ligaments and extends from the medulla oblongata to the lower border of the first lumbar vertebra (Bican et al., 2013). As it is represented in Figure 1, spinal cord is divided in several segments which named: cervical, thoracic, lumbar, sacral, and coccygeal. At each segmental level, ventral and dorsal rootlets give rise to pairs of ventral and dorsal roots, that are combined forming the pairs of spinal nerves (Nieuwenhuys et al., 2008).

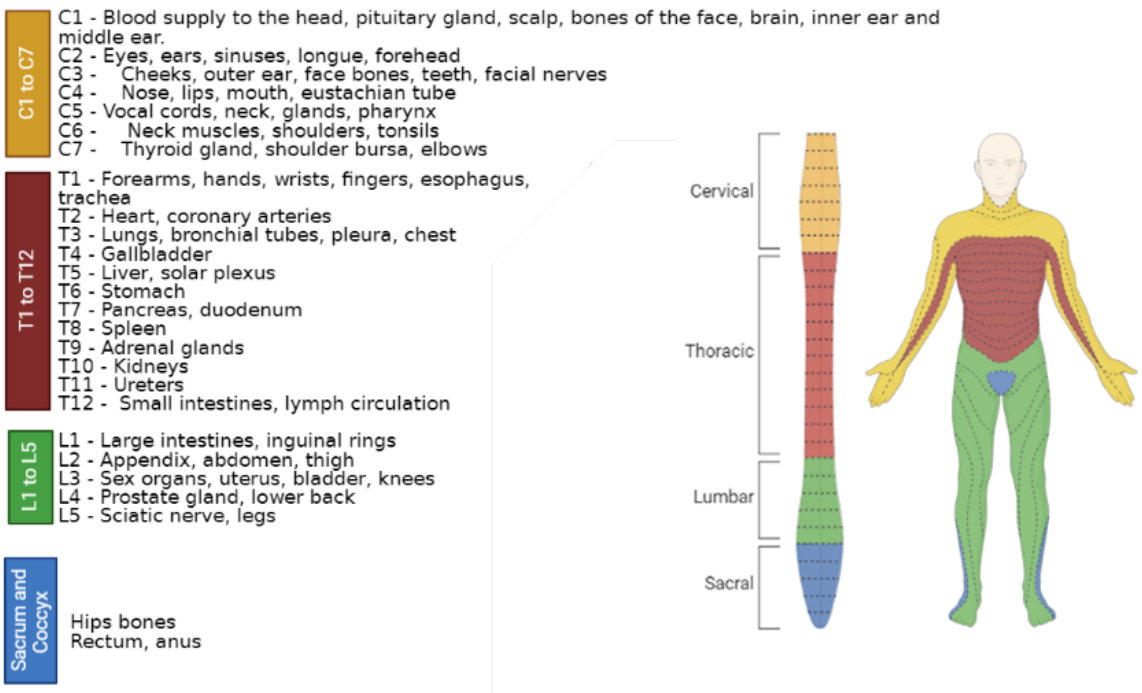

Figure 1. Schematic Representation of Spinal Cord Segments in human. From "Schematic Representation of Spinal Cord Segments in human," by M. Sánchez, 2021, *Toll like receptors in spinal cord derived neural precursor cells: implications on spinal cord injury and cell transplantation,* p.33.

Spinal cord is composed by a gray and a white matter. The gray matter has a butterfly-shape structure and is composed mainly of neurons and their somata, interneurons and glial cells. Surrounding the gray matter, it is located the white matter, which is composed of long projections of myelinated axons setting up the ascending tracts and descending tracts of the spinal cord, that transferring motor, sensory and autonomic information between brain and peripheral nervous system (Nieuwenhuys et al., 2008).

The ascending tracts convey sensory information from PNS to CNS areas, and are essential for posture, walking and information processing about noxious stimuli. While the descending tracts run from the brain and brainstem to the spinal cord and are involved in motor control (Vogelaar et al., 2016). Due to this information transfer, the spinal cord controls the voluntary muscles of the trunk and upper and lower extremities and get sensory inputs from these areas of the body (Bican et al., 2013).

1.2 CORTICOSPINAL TRACT

Corticospinal tract (CST) is the largest and most significant descending tract of the spinal cord, being the principal motor pathway for voluntary movements. Most of CST axons originate from pyramidal cells located in the inferior part of cortical layer V in the primary motor and sensory cortex (M1 and S1 respectively), while other corticalregions make smaller contributions (Nudo et al., 1990).

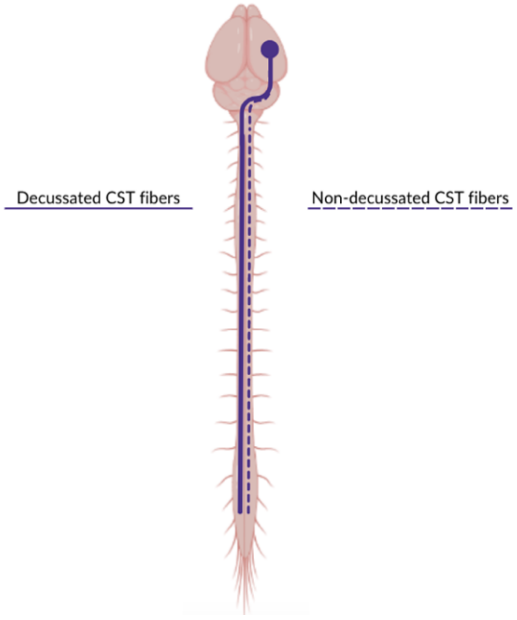

Figure 2. Graphic representation of the decussated Corticospinal fibers in rat.

The trajectory of CST axons from the neocortex to the caudal medulla is conserved across mammals. After leaving the neocortex, CST axons form bundles and run through the internal capsule and cerebral peduncles in a ventral position, drawing the pyramidal shape of neurons, until they reach the caudal part of the medulla. Then, at the junction between the brainstem and spinal cord, almost the 90% of CST axons cross the

midline and pass from a ventral to a dorsal position, decussating in the lower medulla (Figure 2). The tract has also an 8% nondecussating descending fibers form the anterior corticospinal tract and 2% of noncrossing fibers which generate the uncrossed lateral corticospinal tract (Welniarz et al., 2017; Bican et al., 2013).

2. SPINAL CORD INJURY

Spinal cord injury (SCI) is a destructive neurological and pathological state that causes major motor, sensory and autonomic dysfunctions. Unfortunately, its global prevalence has been increasing over the last 30 years and its available treatments are deficient, achieving only palliative effects (Khorasanizadeh, et al., 2019).

The SCI lesions are not homogeneous, because lots of factors can interfere in the complexity of the pathophysiology such as the cause of the injury or the size of the fibrotic scar. Also, it must consider that normal spinal cord physiology involves interactions among many cell types such as astrocytes, neurons, microglia, and oligodendrocytes, which carry out divergent functions. Therefore, after SCI cells present different repair and regeneration mechanisms as well, presenting disorganized interactions and leading to an impaired spinal recovery. The heterogeneous post-SCI landscape conformed by complex pathophysiological consequences together with the intrinsic limitations on spontaneous regenerative capacities of adult CNS neurons, are the main reasons for the poor knowledge and failure of SCI neural recovery (O'Shea et al., 2017). Consequently, understanding the requirements for achieve or improve the regeneration of the neural interactions, its essential for the development of appropriate recovery treatments.

2.1 PATHOPHYSIOLOGY

The SCI pathophysiology can be split into two phases (McDonald et al., 2002). The so-called primary injury is caused by the physical trauma inflicted to the spinal cord, with fragmented bones and spinal ligament tearing. It leads to massive neural parenchyma death, with destruction of glial membrane, interruption of nerve plexus and hemorrhage episodes. The injury severity and the prognosis of further neurological

dysfunction will be determined by the extension of the affected area and the duration of this traumatic event (Anjum et al., 2004).

The post-trauma or secondary injury phase is characterized by a cascade of events activated by the biochemical, mechanical, and physiological changes occurred within neural network, which is going to expand injured zone and intensify neurological deficits. These incidents are interrelated, each causing or facilitating the next one. Also, occasionally, multiple events occur simultaneously and cause complex episodes, thus rendering this illness such difficult to treat. Even so, the secondary injury can be divided into three phases: acute, sub-acute and chronic injury (Figure 3) (Yip et al., 2012; Norenberg et al., 2004)

The clinical manifestation of the acute phase includes vascular damage, ionic imbalance, excitotoxicity, free radical production, increased calcium influx, lipid peroxidation, edema, and necrosis. Also, the disruption of the blood-spinal cord barrier, leads to the infiltration of monocytes, neutrophils, T and B lymphocytic cells and macrophages within injury site; triggering the release of inflammatory cytokines, which are going to remain with elevated levels up to 4 days after injury and will promote the inflammation into the neurons, causing neurotoxicity instead of neuroprotection (Nakamura et al., 2003).

If the acute phase persists, then it initiates the sub-acute secondary injury phase, which is characterized by neuronal apoptosis, axonal demyelination, Wallerian degeneration, axonal remodeling, and scar formation. Finally, the sub-acute phase leads to the chronic injury of SCI with the formation of cystic cavity, axonal dieback, and maturation of the fibrotic scar (Anjum et al., 2004).

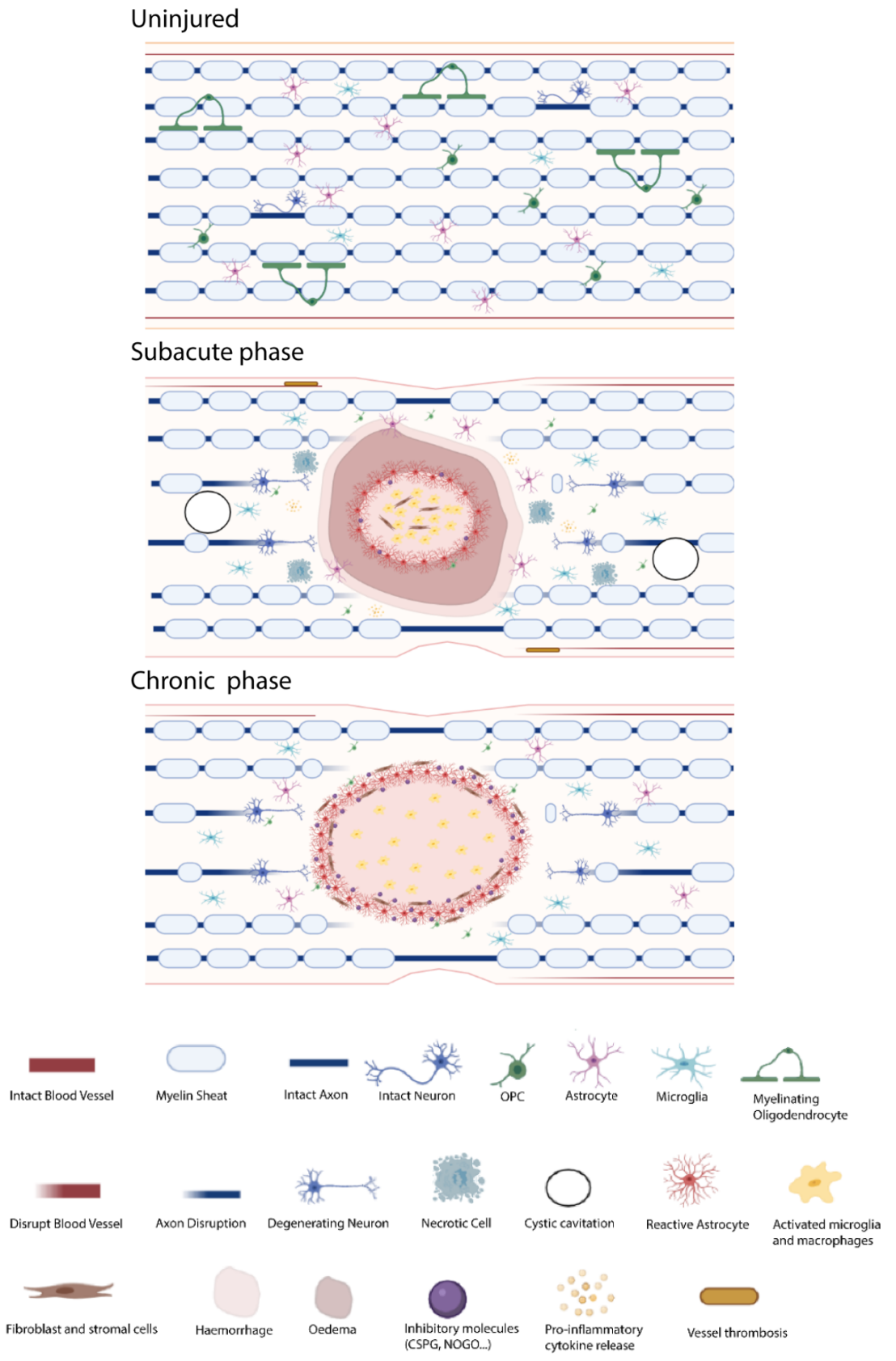

Figure 3. Graphic representation of pathophysiology phases after spinal cord injury. From "The pathophysiology of the spinal cord before and after injury," by M. Sánchez, 2021, *Toll like receptors in spinal cord derived neural precursor cells: implications on spinal cord injury and cell transplantation,* p.39,40.

3. REGENERATIVE MECHANISMS AFTER SPINAL CORD INJURY

3.1 AXONAL REGENERATION

After SCI, CST axons located caudal to the lesion site undergo rapid fragmentation and anterograde degeneration, whereas the rostral to the lesion CST axons present a slow and progressive retrograde degeneration (Hill et al., 2001). As stated before, there are cellular and molecular mechanisms following SCI that compromise axonal regeneration and neuroplasticity.

After axotomy, CNS presents a poor axonal regeneration because it is affected by a combination of extrinsic and intrinsic inhibitors (understanding “intrinsic” as the intraneuronal signaling which is independent of neuronal environment).

Mainly there are two types of extrinsic factors which avoid the CNS regeneration. On one hand, the formed scar presents chondroitin sulfate proteoglycans (CSPGs) as NG2 (Jones et al., 2002; Davies et al., 2004) which are upregulated among others, by reactive astrocytes, leading to an actively impenetrable matrix for axonal growth (Liuzzi et al., 1987; McMillian et al., 1994).

On the other hand, myelin debris contain inhibitory molecules (Fawcett & Asher, 1999) as membrane proteins NI-35 and NI-250 (Raineteau et al., 1999) or myelin associated glycoproteins (MAG) (McKerracher et al., 1994), which are slowly removed due to the low phagocytic activity of CNS macrophage cells. The accumulation and remain of these molecules, activate inhibitory signaling pathways, activating molecules as the small GTPase ras homolog gene family member A (RhoA), that in turn, activates Rho-associated coiled-coil containing protein kinase 2 (ROCK2) which is related with the stop of neurites growth (Schmandke et al., 2007). In addition, there are also axon regeneration inhibitors (ARIs) which are not present in either myelin or fibrotic scar, as the repulsive guidance molecule (RGM) (Hata et al., 2006) or semaphorin 3A (Sema3A) (Gallo, 2006); and activates also RhoA-kinase pathway.

Pre-clinical strategies neutralizing extrinsic inhibitory factors only modestly increased axon regeneration, and in only a sub-population of neurons (He & Jin, 2016; Mar et al., 2014), suggesting that intrinsic factors influence more in the CNS regeneration failure.

