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URINARY DYSFUNCTION AFTER SPINAL CORD INJURY

RESOLVING NEURONAL MECHANISMS BEHIND NEUROGENIC DETRUSOR OVERACTIVITY

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I. Chambel SS, Cruz CD (2023) *Axonal growth inhibitors and their receptors in spinal cord injury: from biology to clinical translation*. Neural Regeneration Research 8(12) 2573-2581.

II. Chambel SS, Ferreira A, Oliveira R, Miranda R, Vale L, Reguenga C, Schwab ME, Cruz CD (2022) *Development of Neurogenic Detrusor Overactivity after Thoracic Spinal Cord Injury Is Accompanied by Time-Dependent Changes in Lumbosacral Expression of Axonal Growth Regulators*. International Journal of Molecular Sciences 23(15): 8667.

III. Chambel SS, Ferreira A, Charrua A, Reguenga C, Cruz CD (2023) *Effects of tropomyosin-related kinase A inhibition to treat neurogenic detrusor overactivity in rats with a traumatic spinal cord injury* - ***Submitted***.

À Professora Doutora Célia Duarte Cruz

Aos meus Pais

Prólogo

"Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less."
- Marie Curie

"It is a capital mistake to theorize before one has data.
Insensibly, one begins to twist facts to suit theories, instead of theories to suit facts."
- Sir Arthur Conan Doyle

Finda esta longa etapa de desafios e aprendizagens, cumpre-me expressar a minha gratidão a todas as pessoas que contribuíram para a realização desta tese de doutoramento.

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formação, tanto enquanto pessoa, como neurocientista e, por isso, ser-lhe-ei sempre grata.

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List of abbreviations

ACh – Acetylcholine

Akt – Protein kinase B

ASIA – American spinal cord injury association

ATF3 - Activating transcription factor 3

ATP – Adenosine triphosphate

BDNF – Brain-derived neurotrophic factor

BSCB – Brain-spinal cord barrier

BoNT/A – Botulinum toxin type A

CGRP – Calcitonin gene-related peptide

CNS – Central nervous system

CRMP2 - Collapsin response mediator protein 2

CSPG – Chondroitin sulphate proteoglycans

CST – Corticospinal tract

DAMP – Damage-associated molecular patterns

DMSO - Dimethyl sulfoxide

DRG – Dorsal root ganglia

DSD – Detrusor-sphincter dyssynergia

ECM – Extracellular matrix

ERK - Extracellular signal-regulated kinase

EUS – External urethral sphincter

GAG – Glycosaminoglycan

GAP43 – Growth associated protein-43

GAPDH - Glyceraldehyde-3-phosphate dehydrogenase

GFAP – Glial fibrillary acidic protein

GSK-3 – Glycogen synthase kinase-3

HPRT1 - Hypoxanthine phosphoribosyltransferase 1

JNK - c-Jun N-terminal kinases

LAR - Leukocyte-antigen-related-like

LIMK - LIM kinase-1

LINGO-1 - Leucine rich repeat and Immunoglobin-like domain-containing protein 1

LRP1 - Low density lipoprotein receptor-related protein 1

LUT – Lower urinary tract

LZK - Leucine zipper-bearing kinase

m6A – N6-methyladenosine

MAG – Myelin-associated glycoprotein

MAI – Myelin-associated inhibitor

MAP - Mitogen-activated protein

MAPKK - Mitogen-activated protein kinase kinase

METTL14 - N6-adenosine-methyltransferase catalytic subunit 14

METTL3 – N6-adenosine-methyltransferase catalytic subunit 3

MMP-9 – Matrix metallopeptidase 9

MPO – Myeloperoxidase

MYPT1 - Myosin phosphatase target subunit 1

NA – Noradrenaline

NDO – Neurogenic detrusor overactivity

NG2 – Neural/glial antigen 2

NGF – Nerve growth factor

NgR1 – Nogo receptor 1

NgR2 – Nogo receptor 2

NgR3 – Nogo receptor 3

NO – Nitric oxide

Nogo-A - Neurite outgrowth inhibitor A

NT-3 – Neurotrophin-3

OMgp - Oligodendrocyte myelin glycoprotein

OPC – Oligodendrocyte progenitor cell

PACAP - Pituitary adenylate-cyclase-activating polypeptide

PAG – Periaqueductal gray

PIR-B - Paired immunoglobulin-like receptor B

PMC – Pontine micturition centre

pMYPT1 – Phosphorylated myosin phosphatase target subunit 1

POSH - Plenty of SH3s

RGMa - Repulsive guidance molecule A

RhoA – small GTPase ras homolog gene family member A

RPTPσ - Receptor type of protein tyrosine phosphatase sigma

RTX – Resiniferatoxin

S1PR2 - Sphingosine-1-phosphate receptor 2

SCI – Spinal cord injury

SP – Substance P

TBP - TATA-binding protein

TrKA - Tropomyosin receptor kinase A

TrkB - Tropomyosin receptor kinase B

TROY - Tumor necrosis factor receptor superfamily, member 19

TRPA1 - Transient receptor potential ankyrin 1

TRPM8 - Transient receptor potential cation channel subfamily M member 8

TRPV1 - Transient receptor potential vanilloid 1

TSPAN3 - Tetraspanin 3

VIP - Vasoactive intestinal peptide

YWHAZ - Tryptophan 5-monooxygenase activation protein, zeta

Abstract

Urinary dysfunction is one of the most debilitating and life-altering consequences of traumatic spinal cord injury (SCI). The lower urinary tract (LUT) is responsible for storage and voluntary release of urine. Fluctuation between continence and micturition depends on intricate neuronal pathways involving neurons located in the peripheral and central nervous system, including the spinal cord, which harbors interneurons involved in signal processing, and ascending and descending tracts. When a SCI occurs rostrally to the lumbosacral spinal cord, tracts that convey input to and from the LUT are interrupted. The spinal tissue undergoes massive rearrangements, eventually leading to neurogenic detrusor overactivity (NDO). A key event in this neuroplasticity is the abnormal sprouting of axonal afferents at the lumbosacral cord. It is interesting that axonal regrowth at the lesion site is virtually non-existent, while at the lumbosacral spinal cord level, sensory afferents expand without apparent restrictions. The molecular intervenients in this maladaptive neuroplastic mechanism are still poorly understood and were the main focus of this thesis, which includes three publications.

Publication I, a literature review on the extrinsic mechanisms of inhibition of axonal growth after spinal cord injury, focused on myelin and chondroitin-sulphate proteoglycans (CSPG)-associated molecules, their receptors and the downstream pathways involved in failure to regenerate synaptic connections. Additionally, the molecular manipulation of these inhibitors and their receptors in pre-clinical and clinical studies to treat spinal cord injuries was also discussed. *Chambel SS, Cruz CD (2023). Axonal growth inhibitors and their receptors in spinal cord injury: from biology to clinical translation. Neural Regen Res. 18(12):2573-2581.*

Publication II focused on uncovering the molecular mechanisms behind NDO emergence after SCI, using a rat model of complete thoracic spinal cord transection. Protein analysis of the extrinsic environment within the lumbosacral cord after SCI revealed the upregulation of some (but not all) inhibitors of axonal growth, namely Phosphacan and Neurocan, two CSPGs, and Nogo-A, a myelin-associated inhibitor, at 7 days-post lesion. This coincided with upregulation of growth-associated protein 43 (GAP43), a marker of axonal sprouting, at the lumbosacral spinal cord. qPCR analysis of the mRNA expression levels of receptors of those inhibitory molecules in lumbosacral dorsal root ganglia (DRG) neurons revealed a time-dependent downregulation in mRNA expression. As the growth of sensory afferents is nerve growth factor (NGF)-dependent, *in vitro* assays were performed to evaluate the contribution of this neurotrophin in the downregulation of these receptors. Cultured lumbosacral DRG neurons from SCI rats displayed a downregulation of some of the receptors studied in an NGF-concentration dependent manner, highlighting NGF as a putative therapeutic tool to control maladaptive neuroplasticity in the lumbosacral cord after SCI. *Chambel SS, Ferreira A, Oliveira R, Miranda R, Vale L, Reguenga C, Schwab ME, Cruz CD (2022). Development of Neurogenic Detrusor Overactivity after Thoracic Spinal Cord Injury Is Accompanied by Time-Dependent Changes in Lumbosacral Expression of Axonal Growth Regulators. Int J Mol Sci. 23(15):8667.*

Publication III evaluated how early NGF modulation, via inhibition of the high affinity receptor tropomyosin-related kinase A (TrkA), influenced the emergence of NDO in a rat model of spinal cord transection and whether this modulation altered the inhibitory environment detected in the lumbosacral spinal cord. Observations indicate that chronic NGF modulation, initiated immediately after spinal lesion, protected bladder function in SCI animals. This is evidenced by improvement of bladder scores and

cystometric parameters, resulting in an overall better voiding efficiency. Moreover, the improvement in bladder function was accompanied by a control of peripheral neuroplasticity, as the bladder GAP43 levels remained low, and calcitonin gene-related peptide (CGRP) expression was downregulated in animals receiving oral TrkA inhibitor. In the lumbosacral spinal cord, levels of inhibitory Phosphacan, Neurocan and Nogo-A were upregulated by NGF modulation, likely regulating neural plasticity associated with NDO. However, care should be taken with the use of dimethyl sulfoxide (DMSO) as a vehicle for drug administration in SCI models, as DMSO by itself was not devoid of effects. Early NGF modulation rose as a potential therapeutic strategy to overcome the molecular cascade that leads to NDO emergence after a spinal cord injury. *Chambel SS, Ferreira A, Charrua A, Reguenga C, Cruz CD (Submitted). Effects of tropomyosin-related kinase A inhibition to treat neurogenic detrusor overactivity in rats with a traumatic spinal cord injury.*

Resumo

A disfunção urinária é uma das consequências mais debilitantes e que alteram a vida após uma lesão traumática da medula espinhal (SCI). O trato urinário inferior (LUT) é responsável pelo armazenamento e libertação voluntária de urina. O equilíbrio entre continência e micção depende de complexas vias neuronais que envolvem neurónios localizados no sistema nervoso periférico e central, incluindo a medula espinhal, composta por interneurónios envolvidos no processamento de sinal, e vias neuroniais ascendentes e descendentes. Aquando de uma lesão medular rostral à medula espinhal lumbo-sagrada, os tratos neuroniais que transmitem informações de e para o LUT são interrompidos. O tecido da medula espinhal sofre rearranjos profundos que levam ao eventual aparecimento de hiperatividade neurogénica do detrusor (NDO). Um evento chave nesta neuroplasticidade é o crescimento anormal das terminações centrais dos neurónios aferentes ao nível da medula lumbo-sagrada. De facto, enquanto a regeneração axonal no local da lesão é praticamente inexistente, ao nível da medula espinhal lumbo-sagrada os aferentes sensitivos expandem-se sem restrições aparentes. Os intervenientes moleculares neste mecanismo neuroplástico são ainda pouco compreendidos e foram, por isso, o foco principal desta tese, que inclui três publicações.

A ***Publicação I***, uma revisão da literatura sobre os mecanismos extrínsecos de inibição do crescimento axonal depois de lesão medular, debruçou-se sobre moléculas inibitórias associadas à mielina e aos proteoglicanos associados à condroitina (CSPGs), os seus receptores e as vias de sinalização a jusante envolvidas na ausência de mecanismos regenerativos. Adicionalmente, a manipulação molecular destas moléculas inibitórias e dos seus receptores em investigação pré-clínica e clínica para o tratamento

de lesões medulares foi também abordada. *Chambel SS, Cruz CD (2023). Axonal growth inhibitors and their receptors in spinal cord injury: from biology to clinical translation. Neural Regen Res. 18(12):2573-2581.*

A ***Publicação II*** debruçou-se sobre os mecanismos moleculares envolvidos no aparecimento da NDO após uma lesão medular, recorrendo ao modelo de transecção completa da medula espinhal em rato. A análise proteica do ambiente extrínseco na medula lumbo-sagrada após SCI mostrou sobre-regulação de algumas (mas não de todas) moléculas inibitórias ao crescimento axonal, nomeadamente de Phosphacan e Neurocan, dois CSPGs, e de Nogo-A, uma molécula inibitória associada à mielina, aos 7 dias após lesão. Este aumento coincidiu com a sobre-regulação de *growth-associated protein 43* (GAP43), um marcador de *sprouting* axonal, na medula lumbo-sagrada. A análise por qPCR dos níveis de expressão de mRNA dos receptores destas moléculas inibitórias nos neurónios dos gânglios dorsais raquidianos (DRG) lumbo-sagrados revelou uma sub-regulação dependente do tempo nos níveis de mRNA. Uma vez que o crescimento de aferentes sensitivos é dependente da presença de factor de crescimento nervoso (NGF), foram feitos ensaios *in vitro* de forma a avaliar a contribuição desta neurotrofina na sub-regulação destes receptores. Neurónios de DRG lumbo-sagrados em cultura, provenientes de ratos com SCI, mostraram uma sub-regulação dependente da concentração de NGF no meio de cultura de alguns dos receptores estudados, evidenciando o NGF como um putativo alvo terapêutico no controlo da neuroplasticidade maladaptativa na medula lumbo-sagrada depois de SCI. *Chambel SS, Ferreira A, Oliveira R, Miranda R, Vale L, Reguenga C, Schwab ME, Cruz CD (2022). Development of Neurogenic Detrusor Overactivity after Thoracic Spinal Cord Injury Is Accompanied by Time-*

Dependent Changes in Lumbosacral Expression of Axonal Growth Regulators. Int J Mol Sci. 23(15):8667.

A ***Publicação III*** avaliou como a modulação precoce do NGF, via inibição do seu receptor de alta afinidade *tropomyosin-related kinase A* (TrkA), influencia o aparecimento de NDO num modelo de rato com transecção da medula espinhal e se esta modulação altera o ambiente inibitório da medula lumbo-sagrada. Os resultados indicaram que a modulação crónica de NGF, iniciada imediatamente após lesão medular, protegeu a função vesical em animais com SCI. Este resultado foi evidenciado pela melhoria dos scores vesicais e parâmetros cistométricos, resultando numa maior eficiência de micção. Adicionalmente, a melhoria da função vesical foi acompanhada por um controlo da neuroplasticidade periférica, evidenciado pelos níveis baixos de GAP43 na bexiga, e pela sub-regulação da expressão de *calcitonin gene-related peptide* (CGRP) na bexiga de animais que receberam o inibidor de TrkA. Na medula lumbo-sagrada, os níveis de Phosphacan, Neurocan e Nogo-A encontravam-se sobre-regulados pela modulação do NGF, um sinal de provável regulação da plasticidade neuronal associada à NDO. Deverá ter-se em atenção o uso de *dimethyl sulfoxide* (DMSO) como veículo para administração de fármacos em modelos de SCI, uma vez que a administração de DMSO por si só não foi desprovida de efeitos. A modulação precoce de NGF surgiu, assim, como uma potencial estratégia terapêutica para suplantar a cascata molecular que leva ao aparecimento de NDO após lesão medular. *Chambel SS, Ferreira A, Charrua A, Reguenga C, Cruz CD (Submitted). Effects of tropomyosin-related kinase A inhibition to treat neurogenic detrusor overactivity in rats with a traumatic spinal cord injury.*

State of the art

Based on Chambel SS, Cruz CD (2023) *Axonal growth inhibitors and their receptors in spinal cord injury: from biology to clinical translation*. Neural Regeneration Research 8(12) 2573-2581.

1. Neurophysiology of the lower urinary tract

1.1 Central nervous system control of the lower urinary tract

The lower urinary tract (LUT) comprises the urinary bladder and the urethra, regulated by a network of intricate neuronal circuits located in the brain, spinal cord and peripheral ganglia (Fowler et al., 2008; Fowler and Griffiths, 2010). Micturition, the process by which urine is stored and periodically released, depends on the coordinated activity between the bladder, the urine reservoir, and the outlet structure, formed by the bladder neck, the urethra and the external urethral sphincter (EUS) (Fowler et al., 2008). In newborns and during early infancy, micturition is a reflex mechanism dependent on neuronal pathways located exclusively in the lumbosacral spinal cord. As the central nervous system (CNS) matures, voluntary control of micturition is acquired (de Groat, 2002). The micturition neural circuitry comprises four basic components: primary afferent neurons, spinal interneurons, supraspinal centres, and spinal efferent neurons.

1.1.1. Afferent innervation

Sensory afferent fibres carry sensory information from the LUT and pelvic floor to the lumbosacral spinal cord, and run in the pudendal, pelvic and hypogastric nerves. Bladder fullness is conveyed to the spinal cord through the pelvic and hypogastric nerves, while urethral and bladder neck sensory input is carried in the pudendal and hypogastric nerves. Sensory afferents innervating the LUT are glutamatergic in nature (Morgan et al., 1981; De Biasi and Rustioni, 1988; Keast and Stephensen, 2000). Their cell bodies are located in caudal lumbosacral dorsal root ganglia (DRG) and can be divided in thinly myelinated Aδ-fibres and unmyelinated C-fibres (Hulsebosch and Coggeshall, 1982).

These sensory fibres project to the superficial laminae of the lumbosacral spinal cord, where they establish synaptic contacts with interneurons involved in local spinal reflexes and with projection neurons that convey information to higher brain centres (Fowler et al., 2008). Aδ-fibres respond to passive distension and active detrusor contraction (de Groat and Yoshimura, 2009), being silent when the bladder is empty, but becoming active as the bladder fills (de Groat and Yoshimura, 2015). C-fibres, on the other hand, are generally mechano-insensitive or "silent C-fibres", responding only to noxious or inflammatory stimuli (Fowler et al., 2008). They are activated by high intensity stimuli and lower their activation threshold in pathological conditions (de Groat and Yoshimura, 2015). C-fibres are peptidergic in nature, expressing substance P (SP), calcitonin-gene related peptide (CGRP), pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) and have a widespread distribution within the bladder wall, being more prominent in the mucosa. C-fibre afferents also express several receptors such as tropomyosin receptor kinase A (TrkA) and tropomyosin receptor kinase B (TrkB), the high-affinity receptors for nerve growth factor (NGF) and brain-derived growth factor (BDNF), respectively; transient receptor potential vanilloid 1 (TRPV1); transient receptor potential ankyrin 1 (TRPA1) ; transient receptor potential cation channel subfamily M member 8 (TRPM8); and purinergic receptors, such as P2X2, P2X3 and P2Y, that can be activated by adenosine triphosphate (ATP) (de Groat and Yoshimura, 2001, 2015).

1.1.2. Spinal cord neurons

Spinal interneurons involved in neuronal circuits relevant for micturition control are present in laminae I and V, the dorsal commissure and intermediolateral grey matter (de

Groat et al., 2015). These second-order neurons, either inhibitory or excitatory in nature, can participate in segmental spinal reflexes (de Groat et al., 1998). Along with interneurons, projection neurons are also important as they relay information to supraspinal centres via the spinothalamic tract (de Groat et al., 2015).

1.1.3. Supraspinal control of micturition

Brain control of micturition relies on several neuronal populations that are directly or indirectly involved in bladder, urethra and urethral sphincter control. Specific brain centres for micturition include the neurons of Barrington's nucleus or pontine micturition centre (PMC), neurons of the periaqueductal grey (PAG), cell clusters in the caudal and preoptic hypothalamus and neurons in the cerebral cortex. Other brain centres, with diffuse spinal projections, include serotonergic neurons of the medullary raphe nuclei, noradrenergic neurons of the locus coeruleus and the noradrenergic A5 cell group in the brain stem (Fowler et al., 2008; Fowler and Griffiths, 2010). These brain areas share interconnections and project to the lumbosacral spinal cord, allowing the physiological control of micturition.

1.1.4. Efferent innervation

The efferent control of the LUT relies on sympathetic and parasympathetic nerves of the autonomic nervous system and motor fibres of the somatic nervous system (de Groat et al., 2015). Parasympathetic preganglionic fibres arise from neurons located in sacral spinal segments. These neurons send long projections along the lateral funiculus, the lateral dorsal horn and the dorsal commissure, running in the pelvic nerves until they synapse with postganglionic neurons located in the pelvic plexus, in the rat, or

intramurally in the bladder, in humans and the guinea pig (Wada et al., 2022). These fibres innervate the bladder smooth muscle, releasing acethylcholine (ACh) that acts on muscarinic receptors. They can also release ATP, that binds to purinergic receptors (Birder et al., 2009). Some parasympathetic nerve fibres also innervate the urethra, inducing relaxation of the urethral smooth muscle, possibly through the release of nitric oxide (NO) (Andersson et al., 1992; Bennett et al., 1995) (**Figure 1A-B**).

Sympathetic preganglionic neurons are located in the intermediolateral column of the thoracolumbar cord (T11-L2) in humans (Wada et al., 2022). Their fibres run in the hypogastric nerve and in the inferior mesenteric plexus and synapse with postganglionic neurons in inferior mesenteric ganglia. Axons emerging from postganglionic neurons located in these ganglia innervate the bladder smooth muscle, inducing its relaxation via noradrenaline (NA) release and activation of β3 adrenergic receptors (Andersson and Arner, 2004). Sympathetic fibres also run through the paravertebral chain, entering the pelvic nerve and innervating the bladder neck and urethra, activating α1 adrenergic receptors in bladder ganglia (Fowler et al., 2008), inducing urethral smooth muscle contraction (**Figure 1A-B**).

Finally, somatic cholinergic motor fibres, excitatory in nature, arise in sacral motor neurons of the Onuf's nucleus and run through pudendal nerves until they synapse with neurons that innervate the striated muscle of the EUS, releasing ACh and contributing to urethral contraction through maintenance of EUS closure and the activation of nicotinic receptors. Another set of motor neurons, located at the same spinal level, have their axons innervate the pelvic floor musculature (Fowler et al., 2008) (**Figure 1A-B**).