One of the early intrinsic manifestations of axonal degeneration which preclude axonal regeneration in the CNS is the presence of swellings formed by the lesioned axons, the so-called retraction bulbs. As Figure 4 shows, these structures have oval shape and their microtubules disorganized and dispersed (Ramon y Cajal, 1928; Li & Raisman, 1995; Hill et al., 2001). Said disorganization do not allow axonal extension, leading to the impaired regeneration of axons that show this abnormal morphology (Kabir et al., 2001; Schaefer et al., 2002). Unfortunately, the intracellular and molecular mechanisms involved in retraction bulbs formation and its growth disability is still unknown.

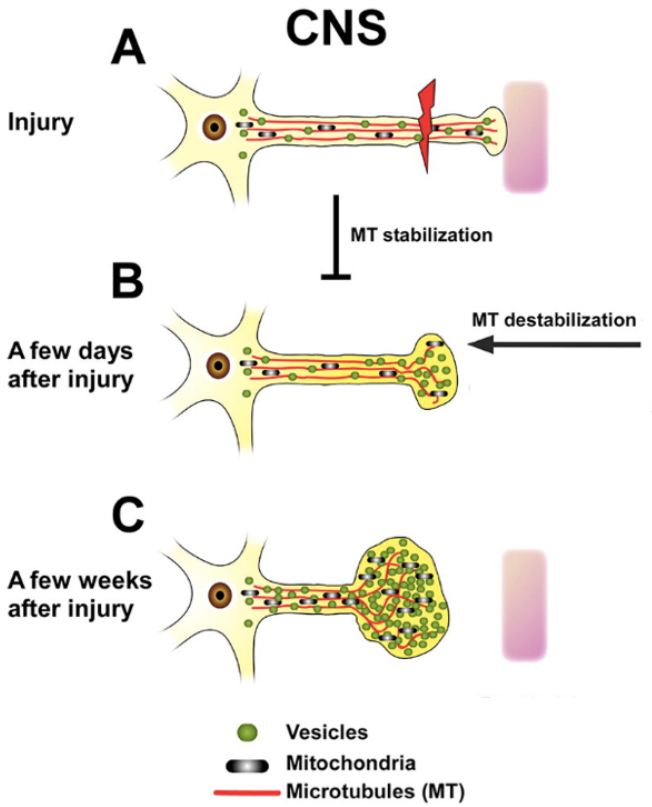

Figure 4: Graphic representation of retraction bulbs formation. Adapted from "Model for retraction bulb formation versus growth cone-mediated regeneration", by Ertürk, A., Hellal, F., Enes, J., & Bradke, F, 2007, *Disorganized microtubules underlie the formation of retraction bulbs and the failure of axonal regeneration, the Journal of Neuroscience*, 27 (34), p. 11. Copyright 2007 Society for Neuroscience.

Otherwise, it is known that intrinsic regeneration is mainly limited by the presence of axonal growth repressors as phosphatase and tensin homolog (Pten) (Liu et al., 2010; Park et al., 2008) or glycogen synthase kinase 3β (GSK3β) (Liz et al., 2014). In addition to the low levels of injury signaling machinery and the reduced expression of

regeneration-associated genes (RAGs). These endogenous mechanisms are deficient because the regenerative potential of the CNS neurons presented on the development stage declines progressively during adulthood. Owing this fact, last's years studies were focused on the searching of multiple molecules which are able to regulate intrinsic regeneration in adult CNS neurons. Some of these studied intrinsic molecules are: GAP43 (Fernandes et al., 1999), rapidly accelerated fibrosarcoma kinase (B-RAF) (O'Donovan et al., 2014), c-myc (Belin et al., 2015), α9 integrin (Andrews et al., 2009), cAMP response element binding protein (CREB) (Gao et al., 2004) and histone acetyl-transferase p300/CBP-associated factor (PCAF) (Puttagunta et al., 2014).

Regarding the CST axonal regeneration concretely, many genetic studies highlight as beneficial the deletion of the phosphatase and tensin homolog (Pten*)* (Liu et al., 2010; Geoffroy et al., 2016; Nakamura et al., 2021; Danilov & Steward, 2015; Du et al., 2015; Zukor et al., 2013; Lewandowski & Steward, 2014) while propose the overexpression of one of the following molecules: *Klf7* (Blackmore et al., 2012; Wang et al., 2017), *Sox11* (Wang et al., 2015), *Lkb1* (Ohtake et al., 2019), *Lin28a* (Nathan et al., 2020), and (*Stat3*) (Lang et al., 2013). Also, there are pharmacological studies that expose the administration of other molecules as beneficious for axonal CST regeneration such as: C3 coenzyme (Dergham et al., 2002), Lithium as inhibitor of glycogen synthase kinase (GSK3β) (Dill et al., 2008), and *Pten* antagonist peptides (PAP) (Ohtake et al., 2014; Bhowmick & Abdul-Muneer, 2021).

3.2 CORTICOSPINAL TRACT SPROUTING

Despite failure in elongation and reinnervation of lesioned axons, exists a plasticity mechanism that could bypass the injury, achieving functional recovery of CST axons after SCI: the so-called sprouting.

Sprouting consist in a spontaneous structural reorganization of injured or uninjured axons, that achieve new synaptic innervations to recover functionality. The first plasticity case occurs when new axonal branches extend out from CST damaged axons to several spinal segments located surrounding the lesion site, forming contacts with spinal interneurons, and restoring the innervation beyond the injury. The second plasticity case occurs when damage itself to synaptic pathways induces compensatory growth of

new connections from nearby to lesion undamaged axons (Weidner et al., 2001; Tuszynski & Steward, 2012).

Regarding sprouting, many studies have demonstrated that the CST has the capacity to spontaneously sprout rostrally and caudally to a spinal cord lesion (Fouad et al., 2001, 2011; Hill et al., 2001; Bareyre et al., 2004; Ghosh et al., 2009; Onifer et al., 2011), but this recovery mechanisms is limited, thus it is not enough to achieve rehabilitation on SCI patients. Therefore, actual studies are focus on induce more sprouting capacity after SCI, by different strategies such as electrical stimulation (Carmel et al., 2014; Song et al., 2016).

4. NEUREGULINS

Neuregulins (NRGs) are a family of transmembrane growth factors encoded by four genes (NRG1, NRG2, NRG3 and NRG4), those have signaling pathways which regulate cell-cell interactions, playing critical roles in the development and maintenance of the central and peripheral nervous systems (Rahman-Enyart et al., 2020; Falls, 2003).

Alternative both splicing and promoter usage; give rise to neuregulin genes to has many protein products, reaching more than 30 diverse isoforms which could be soluble or membrane bound, and are going to present different domains as: immunoglobulin (Ig)-like domains, kringle domains or cysteine-rich domains. However, all NRGs share a single extracellular epidermal growth factor (EGF)-like domain which is described to be necessary and sufficient for the activation of a set of receptor protein tyrosine kinases pertained to the ErbB family (ErbB1–4) (Lemke, 1996; Rahman-Enyart et al., 2020).

All NRG signals appear to be transduced through the ErbB receptors family. Nevertheless, in addition to the canonical forward signaling via ErbB family receptors, the membrane-bound form of NRG1 type III (NRG1-III) has a reverse signaling pathway which is activated by γ-secretase, a protease which cleaves NRG1-III of the membrane, releasing the intracellular domain NRG1-ICD, which translocates to the nucleus and regulates the gene expression (Figure 5) (Bao et al., 2003, 2004).

There are multiple functions associated with NRGs. Specifically, there are two bioactivities which have been appreciated as regulators of neural development. The first

one is the capacity to stimulate the proliferation of Schwann cells and astrocytes, which led to name the protein as glial growth factor (GGF). The second one is the role, as a trophic factor, to enhance the expression of specific differentiation-genes of skeletal muscle development (Lemke, 1996; Gassmann & Lemke, 1997).

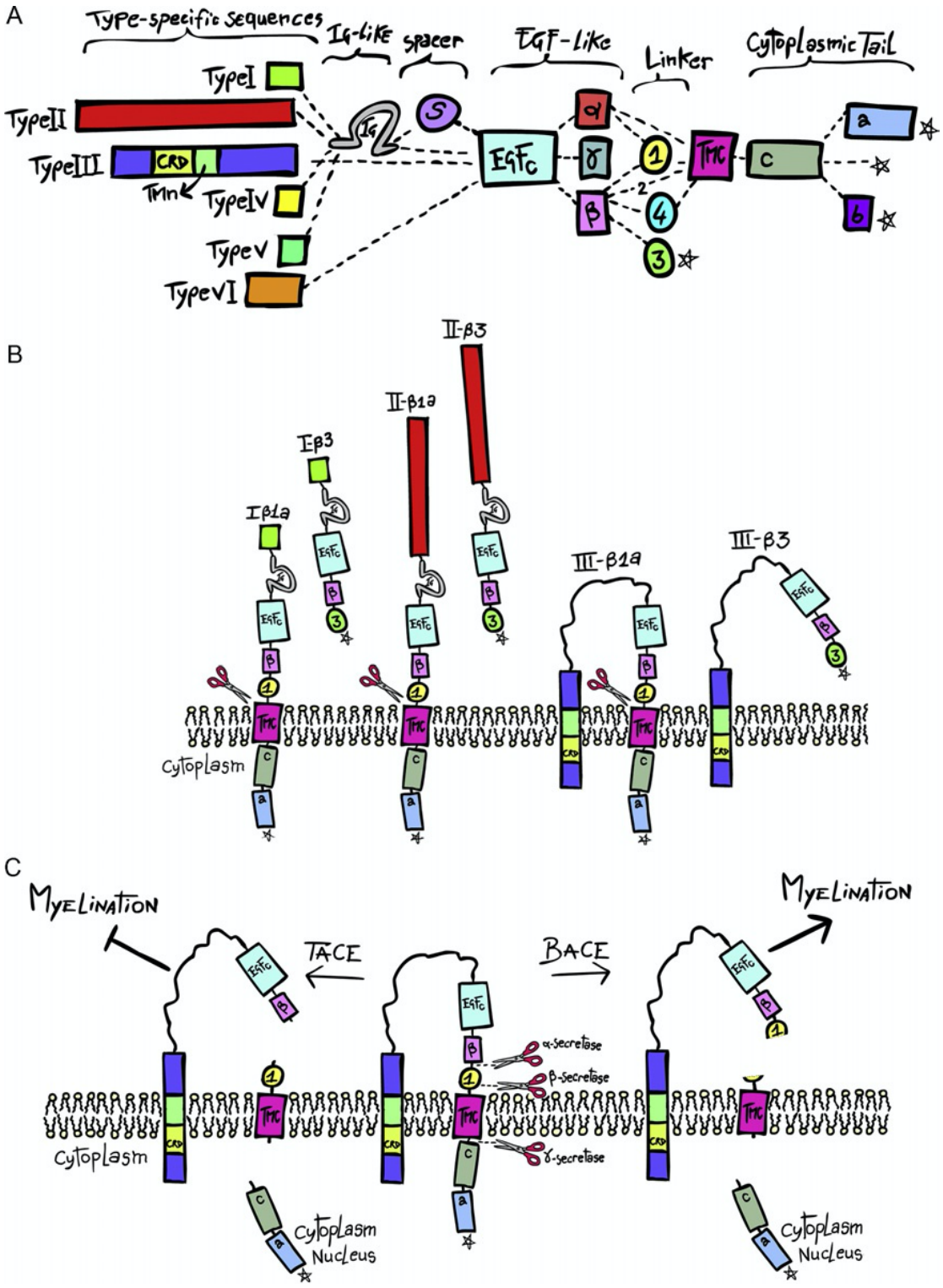

Figure 5. NRG1 isoforms and their signaling activation pathways.

A) Six types of NRG1 are described (I–VI), which differ for N-terminal exons. NRG1-types I, II, IV, and V (with or without a spacer region) are characterized by an immunoglobulin (Ig)-like domain, located between the N-terminal sequence and the EGF-like domain; NRG1-types III and VI present an N-terminal region connected directly to the EGF-like domain. The type III N-terminal sequence contains a cystein-rich domain (CRD) with an additional transmembrane domain (TMn). Variants derive also by splicing in the linker and in the cytoplasmic tail; the C-terminal transmembrane domain (TMc) is located between these two regions. The great number of NRG1 isoforms derives also from the alternative use of different exons located downstream the EGF-like domain: the first exon can be α or β or can be missing (isoform g); the second exon can be 1 or 4 or 3 (followed by a stop codon) or can be missing (isoform 2); the third exon can be a transmembrane domain (TMc), followed by a cytoplasmic exon c, that can be followed by a stop codon (isoform c), by exon a or exon b.

B) In this scheme is reported the structure of those isoforms (I, II, and III, β1a and β3). Many soluble and mature NRG1 proteins are produced in the form of transmembrane precursors and are generated by the cleavage by different secretases, except in the case of NRG1-β3 isoforms, which are already released as soluble mature proteins for autocrine/paracrine interactions (type I/II) or as transmembrane mature proteins for juxtracrine interactions (type III).

C) NRG1-type III can be cleaved by α-secretases belonging to tumor necrosis factor-α-converting enzyme (TACE) or a disintegrin and metalloprotease (ADAM) family (e.g., TACE/ ADAM17) or by β-secretases (BACE-1/β-site of amyloid precursor protein-cleaving enzyme). BACE-1 cleavage stimulates myelination (Hu et al., 2006; Willem et al., 2006) and remyelination (Hu et al., 2008); TACE cleavage inhibits myelination (La Marca et al., 2011). A second cleavage by a γ-secretase-dependent protease, in those NRG1 isoforms containing the TMc, generates a cytoplasmic fragment that can translocate into the nucleus and influence gene transcription (Bao et al., 2004).

From “NRG1 isoforms”, by Gambarotta, G., Fregnan, F., Gnavi, S., & Perroteau, I, 2013, *Neuregulin 1 role in Schwann cell regulation and potential applications to promote peripheral nerve regeneration.* International review of neurobiology, 108, p. 225. doi.org/10.1016/B978-0-12-410499-0.00009-5. Copyright 2013 by Elsevier Inc.

4.1 NEUREGULIN-1 IN SPINAL CORD INJURY

NRG1 and its isoforms have been the most extensively characterized of the NRGs. The three structural characteristics that differentiate isoforms regarding in vivo functions and cell biological properties are: (1) the type of EGF-like domain (α or β), (2) if there are initially synthesized as a transmembrane or non-membrane protein and (3) the N-terminal sequence (type I, II, or III), which determines if the proteins generate mainly paracrine signals (I and II), or juxtracrine signals (III) (Figure 5) (Falls, 2003).

Regarding individual functional roles of NRG1 isoforms, (Meyer et al., 1997) studied that type I NRG1 (NRG1-I) is the predominant isoform expressed in early embryogenesis. It is expressed in cephalic mesenchyme and cranial ganglia as well as the endocardium, been essential for survival and/or development of neural crest-derived sensory neurons in cranial ganglia. In contrast, NRG1 types II (NRG1-II) and III (NRG1-III) are first detected at midgestation state. Despite genetic analysis does not reveal essential roles of NRG1-II, it is established that NRG1-III is expressed in different sensory and motor neurons and been essential to drove first initial development of Schwann cell precursors, which an essential function into myelination. Consistently, (Falls, 2003) describes NRG1-III isoform as “the major NRG isoforms that act as “glial growth factors” in vivo”, been studied that it can determine the myelin ensheathment fate of axons (Michailov et al., 2004; Taveggia et al., 2005).

Considering its roles in the development and maintenance of the central and peripheral nervous systems, several studies have been carried out to elucidate further functions of NRG1 protein which could be effective in the recovery after a spinal cord injury. Thanks to the conclusions of some of these studies, it is known that: (1) NRG1 plays an essential role in axonal **remyelination** and functional repair after SCI, by promoting oligodendrocyte differentiation (Ding et al., 2021), Schwann cells differentiation (Fricker et al., 2011) and also trans-differentiation of central precursor cells into peripheral nervous system-like Schwann cells (Bartus et al., 2016); (2) NRG1 has a positive **immunomodulatory** role that improves neurological recovery in the acute phase of SCI, taking advantage of the beneficial properties of activated glia (Alizadeh et al., 2017) and inflammatory cells by stimulating a supportive environment for repair and regeneration (Alizadeh et al., 2018); and (3) NRG-1β1 has the potential to

modulate mitochondrial and intracellular pathways in neurons, attenuating neural death after SCI (Shahsavani et al., 2021).