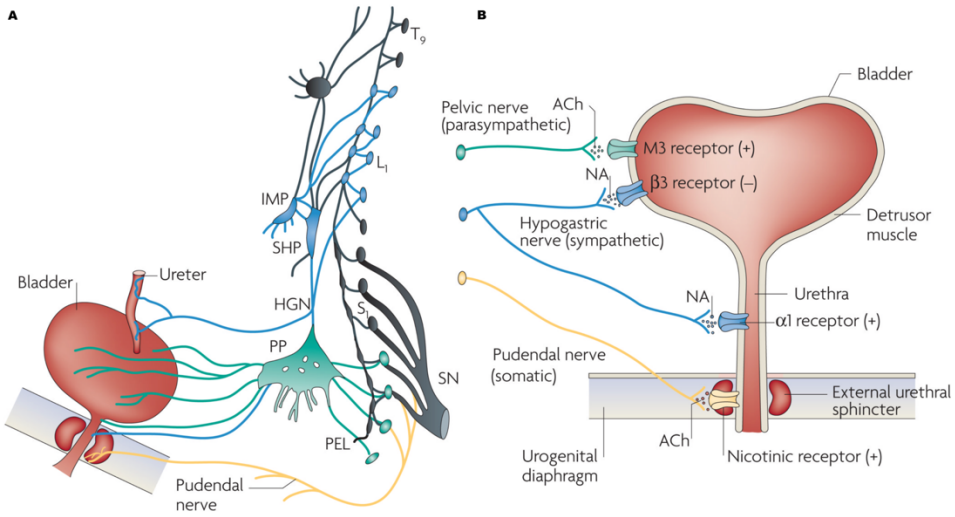

Figure 1. Efferent innervation of the lower urinary tract. A. Innervation of the female lower urinary tract. In blue, sympathetic fibres from the T11-L2 spinal segments run through the inferior mesenteric plexus (IMP) and the hypogastric nerve (HGN) or through the paravertebral chain, entering the pelvic nerves at the base of the bladder and urethra. Parasympathetic preganglionic fibres, in green, arise from the S2-S4 spinal segments and run through sacral roots and pelvic nerves (PEL) until they reach the ganglia in the pelvic plexus (PP) and in the bladder wall. Somatic motor nerves, in yellow, arise from S2-S4 motor neurons and run through the pudendal nerves to innervate the striated muscle of the external urethral sphincter. **B.** Efferent pathways and neurotransmitter mechanisms of the lower urinary tract. Parasympathetic postganglionic axons in the pelvic nerve release acetylcholine (ACh), which causes bladder contraction by stimulating M3 muscarinic receptors in the bladder smooth muscle. Sympathetic postganglionic neurons release noradrenalin (NA) that activates β3 adrenergic receptors to relax the bladder smooth muscle and α1 adrenergic receptors to contract the urethral smooth muscle. Somatic axons in the pudendal nerve release ACh that induces the contraction of the external urethral sphincter by the activation of nicotinic cholinergic receptors. *Adapted from Fowler et al., 2008.*

2. Micturition – storage and voiding

The LUT operates in an on-off switch manner, alternating between voiding and continence and maintaining a reciprocal relationship between the urinary bladder and urethral outlet. Bladder filling relies on mechanisms primarily organized in the spinal cord, whereas voiding is dependent on supraspinal centres. Under physiological conditions, intravesical pressures during the storage phase are low and remain constant, when the bladder volume is below the threshold for voiding induction. During continence, bladder distension induces low-level vesical afferent firing, while parasympathetic innervation of the detrusor muscle is inhibited and sympathetic

innervation through the hypogastric nerve to the bladder outlet (the bladder base and the urethra) and the pudendal nerve to the EUS is activated (Fowler et al., 2008; de Groat and Yoshimura, 2015) (**Figure 2A**). This process is mainly organized by spinal reflexes that are regulated in response to bladder afferent activity, which is conveyed through pelvic nerves and organized by interneurons of the spinal cord. This mechanism is called guarding reflex and promotes continence. Additionally, the L-region of the rostral pons (the pontine storage centre) might increase striated urethral sphincter activity, further contributing to continence (Fowler et al., 2008).

During voiding, intense bladder afferent firing in the pelvic nerve ascends through projection neurons, activating spinobulbo reflex pathways to the PAG. In turn, the PAG provides an excitatory input to the PMC. Excitation of the PMC activates descending pathways that innervate the detrusor and urethral smooth muscle, causing urethral relaxation and activation of the sacral parasympathetic outflow, resulting in bladder contraction, increase in intravesical pressure and urine voiding (**Figure 2B**).

In normal conditions, voiding is under strict voluntary control, enabling one to void at socially acceptable times and places. The PAG plays a critical role on voiding control, which is dependent on two aspects: the conscious registration of bladder filling and to which extent is acceptable to keep delaying voiding; and manipulation of the voiding reflex. The PAG not also receives and processes ascending bladder input and sends this information to the PMC and to higher brain centres, but also receives projections from these brain centres, such as the prefrontal cortex. These higher brain structures are able to inhibit the excitatory input to the PMC and thus prevent voiding. When voiding is desired, the prefrontal cortex interrupts the tonic suppression of PAG input to the PMC and urine voiding occurs (Fowler et al., 2008).

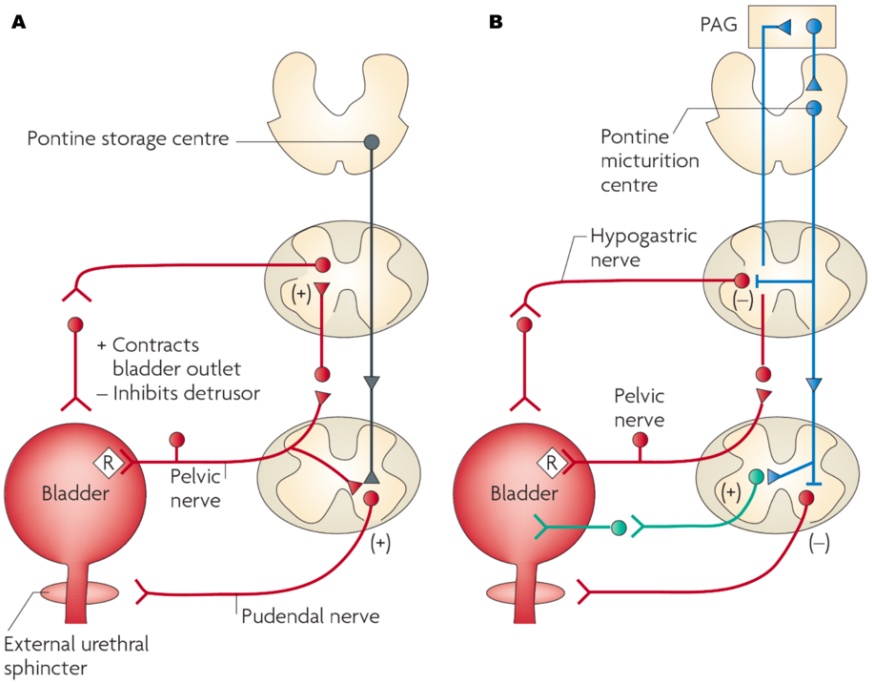

Figure 2. Regulation of continence and micturition. A. Urine storage. During the urine storage phase, bladder distension creates a low-level vesical afferent firing that stimulates the sympathetic outflow in the hypogastric nerve to the bladder outlet and the pudendal outflow to the external urethral sphincter. These represent spinal reflexes that maintain continence. Sympathetic innervation also inhibits contraction of the detrusor smooth muscle and modulates neurotransmission in the bladder ganglia (represented in red). A region in the pontine storage centre might increase striated urethral sphincter activity (represented in grey). **B.** Voiding reflexes. During urine expulsion, intense bladder afferent firing in the pelvic nerve activates spinobulbospinal reflex pathways (represented in blue) that go up to the pontine micturition centre. This, in turn, stimulates the parasympathetic outflow to the bladder and to the urethral smooth muscle (represented in green) and inhibits the sympathetic and pudendal outflow to the urethra (represented in red). The diagram represents the micturition reflex and does not acknowledge the generation of conscious bladder voiding that involves cerebral regions above the periaqueductal grey region. *Adapted from Fowler et al., 2008.*

3. Spinal cord injury and alterations to the lower urinary tract

Normal bodily function requires an intact CNS, comprising the brain and the spinal cord. The spinal cord is a complex structure that functions as a relay and processing centre. It conveys peripherally generated sensory input to supraspinal structures which, in turn, generate motor responses that are transmitted to effector organs by the spinal cord. Additionally, the spinal cord is also able to generate reflexes, the spinal reflexes.

Therefore, it comes as no surprise that spinal cord injury (SCI) disrupts spinal cord function, with massive consequences in sensorimotor function.

According to the American Spinal Cord Injury Association (ASIA), SCI can be classified according to a scale that defines the degree of neurological impairment: A (total sensorimotor loss below the lesion, including no sacral feeling); B (sensory but no motor function below the lesion); C (partial motor preservation, but the majority of muscles have a grade of less than 3); and D (the majority of muscle groups have a grade of 3 or higher below the lesion) (Roberts et al., 2017). Nowadays, the most common spinal injury is incomplete tetraplegia (45%), followed by incomplete paraplegia (21.3%), total paraplegia (20%), and complete tetraplegia (13.3%). Less than 1% of patients fully recover by the time they are discharged from the hospital (NSCISC, 2017). SCI can result from contusion, generally caused by a sudden impact to the cord and resulting in bruising and bleeding; compression, which involves prolonged external pressure to the cord; and laceration, when sharp objects or bone fragments penetrate the cord.

3.1. Incidence and prevalence of spinal cord injuries

The function, dynamic and composition of the spinal cord is significantly altered by SCI, causing sensorimotor and autonomic impairments. Estimates from the World Health Organization calculate the occurrence of 250 000 to 500 000 new cases of SCI in the world each year, with a prevalence between 250 and 906 cases per million (Singh et al., 2014; Srikandarajah et al., 2023). The most common causes of SCI include motor accidents, falls, sports, violent events and military service. The epidemiological features of traumatic spinal injuries have changed in the last few decades. In the past, SCI occurred mostly in young individuals, due to sports or motor vehicle-related accidents,

but nowadays a bimodal distribution can be observed: an age peak occurs between 15-29 years of age and another in persons over 50 years old due to falls, mostly associated with degenerative diseases (Chen et al., 2016). In most cases, SCI occurs in the cervical level of the cord, representing more than 50% of cases, followed by injuries at the thoracic level (35%) and lumbar region (11%) (Alizadeh et al., 2019).

3.2. Management of spinal cord injuries

Due to advances in surgical techniques and medical management, life expectancy has greatly improved and can now span several decades after spinal lesion (Pizzolato et al., 2021). Treatment of spinal cord injuries involves a multidisciplinary approach that depends on numerous factors, such as the type, severity, and location of the injury, but also the age and overall health of the patient. In the case of traumatic spinal cord injuries, spinal immobilization is the first step of injury management (Ahn et al., 2011). In high cervical injuries, diaphragmatic innervation can be affected and patients may need immediate respiratory and cardiovascular interventions upon hospital arrival. Another phenomenon commonly seen in cervical and upper thoracic spinal cord injuries is neurogenic shock. This critical condition results from the disruption of normal sympathetic innervation, leading to decreased systemic vascular resistance, profound hypotension, bradycardia and unopposed vagal tone, conditions that also require immediate medical attention (Sandean, 2020). In the **acute phase**, SCI management may include the administration of the anti-inflammatory corticosteroid and antioxidant drug methylprednisolone. Methylprednisolone was approved as the standard of care for acute SCI worldwide decades ago, as it promotes blood flow through the reduction of calcium influx and decrease in lipid peroxidation (Bracken et al., 1997). However, in

more recent years, its use has been linked with increased incidence of pneumonia or thrombosis in SCI patients, resulting in the discontinuation of its use in acute SCI patients (Pizzolato et al., 2021).

Patients that present with spinal compression injuries may require decompressive spine surgery to relieve pressure on the spinal cord. These interventions allow re-establishment of blood flow and stabilization of the cord and vertebrae to reduce secondary injury and promote recovery (Badhiwala et al., 2018). As soon as patients are stable, early rehabilitation protocols begin to be implemented to maximize improvement in functional outcomes. Nevertheless, full functional recovery is still lacking in these patients, with quality of life being severely affected in the long-term. Several clinical trials to promote regeneration and functional recovery after SCI are undergoing, but currently none has been approved for clinical use.

During the **chronic phase** of injury, patients continue to undergo rehabilitation and acquire new skills to facilitate independent post-discharge living, including the use of wheelchairs, braces, and orthotics to improve mobility (Srikandarajah et al., 2023). Pharmacological interventions, such as baclofen, gabapentin and botulinum toxin type A (BoNT/A), can also be chronically employed to manage long-lasting secondary injury-related complications, including spasticity, neuropathic pain and urinary dysfunction (Bhagwani, 2022). Recently, great advances have been made in post-SCI neurorehabilitation. Targeted neural stimulation of both the brain and lumbosacral spinal cord via implantation of digital bridges have allowed a chronic SCI patient to produce movements such as walking, standing and stair climbing and, in combination with neurorehabilitation, to trigger the reorganization of neuronal pathways, further enhancing neurological recovery (Lorach et al., 2023).