These played roles of NRG1 on myelinated or immune cells leads generally to a neuroprotective effect on neurons, suggesting that the treatment with NRG1 could improve the recovery out-comes following SCI. In fact, Joung et al. (2010) used recombinant adenoviruses which express the secreted form of NRG β-EGF-like domain of NRG1-I, to achieve recovery improvements after an injured PNS, specifically after an axotomy model of sciatic nerve damage. As results, it is displayed an augmented expression of neurofilaments, GAP43 and S100 in the distal stump of the injury site and an increased length of regenerating axons, leading also to sensory and motor functions improvement. These results suggest a therapeutic potential for β-EGF-like domain of NRG1 in the treatment of peripheral nerve injury. Nevertheless, further studies are needed to know the possible functionality of NRG1 in the axonal regeneration of mature CNS axons after SCI.

OBJECTIVES

Aim of study is to elucidate whether overexpression of NRG1-III at the CST can play any role on its regenerative response in a model of rat SCI.

To achieve the main aim, the following experimental objectives were established:

1. To assess NRG1-III overexpression intrinsic role on CST regeneration at the injured spinal cord, studying:
   1.1 The distance of axonal projections to the lesion.
   1.2 GAP43 growth factor expression on axonal projections.
   1.3 CST sprouting.

2. To assess NRG1-III overexpression intrinsic role on CST degenerative or retractive response at the injured site of the spinal cord:
   2.1 Studying axonal projections morphology.

3. To assess NRG1-III overexpression extrinsic role on CST myelination or demyelination at the injured site of the spinal cord:
   3.1 Studying the oligodendrocyte population adjacent to CST.
   3.2 Studying the levels of Myelin basic protein (MBP) expression.

MATERIALS AND METHODS

All the surgical interventions, the subsequent care of the animals and the handling of microscope for image acquisition, were carried out by the members of the research team led by Dr Moreno. Nevertheless, the entire methodology process is going to be described.

EXPERIMENTAL ANIMALS

Female rats Sprague Dawley 2 months old (n=6) were used for the experiments and were housed in a barrier facility with a 12 h light/dark cycle with free access to food and water. The maintenance, manipulations, and surgeries of all the animals were in accordance with the Spanish Regulations in force for the Protection of Experimental Animals (Real Decreto 118/2021, of the Ministerio de Presidencia, Relaciones con las Cortes y Memoria Democrática). These animals had also received the approval of the Ethics and Animal Experimentation committee of the Príncipe Felipe Research Centre. The study was done in accordance with the ARRIVE guidelines for reporting in vivo animal experiments and the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals (2020).

ANTEROGRADE TRACING AND NEUREGULIN-1 OVEREXPRESSION

The experimental procedure is briefly summarized in Figure 6. Anterograde tracing of the CST was performed by stereotaxically injecting viral vectors cited in table 1 into the motor cortex of the rats (the following coordinates were used: Bregma—AP: 0, +2; ML: +1.5, -1.5; DV: -1.4). The fluorescent proteins expressed by the viruses will act as reporters, marking the tract of interest and making it visible by fluorescent microscopy in tissue analysis.

Viral vectors

Adenovirus	Reference
AAV_Syp_RFP (1ul (1/1,25))	Ceded by the viral production unit, Barcelona
AAV_Syp_GFP (1ul (1/4))	Ceded by the viral production unit, Barcelona
AAV_Syp_NRG1-III (1ul)	Ceded by the viral production unit, Barcelona

Table 1. Viral vectors used to perform the anterograde tracing and Neuregulin-1-III overexpression.

The cerebral left hemisphere was treated as the experimental condition: performing a co-transfection of two viral vectors: a vector carrying the NRG1 gene in its isoform 3 (NRG1-III), which express the protein of interest; and another vector with the green fluorescent protein (GFP), which served as a reporter protein. Although NRG1-III and GFP were not located in the same viral vector, it is assumed that there will be a co-transfection of both vectors, thus this portion will fluoresce in green (488 nm) to be detected in the immunofluorescence. On the other hand, the cerebral right hemisphere was treated as the control condition, performing a transfection of a viral vector with the red fluorescent protein (RFP), thus this portion of the CST will fluoresce in red (555 nm).

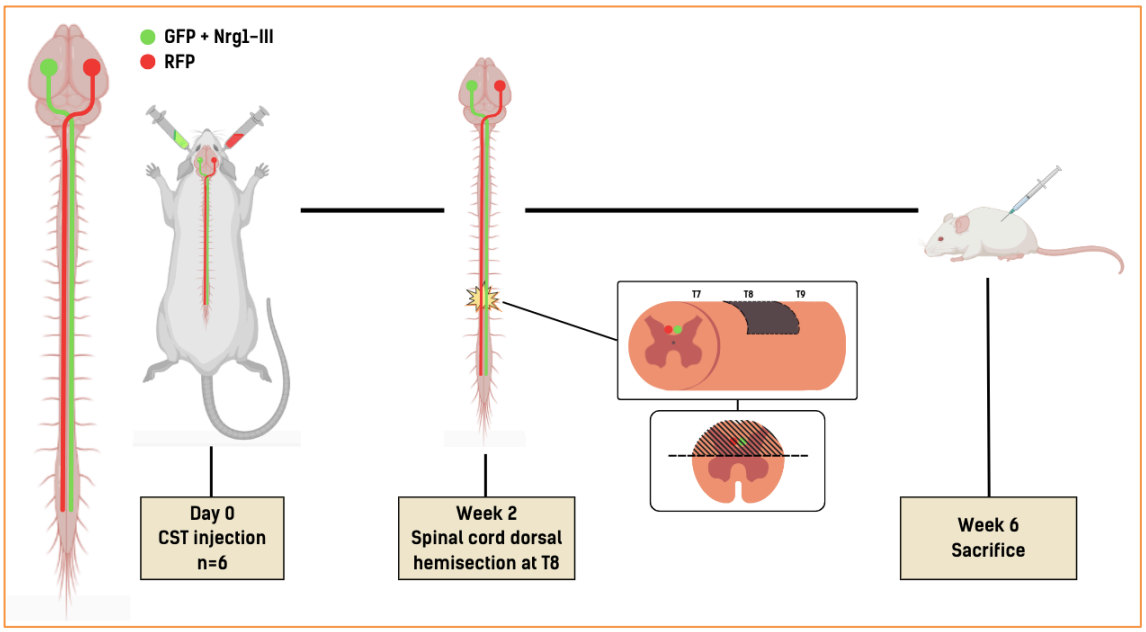

Figure 6. Graphic representation of the experimental procedure.

GENERATION OF THE SPINAL CORD INJURY MODEL

Two weeks after tracing the CST, a dorsal hemisection of the spinal cord was performed. Briefly, the spinal cord was exposed by carrying out a laminectomy at the thoracic level (T8) followed by a dorsal incision of 1 mm approximately by using a microsurgical knife. Following surgery, bladder expression was performed twice a day until reflexive bladder emptying was restored. Also, well-being checks were carried out including soft bedding in all cages, the provision of accessible food and hydration gel.

HISTOLOGY

Four weeks after the lesion the rats were irreversibly anesthetized by an intraperitoneal injection of 3 times the therapeutically dose of pentobarbital (this is final concentration of 150-200 mg/kg) diluted 1:10 with saline serum. Subsequently, rats were transcardially perfused with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde (PFA) in 0.1 M PBS. The spinal cord and brain samples obtained were post-fixed in 4% PFA for 24 hours. After this step, the samples were passed through sucrose 30% before freezing OCT blocks. Although in this study we will only focus on the analysis of the spinal cord samples.

The spinal cords were cut with blade into two sections of interest: the first one consists in 1 cm long of the cervical part and the second one is the portion for the study of the lesion, cutting as from 1 cm before the lesion. These two samples were then included in blocks with Optimum Cutting Temperature (OCT) medium.

Before the OCT inclusion, it is important to know which is the anatomical plane (Figure 7) of interest for the analysis, since according to this, the spatial position of the sample in the block will be different. The rostral-caudal and dorsal-ventral positions must be also considered.

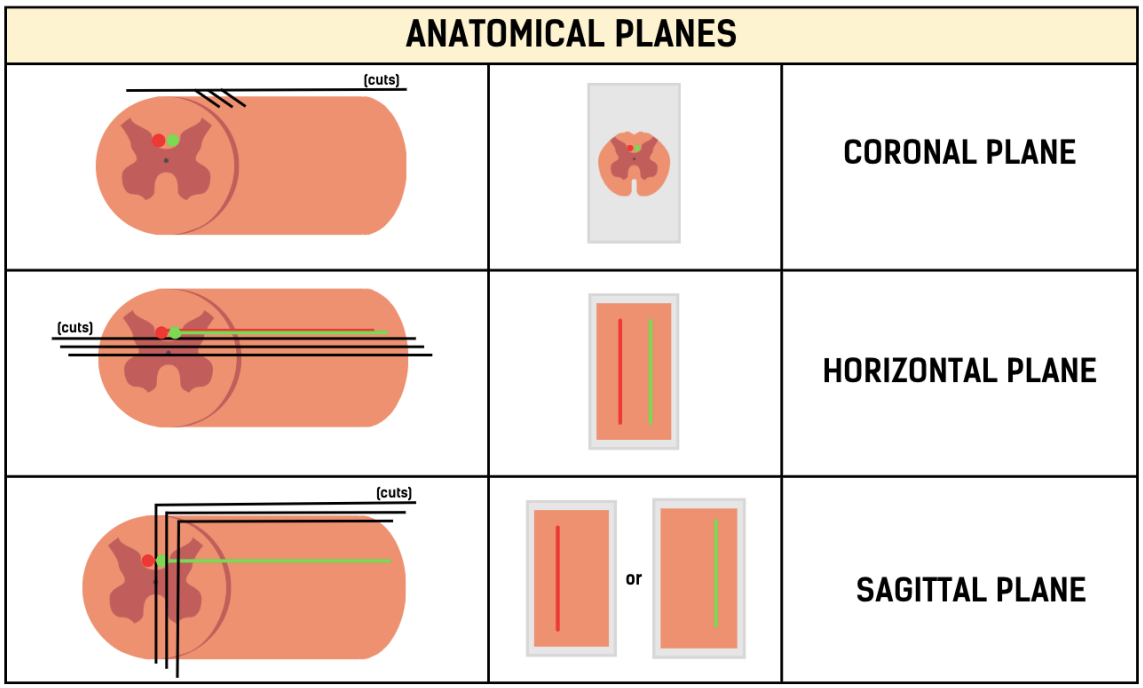

Figure 7. Graphic representation of anatomical planes in spinal cord and their corresponding slices.

All the spinal cord sections were cut on a cryostat with a thickness of 20 µm, and three to four cuts were mounted on each slide following a serial pattern. The serial pattern of the cut process allows a representative visualization of the entire spinal cord throughout the series, thus avoiding the analysis of all the cuts obtained. The slides should be kept frozen (-20ºC).

In this case, the cervical sections of the spinal cord were cut in coronal as Figure 7 represents. These samples were used to verify the tracing and quantify the relative positive staining (GFP/RFP) in each CST part. These data were collected to normalize the labelling in the rest of the samples, relativizing each animal with his own.

On the other hand, the sections to study the lesion of the spinal cord were cut in different longitudinal planes. To note, at the beginning of the analysis, in the first two animals, the spinal cords were dissectioned into sagittal plane. However, the post-analysis of these samples revealed that this kind of anatomical plane was not the best option for the experiment, since the analysis required the comparation of the two parts of the CST: (NRG1-III- GFP) and (Control- RFP), therefore, it was impossible to show both tracts in the same sample, unless it was a specific medial part of the spinal cord, more clearly represented in Figure 7. Therefore, the rest of the spinal cords were cut into the horizontal plane, which made possible the comparison of both portions (NRG1-III- GFP together with Control- RFP) visualized in each sample.

IMMUNOFLUORESCENCE ASSAY

Once the histological sections were obtained, immunofluorescence assays (IFAs) are performed to detect in the samples the proteins of interest. It is crucial to take in account that the slides must be protected from the light, as much as possible in all the following steps, because the fluorescent labelling present in the tissues could be affected by prolonged light exposure, interfering in the subsequent analyses. Also, before the start, it is important to mark off the edges of the slides with a hydrophobic marker, which will prevent solutions from overflowing.

Firstly, the slides were tempered at room temperature for 30 minutes and fixed with 4% PFA in PBS 1x for 30 minutes at room temperature. Then, at the end of the fixation period, the slides were rinsed with PBST consisting of PBS 1x and 0.05% of Tween 20 for 10 minutes, 3 replicates.

Secondly, it is necessary to permeate the cells so the antibody can reach its antigen and to block the prospective nonspecific binding sites of the tissue. To achieve it, a blocking solution is prepared with PBST and 2% of Horse Serum (HS). The sample must incubate one hour at room temperature with the blocking solution.

The next step is the incubation with the primary antibodies, which are going to be diluted in blocking solution (according to the manufacturer's instructions). The species of the primary antibodies used, and its concentrations were specificize in Table 2. These antibodies will amplify the fluorescent signals of the proteins, and its incubation will be overnight at 4ºC in the cold chamber.

Once the incubation is finished, the slides were rinsed with PBST for 5 minutes, 5 times. Then, specific fluorescent secondary antibodies were added (diluted also in blocking solution as primary ones). The species of the secondary antibodies used, and their concentrations were specified in Table 2. The incubation time is 1 hour and 30 minutes at room temperature. This time in this step is important because if the incubation lasts for more than 2 hours, non-specific bindings could arise.

Primary antibodies

Antibody	Species	Dilution	Reference
Anti-GFP	Chicken	1:1000	ab13970 Abcam
Anti-RFP	Guinea Pig	1:1000	390004 Synaptic System
Anti-Beta-3 Tubulin	Mouse	1:400	ab78078 Sigma
Anti-GAP43	Rabbit	1:400	GTX101535 Gen TEX
Anti-Olig2	Rabbit	1:400	AB9610 Sigma
Anti-MBP	Rabbit	1:200	ab69863 Abcam

Secondary antibodies

Antibody	Species	Dilution	Reference
Anti-Chicken (488)	Goat	1:400	A11039 Invitrogen
Anti-Guinea Pig (555)	Goat	1:400	A21435 Invitrogen
Anti-Mouse (647)	Goat	1:400	A21236 Invitrogen
Anti-Rabbit (647)	Goat	1:400	A21244 Invitrogen

Table 2. Primary and Secondary Antibodies used to perform the Immunofluorescence assays.

After the incubation period, a PBST solution with a concentration of 1/1000 DAPI (4',6-diamidino-2-phenylindole) was added during 5 minutes at room temperature, after which 5 PBST washes were done. DAPI is a DNA-specific probe which forms a fluorescent complex by attaching in the minor grove of A-T rich sequences of DNA, thus marking the cell nuclei. The use of DAPI allows the identification of the independent cells and thus, the cell density will show the cytoarchitecture (morphology of the different structures) of the tissue.

Before putting the coverslips, the hydrophobic marker was carefully removed with acetone and the slides were washed after those three times with PBST. Then, the sections were covered by Mowiol (mounting medium) and the coverslips placed. Finally, the slides were dried completely and stored at 4ºC in the dark.