3.3. Changes in micturition after spinal cord injury – neurogenic detrusor overactivity and detrusor-sphincter dyssynergia

Irrespective of age group, cause or lesion severity, SCI has dramatic consequences in patients' lives. SCI-induced loss of voluntary control over bladder function is particularly devastating, affecting approximately 90% of SCI patients and dramatically impacting their dignity, independence and quality of life. In fact, urinary incontinence is pinpointed as one of the most incapacitating aspects of everyday life post-injury (Simpson et al., 2012; Braaf et al., 2017), due to the need to permanently be in close proximity to a toilet room, the fear of suffering an urinary incontinence episode and the dependence of caregivers, if hand movement is impaired. Moreover, urinary dysfunction after SCI is associated with urosepsis, incontinence and a high risk of deteriorating renal function due to loss of coordination between the bladder and the sphincter, the so-called detrusor-sphincter dyssynergia (DSD). Early SCI management focuses on the protection of the upper urinary tract in order to prevent life-threatening kidney failure (Panicker et al., 2015). Afterwards, the main concerns include recurrent urinary infections and urinary incontinence.

Due to its dependence from the CNS, voluntary control over LUT function is lost after a traumatic spinal cord injury rostral to the lumbosacral spinal cord, as supraspinal control of micturition is disturbed. SCI is usually followed by the spinal shock period. Spinal shock refers to the acute loss of motor, sensory, and reflex functions below the injury level, resulting in little or no bladder reflex activity and urine retention in the absence of catheterization (Ditunno et al., 2004; Bywater et al., 2018; Anderson et al., 2023) (**Figure 3B**). Weeks, months or even years after a SCI, a new micturition reflex, exclusively located at the lumbosacral spinal cord and independent from supraspinal

centres, is established. This new reflex is the main generator of neurogenic detrusor overactivity (NDO) (**Figure 3C**). It is estimated that nearly 95% of SCI patients develop NDO, a pathological condition characterized by strong, frequent and involuntary detrusor contractions that result in urinary incontinence (de Groat and Yoshimura, 2006). It is defined by the International Continence Society as "involuntary detrusor muscle contractions occurring near or at the maximum cystometric capacity, in the setting of a clinically relevant neurologic disease. These contractions generally cannot be suppressed, resulting in urinary incontinence or even reflex bladder emptying (reflex voiding)" (Gajewski et al., 2018). NDO is often concurrent with DSD, the loss of coordination between the bladder and the EUS. DSD is characterized by periods of very high and extremely dangerous intravesical pressures that may produce significant damage to the upper urinary tract.

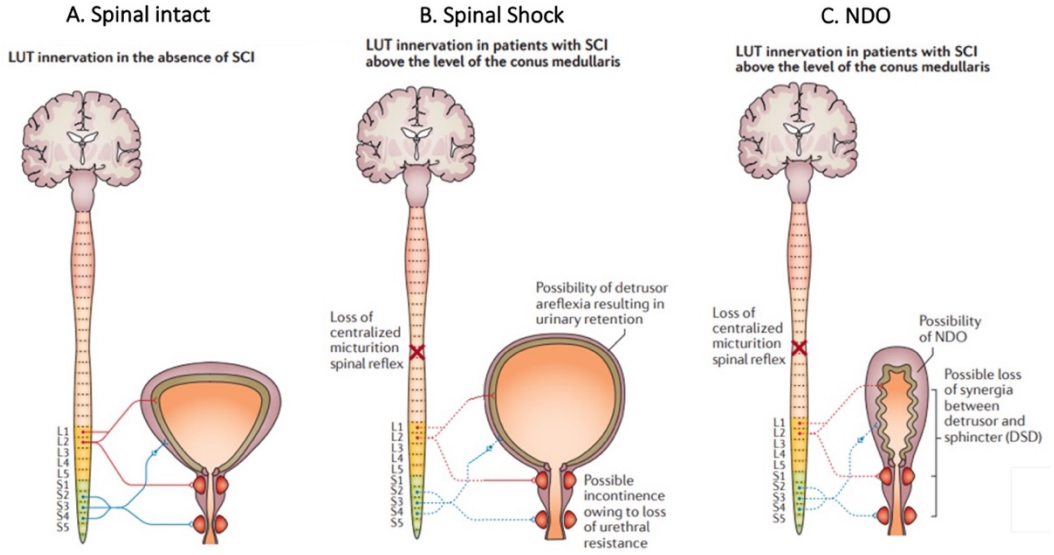

Figure 3. Evolution of the lower urinary tract after a spinal cord injury. A. Intact innervation of the lower urinary tract. B. Innervation of the lower urinary tract after a spinal cord injury (SCI). Immediately after injury, spinal shock sets and lack of supraspinal input to the lower urinary tract renders the bladder becomes acontractile and urine is retained. C. Some time after SCI, a new micturition reflex, located in the lumbosacral spinal cord and independent from supraspinal centres, is established, termed neurogenic detrusor overactivity (NDO). NDO might be accompanied by detrusor-sphincter dyssynergia (DSD), leading to a lack of coordination between bladder contractions and external urethral sphincter relaxation, resulting in urine retention or urinary incontinence. *Adapted from Wyndaele, 2016.*

In addition to lack of supraspinal input, development of NDO and DSD is linked to neuroplastic rearrangements within the lumbosacral spinal cord that include axonal sprouting, synaptic rearrangement, alterations in neurotransmitter synthesis and modifications in sensory afferents innervation of the LUT (Krenz and Weaver, 1998; Yoshimura, 1999; Wada et al., 2022). These compensatory processes course with elevated levels of neurotrophins in the bladder and spinal cord, such as NGF and BDNF (Yoshimura et al., 2006; Frias et al., 2015; Shimizu et al., 2018; Wada et al., 2018). Several studies have indicated that NDO development courses with abnormal sprouting of bladder sensory peptidergic afferents at the lumbosacral spinal cord (Vizzard, 1999; Zinck et al., 2007; Frias et al., 2015). These afferents are peptidergic unmyelinated C-fibres that, under physiological conditions, have little to no relevance to micturition, but become responsive after SCI. Their plasticity is key in the emergence of the new micturition reflex that causes urinary incontinence (Häbler et al., 1990). Nevertheless, the fine molecular mechanisms regulating this aberrant axonal growth are still mostly unresolved.

3.4. Management of bladder dysfunction after spinal cord injury

Currently, there are no preventive measures to avoid the development of urinary dysfunction following SCI, and available treatment options can only be employed once NDO and/or DSD have developed. One of the top priorities for patients is to prevent urine leakage and, for that, some pharmacological approaches to reduce detrusor contractions and improve urinary incontinence are currently available. Nonetheless, these treatments are not exempt from secondary effects and must be continuously

combined with intermittent catheterisation for timely urine removal, to prevent urinary tract damage and improve long-term outcomes (Panicker et al., 2015).

Antimuscarinic drugs are typically the first-line choice for treating NDO. These agents block M2 and M3 muscarinic receptors present on the urothelium and in the detrusor smooth muscle, preventing ACh-mediated bladder contraction by inhibition of parasympathetic pathways (Andersson, 2011). The most established, effective, and well-tolerated antimuscarinic drugs include oxybutynin, trospium, tolterodine and propiverine. Darifenacin and solifenacin are more recent formulations of antimuscarinic drugs, effective in the treatment of NDO secondary to SCI (Blok et al., 2022). While being able to increase bladder capacity and reduce episodes of urinary incontinence in some patients, antimuscarinic drugs may also present major side-effects such as dry mouth, blurred vision, constipation, tachycardia, and cognitive impairment (Romo et al., 2018). β3-adrenergic receptor agonists, such as mirabegron, have produced improvements in LUT symptoms in SCI patients, but significant effects on detrusor pressure or cystometric capacity were not demonstrated (Blok et al., 2022). Vanilloids, such as capsaicin and resiniferatoxin (RTX), are agonists of the TRPV1 receptor, and their administration to treat NDO results in long-lasting desensitization of C-fibres (Giannantoni et al., 2004). Despite this, currently there is no clinical indication for the use of both formulations, due to their highly irritant proprieties, in the case of capsaicin, and to the impossibility to produce stable and affordable RTX formulations (Blok et al., 2022). Presently, BoNT/A injections in the detrusor muscle are the golden-standard treatment for NDO following SCI for patients that are refractory to pharmacological interventions (Panicker et al., 2015). BoNT/A causes long-lasting chemical denervation of parasympathetic cholinergic, sympathetic adrenergic and sensory peptidergic fibres in the bladder smooth muscle by

blocking vesicle-mediated neurotransmission (Coelho et al., 2010; Coelho et al., 2012). BTX effects are long-lasting (up to nine months), repeated injections seem to be possible without loss of efficacy and improvements in LUT function are evident, reducing bladder contractions and the frequency of episodes of incontinence (Ginsberg et al., 2013; Kennelly et al., 2015). The most frequent side effects include urinary retention, recurrent urinary infections, and haematuria (Blok et al., 2022).

3.5. NGF in the development of neurogenic detrusor overactivity after spinal cord injury

NGF is a neurotrophin essential for survival, growth and maintenance of small-diameter, primary sensory and sympathetic neurons during development. Sources of NGF include glial cells and neurons (Brown et al., 2004, 2007; Althaus et al., 2008) and peripheral tissues such as the bladder smooth muscle (Steers et al., 1991; Steers and Tuttle, 2006) and the urothelium (Birder et al., 2010). NGF exerts most of its effects upon binding to its high affinity receptor TrkA or, in alternative, to the low-affinity p75 neurotrophin receptor (p75) (Mizumura and Murase, 2015; Denk et al., 2017). When NGF binds to TrkA, intracellular signalling pathways, such as the Extracellular signal-regulated kinase (ERK) and Akt pathways are activated, resulting in the transcription of pro-survival genes and blockade of cell death pathways (Coelho et al., 2019).

In the adult, NGF can be upregulated under pathological conditions, such as spinal cord injury (Yoshizawa et al., 2014; Kadekawa et al., 2017a), where it plays a role in both recovery and dysfunction. NGF is upregulated both at rostral and caudal spinal segments (in relation to the injury site) and in DRG neurons (Murakami et al., 2002; Seki et al., 2002; Brown et al., 2007), promoting some regeneration processes and protecting

spared neuronal circuits (Grill et al., 1997; Krenz and Weaver, 2000; Ramer et al., 2000; Romero et al., 2000). Conversely, NGF-dependent sprouting has been associated with severe SCI outcomes, such as autonomic dysreflexia and urinary dysfunction (Krenz et al., 1999; Seki et al., 2002; Cameron et al., 2006; Yoshimura et al., 2006; Wada et al., 2018).

The involvement of NGF in SCI-induced bladder impairment has been widely explored. NGF is known to increase in the bladder after SCI in response to bladder overdistension which, in turn, leads to increased C-fibre excitability and NDO (Yoshimura et al., 2006) (**Figure 4**). This role of NGF in urinary dysfunction after SCI was demonstrated in rodent models. On one hand, urinary dysfunction can be reversed by NGF neutralization, supressing both NDO (Seki et al., 2002) and DSD (Seki et al., 2004), while bladder NGF administration causes bladder hyperactivity (Yoshimura et al., 2006). When C-fibres bladder afferents are desensitized with capsaicin (Cheng et al., 1995; Kadekawa et al., 2017b) or RTX administration (Oliveira et al., 2019), bladder overactivity is inhibited. Approximately half of sensory C-fibre bladder afferents express TrkA (Averill et al., 1995) and this expression increases in bladder afferent neurons after SCI in rodent models (Vizzard, 2000; Qiao and Vizzard, 2002). NGF is a likely candidate involved in maladaptive neuroplasticity leading to autonomic dysreflexia (Cameron et al., 2006), chronic neuropathic pain (Lin et al., 2014; Fu et al., 2023) and urinary impairment (Seki et al., 2002; Seki et al., 2004). In fact, it has been demonstrated that in SCI rodents, NGF regulates C-fibers hyperexcitability (Kadekawa et al., 2017b; Shimizu et al., 2018) and potentiates axonal sprouting (Marsh et al., 2002; Lin et al., 2014), leading to urinary dysfunction. Other NGF-driven mechanisms, such as control of gene expression, may also be relevant but remain to be explored.

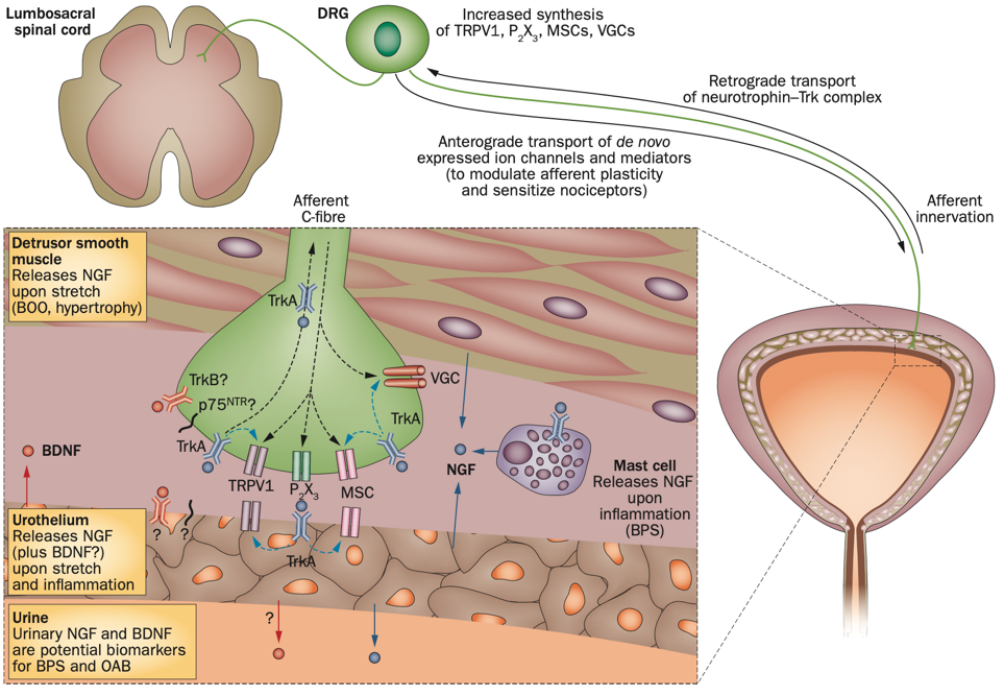

Figure 4. Peripheral mechanisms involved in the neurotrophin-mediated development of bladder overactivity. In the urinary bladder, nerve growth factor (NGF) can be produced by several cell types, including the urothelium, mast cells and detrusor smooth muscle cells, upon distension or inflammation. NGF binding to tropomyosin related kinase A (TrkA) receptors on the urothelium might activate urothelial sensory ion channels, such as transient receptor potential vanilloid 1 (TRPV1), or increase TRPV1 and mechanosensory channels (MSC) expression. Increased TRPV1 and MSC activity stimulate the release of urothelial mediators, such as ATP, which, in turn, sensitize the underlying afferents. Moreover, NGF activates TrkA receptors expressed on sub-urothelial afferent C-fibre terminals, directly sensitizing neuronal TRPV1, MSCs and voltage-gated ion channels. This NGF-TrkA complex is then internalized and retrogradely transported to cell bodies in lumbosacral dorsal root ganglia (DRG) neurons, where *de novo* transcription of TRPV1, MSCs, voltage-gated ion channels (VGCs) and additional sensory ion channels (including purinergic P2X3 receptor for ATP) is initiated. These newly synthesized ion channels are anterogradely transported back to afferent terminals to contribute to peripheral hypersensitivity. Besides NGF, the urothelium also produces BDNF. BDNF's high affinity receptor tropomyosin receptor kinase B (TrkB) and p75 are expressed both on the urothelium and bladder sensory afferent terminals. *Adapted from Ochodnicky et al., 2012.*

4. Pathophysiology of scar formation and failure to regenerate after spinal cord injury

After a spinal cord injury, remodelling and scaring of damaged tissue begins immediately and spans throughout the remainder of life. The **acute post-injury phase** spans the first 72h following trauma, when a cascade of pathological events ensues.

Mechanical trauma to the spinal cord causes tissue damage that immediately disrupts the spinal cord vasculature resulting in haemorrhage, oedema and swelling. Platelets invade the cord and cause vasospasm, resulting in ischemia and glutamate release (Mautes et al., 2000). In response to injury, stromal cells, astrocytes, oligodendrocytes, oligodendrocyte progenitor cells (OPCs), microglia and immune cells are recruited to the injury site and a series of molecular events are set in motion (**Figure 5**).

Neutrophils are the first immune responders after a spinal cord injury, infiltrating the damaged tissue within 1 hour and becoming abundant 12-24h post-lesion. Neutrophils release pro-inflammatory molecules, such as myeloperoxidase (MPO) and matrix metallopeptidase 9 (MMP-9), that exacerbate the permeabilization of the blood-spinal cord barrier (BSCB), allowing immune cell access to the CNS (Shapiro, 1998; Rosell et al., 2008). Damage to the spinal tissue catalyses the release of molecules known as damage-associated molecular patterns (DAMPs), which lead to disturbances in ionic homeostasis, oxidative stress and excitotoxicity. This results in upregulation of pro-inflammatory cytokines and chemokines by neurons and glial cells and further recruitment of immune cells (Didangelos et al., 2016; Yang et al., 2017; Tran et al., 2018; Bradbury and Burnside, 2019), as resident microglial cells. These, in response to injury, assume an amoeboid shape, retracting their processes and becoming phenotypically indistinguishable from invading monocyte-derived macrophages. Together, both monocyte- and microglia-derived macrophages repopulate the lesion core (Bellver-Landete et al., 2019) and assume a pro-inflammatory or a pro-repair phenotype termed, respectively, M1 and M2. This initial mixed M1/M2 phenotype is short-lived, as M2 macrophages tend to dissipate a few days after spinal cord injury (Kigerl et al., 2009) while M1 macrophages remain at the injury site indefinitely (Donnelly and Popovich,

2008), prolonging inflammation and stimulating recruitment of other immune cells (**Figure 5**).

In addition to innate immune cells, adaptive γδ T-cells are recruited shortly after injury and further contribute to the lesion worsening by releasing other pro-inflammatory cytokines (Sun et al., 2018). Leucocytes are also involved in DAMP presentation to T- and B-cells, leading to their expansion (Bradbury and Burnside, 2019). B-cells are also involved in the production of large amounts of auto-antibodies that accumulate at sites of axon pathology and demyelination, further fuelling immune responses (Ankeny et al., 2009).

Since no effective resolution occurs at the injury site, a **chronic spinal cord injury** scar is formed, sealing the damaged tissue. Astrocytes become reactive, hypertrophic and upregulate the expression of a series of molecules, including glial fibrillary acidic protein (GFAP), nestin and vimentin and the extracellular matrix (ECM) components chondroitin sulphate proteoglycans (CSPGs) in a process described as reactive astrocytosis (Tran et al., 2018). The scar that subsequently forms has two main components: the *lesion core*, with a fluid-filled cavity surrounded by fibroblasts, endothelial cells and macrophages, that gives rise to a highly inhibitory environment for regeneration, the fibrotic scar, and; an *astrocytic border* that seals the injury site, restricting immune cell migration into the CNS and sparing non-damaged tissue, therefore allowing re-establishment of tissue homeostasis (O'Shea et al., 2017; Zheng and Tuszynski, 2023) (**Figure 5**). The true role of astrocytic borders is still not fully understood. In the past, astrocyte borders had been solely associated with axonal failure to regenerate after a CNS lesion, as reactive astrocytes secrete CSPGs, traditionally seen as important inhibitors of axonal regeneration (Silver and Miller, 2004). In recent years, however, scar-forming astrocytes

were shown to support post-SCI regeneration by expressing axon-growth supporting molecules and concentrating trophic factors that aid in tissue regeneration (Anderson et al., 2016; Anderson et al., 2018).

OPCs are also involved in this failure to regenerate process after SCI. OPCs proliferate at the injury border and upregulate the expression of the Neuron-glial antigen 2 (NG2) chondroitin sulfate proteoglycan and of GFAP, indicating that, at some point, they can also differentiate into a *de novo* astrocyte population (Levine, 2016; Bradbury and Burnside, 2019), further exacerbating reactive astrocytosis.

Importantly, damaged axons suffer Wallerian degeneration, which contributes to the deposition of myelin-debris and expression of myelin-associated inhibitors (MAIs), such as Neurite outgrowth inhibitor A (Nogo-A), myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp) that, together with CSPGs, act to inhibit neuroplasticity and neuronal regeneration. Therefore, full or even partial recovery of function is often not achieved after a CNS injury, prompting maladaptive neuroplasticity events and leading to the emergence of co-morbidities, such as chronic neuropathic pain and urinary dysfunction.