IMAGE ADQUISITON AND PROCESSING

Once the immunofluorescence (IF) protocol is finished, the slides are available to be observed and processed by the vertical fluorescence ZEISS ApoTome microscope (Carl Zeiss). The images were obtained with a 10x achromatic objective, were acquired from a tile of images per each sample and were then analyzed using Fiji: an open-source image processing package based on ImageJ2.

Firstly, to normalize and relativize all the data related with the labelling and infection of GFP and RFP that had each animal, it was performed a relative positive staining quantification in the cervical samples of the spinal cord, which, as mentioned before, were cut into the coronal plane. For the assessment of the relative positive area, comparable regions of interest (ROIs) of the tiled images were selected in Fiji and thresholded to quantify the ratio of thresholded area to the total area of the ROI. This relative positive staining quantification was performed also to analyze the sprouting and the Myelin basic protein (MBP) expression.

Secondly, for the assessment of the number of retraction bulbs and olygodendrocytes, the total area of comparable regions of interest (ROIs) and the number and size of thresholded objects were quantified with Fiji function “Analyze particles”. For the assessment of GAP43 distribution and expression, GAP43/GFP and GAP43/RFP ratios was assessed by the Mander coefficient given by the Colocalization test of Fiji. Finally,

the distance of the axons to the lesion were assessed with the Fiji program, measuring the minimum distance of the axons to the lesion epicenter.

STATISTICAL ANALYSIS

There were animals that had to be discarded on the analysis, as study (NRG1-III- GFP) and control (Control- RFP) condition were not comparable, due to sagittal anatomical plane selection or to lack of RFP tracing.

The numerical values were reported as mean ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism Version 8.0.2. Paired *t*-test was used for comparisons between the treated (NRG1-III- GFP) and the untreated (RFP) portion of the CST of each animal; and differences were considered significant * at $p < 0,05$, ** at $p < 0,01$ and *** at $p < 0,001$. Also, linear correlations were performed to know if there was a significant correlation between different variables.

FIGURE LAYOUT

The graphs show on figures were plotted in GraphPad Prism Version 8.0.2 software (https://www.graphpad.com/). The design and assembly of such figures were conducted with Adobe Photoshop C6 and Adobe InDesign CS2. The images and graph representations presented were modified from images from Biorender Basic (https://app.biorender.com/) and Canva (https://www.canva.com/).

RESULTS

AXONAL PROJECTIONS FROM NRG1-III- OVEREXPRESSED NEURONS SHOW LARGER DISTANCE TO THE LESION AREA

To accomplish the study of NRG1-III role on CNS after SCI, an anterograde tracing of the CST was performed by stereotaxically injecting viral vectors (Table 1) into rat motor cortex as specified in Material and Methods. This methodology allows to study two different conditions in the CST: an experimental condition in which NRG1-III is overexpressed in the motor cortex of the left hemisphere, which is observed by the green fluorescence protein (GFP) staining (NRG1-III- GFP); and a control condition on the motor cortex of the right hemisphere which expresses the red fluorescence protein (RFP) (Control- RFP). CST analyses were carried out by IFAs which were also specified in Materials and Methods, and which allowed to observe the fluorescence of GFP and RFP.

First, to analyze the intrinsic effects caused by NRG1-III after SCI, the morphology presented by the different fibers of the CST portions is observed, such as their axonal length or the presence of retraction bulbs (described on Introduction, Figure 4). The histological slices of the spinal cord were cut in horizontal plane as it is represented in Figure 8A, making possible the comparison of the two parts of the CST: RFP- positive control axons and GFP- positive axons arising from neurons that overexpress NRG1-III (Figure 8B).

To compare the axonal length of both conditions after SCI, it was measured the shorter distance of the fibers to the lesion area. Specifically, dorso-ventral sections where the lesion is properly visualized, were selected for this study. As a result, Figure 8C indicates differences between NRG1-III- GFP and Control- RFP axons regarding the distance to the lesion. NRG1-III- GFP axons show a larger distance to the lesion (arrow) compared with Control- RFP axons (arrowhead), which are located immediately next to the lesion. Quantification of these results shows a significantly difference between the groups (Figure 8D). This divergence could be given either by a higher degeneration or a lower regeneration capacity of the fibers with NRG1-III overexpression.

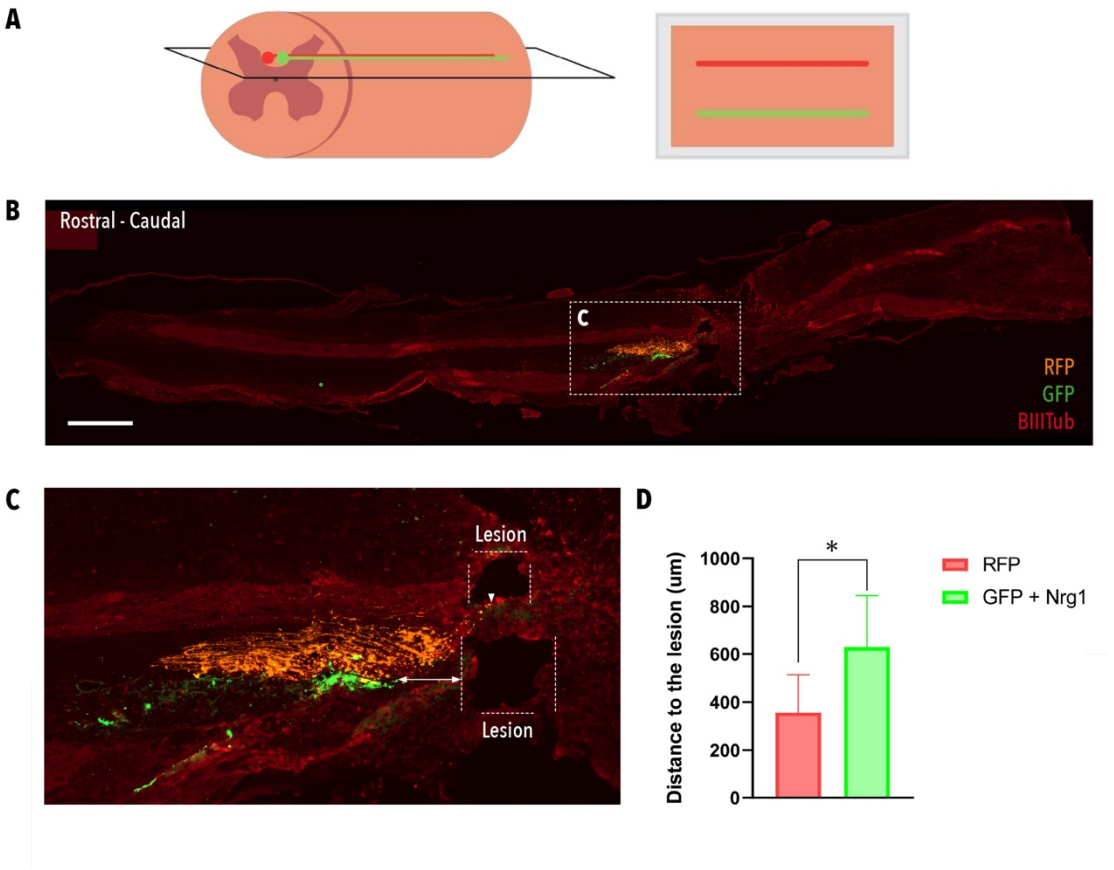

Figure 8. Axonal projections from NRG1-III- overexpressed neurons show a larger distance to the lesion area. A) Graphic representation of the selected anatomical plane (horizontal) in spinal cord and its slice. B) Entire histological slice. Immunofluorescence assay of the CST fibers with antibodies to GFP, RFP and BIIITub. Scale bar, 1000μm. C) Lesion traced by discontinue lines. Arrow, shorter distance of NRG1-III- GFP axons to the lesion area. Arrowhead, shorter distance of Control- RFP axons to the lesion area. D) Distance of NRG1-III- GFP or RFP axons to the lesion area (μm). Data are presented as mean of measured distances (n=4). *, $P < 0,05$, Student's t test.

GAP 43 SHOW MORE EXPRESSION ON AXONAL PROJECTIONS FROM NRG1-III- OVEREXPRESSED NEURONS

As stated in the Introduction, GAP43 is an intrinsic molecule associated with axonal growth and neuronal plasticity which can regulate axonal regeneration in adult CNS neurons (Fernandes et al., 1999). In addition, it was studied that one of the NRG1 isoforms (NRG-1β) has a role on the promotion of the protein expression of GAP43 in dorsal root ganglion neurons (Li et al., 2013). Regarding this data, GAP43 protein expression on CST fibers was analyzed on the present study.

As a result, the image of the IHC test (Figure 9) allows the visualization of the prospective differences among NRG1-III- GFP and Control- RFP axons. This difference is more visible on Figure 9D and 9E, which show a colocalization map created by the Colocalization test of Fiji program described in Material and Methods. Quantitative data (Figure 9F) indicate that, surprisingly, NRG1-III- GFP portion show greater expression of GAP43 with statistically significant values.

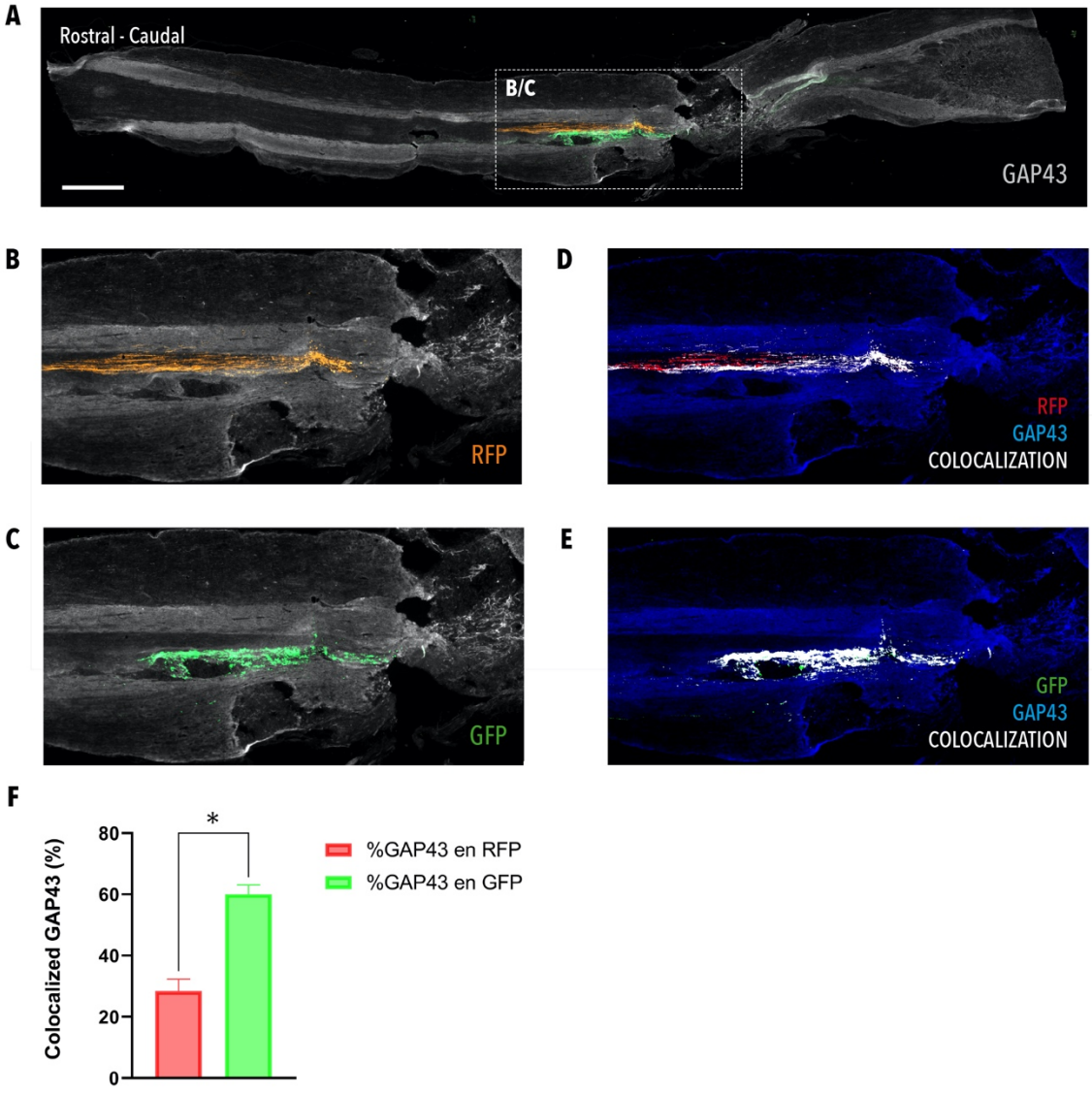

Figure 9. Axonal projections from NRG1-III- overexpressed neurons show more expression of GAP43. A) Entire histological slice. Immunofluorescence assay of the CST fibers with antibodies to GFP, RFP and GAP43. Scale bar, 1000μm. B, C) GAP43 expressed by Control- RFP axons and NRG1-III- GFP axons respectively. D, E) Colocalization test of GAP43 staining with RFP or GFP staining respectively. F) GAP43 colocalization (%) with RFP or GFP staining. Data are presented as mean percentages of colocalized GAP43 staining (n=3) in RFP or GFP staining. *, $P < 0,05$, Student's t test.

NRG1-III- OVEREXPRESSED NEURONS SHOW LESS SPROUTED AXONS

Although Figure 8D showed higher degenerative features upon NRG1-III, it was evaluated if whether a compensatory neuroplasticity by rostral sprouting could be contributing for neuronal rewiring and new synaptic activity.

To analyze the sprouting, histological slices of the spinal cord were cut into the coronal plane as it is represented in Figure 10A, making possible to visualize the gray matter (Figure 10B), which was defined (Figure 10C and 10D) in order to select only the sprouted corticospinal axons on each CST portion (NRG1-III- GFP or Control- RFP) (Figure 10E). As a result, NRG1-III- GFP axons show lower sprouting in the greymatter than Control- RFP axons (Figure 10F), although this difference is not statistically significant.

Besides data presented (Figure 10F), to know if distance of axonal projections to the lesion was related with the showed sprouting, linear correlations were performed between these variables. As result, Figure 11 shows non correlation, meaning that variables were independent, although it was not statistically significant.