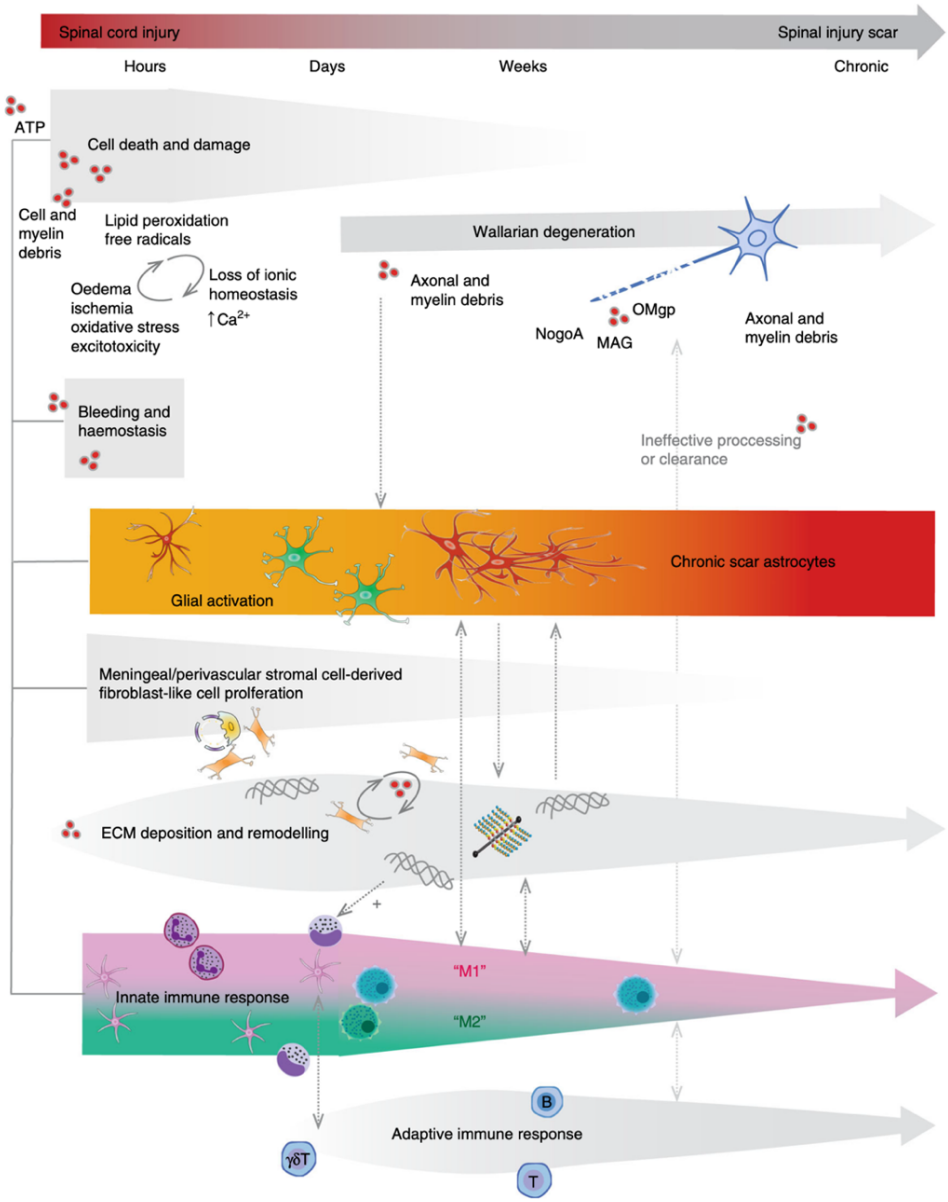

Figure 5. Time course of progressive scar pathology after spinal injury. Following a traumatic spinal cord injury, acute cell death and damage triggers release of cell- and blood-derived damage-associated molecular patterns (DAMP) and ATP, dysregulation of ionic homeostasis, oxidative stress and excitotoxicity, representing potent stimuli for triggering glial cell activation, stromal cell proliferation, deposition of extracellular matrix (ECM) and recruitment of circulating innate immune cells. A few days following injury, monocyte-derived macrophage/microglia adopt a predominantly M1 phenotype which does not favour injury resolution. Proinflammatory innate responders also present DAMP-derived antigens to T and B-cells. B cells, in turn, may present antigens to T-cells, triggering their expansion. During this time, reactive astrocytes proliferate, hypertrophy and overlap, in an attempt to contain the injury zone and spare healthy surrounding tissue. Astrocytes also secrete chondroitin sulphate proteoglycans (CSPGs), responsible for downregulating neuronal plasticity. Wallerian degeneration of axonal tracts contributes to continued deposition of myelin debris, which are ineffectively processed by immune cells, resulting in the deposition of myelin-associated molecules, such as Nogo-A, MAG and OMgp, which further inhibit neuronal regrowth. Ongoing Wallerian degeneration at later post-injury stages further promotes gliosis and neuroinflammation. The dynamic interactions between these different cell types at the injury site eventually leads to ECM remodelling and reactive cellular and extracellular changes, culminating in the formation of the spinal injury scar. *Adapted from Bradbury and Burnside, 2019.*

5. Failure to regenerate vs. maladaptive sprouting: inhibitory molecules of axonal growth

In the adult mammalian nervous system, after a traumatic spinal cord injury, injured neurons display a very limited ability to regrow and reestablish functional synaptic connections. It is important to establish the difference between *regeneration* and *sprouting*, the two forms of growth following an injury. After a CNS injury, distal segments of damaged neurons degenerate over time due to lack of supraspinal input, while the proximal axonal portion, whilst suffering retraction, is still supported by the cell body and able to regrow and thus *regenerate*. While *sprouting* also occurs in response to injury, it is characterized by axonal growth of uninjured neurons that extend their branches into a denervated region, and does not require growth into or around the lesion. Sprouting also occurs spontaneously following injury and is thus easier to achieve, compared to regeneration.

Three complementary theories are currently accepted to elucidate the lack of regeneration ability of the CNS following an injury: **(1)** the reduced inherent ability of the CNS towards regeneration; **(2)** the presence of extrinsic factors that inhibit growth; and **(3)** the lack of growth factors to promote proper regrowth (Zheng and Tuszynski, 2023). In this section, we will focus on extrinsic inhibitors of axonal growth following spinal cord injury and their role in maladaptive processes away from the lesion site. Given the spinal cord's lack of effective resolution of inflammatory processes and cellular recruitment following a lesion, the scar formed at the injury site plays an important role in the containment of the damaged tissue, minimizing secondary injury (Sofroniew, 2015). However, the deposition of CSPGs by astrocytes and OPCs at the astrocytic border and the release of myelin debris and expression of myelin-associated

inhibitory molecules by damaged axons account for the lack of spinal regeneration. These inhibitory molecules bind to an array of receptors and, together with their downstream effectors, compromise post-injury tissue repair.

5.1 Myelin-associated inhibitors of axonal growth

Inhibition of axon outgrowth by CNS myelin has been demonstrated decades ago (Schwab and Thoenen, 1985; Caroni and Schwab, 1988b; Savio and Schwab, 1989) and early studies showed the presence of specific proteins in the myelin sheath with inhibitory properties for sprouting axons. While some remain to be identified, Nogo-A, MAG and OMgp are now widely known as strong blockers of neurite outgrowth.

i. Nogo-A

Nogo is a member of the reticulon (Rtn)-family of membrane proteins, expressed in three isoforms: Nogo-A, -B and -C. These isoforms are derived from alternate promoter and splicing, but exhibit a common C-terminal domain related to the Rtn-family of proteins (GrandPré et al., 2000; Oertle and Schwab, 2003; Oertle et al., 2003). Nogo-A (*Rtn4A*) is expressed by oligodendrocytes that surround myelinated axons in the adult CNS (Caroni and Schwab, 1988b, a) and subpopulations of central and peripheral neurons (Chen et al., 2000). Nogo-A is present in the endoplasmic reticulum and cell membrane (Schwab, 2010) and encompasses two extracellular inhibitory domains: Nogo-66 and Nogo-Δ20. Nogo-66 interacts with Nogo receptor 1 (NgR1) (Fournier et al., 2001), that forms a receptor complex with leucine rich repeat and immunoglobulin-like domain-containing NgR interacting protein 1 (Lingo-1) (Mi et al., 2004) and p75 (McMahon et al., 1994) and/or TROY (*Tnfrs19*) (Park et al., 2005; Shao et al., 2005).

Nogo-66 can also bind to the paired immunoglobulin-like receptor B (Pir-B) (Atwal et al., 2008). Nogo-Δ20 binds to membrane protein tetraspanin-3 (TSPAN3) (Thiede-Stan et al., 2015), which co-clusters with sphingosine-1 phosphate receptor 2 (S1PR2) (Kempf et al., 2014) (**Figure 6**).

In vivo studies using antibodies to block Nogo-A in rodents were initiated in the 1990's. Anti-Nogo-A antibody therapies induced regeneration of damaged tracts (Schnell and Schwab, 1990; Brösamle et al., 2000) and improved sensorimotor function after SCI (Schnell and Schwab, 1990, 1993; Bregman et al., 1995; Z'Graggen et al., 1998; Raineteau et al., 1999; Merkler et al., 2001; Liebscher et al., 2005; Freund et al., 2006; Freund et al., 2007). Anti-Nogo-A antibodies were also effective in restoring autonomous bladder voiding in SCI rats (Liebscher et al., 2005) and preventing the development of urinary dysfunction, in particular DSD (Schneider et al., 2019; Sartori et al., 2020) in rats with incomplete SCI. Moreover, when combined with intense locomotor training, anti-Nogo-A therapy enhanced locomotor recovery of rats with incomplete but severe hemisection lesions, in comparison to single interventions (Chen et al., 2017). The promising effects of anti-Nogo-A therapy have also been demonstrated in non-human primates (Fouad et al., 2004; Freund et al., 2007; Freund et al., 2009; Hoogewoud et al., 2013) and clinical combined application of anti-Nogo-A antibodies and physical rehabilitation for acute cervical spinal injuries were investigated in a multicentric, European, randomized, double-blind and placebo-controlled phase II trial that was closed in 2022 with more than 110 patients enrolled. Preliminary results from the study suggest improved recovery profiles of sensorimotor functions in patients receiving anti-Nogo, compared to placebo (nisci-2020.eu).