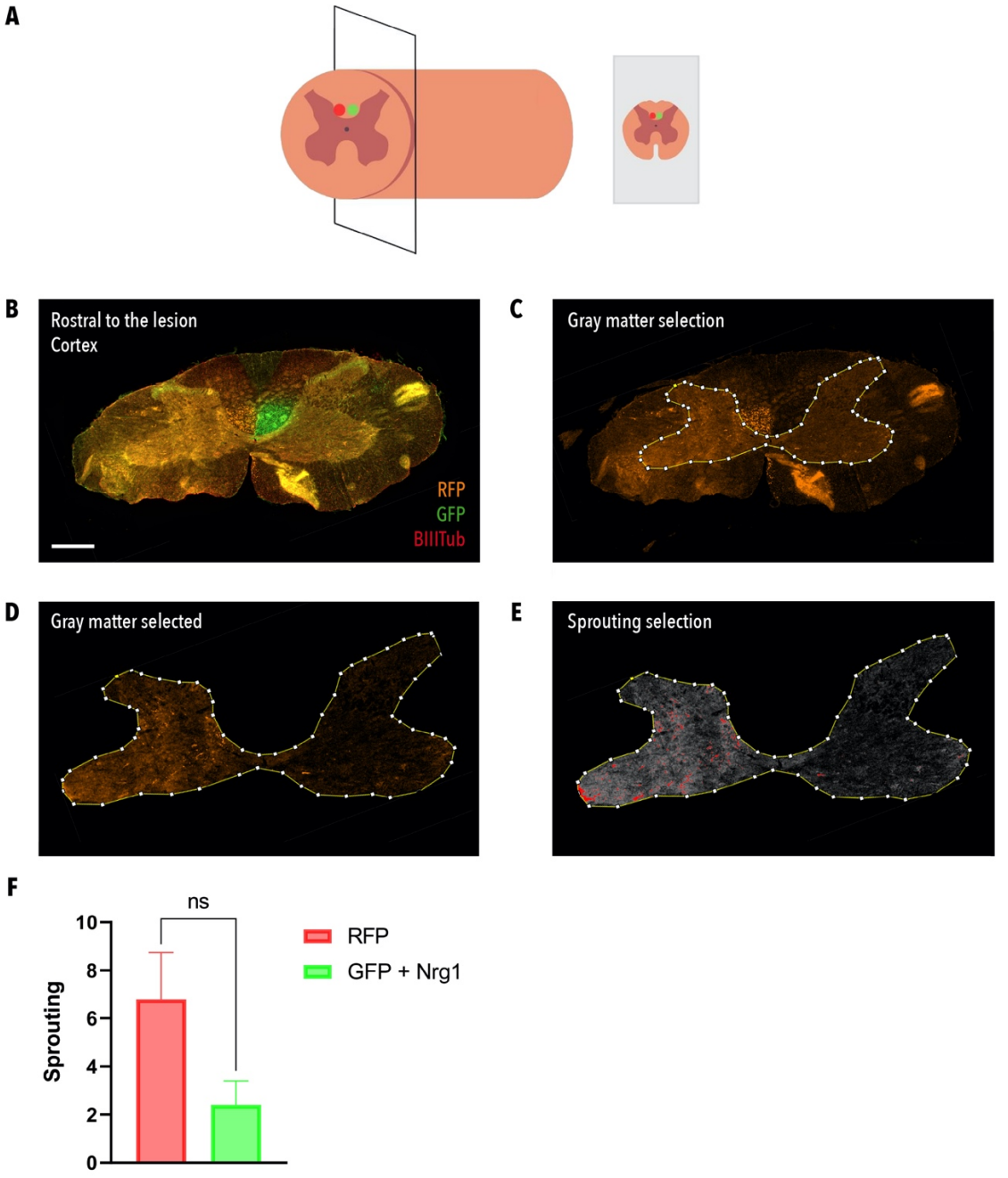

Figure 10. NRG1-III- overexpressed neurons show less sprouting. A) Graphic representation of the selected anatomical plane (coronal) in spinal cord and its slice. B) Entire histological slice. Immunofluorescence assay of the cervical spinal cord with antibodies to GFP, RFP and BIIITub. Scale bar, 500μm. C, D) Gray matter traced by a dotted line. E) Selection of the Control- RFP sprouted axons area. F) Sprouting. Data are presented as mean of GFP/RFP sprouted area (n=3) relative to GFP/RFP area of the dorsomedial portion of the CST. Same procedure was done to acquired NRG1-III- GFP sprouted area data. (Data not shown).

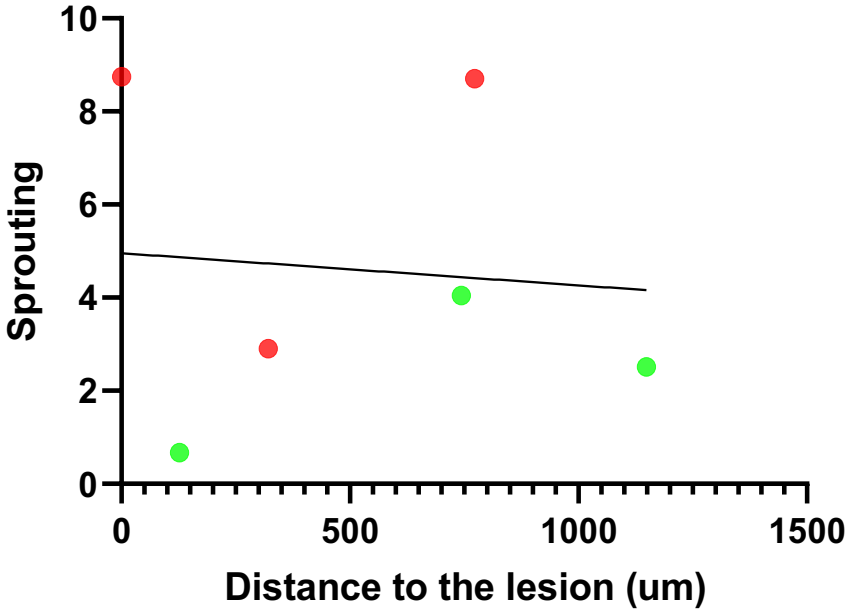

Figure 11. Linear correlation graphic between distance of axonal projections to the lesion and sprouting data. Green and red dots represent data from NRG1-III- GFP portion and Control-RFP portion respectively. X axis shows distance to the lesion data (μm) (n=3) and Y axis shows sprouting data (n=3).

AXONAL PROJECTIONS FROM NRG1-III- OVEREXPRESSED NEURONS SHOW MORE RETRACTION BULBS

Retraction bulbs are the morphological hallmarks of lesioned axons that do not regenerate as Ramon y Cajal (1928) stated. Owing this fact, morphology presented by the NRG1-III- GFP axons is also analyzed in this study, as an indicator of axonal state in terms of degeneration.

As described before, horizontal sections were taken so the two parts of the CST are shown in parallel (Figure 12A), which allowed the visualization of the prospective differences among Control- RFP and NRG1-III- GFP axons (Figure 12B and 12C, respectively). Axons overexpressing NRG1-III present higher density of retraction bulbs with statistically significant values (Figure 12D), which indicates a higher retraction and degeneration of these axons.

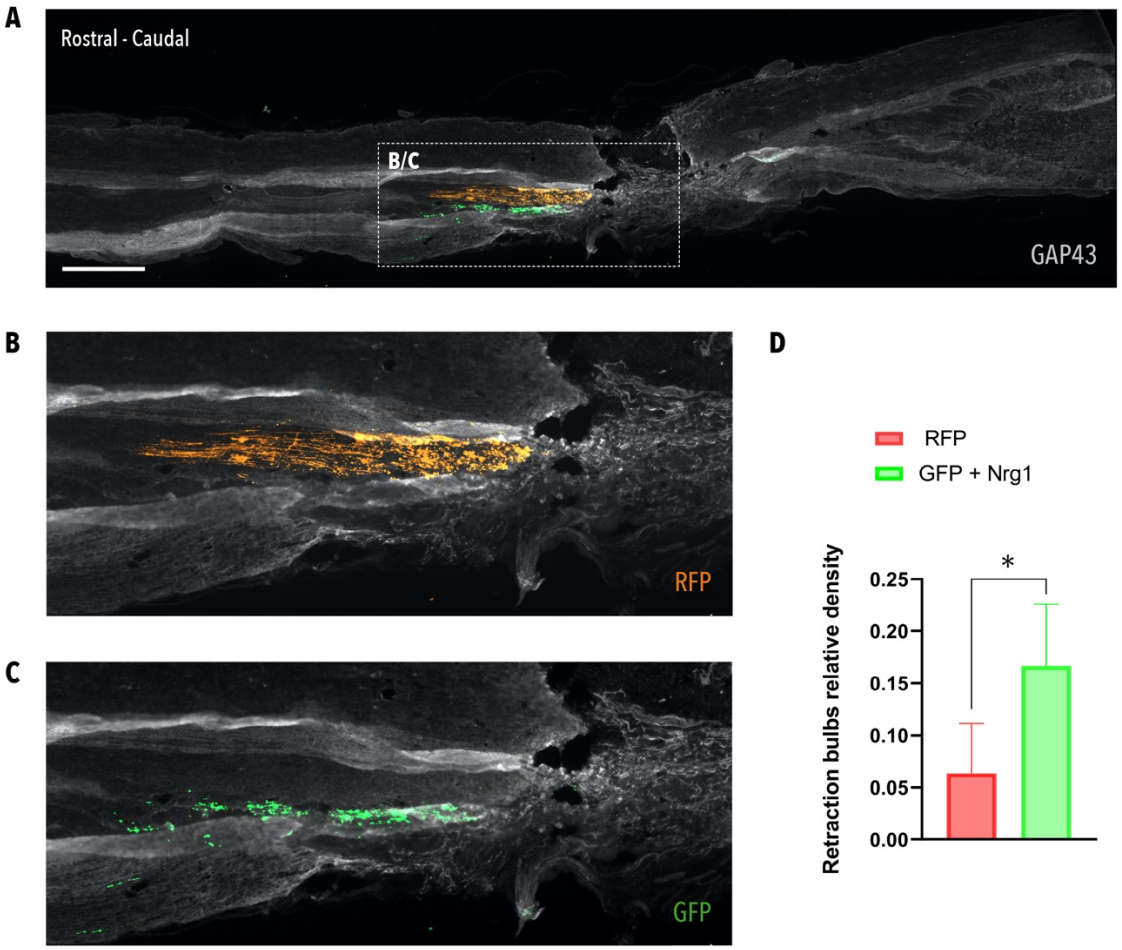

Figure 12. Axons that come from NRG1-III overexpression neurons show more retraction bulbs. A) Entire histological slice. Immunofluorescence assay of the CST fibers with antibodies to GFP, RFP and GAP43. Scale bar, 1000μm. B, C) Retracted Control- RFP axons and NRG1-III-GFP axons respectively. D) Retraction bulbs relative density. Data are presented as mean of the quantified GFP/RFP retraction bulbs (n=3) relative to GFP/RFP area of the dorsomedial portion of the CST. *, $P < 0,05$, Student's t test.

This, together with the axonal distance to the lesion and the lower sprouting, could be indicating that NRG1-III is involved in the lack of preservation of the axonal CST fibers.

OLIGODENDROCYTES ADJACENT TO NRG1-III- OVEREXPRESSED NEURONS SHOW LOW DENSITY

After analyzing the intrinsic effects caused by the overexpression of NRG1-III after SCI, extrinsic effects were also studied. It is well-established that NRG1 promotes the generation and preservation of myelinating precursor cells, in both Schawn cells at PNS (Bartus et al., 2016; Lee et al., 2020) and oligodendrocytes at developmental stage of CNS (Calaora et al., 2001; Canoll et al., 1996, 1999). The myelination carried out by these mature cells will promote axonal growth and functional recovery of fibers. Accordingly, oligodendrocytes density was analyzed on this study (Figure 13), to know the extrinsic role of NRG1-III at its isoform 3, which as stated in Introduction, has a juxtracrine signaling pathway.

As Figure 13B and 13C shows oligodendrocytes were identified by using an antibody recognizing Olig2 protein, thus allowing their density quantification. The displayed data (Figure 13D) indicate with statistically significant values that adjacent to the CST tract oligodendrocytes shown lower density in NRG1-III overexpressed neurons.

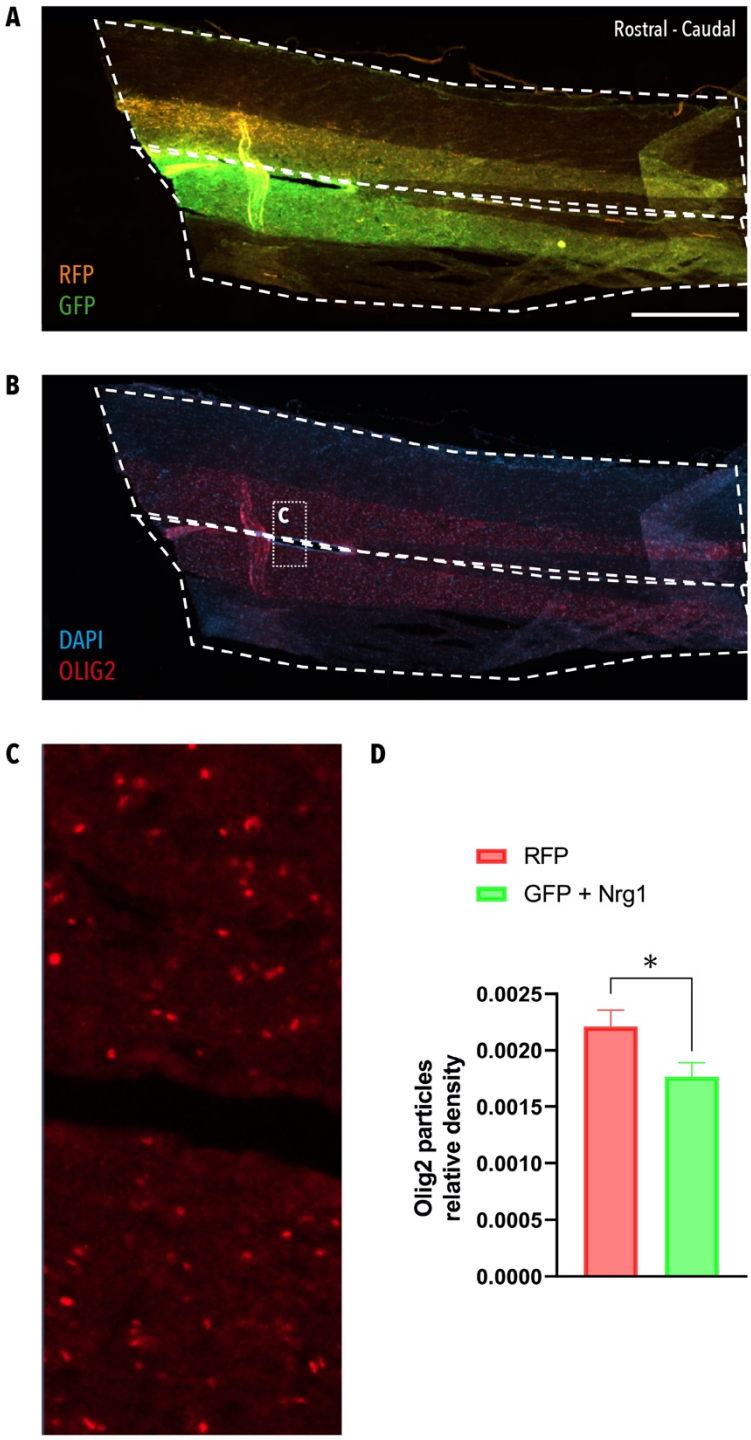

Figure 13. Oligodendrocytes adjacent to NRG1-III- overexpressed neurons show low density. A, B) Immunofluorescence assay of the CST fibers with antibodies to GFP and RFP, or to Olig2 and DAPI respectively. Scale bar, 1000μm. C) Olig 2- positive particles. D) Olig2 particles relative density. Data are presented as mean of the quantified oligodendrocytes (n=3) relative to GFP/RFP area of the dorsomedial portion of the CST. *, P < 0,05, Student's t test.

MYELIN BASIC PROTEIN EXPRESSION SHOW NO DIFFERENCE ON AXONAL PROJECTIONS FROM NRG1-III- OVEREXPRESSED NEURONS

Due to know if the observed difference in density value of oligodendrocytes (Figure 13 D) leads to a variance in the myelination levels of the fibers, expression of myelin basic protein (MBP) was studied.

As a result, Figure 14 data indicates that myelination degree measured as the relative changes on MBP detection, do not vary in the portion that overexpresses NRG1-III, which could indicate that not all the oligodendrocytes marked by Olig2 antibodies (Figure 13C) are fully preserved in terms of their myelinating functionality.

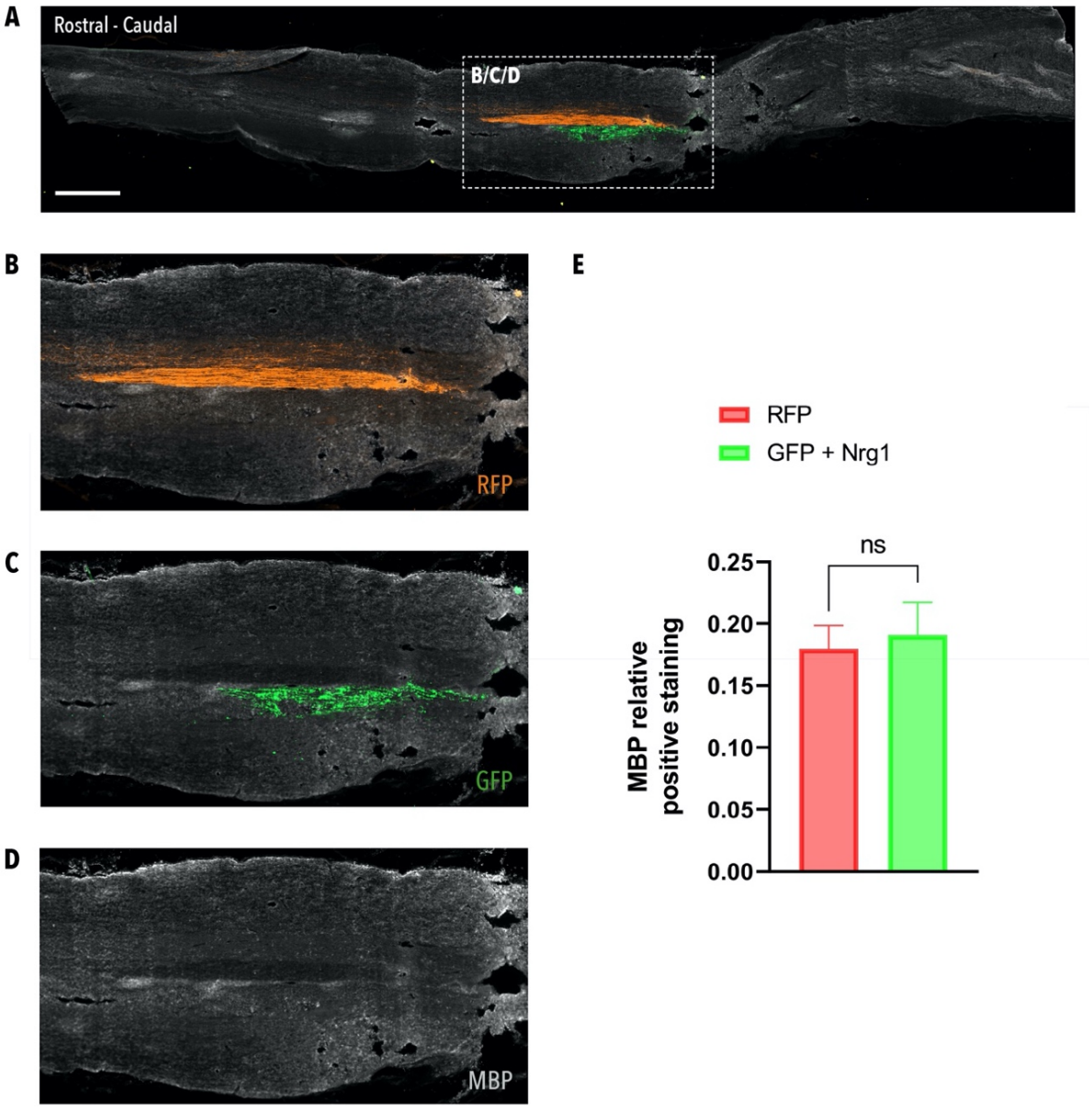

Figure 14. MBP expression show no difference on axonal projections from NRG1-III-overexpressed neurons. A) Entire histological slice. Immunofluorescence assay of CST fibers with antibodies to GFP, RFP and MBP. Scale bar, 1000μm. B, C, D) Immunofluorescence assay of MBP with Control- RFP axons or NRG1-III- GFP axons, and MBP itself respectively. D) MBP relative positive staining. Data are presented as mean of the MBP expression area (n=3) relative to GFP/RFP area of the dorsomedial portion of the CST.

DISCUSSION

To assess NRG1-III intrinsic role on CST regenerative response, several analyses are performed. First, distance of CST axonal projections to the lesion is measured getting, surprisingly, that NRG1-III axons show larger distance to the lesion with statistically significant values (Figure 8), indicating that NRG1-III overexpression is causing either less regeneration or more degeneration on CST fibers. As stated in Introduction, little is known regarding the intrinsic role of NRG1 on spinal cord CNS axonal regeneration, despite this fact it should be considered that in contrast to our results, Joung et al. (2010) describes secretion of EGF-like domain of NRG1β-1 (heregulinβ) promotes xonal growth and functional recovery of injured sciatic nerve, consistently, (Rahman-Enyart et al., 2020) describes that NRG1 is capable to influence dendritic and axonal growth at early development stages in GABAergic neurons grown in vitro; and (Vaskovsky et al., 2000) describes that ErbB-4 activation promotes Neurite Outgrowth in PC12 Cells.

Secondly, analysis of GAP43 is done to clarify if NRG1-III overexpression is causing lower regeneration or higher degeneration. As result NRG1-III- GFP axons show greater expression of GAP43 with statistically significant values (Figure 9). These results are unexpected results since GAP43 is a well-studied growth factor with axonal regeneration implications (Fernandes et al., 1999; Jang et al., 2021; Agarwal et al., 2022). So, if it assumed that NRG1-III signaling is promoting detrimental effects itself by promoting more degeneration, GAP43 expression data could be showing that higher expression of growth factor on NRG1-III- GFP is a regulatory response against degenerative process, with the aim to achieve axonal preservation or regeneration. Otherwise, NRG1-III overexpression could be promoting GAP43 expression (Li et al., 2013; Vaskovsky et al., 2000) without appreciable results in axonal outgrowth.

Nevertheless, is important to mention that all the realized analyses are describing features at one specific state of the SCI, which as stated in Introduction, present a heterogeneous pathophysiology with highly dynamic situations on which some cell responses can be helpful on first instance but not on later states. Regarding this fact, and in view that SCI is performed two weeks after overexpression of NRG1-III and four weeks before sacrifice; is possible that NRG1-III or GAP43 expression has positive effects in the first instance, but its sustained overexpression causes non-beneficial aberrations in the fibers. This hypothesis could be studied if experimental conditions are

repeated but in earlier state, in order to assess actual effects of NRG1-III with respect to pathophysiology evolution.

Thirdly, analysis of sprouting is done to assess if lack of preservation of NRG1-III- GFP axonal projections is due to the promotion of higher plasticity. As result, NRG1-III- GFP axons show lower sprouting in the gray matter (Figure 10). In addition, also linear correlation analysis shows that measurements of CST axonal projections distance are independent of sprouting, and vice-versa (Figure 11). Thus, both results, together with axonal distance and GAP43 expression, confirms lack of preservation or regenerative responses of NRG1-III signaling on CST after SCI, at observed state.

Another factor that should be considered, is the age of rats at dorsal T8 hemisection (SCI) time, which was did it in an adult stage (2 months) instead of in an embryonic state, at which it is established that NRG1 signaling plays an important role on the promotion of neurite outgrowth (Vaskovsky et al., 2000). Owing this fact, could be supposed that NRG1-III overexpression would present more plasticity or sprouting if SCI has been done at an earlier state.

Lastly, to elucidate if NRG1-III- GFP axons are showing degeneration added to lack of preservation and regenerative response, axonal CST projections morphology is studied. As result, NRG1-III- GFP present higher density of retraction bulbs with statistically significant values (Figure 12). Therefore, in view of retraction bulbs are one of the intrinsic manifestations of axonal degeneration which preclude axonal regeneration in CNS (Ramon y Cajal, 1928; Li & Raisman, 1995; Hill et al., 2001), analysis results confirm a higher retraction and degeneration of these axons.

Considering all previous results, NRG1-III overexpression intrinsic role after SCI on adult CST is related with less preservation or regeneration response added to higher axonal retraction and degeneration, which feature is not clear if either is a direct consequence of axonal preservation and regeneration lack or if is also related with NRG1-III signaling which can be promoting an aberrant degeneration. These described effects were surprising allowing for the several studies which describes that NRG1 signaling promotes preservation (Shahsavani et al., 2021), synaptic plasticity (Mei & Xiong, 2008), neurites outgrowth (Vaskovsky et al., 2000) and regeneration (Joung et al., 2010; Rahman-Enyart et al., 2020).

As hypothesis, it is possible that, in this study, both conditions: NRG1-III- GFP and Control-RFP, were influencing each other since both were arising on the same animal. Thus, it would be convenient to repeat the experiment studying NRG1-III overexpression condition on animals apart from Control condition animals. In such manner, it will be possible to clarify NRG1-III role itself. In addition, it would be convenient also to make the experiment without SCI, to assess if NRG1-III overexpression cause aberrant degeneration in healthy conditions.

On the other hand, to assess NRG1-III extrinsic role on CST myelination or demyelination, two analyses are performed in this study. First, oligodendrocyte population adjacent to CST are studied since they represent the myelinating cells in the CNS (Calaora et al., 2001; Canoll et al., 1996, 1999), and it is well-established that NRG1 promotes their proliferation and preservation (Gauthier et al., 2013; Santhosh et al., 2017; Cui et al., 2023). Nevertheless, study's results shown that surprisingly, oligodendrocytes levels were lower in NRG1-III- GFP condition (Figure 13), which could be interpreted as cause factor of lower regeneration presented by NRG1-III- GFP axons along results of the different stated analysis. Even so, it would be convenient to study if NRG1-III is promoting oligodendrocytes proliferation by analyzing by IHC the presence of oligodendrocytes precursor cells (OPCs), using Ki67+ antibody as (Hu et al., 2021) performed.

However, if it assumed that higher degeneration or lower regeneration capacity showed by NRG1-III- GFP axons can be an effect caused by failure of myelination signaling pathways, it should be considered that NRG1-III is mainly controlled by juxtracrine or contact-dependent signaling pathways (Gambarotta et al., 2013), but even so, it can be also controlled in a paracrine manner, cleaving the ectodomain by different proteolytic proteins as ADAM10, ADAM17 and BACE1; which can be leading to failure of NRG1-III function. Consistently, it was studied that "TACE (ADAM17) cleaves neuregulin-1 (NRG1) type III in the epidermal growth factor domain, probably inactivating it and thereby negatively regulating PNS myelination" (La Marca et al., 2011). So, regarding this fact, degenerative cues presented on NRG1-III- GFP axons could be caused by ADAM 17 action. This hypothesis can be test it analyzing by IHC the presence of this protein, using an Anti-TACE/ADAM17 antibody.

Nevertheless, even with low oligodendrocyte's levels that NRG1-III- GFP condition shows, thanks to the comparison of MBP levels (Figure 14), it can be shown that NRG1-III- GFP portion tends to present the same levels of myelinating activity as Control- RFP condition, which could be indicating that NRG1-III is promoting the

survivance or preservation of functional oligodendrocytes only. Even so, to study more accurate myelin state and myelination oligodendrocyte's function, it would be convenient to analyze spinal cord trough electron microscopy (EM) as (Weil et al., 2019), being allowed to visualize myelin sheath thickness.

Finally, another factor that should be considered regarding extrinsic role of NRG1-III is the influence of the inflammatory landscape. In view of this fact, it would be convenient to study microglia and astrocytes states by analyzing by IHC the presence of Iba1 and GFAP antibodies respectively. It is important to study these cells since are key regulators of inflammatory responses in CNS (Kwon & Koh, 2020) and its state and roles are also determinant on CNS recovery after SCI. In fact, (Talbott et al., 2005) studied that OPCs fail to remyelinate the demyelinated adult rat spinal cord in the absence of astrocytes.

As hypothesis, instead of having an inactivated NRG1-III signaling is possible that NRG1-III overexpression is leading to an aberrant overactivation of ErbB receptors, that could be causing deleterious outcomes such as axonal degeneration stated due to inflammation. Consistently, (Hu et al., 2021) studied that "ErbB ligand-induced cell death mostly occurs to cells with high ErbB receptor expression" and "ErbB overactivation induced primary mature oligodendrocytes necroptosis, causing inflammatory demyelination".

To sum up, study's results shown that NRG1-III overexpression on CST on adult female rats after SCI, show: CST axonal projections with larger distance to the lesion area with unexpected higher GAP43 expression, less sprouting and more density of retraction bulbs; as intrinsic factors. Indicating less axonal preservation or regeneration response, which is added to higher axonal retraction and degeneration, remaining unclear if axonal degeneration is a direct consequence of axonal preservation and regeneration failure, or if it is related with NRG1-III signaling itself, which can be promoting an aberrant degeneration. Meanwhile, NRG1-III extrinsic role on CST myelination or demyelination is also unclear since analyses results show inconsistent data such as less density of adjacent oligodendrocytes but no difference on MBP expression, suggesting that more accurate analyses are required to take conclusions. Therefore, together analyzed hallmarks may be due to either detrimental intrinsic or extrinsic effects (or both) caused by NRG1-III overexpression. Even so, it is important to highlight those further analyses and studies are needed to determine more accurate the actual role of NRG1-III overexpression in CST after SCI.

CONCLUSIONS

After performing different analysis to characterize prospective role of NRG1-III overexpression on CST, study's conclusions are:

- NRG1-III overexpression show less regenerative or preservative response on CST. Given that:
  - Axonal projections from NRG1-III- overexpressed neurons show larger distance to the lesion area.
  - GAP 43 show more expression on axonal projections from NRG1-III- overexpressed neurons.
  - NRG1-III- overexpressed neurons show less sprouted axons.

- NRG1-III overexpression show more degenerative or retractive response on CST. Given that:
  - Axonal projections from NRG1-III- overexpressed neurons show more retraction bulbs.

- NRG1-III overexpression on CST show low density of adjacent oligodendrocytes and does not significantly modify the MBP expression, however its myelination or demyelination role needs to be further evaluated.