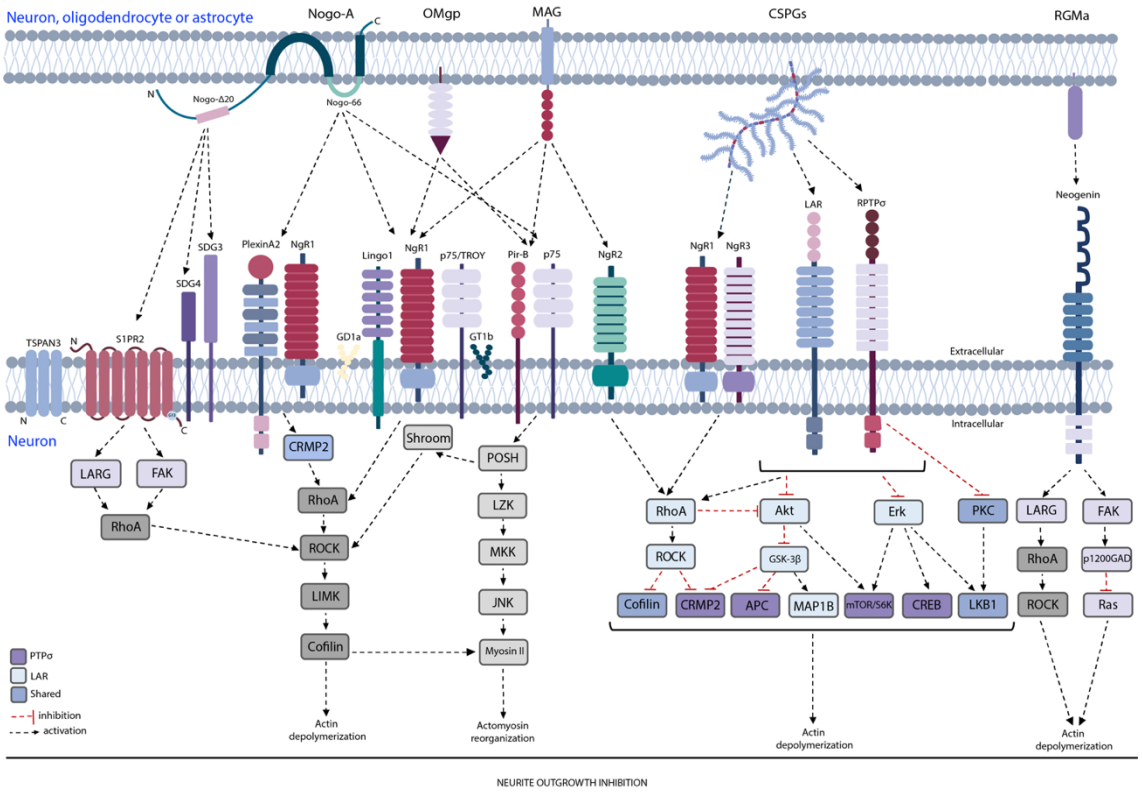

Figure 6. Molecular mechanisms of inhibitory and repulsive molecules signalling. Myelin-associated inhibitors Neurite outgrowth inhibitor A (Nogo-A), through its Nogo-66 inhibitory domain, oligodendrocyte myelin glycoprotein (OMgp) and myelin-associated glycoprotein (MAG) collectively signal through the receptor complexes Nogo receptor 1 (NgR1), leucine-rich repeat and Ig domain containing 1 (Lingo-1) and p75 neurotrophin receptor/tumour necrosis factor receptor superfamily member 19 (p75/TROY), and through the NgR1 and paired immunoglobulin-like receptor B (Pir-B). MAG can also signal through the NgR1 homolog Nogo receptor 2 (NgR2) and via the gangliosides GD1a and GT1b. Additionally, Nogo-66 signals through the NgR1, PlexinA2 and Collapsin response mediator protein 2 (CRMP2). Nogo-Δ20, another Nogo-A inhibitory domain, signals through the receptor complex composed by Tetraspanin 3 (TSPAN3), Sphingosine-1-Phosphate Receptor 2 (S1PR2) and the syndecans SDG3 and SDG4, either via Leukemia-associated Rho guanine nucleotide exchange factor (LARG) or focal adhesion kinase (FAK) signalling, both converging to the Ras Homolog Family Member A/Rho-associated protein kinase (RhoA/ROCK) signalling pathway. NgR1, together with Lingo-1 and p75/Troy signals through RhoA/ROCK, LIM kinase (LIMK) and Cofilin, the same signalling pathway followed by PlexinA2, NgR1 and CRMP2 complex. NgR1 and Pir-B signalling can activate plenty of sarcoma (src) homology domain 3 (SH3) (POSH) through an unknown mechanism. POSH, in its turn, can activate the RhoA/ROCK signalling through Shroom or can activate a kinase cascade via leucine zipper kinase (LZK), mitogen-activated protein (MAP) kinase kinase (MKK), Jun N-terminal kinase (JNK) and Myosin II. LOTUS, an endogenous NgR1 inhibitor, can bind to this receptor and suppress axonal growth inhibition. Chondroitin sulphate proteoglycans (CSPGs) can induce growth inhibition by binding to NgR1 and NgR3 receptors, as well as receptor protein tyrosine phosphatase σ (RPTPσ) and leucocyte common antigen related (LAR). When activated, these receptors activate the RhoA/ROCK signalling pathway and, in the case of RPTPσ and LAR, inactivate Akt and Erk pathways to inhibit axonal growth. When the repulsive molecule RGMa binds to its receptor Neogenin, the RhoA/ROCK pathway can be activated through LARG activation; in parallel, RGMa/Neogenin signalling can go inhibit FAK and p120GAP and also induce actin depolymerization and inhibition of neuronal outgrowth. *Adapted from Chambel and Cruz, 2023.*

ii. *MAG*

MAG is a type-1 transmembrane protein and a member of the Siglec family of sialic acid binding proteins and the first myelin-associated inhibitor to be identified (McKerracher et al., 1994; Mukhopadhyay et al., 1994). MAG comprises two isoforms: large (L)-MAG, mainly expressed in the PNS, and small (S)-MAG, predominant in the CNS (Salzer et al., 1987; Miescher et al., 1997). MAG is expressed by myelinating glia, PNS Schwann cells and CNS oligodendrocytes (Quarles, 2007). It plays a dual role according to the developmental stage: MAG induces axonal growth in the embryo (Johnson et al., 1989; Turnley and Bartlett, 1998) but neurite outgrowth of adult neurons is inhibited by MAG (McKerracher et al., 1994; Mukhopadhyay et al., 1994; Shen et al., 1998), a switch that is age- and neuron type-dependent (DeBellard et al., 1996; Shen et al., 1998). MAG exerts its effect by binding independently to several receptors, including the GPI-anchored NgR1 and its homolog Nogo receptor 2 (NgR2) (Venkatesh et al., 2005). However, NgR receptors appear to play a minor role in outgrowth inhibition by MAG, as sensory neurons from *NgR1/2* double *knockout* mice exposed to MAG are still moderately inhibited (Wörter et al., 2009), indicating the use of alternative receptors that include neuronal gangliosides GD1a and GT1b (Yang et al., 1996; Vyas et al., 2002; Vinson et al., 2003), as well as Pir-B (Atwal et al., 2008), β1-integrin (Goh et al., 2008) and low density lipoprotein receptor-related protein 1 (LRP1) (Stiles et al., 2013) (**Figure 6**). After SCI, *Mag*-null mice show no enhancement in axonal regeneration or locomotion (Bartsch, 1996; Cafferty et al., 2010). Additionally, even though *Mag*-KO mice exhibit sprouting of serotonergic fibers at the lumbar enlargement below the level of hemisection injury, deleting MAG in both hemisection and pyramidotomy models decreases corticospinal tract (CST) sprouting and does not promote its regeneration (Lee

et al., 2010). These findings suggest that MAG may play some sort of neuroprotective role after CNS injury (Lee et al., 2010; Silver, 2010). However, lack of published studies on the role of MAG in CNS regeneration precludes the true understanding its function.

iii. *OMgp*

OMgp is a member of the leucine-rich repeat protein family. It is a membrane-bound protein, connecting to the cell surface via a GPI-anchor. OMgp is expressed by oligodendrocytes and mature CNS neurons, in the glial-axonal border of myelinated axons in the brain and spinal cord (Vourc'h and Andres, 2004; Lee et al., 2009) and by astrocytes (Zhang et al., 2014). Like Nogo-A and MAG, OMgp binds to NgR1/Lingo-1/p75 and/or TROY receptor complex and Pir-B (Wang et al., 2002b; Atwal et al., 2008) (**Figure 6**). Much less is known about OMgp, compared to other myelin inhibitors, but studies have demonstrated its strong inhibitory properties over axonal growth (Kottis et al., 2002; Wang et al., 2002b). After spinal cord injury, OMgp is upregulated at the injury site (Guo et al., 2007) and involved in neurite outgrowth inhibition when it synergizes with MAG and Nogo-A (Ji et al., 2008; Cafferty et al., 2010). No alterations in OMgp expression were detected above and below the injury site after complete spinal cord transection in the rat (Guo et al., 2007; Chambel et al., 2022).

5.2 Scar-associated regulators of axonal growth

i. Chondroitin sulphate proteoglycans

Chondroitin sulphate proteoglycans are a diverse group of glycoproteins, and in the spinal cord they are ubiquitously expressed in the ECM or surface of several cells (Maeda, 2015). CSPGs consist of core proteins to which one or more glycosaminoglycan

(GAG) chains are attached via serine residues. CSPGs are mainly synthesized by astrocytes and neurons, but after spinal cord injury, they can also be expressed by OPCs and macrophages (Jones et al., 2002). CSPGs are named after their core proteins and are divided into lecticans (Versican, Neurocan, Brevican and Aggrecan), Phosphacan, NG2 and the small leucine-rich proteoglycans Decorin and Biglycan (Mironova and Giger, 2013; Bradbury and Burnside, 2019). CSPGs bind to membrane-bound receptors that including receptor-type protein tyrosine phosphatase sigma (RPTPσ) (Shen et al., 2009), leukocyte common antigen-related phosphatase (LAR) (Fisher et al., 2011), NgR1 and Nogo receptor 3 (NgR3) (Dickendesher et al., 2012) (**Figure 6**).

In the normal CNS, CSPGs are expressed during development, regulating axon guidance. In the adult, they modulate synaptic connections and cell migration (Viapiano and Matthews, 2006). After CNS injury, CSPGs are upregulated in the astrocytic border of the lesion scar, inhibiting neurite outgrowth both *in vitro* (Jin et al., 2018) and *in vivo* conditions (Andrews et al., 2012), blocking both axonal sprouting and regeneration of damaged tracts. Alterations in CSPGs levels distant from the injury have also been observed, both in distal lumbar and cervical enlargements (Andrews et al., 2012).

ii. Repulsive guidance molecule A

Repulsive guidance molecule A (RGMa) is part of the repulsive guidance molecules family. RGMa is present in myelin and accumulates in the glial scar after SCI (Schwab et al., 2005), where it is expressed in neurons, oligodendrocytes, astrocytes, activated microglia and macrophages (Mothe et al., 2017). RGMa binds to its neuronal receptor, Neogenin (Neo) (Rajagopalan et al., 2004), inhibiting neurite outgrowth (Tassew et al., 2012; Yamashita, 2019) (Figure 1). RGMa blockade has been showed to promote

regeneration, plasticity and recovery following spinal cord injury in rats (Hata et al., 2006; Mothe et al., 2017; Mothe et al., 2020) and mice (Nakanishi et al., 2019). When administered to non-human primates, elezanumab, an anti-RGMa antibody, promoted neuroprotection, neuroplasticity and recovery of neuromotor function after a hemicompression thoracic spinal injury (Nakagawa et al., 2019; Jacobson et al., 2021). These promising outcomes prompt the administration of elezanumab in a phase II randomized, double-blind, placebo-controlled proof of concept clinical assay that is currently in the recruitment stage.

5.3 Receptors of molecular inhibitors of axonal outgrowth

i. NgR1

Myelin inhibitors are known to induce growth cone collapse after SCI via activation of their surface receptors. This was demonstrated following the identification of NgR1 as a high affinity receptor for both Nogo-A, MAG and OMgp (Fournier et al., 2001; Domeniconi et al., 2002; Liu et al., 2002; Wang et al., 2002b), as dorsal root ganglion (DRG) neurons from *NgR1*-null mice did not show evidence of growth cone collapse when acutely exposed *in vitro* to soluble Nogo-A, MAG and OMgp (Kim et al., 2004; Chivatakarn et al., 2007). *In vivo*, *NgR1* deletion in SCI animals results in improved functional recovery and neuronal regeneration (Kim et al., 2004). NgR1 blockade via a soluble truncated NgR1 fusion protein proved to be effective in treating rats acutely (Li et al., 2004; Wang et al., 2006) and chronically (Wang et al., 2011) after SCI, promoting axonal growth and functional recovery. Pre-clinical studies in non-human primates further demonstrated the efficacy of this fusion protein (Wang et al., 2020) and a clinical trial to assess the safety, tolerability, pharmacokinetics and efficacy of this decoy protein

in chronic cervical SCI patients is currently undergoing. However, targeting Ngr1 to treat SCI might not be sufficient, as this GPI-anchored receptor requires other co-receptors, such as p75 and TROY, to initiate signal transduction.

ii. p75 and TROY

The function of p75 is complex and difficult to discern. When by itself or in association with Trk receptors, p75 can promote cell survival and nerve regeneration, but its activation has also been associated with cell death (Roux and Barker, 2002). Furthermore, p75 is known to co-localize with NgR1 and Pir-B, indicating its role in signaling transduction of myelin inhibitors (Wang et al., 2002a; Wong et al., 2002; Fujita et al., 2011). In a mouse model of spinal contusion, p75 was shown to be essential for neuronal cell survival and locomotor recovery (Chu et al., 2007), while another study showed that genetic deletion or blockade with Fc-fusion protein promoted axonal regeneration after SCI in mice (Song et al., 2004), suggesting that p75 signaling could be compensated by other co-receptors. Another study used a different Fc-fusion protein, which also blocked p75. In the same model, this resulted in axonal regeneration and functional recovery (Wang et al., 2015). These conflicting results could be explained by differences in experimental timepoints for treatment delivery (2 versus 6 weeks post-injury) and might reveal species-dependent differences. In contrast, the role of p75 signaling is rather clear in urinary dysfunction after SCI. Systemic administration to mice of LM11A-31, an agent that blocks p75 activation by pro-NGF, prevented urothelial hyperplasia and detrusor hypertrophy, reduced bladder pressure and hyperreflexia, increased bladder capacity and led to earlier development of automatic micturition (Ryu et al., 2018). Another study using LM11A-31 ameliorated neurogenic detrusor

overactivity and DSD, increasing voiding efficiency together with preservation of the urothelial layer (Zabbarova et al., 2018).