REFERENCES

Agarwal, G., Roy, A., Kumar, H., & Srivastava, A. (2022). Graphene-collagen cryogel controls neuroinflammation and fosters accelerated axonal regeneration in spinal cord injury. Biomaterials advances, 139, 212971. https://doi.org/10.1016/j.bioadv.2022.212971

Alizadeh, A., Dyck, S. M., Kataria, H., Shahriary, G. M., Nguyen, D. H., Santhosh, K. T., & Karimi-Abdolrezaee, S. (2017). Neuregulin-1 positively modulates glial response and improves neurological recovery following traumatic spinal cord injury. Glia, 65(7), 1152–1175. https://doi.org/10.1002/glia.23150

Alizadeh, A., Santhosh, K. T., Kataria, H., Gounni, A. S., & Karimi-Abdolrezaee, S. (2018). Neuregulin-1 elicits a regulatory immune response following traumatic spinal cord injury. Journal of neuroinflammation, 15(1), 53. https://doi.org/10.1186/s12974-018-1093-9

Andrews, M. R., Czvitkovich, S., Dassie, E., Vogelaar, C. F., Faissner, A., Blits, B., Gage, F. H., ffrench-Constant, C., & Fawcett, J. W. (2009). Alpha9 integrin promotes neurite outgrowth on tenascin-C and enhances sensory axon regeneration. The Journal of neuroscience : the official journal of the Society for Neuroscience, 29(17), 5546–5557. https://doi.org/10.1523/JNEUROSCI.0759-09.2009

Anjum, A., Yazid, M. D., Fauzi Daud, M., Idris, J., Ng, A. M. H., Selvi Naicker, A., Ismail, O. H. R., Athi Kumar, R. K., & Lokanathan, Y. (2020). Spinal Cord Injury: Pathophysiology, Multimolecular Interactions, and Underlying Recovery Mechanisms. International journal of molecular sciences, 21(20), 7533. https://doi.org/10.3390/ijms21207533

Bao, J., Wolpowitz, D., Role, L. W., & Talmage, D. A. (2003). Back signaling by the Nrg-1 intracellular domain. The Journal of cell biology, 161(6), 1133–1141. https://doi.org/10.1083/jcb.200212085

Bao, J., Lin, H., Ouyang, Y., Lei, D., Osman, A., Kim, T. W., Mei, L., Dai, P., Ohlemiller, K. K., & Ambron, R. T. (2004). Activity-dependent transcription regulation of PSD-95 by neuregulin-1 and Eos. Nature neuroscience, 7(11), 1250–1258. https://doi.org/10.1038/nn1342

Bareyre, F. M., Kerschensteiner, M., Raineteau, O., Mettenleiter, T. C., Weinmann, O., & Schwab, M. E. (2004). The injured spinal cord spontaneously forms a new intraspinal circuit in adult rats. Nature neuroscience, 7(3), 269–277. https://doi.org/10.1038/nn1195

Bartus, K., Galino, J., James, N. D., Hernandez-Miranda, L. R., Dawes, J. M., Fricker, F. R., Garratt, A. N., McMahon, S. B., Ramer, M. S., Birchmeier, C., Bennett, D. L., & Bradbury, E. J. (2016). Neuregulin-1 controls an endogenous repair mechanism after spinal cord injury. Brain : a journal of neurology, 139(Pt 5), 1394–1416. https://doi.org/10.1093/brain/aww039

Belin, S., Nawabi, H., Wang, C., Tang, S., Latremoliere, A., Warren, P., Schorle, H., Uncu, C., Woolf, C. J., He, Z., & Steen, J. A. (2015). Injury-induced decline of intrinsic regenerative ability revealed by quantitative proteomics. Neuron, 86(4), 1000–1014. https://doi.org/10.1016/j.neuron.2015.03.060

Bhowmick, S., & Abdul-Muneer, P. M. (2021). PTEN Blocking Stimulates Corticospinal and Raphespinal Axonal Regeneration and Promotes Functional Recovery After Spinal Cord Injury. Journal of neuropathology and experimental neurology, 80(2), 169–181. https://doi.org/10.1093/jnen/nlaa147

Bican, O., Minagar, A., & Pruitt, A. A. (2013). The spinal cord: a review of functional neuroanatomy. Neurologic clinics, 31(1), 1–18. https://doi.org/10.1016/j.ncl.2012.09.009

Blackmore, M. G., Wang, Z., Lerch, J. K., Motti, D., Zhang, Y. P., Shields, C. B., Lee, J. K., Goldberg, J. L., Lemmon, V. P., & Bixby, J. L. (2012). Krüppel-like Factor 7 engineered for transcriptional activation promotes axon regeneration in the adult corticospinal tract. Proceedings of the National Academy of Sciences of the United States of America, 109(19), 7517–7522. https://doi.org/10.1073/pnas.1120684109

Calaora, V., Rogister, B., Bismuth, K., Murray, K., Brandt, H., Leprince, P., Marchionni, M., & Dubois-Dalcq, M. (2001). Neuregulin signaling regulates neural precursor growth and the generation of oligodendrocytes in vitro. The Journal of neuroscience : the official journal of the Society for Neuroscience, 21(13), 4740–4751. https://doi.org/10.1523/JNEUROSCI.21-13-04740.2001

Canoll, P. D., Musacchio, J. M., Hardy, R., Reynolds, R., Marchionni, M. A., & Salzer, J. L. (1996). GGF/neuregulin is a neuronal signal that promotes the proliferation and survival and inhibits the differentiation of oligodendrocyte progenitors. Neuron, 17(2), 229–243. https://doi.org/10.1016/s0896-6273(00)80155-5

Canoll, P. D., Kraemer, R., Teng, K. K., Marchionni, M. A., & Salzer, J. L. (1999). GGF/neuregulin induces a phenotypic reversion of oligodendrocytes. Molecular and cellular neurosciences, 13(2), 79–94. https://doi.org/10.1006/mcne.1998.0733

Carmel, J. B., & Martin, J. H. (2014). Motor cortex electrical stimulation augments sprouting of the corticospinal tract and promotes recovery of motor function. Frontiers in integrative neuroscience, 8, 51. https://doi.org/10.3389/fnint.2014.00051

Cui, M. Y., Fu, Y. Q., Li, Z. L., Zheng, Y., Yu, Y., Zhang, C., Zhang, Y. Q., Gao, B. R., Chen, W. Y., Lee, Y. L., Won, M. H., Liao, M., Jian, Y., & Chen, B. H. (2023). Neuregulin-1/PI3K signaling effects on oligodendrocyte proliferation, remyelination and behaviors deficit in a male mouse model of ischemic stroke. Experimental neurology, 362, 114323. https://doi.org/10.1016/j.expneurol.2023.114323

Danilov, C. A., & Steward, O. (2015). Conditional genetic deletion of PTEN after a spinal cord injury enhances regenerative growth of CST axons and motor function recovery in mice. Experimental neurology, 266, 147–160. https://doi.org/10.1016/j.expneurol.2015.02.012

Davies, J. E., Tang, X., Denning, J. W., Archibald, S. J., & Davies, S. J. (2004). Decorin suppresses neurocan, brevican, phosphacan and NG2 expression and promotes axon growth across adult rat spinal cord injuries. The European journal of neuroscience, 19(5), 1226–1242. https://doi.org/10.1111/j.1460-9568.2004.03184.x

Dergham, P., Ellezam, B., Essagian, C., Avedissian, H., Lubell, W. D., & McKerracher, L. (2002). Rho signaling pathway targeted to promote spinal cord repair. The Journal of neuroscience : the official journal of the Society for Neuroscience, 22(15), 6570–6577. https://doi.org/10.1523/JNEUROSCI.22-15-06570.2002

Dill, J., Wang, H., Zhou, F., & Li, S. (2008). Inactivation of glycogen synthase kinase 3 promotes axonal growth and recovery in the CNS. The Journal of neuroscience : the official journal of the Society for Neuroscience, 28(36), 8914–8928. https://doi.org/10.1523/JNEUROSCI.1178-08.2008

Ding, Z., Dai, C., Zhong, L., Liu, R., Gao, W., Zhang, H., & Yin, Z. (2021). Neuregulin-1 converts reactive astrocytes toward oligodendrocyte lineage cells via upregulating the PI3K-AKT-mTOR pathway to repair spinal cord injury. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie, 134, 111168. https://doi.org/10.1016/j.biopha.2020.111168

Du, K., Zheng, S., Zhang, Q., Li, S., Gao, X., Wang, J., Jiang, L., & Liu, K. (2015). Pten Deletion Promotes Regrowth of Corticospinal Tract Axons 1 Year after Spinal Cord Injury. The Journal of neuroscience : the official journal of the Society for Neuroscience, 35(26), 9754–9763. https://doi.org/10.1523/JNEUROSCI.3637-14.2015

Ertürk, A., Hellal, F., Enes, J., & Bradke, F. (2007). Disorganized microtubules underlie the formation of retraction bulbs and the failure of axonal regeneration. The Journal of neuroscience : the official journal of the Society for Neuroscience, 27(34), 9169–9180. https://doi.org/10.1523/JNEUROSCI.0612-07.2007

Falls, D. L. (2003). Neuregulins: functions, forms, and signaling strategies. Experimental cell research, 284(1), 14–30. https://doi.org/10.1016/s0014-4827(02)00102-7

Farley, A., Johnstone, C., Hendry, C., & McLafferty, E. (2014). Nervous system: part 1. Nursing standard (Royal College of Nursing (Great Britain) : 1987), 28(31), 46–51. https://doi.org/10.7748/ns2014.04.28.31.46.e7004

Fawcett, J. W., & Asher, R. A. (1999). The glial scar and central nervous system repair. Brain research bulletin, 49(6), 377–391. https://doi.org/10.1016/s0361-9230(99)00072-6

Fernandes, K. J., Fan, D. P., Tsui, B. J., Cassar, S. L., & Tetzlaff, W. (1999). Influence of the axotomy to cell body distance in rat rubrospinal and spinal motoneurons: differential regulation of GAP-43, tubulins, and neurofilament-M. The Journal of comparative neurology, 414(4), 495–510. https://doi.org/10.1002/(sici)1096-9861(19991129)414:4<495::aid-cne6>3.0.co;2-s

Fouad, K., Pedersen, V., Schwab, M. E., & Brösamle, C. (2001). Cervical sprouting of corticospinal fibers after thoracic spinal cord injury accompanies shifts in evoked motor responses. Current biology : CB, 11(22), 1766–1770. https://doi.org/10.1016/s0960-9822(01)00535-8

Fouad, K., Krajacic, A., & Tetzlaff, W. (2011). Spinal cord injury and plasticity: opportunities and challenges. Brain research bulletin, 84(4-5), 337–342. https://doi.org/10.1016/j.brainresbull.2010.04.017

Gallo G. (2006). RhoA-kinase coordinates F-actin organization and myosin II activity during semaphorin-3A-induced axon retraction. Journal of cell science, 119(Pt 16), 3413–3423. https://doi.org/10.1242/jcs.03084

Gambarotta, G., Fregnan, F., Gnavi, S., & Perroteau, I. (2013). Neuregulin 1 role in Schwann cell regulation and potential applications to promote peripheral nerve regeneration. International review of neurobiology, 108, 223–256. https://doi.org/10.1016/B978-0-12-410499-0.00009-5

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

Gassmann, M., & Lemke, G. (1997). Neuregulins and neuregulin receptors in neural development. Current opinion in neurobiology, 7(1), 87–92. https://doi.org/10.1016/s0959-4388(97)80125-0

Gauthier, M. K., Kosciuczyk, K., Tapley, L., & Karimi-Abdolrezaee, S. (2013). Dysregulation of the neuregulin-1-ErbB network modulates endogenous oligodendrocyte differentiation and preservation after spinal cord injury. The European journal of neuroscience, 38(5), 2693–2715. https://doi.org/10.1111/ejn.12268

Geoffroy, C. G., Hilton, B. J., Tetzlaff, W., & Zheng, B. (2016). Evidence for an Age-Dependent Decline in Axon Regeneration in the Adult Mammalian Central Nervous System. Cell reports, 15(2), 238–246. https://doi.org/10.1016/j.celrep.2016.03.028

Ghosh, A., Sydekum, E., Haiss, F., Peduzzi, S., Zörner, B., Schneider, R., Baltes, C., Rudin, M., Weber, B., & Schwab, M. E. (2009). Functional and anatomical reorganization of the sensory-motor cortex after incomplete spinal cord injury in adult rats. The Journal of neuroscience : the official journal of the Society for Neuroscience, 29(39), 12210–12219. https://doi.org/10.1523/JNEUROSCI.1828-09.2009

Hata, K., Fujitani, M., Yasuda, Y., Doya, H., Saito, T., Yamagishi, S., Mueller, B. K., & Yamashita, T. (2006). RGMa inhibition promotes axonal growth and recovery after spinal cord injury. The Journal of cell biology, 173(1), 47–58. https://doi.org/10.1083/jcb.200508143

He, Z., & Jin, Y. (2016). Intrinsic Control of Axon Regeneration. Neuron, 90(3), 437–451. https://doi.org/10.1016/j.neuron.2016.04.022

Hill, C. E., Beattie, M. S., & Bresnahan, J. C. (2001). Degeneration and sprouting of identified descending supraspinal axons after contusive spinal cord injury in the rat. Experimental neurology, 171(1), 153–169. https://doi.org/10.1006/exnr.2001.7734

Hu, X., Hicks, C. W., He, W., Wong, P., Macklin, W. B., Trapp, B. D., & Yan, R. (2006). Bace1 modulates myelination in the central and peripheral nervous system. Nature neuroscience, 9(12), 1520–1525. https://doi.org/10.1038/nn1797

Hu, X., He, W., Diaconu, C., Tang, X., Kidd, G. J., Macklin, W. B., Trapp, B. D., & Yan, R. (2008). Genetic deletion of BACE1 in mice affects remyelination of sciatic nerves. FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 22(8), 2970–2980. https://doi.org/10.1096/fj.08-106666

Hu, X., Xiao, G., He, L., Niu, X., Li, H., Lou, T., Hu, Q., Yang, Y., Xu, Q., Wei, Z., Qiu, M., Tanaka, K. F., Shen, Y., & Tao, Y. (2021). Sustained ErbB Activation Causes Demyelination and Hypomyelination by Driving Necroptosis of Mature Oligodendrocytes and Apoptosis of Oligodendrocyte Precursor Cells. The Journal of neuroscience : the official journal of the Society for Neuroscience, 41(48), 9872–9890. https://doi.org/10.1523/JNEUROSCI.2922-20.2021

Jang, E. H., Bae, Y. H., Yang, E. M., Gim, Y., Suh, H. J., Kim, S., Park, S. M., Park, J. B., & Hur, E. M. (2021). Comparing axon regeneration in male and female mice after peripheral nerve injury. Journal of neuroscience research, 99(11), 2874–2887. https://doi.org/10.1002/jnr.24955

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. The Journal of neuroscience : the official journal of the Society for Neuroscience, 22(7), 2792–2803. https://doi.org/10.1523/JNEUROSCI.22-07-02792.2002

Joung, I., Yoo, M., Woo, J. H., Chang, C. Y., Heo, H., & Kwon, Y. K. (2010). Secretion of EGF-like domain of heregulinβ promotes axonal growth and functional recovery of injured sciatic nerve. Molecules and cells, 30(5), 477–484. https://doi.org/10.1007/s10059-010-0137-5

Kabir, N., Schaefer, A. W., Nakhost, A., Sossin, W. S., & Forscher, P. (2001). Protein kinase C activation promotes microtubule advance in neuronal growth cones by increasing average microtubule growth lifetimes. The Journal of cell biology, 152(5), 1033–1044. https://doi.org/10.1083/jcb.152.5.1033

Khorasanizadeh, M., Yousefifard, M., Eskian, M., Lu, Y., Chalangari, M., Harrop, J. S., Jazayeri, S. B., Seyedpour, S., Khodaei, B., Hosseini, M., & Rahimi-Movaghar, V. (2019). Neurological recovery following traumatic spinal cord injury: a systematic review and meta-analysis. Journal of neurosurgery. Spine, 1–17. Advance online publication. https://doi.org/10.3171/2018.10.SPINE18802

Kwon, H. S., & Koh, S. H. (2020). Neuroinflammation in neurodegenerative disorders: the roles of microglia and astrocytes. Translational neurodegeneration, 9(1), 42. https://doi.org/10.1186/s40035-020-00221-2

La Marca, R., Cerri, F., Horiuchi, K., Bachi, A., Feltri, M. L., Wrabetz, L., Blobel, C. P., Quattrini, A., Salzer, J. L., & Taveggia, C. (2011). TACE (ADAM17) inhibits Schwann cell myelination. Nature neuroscience, 14(7), 857–865. https://doi.org/10.1038/nn.2849

Lang, C., Bradley, P. M., Jacobi, A., Kerschensteiner, M., & Bareyre, F. M. (2013). STAT3 promotes corticospinal remodelling and functional recovery after spinal cord injury. EMBO reports, 14(10), 931–937. https://doi.org/10.1038/embor.2013.117

Lee, D. W., Kim, E., Jeong, I., Kim, H. K., Kim, S., & Park, H. C. (2020). Schwann cells selectively myelinate primary motor axons via neuregulin-ErbB signaling. Glia, 68(12), 2585–2600. https://doi.org/10.1002/glia.23871

Lemke G. (1996). Neuregulins in development. Molecular and cellular neurosciences, 7(4), 247–262. https://doi.org/10.1006/mcne.1996.0019

Lewandowski, G., & Steward, O. (2014). AAVshRNA-mediated suppression of PTEN in adult rats in combination with salmon fibrin administration enables regenerative growth of corticospinal axons and enhances recovery of voluntary motor function after cervical spinal cord injury. The Journal of neuroscience : the official journal of the Society for Neuroscience, 34(30), 9951–9962. https://doi.org/10.1523/JNEUROSCI.1996-14.2014

Li, Y., & Raisman, G. (1995). Sprouts from cut corticospinal axons persist in the presence of astrocytic scarring in long-term lesions of the adult rat spinal cord. Experimental neurology, 134(1), 102–111. https://doi.org/10.1006/exnr.1995.1041

Li, Y., Li, H., Liu, G., & Liu, Z. (2013). Effects of neuregulin-1β on growth-associated protein 43 expression in dorsal root ganglion neurons with excitotoxicity induced by glutamate in vitro. Neuroscience research, 76(1-2), 22–30. https://doi.org/10.1016/j.neures.2013.02.012

Liz, M. A., Mar, F. M., Santos, T. E., Pimentel, H. I., Marques, A. M., Morgado, M. M., Vieira, S., Sousa, V. F., Pemble, H., Wittmann, T., Sutherland, C., Woodgett, J. R., & Sousa, M. M. (2014). Neuronal deletion of GSK3β increases microtubule speed in the growth cone and enhances axon regeneration via CRMP-2 and independently of MAP1B and CLASP2. BMC biology, 12, 47. https://doi.org/10.1186/1741-7007-12-47

Liu, K., Lu, Y., Lee, J. K., Samara, R., Willenberg, R., Sears-Kraxberger, I., Tedeschi, A., Park, K. K., Jin, D., Cai, B., Xu, B., Connolly, L., Steward, O., Zheng, B., & He, Z. (2010). PTEN deletion enhances the regenerative ability of adult corticospinal neurons. Nature neuroscience, 13(9), 1075–1081. https://doi.org/10.1038/nn.2603

Liuzzi, F. J., & Lasek, R. J. (1987). Astrocytes block axonal regeneration in mammals by activating the physiological stop pathway. Science (New York, N.Y.), 237(4815), 642–645. https://doi.org/10.1126/science.3603044

Mar, F. M., Bonni, A., & Sousa, M. M. (2014). Cell intrinsic control of axon regeneration. EMBO reports, 15(3), 254–263. https://doi.org/10.1002/embr.201337723

McDonald, J. W., & Sadowsky, C. (2002). Spinal-cord injury. Lancet (London, England), 359(9304), 417–425. https://doi.org/10.1016/S0140-6736(02)07603-1

McKerracher, L., David, S., Jackson, D. L., Kottis, V., Dunn, R. J., & Braun, P. E. (1994). Identification of myelin-associated glycoprotein as a major myelin-derived inhibitor of neurite growth. Neuron, 13(4), 805–811. https://doi.org/10.1016/0896-6273(94)90247-x

McMillian, M. K., Thai, L., Hong, J. S., O'Callaghan, J. P., & Pennypacker, K. R. (1994). Brain injury in a dish: a model for reactive gliosis. Trends in neurosciences, 17(4), 138–142. https://doi.org/10.1016/0166-2236(94)90086-8

Mei, L., & Xiong, W. C. (2008). Neuregulin 1 in neural development, synaptic plasticity and schizophrenia. Nature reviews. Neuroscience, 9(6), 437–452. https://doi.org/10.1038/nrn2392

Meyer, D., Yamaai, T., Garratt, A., Riethmacher-Sonnenberg, E., Kane, D., Theill, L. E., & Birchmeier, C. (1997). Isoform-specific expression and function of neuregulin. Development (Cambridge, England), 124(18), 3575–3586. https://doi.org/10.1242/dev.124.18.3575

Michailov, G. V., Sereda, M. W., Brinkmann, B. G., Fischer, T. M., Haug, B., Birchmeier, C., Role, L., Lai, C., Schwab, M. H., & Nave, K. A. (2004). Axonal neuregulin-1 regulates myelin sheath thickness. Science (New York, N.Y.), 304(5671), 700–703. https://doi.org/10.1126/science.1095862

Modrak, M., Talukder, M. A. H., Gurgenashvili, K., Noble, M., & Elfar, J. C. (2020). Peripheral nerve injury and myelination: Potential therapeutic strategies. Journal of neuroscience research, 98(5), 780–795. https://doi.org/10.1002/jnr.24538

Nakamura, M., Houghtling, R. A., MacArthur, L., Bayer, B. M., & Bregman, B. S. (2003). Differences in cytokine gene expression profile between acute and secondary injury in adult rat spinal cord. Experimental neurology, 184(1), 313–325. https://doi.org/10.1016/s0014-4886(03)00361-3

Nakamura, Y., Ueno, M., Niehaus, J. K., Lang, R. A., Zheng, Y., & Yoshida, Y. (2021). Modulation of Both Intrinsic and Extrinsic Factors Additively Promotes Rewiring of Corticospinal Circuits after Spinal Cord Injury. The Journal of neuroscience : the official journal of the Society for Neuroscience, 41(50), 10247–10260. https://doi.org/10.1523/JNEUROSCI.2649-20.2021

Nathan, F. M., Ohtake, Y., Wang, S., Jiang, X., Sami, A., Guo, H., Zhou, F. Q., & Li, S. (2020). Upregulating Lin28a Promotes Axon Regeneration in Adult Mice with Optic Nerve and Spinal Cord Injury. Molecular therapy : the journal of the American Society of Gene Therapy, 28(8), 1902–1917. https://doi.org/10.1016/j.ymthe.2020.04.010

Nieuwenhuys, R., Voogd, J., & Van Huijzen, C. (2008). Topography of Spinal Cord, Brain Stem and Cerebellum. In: The Human Central Nervous System. Springer, Berlín, Heidelberg. https://doi.org/10.1007/978-3-540-34686-9_6

Norenberg, M. D., Smith, J., & Marcillo, A. (2004). The pathology of human spinal cord injury: defining the problems. Journal of neurotrauma, 21(4), 429–440. https://doi.org/10.1089/089771504323004575

Nudo, R. J., & Masterton, R. B. (1990). Descending pathways to the spinal cord, III: Sites of origin of the corticospinal tract. The Journal of comparative neurology, 296(4), 559–583. https://doi.org/10.1002/cne.902960405

O'Donovan, K. J., Ma, K., Guo, H., Wang, C., Sun, F., Han, S. B., Kim, H., Wong, J. K., Charron, J., Zou, H., Son, Y. J., He, Z., & Zhong, J. (2014). B-RAF kinase drives developmental axon growth and promotes axon regeneration in the injured mature CNS. The Journal of experimental medicine, 211(5), 801–814. https://doi.org/10.1084/jem.20131780

Ohtake, Y., Park, D., Abdul-Muneer, P. M., Li, H., Xu, B., Sharma, K., Smith, G. M., Selzer, M. E., & Li, S. (2014). The effect of systemic PTEN antagonist peptides on axon growth and functional recovery after spinal cord injury. Biomaterials, 35(16), 4610–4626. https://doi.org/10.1016/j.biomaterials.2014.02.037

Ohtake, Y., Sami, A., Jiang, X., Horiuchi, M., Slattery, K., Ma, L., Smith, G. M., Selzer, M. E., Muramatsu, S. I., & Li, S. (2019). Promoting Axon Regeneration in Adult CNS by Targeting Liver Kinase B1. Molecular therapy : the journal of the American Society of Gene Therapy, 27(1), 102–117. https://doi.org/10.1016/j.ymthe.2018.10.019

Onifer, S. M., Smith, G. M., & Fouad, K. (2011). Plasticity after spinal cord injury: relevance to recovery and approaches to facilitate it. Neurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics, 8(2), 283–293. https://doi.org/10.1007/s13311-011-0034-4

O'Shea, T. M., Burda, J. E., & Sofroniew, M. V. (2017). Cell biology of spinal cord injury and repair. The Journal of clinical investigation, 127(9), 3259–3270. https://doi.org/10.1172/JCI90608

Park, K. K., Liu, K., Hu, Y., Smith, P. D., Wang, C., Cai, B., Xu, B., Connolly, L., Kramvis, I., Sahin, M., & He, Z. (2008). Promoting axon regeneration in the adult CNS by modulation of the PTEN/mTOR pathway. Science (New York, N.Y.), 322(5903), 963–966. https://doi.org/10.1126/science.1161566

Puttagunta, R., Tedeschi, A., Sória, M. G., Hervera, A., Lindner, R., Rathore, K. I., Gaub, P., Joshi, Y., Nguyen, T., Schmandke, A., Laskowski, C. J., Boutillier, A. L., Bradke, F., & Di Giovanni, S. (2014). PCAF-dependent epigenetic changes promote axonal regeneration in the central nervous system. Nature communications, 5, 3527. https://doi.org/10.1038/ncomms4527

Rahman-Enyart, A., Lai, C., & Prieto, A. L. (2020). Neuregulins 1, 2, and 3 Promote Early Neurite Outgrowth in ErbB4-Expressing Cortical GABAergic Interneurons. Molecular neurobiology, 57(8), 3568–3588. https://doi.org/10.1007/s12035-020-01966-7

Raineteau, O., Z'Graggen, W. J., Thallmair, M., & Schwab, M. E. (1999). Sprouting and regeneration after pyramidotomy and blockade of the myelin-associated neurite growth inhibitors NI 35/250 in adult rats. The European journal of neuroscience, 11(4), 1486–1490. https://doi.org/10.1046/j.1460-9568.1999.00560.x

Ramón y Cajal, S. (1928). Degeneration and regeneration of the nervous system. London: Oxford UP.

Sánchez, M. (2021). Toll like receptors in spinal cord derived neural precursor cells: implications on spinal cord injury and cell transplantation. [PhD thesis, Universitat Politècnica de València]. Riunet repositorio UPV. https://doi.org/10.4995/Thesis/10251/180753

Santhosh, K. T., Alizadeh, A., & Karimi-Abdolrezaee, S. (2017). Design and optimization of PLGA microparticles for controlled and local delivery of Neuregulin-1 in traumatic spinal cord injury. Journal of controlled release : official journal of the Controlled Release Society, 261, 147–162. https://doi.org/10.1016/j.jconrel.2017.06.030

Schaefer, A. W., Kabir, N., & Forscher, P. (2002). Filopodia and actin arcs guide the assembly and transport of two populations of microtubules with unique dynamic parameters in neuronal growth cones. The Journal of cell biology, 158(1), 139–152. https://doi.org/10.1083/jcb.200203038

Schmandke, A., Schmandke, A., & Strittmatter, S. M. (2007). ROCK and Rho: biochemistry and neuronal functions of Rho-associated protein kinases. The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry, 13(5), 454–469. https://doi.org/10.1177/1073858407303611

Shahsavani, N., Alizadeh, A., Kataria, H., & Karimi-Abdolrezaee, S. (2021). Availability of neuregulin-1beta1 protects neurons in spinal cord injury and against glutamate toxicity through caspase dependent and independent mechanisms. Experimental neurology, 345, 113817. https://doi.org/10.1016/j.expneurol.2021.113817

Song, W., Amer, A., Ryan, D., & Martin, J. H. (2016). Combined motor cortex and spinal cord neuromodulation promotes corticospinal system functional and structural plasticity and motor function after injury. Experimental neurology, 277, 46–57. https://doi.org/10.1016/j.expneurol.2015.12.008

Talbott, J. F., Loy, D. N., Liu, Y., Qiu, M. S., Bunge, M. B., Rao, M. S., & Whittemore, S. R. (2005). Endogenous Nkx2.2+/Olig2+ oligodendrocyte precursor cells fail to remyelinate the demyelinated adult rat spinal cord in the absence of astrocytes. Experimental neurology, 192(1), 11–24. https://doi.org/10.1016/j.expneurol.2004.05.038

Taveggia, C., Zanazzi, G., Petrylak, A., Yano, H., Rosenbluth, J., Einheber, S., Xu, X., Esper, R. M., Loeb, J. A., Shrager, P., Chao, M. V., Falls, D. L., Role, L., & Salzer, J. L. (2005). Neuregulin-1 type III determines the ensheathment fate of axons. Neuron, 47(5), 681–694. https://doi.org/10.1016/j.neuron.2005.08.017

Tuszynski, M. H., & Steward, O. (2012). Concepts and methods for the study of axonal regeneration in the CNS. Neuron, 74(5), 777–791. https://doi.org/10.1016/j.neuron.2012.05.006

Vaskovsky, A., Lupowitz, Z., Erlich, S., & Pinkas-Kramarski, R. (2000). ErbB-4 activation promotes neurite outgrowth in PC12 cells. Journal of neurochemistry, 74(3), 979–987. https://doi.org/10.1046/j.1471-4159.2000.0740979.x

Vogelaar, C. F., & Estrada, V. (2016). Experimental Spinal Cord Injury Models in Rodents: Anatomical Correlations and Assessment of Motor Recovery. InTech. doi: http://dx.doi.org/10.5772/62947

Wang, Z., Reynolds, A., Kirry, A., Nienhaus, C., & Blackmore, M. G. (2015). Overexpression of Sox11 promotes corticospinal tract regeneration after spinal injury while interfering with functional recovery. The Journal of neuroscience : the official journal of the Society for Neuroscience, 35(7), 3139–3145. https://doi.org/10.1523/JNEUROSCI.2832-14.2015

Wang, Z., Winsor, K., Nienhaus, C., Hess, E., & Blackmore, M. G. (2017). Combined chondroitinase and KLF7 expression reduce net retraction of sensory and CST axons from sites of spinal injury. Neurobiology of disease, 99, 24–35. https://doi.org/10.1016/j.nbd.2016.12.010

Weidner, N., Ner, A., Salimi, N., & Tuszynski, M. H. (2001). Spontaneous corticospinal axonal plasticity and functional recovery after adult central nervous system injury. Proceedings of the National Academy of Sciences of the United States of America, 98(6), 3513–3518. https://doi.org/10.1073/pnas.051626798

Weil, M. T., Ruhwedel, T., Meschkat, M., Sadowski, B., & Möbius, W. (2019). Transmission Electron Microscopy of Oligodendrocytes and Myelin. Methods in molecular biology (Clifton, N.J.), 1936, 343–375. https://doi.org/10.1007/978-1-4939-9072-6_20

Welniarz, Q., Dusart, I., & Roze, E. (2017). The corticospinal tract: Evolution, development, and human disorders. Developmental neurobiology, 77(7), 810–829. https://doi.org/10.1002/dneu.22455

Willem, M., Garratt, A. N., Novak, B., Citron, M., Kaufmann, S., Rittger, A., DeStrooper, B., Saftig, P., Birchmeier, C., & Haass, C. (2006). Control of peripheral nerve myelination by the beta-secretase BACE1. Science (New York, N.Y.), 314(5799), 664–666. https://doi.org/10.1126/science.1132341

Yip, P.K., & Malaspina, A. (2012). Spinal cord trauma and the molecular point of no return. Mol Neurodegeneration 7, 6. https://doi.org/10.1186/1750-1326-7-6

Zukor, K., Belin, S., Wang, C., Keelan, N., Wang, X., & He, Z. (2013). Short hairpin RNA against PTEN enhances regenerative growth of corticospinal tract axons after spinal cord injury. The Journal of neuroscience : the official journal of the Society for Neuroscience, 33(39), 15350–15361. https://doi.org/10.1523/JNEUROSCI.2510-13.2013

ANNEXES

[1] All figures were created from BioRender and Canva unless a bibliographical reference is specified in the figure caption.