TROY is a functional homolog of p75 and was independently identified by two groups (Park et al., 2005; Shao et al., 2005). Neurons from *Troy*-null mice are less sensitive to myelin inhibitors (Shao et al., 2005) and TROY negatively modulates CNS remyelination. When OPCs submitted to *Troy* knockdown were transplanted into the spinal cord of SCI rats, increased remyelination and recovery were observed (Sun et al., 2014), confirming the role of TROY in blocking axonal growth.

iii. Lingo-1

Lingo-1 is an element of the NgR1/p75 and/or TROY receptor complex (Mi et al., 2004). Lingo-1 activation appears to be critical for CNS myelination, inhibiting oligodendrocyte differentiation and myelination in mice (Jepson et al., 2012) and zebrafish (Yin and Hu, 2014). Lingo-1 blockade leads to increased myelination competence (Mi et al., 2005), while its blockade with a Lingo-1-Fc protein improved neuronal and oligodendrocyte survival, as well as axonal sprouting after SCI (Ji et al., 2006).

iv. Pir-B

Pir-B is a receptor for Nogo-A, MAG and OMgp (Atwal et al., 2008). Genetic ablation or pharmacological blockage of Pir-B *in vitro* promotes neurite outgrowth on substrates rich in myelin inhibitors or CNS myelin (Atwal et al., 2008). After SCI, Pir-B is overexpressed in DRG neurons, sciatic nerves and spinal cord segments (Peng et al.,

2015). Like NgR1, Pir-B can associate with p75 and mediate inhibition of axon growth (Fujita et al., 2011).

v. NgR3, LAR and *RPTPσ*

NgR3, LAR and RPTPσ are the exclusive high-affinity receptors for CSPGs. Several studies using genetic ablation of these receptors demonstrated their role in regulating axonal growth. In cell cultures, CSPGs bind to NgR1 and NgR3 with high affinity, resulting in inhibition of axonal growth (Dickendesher et al., 2012). In contrast, in a *in* vivo mouse model of optic nerve crush injury, genetical deletion of *NgR3* and *NgR1* resulted in full regeneration of damaged tracts. This regeneration was enhanced when RPTP*σ* was also genetically ablated in *NgR1*- and *NgR3*-null mice (Dickendesher et al., 2012), demonstrating the critical role of RPTPσ in CSPG-mediated inhibition. The importance of RPTPσ and LAR in CSPG-mediated axonal inhibition has also been demonstrated in SCI models. Mice in which RPTPσ was genetically eliminated exhibit increased regrowth of sensory and CST axons into the scar and caudal spinal cord, respectively (Shen et al., 2009; Fry et al., 2010). *Lar*-null mice showed improved locomotor function and sprouting of serotonergic and CST axons after SCI (Xu et al., 2015). Deleting genes encoding RPTPσ and LAR in adult neuronal cultures produced synergistic enhancements of axon growth (Ohtake et al., 2016). Furthermore, pharmacological inhibition of RPTPσ and LAR signaling has been showed to improve functional outcomes and sprouting of serotonergic fibers in spinal cord injured rats (Xie et al., 2006; Fisher et al., 2011; Lang et al., 2015) and to promote recovery of diaphragm function after cervical SCI (Urban et al., 2020; Cheng et al., 2021).

5.4 Downstream signalling pathways of inhibitory molecules for axonal growth

Following SCI, damaged neurons extend axons in an attempt to reconnect with other neurons. When their axonal growth cones contact with inhibitory molecules, present in and around the glial scar, the receptors for those molecules are activated, setting in motion downstream signaling pathways involved in cytoskeletal dynamics, the most well studied of which is the Ras homolog family member A (RhoA)/Rho kinase (ROCK) pathway. When activated, RhoA/ROCK promotes F-actin disassembly in growth cones and hinders axon elongation, by preventing microtubule recruitment, which ultimately results in growth cone collapse (Maekawa et al., 1999; Wu et al., 2005). When active, Rho-GTP activates its downstream effector, ROCK. In turn, activated ROCK phosphorylates several downstream effectors, such as LIM kinase (LIMK), collapsin response mediator protein (CRMP2), adducin, myosin light chain, among others, eventually leading to growth cone collapse and neurite outgrowth inhibition (Boghdadi et al., 2018; Kimura et al., 2021).

NgR1-mediated signaling pathway includes RhoA, ROCK, LIMK and Cofilin, eventually resulting in actin depolymerization (Hsieh et al., 2006). Pir-B signals through different but interconnected downstream effectors, including plenty of sarcoma (src) homology domain 3 (SH3) (POSH), that either converges with the ROCK pathway; or activates the leucine zipper kinase (LZK), mitogen-activated protein (MAP) kinase kinase (MAPKK), Jun N-terminal kinase (JNK) and Myosin II, eventually resulting in inhibition of axonal growth (Taylor et al., 2008; Gou et al., 2014; Boghdadi et al., 2018) (**Figure 6**). CSPG activation of RPTPσ and LAR also results in downstream activation of RhoA/ROCK and blockade of Akt and extracellular signal-regulated kinase (ERK) cascades (Ohtake et al., 2016), as described in **Figure 6**.

Myelin inhibitors and CSPGs share several common downstream effectors that can potentially crosstalk and compensate for one another, hence presenting as potential therapeutic targets for SCI treatment. RhoA inhibition has attracted considerable attention and C3 transferase, obtained from *Clostridium botulinum*, has been shown to selectively inhibit RhoA. Tested in several pre-clinical studies, RhoA inactivation with C3 transferase resulted in axonal sprouting and recovery of locomotor function after SCI (Dergham et al., 2002; Dubreuil et al., 2003), by reversing Rho activation and preventing p75-dependent cell death. The clinical application of VX-210, a C3 transferase undergoing clinical trials, was already tested in a phase 2b/3, double-blind, randomized, placebo controlled, multicenter clinical trial (Fehlings et al., 2018). Despite promising pre-clinical data, the final analysis of the VX-210 clinical trials revealed no statistically significant differences between the treatment and placebo groups (Fehlings et al., 2021).

Main goals

As reviewed above, neuronal control of the lower urinary tract is interrupted after a spinal cord injury, disturbing the normal micturition cycle. Spinal cord injury triggers an abnormal sprouting of sensory afferents, which extend their central terminals into the lumbosacral spinal cord in response to increased NGF levels. This leads to the establishment of new neuronal circuits, independent of supraspinal input, and responsible for a new micturition reflex linked to development of NDO. This new spinal reflex is a clear example of maladaptive plasticity, as NDO results in urinary incontinence.

Importantly, while at the lumbosacral spinal cord there is an exaggerated sprouting response from sensory neurons, at the injury site, axonal growth necessary to re-establish synaptic connections is almost absent. This failure to regenerate is associated with various factors, including the upregulation of highly inhibitory extrinsic factors by neurons and glial cells. In the absence of traumatic SCI, the presence of those inhibitory molecules in spinal tissue is low but still relevant to limit sprouting. This suggests that, after a spinal cord injury, the extracellular milieu at the lumbosacral spinal cord might be differently regulated, in comparison with the lesion site, possibly being less inhibitory and allowing axonal growth. In addition to changes in the inhibitory composition of the extracellular environment, it might be possible that expanding central terminals of sensory afferents at the lumbosacral level may become less sensitive to inhibitory cues after traumatic high-level SCI. Both hypotheses might be simultaneously true.

As such, this thesis had the following goals:

Goal 1: To define the extrinsic factors associated with abnormal sprouting of bladder afferents and neurogenic detrusor overactivity emergence after a spinal cord injury.

a) To identify and quantify inhibitory molecules of axonal growth at the lumbosacral spinal cord and their respective receptors in dorsal root ganglia neurons, in spinal intact and spinal cord injured animals at different time points of disease progression (***Publication II***);

b) To identify alterations in the expression of receptors of inhibitory molecules in dorsal root ganglia neurons from spinal intact and spinal cord injured animals exposed to different concentrations of NGF, a key neurotrophin regulating axonal growth of sensory afferents upregulated in the nervous system and bladder after spinal cord injury (***Publication II***);

Goal 2: To investigate the effects of early NGF modulation after SCI on bladder function and axonal growth.

a) To explore the role of early NGF modulation in the emergence of neurogenic detrusor overactivity after spinal cord injury, in spinal intact and spinal cord injured animals at different time points of disease progression (***Publication III***);

b) To identify alterations in the expression of inhibitory molecules of axonal growth in the lumbosacral spinal cord of spinal intact and after SCI when NGF is modulated immediately after the injury (***Publication III***).

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Publications

Publication I

Chambel SS, Cruz CD (2023) Axonal growth inhibitors and their receptors in spinal cord injury: from biology to clinical translation. Neural Regeneration Research 8(12) 2573-2581.

Axonal growth inhibitors and their receptors in spinal cord injury: from biology to clinical translation

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Abstract

Axonal growth inhibitors are released during traumatic injuries to the adult mammalian central nervous system, including after spinal cord injury. These molecules accumulate at the injury site and form a highly inhibitory environment for axonal regeneration. Among these inhibitory molecules, myelin-associated inhibitors, including neurite outgrowth inhibitor A, oligodendrocyte myelin glycoprotein, myelin-associated glycoprotein, chondroitin sulfate proteoglycans and repulsive guidance molecule A are of particular importance. Due to their inhibitory nature, they represent exciting molecular targets to study axonal inhibition and regeneration after central injuries. These molecules are mainly produced by neurons, oligodendrocytes, and astrocytes within the scar and in its immediate vicinity. They exert their effects by binding to specific receptors, localized in the membranes of neurons. Receptors for these inhibitory cues include Nogo receptor 1, leucine-rich repeat, and Ig domain containing 1 and p75 neurotrophin receptor/tumor necrosis factor receptor superfamily member 19 (that form a receptor complex that binds all myelin-associated inhibitors), and also paired immunoglobulin-like receptor B. Chondroitin sulfate proteoglycans and repulsive guidance molecule A bind to Nogo receptor 1, Nogo receptor 3, receptor protein tyrosine phosphatase σ and leucocyte common antigen related phosphatase, and neogenin, respectively. Once activated, these receptors initiate downstream signaling pathways, the most common amongst them being the RhoA/ROCK signaling pathway. These signaling cascades result in actin depolymerization, neurite outgrowth inhibition, and failure to regenerate after spinal cord injury. Currently, there are no approved pharmacological treatments to overcome spinal cord injuries other than physical rehabilitation and management of the array of symptoms brought on by spinal cord injuries. However, several novel therapies aiming to modulate these inhibitory proteins and/or their receptors are under investigation in ongoing clinical trials. Investigation has also been demonstrating that combinatorial therapies of growth inhibitors with other therapies, such as growth factors or stem-cell therapies, produce stronger results and their potential application in the clinics opens new venues in spinal cord injury treatment.

Key Words: chondroitin sulphate proteoglycans; collapsin response mediator protein 2; inhibitory molecules; leucine-rich repeat and Ig domain containing 1; leucocyte common antigen related; myelin-associated glycoprotein; neurite outgrowth inhibitor A; Nogo receptor 1; Nogo receptor 3; oligodendrocyte myelin glycoprotein; p75 neurotrophin receptor; PlexinA2; Ras homolog family member A/Rho-associated protein kinase; receptor protein tyrosine phosphatase σ; repulsive guidance molecule A; spinal cord injury; tumour necrosis factor receptor superfamily member 19

Introduction

In the adult mammalian nervous system, following a traumatic spinal cord injury (SCI), neurons are unable to regenerate synaptic contacts due to the reduced inherent ability of the central nervous system (CNS), the presence of extrinsic factors that inhibit growth and reduced levels of correct growth factors (Zheng and Tuszynski, 2023). Immediately after trauma, damaged nervous tissue undergoes a series of immune, inflammatory and apoptotic events. The inflammatory response is mediated by invading macrophages, resident microglia, and oligodendrocyte progenitor cells (OPCs) that, together with damaged neurons, astrocytes, and myelin sheaths, release myelin-associated inhibitors and chondroitin sulfate proteoglycans (CSPGs) (Filbin, 2003; Boghdadi et al., 2018). The "scar" formed at the injury site seals the damaged tissue, creating an astrocytic outer border that minimizes secondary injury (Sofroniew, 2015). Myelin-associated inhibitors and CSPGs inhibit axonal regeneration upon binding to an array of molecular receptors and, together with their downstream effectors, compromise post-SCI tissue repair. Because regeneration of damaged circuits is blocked, SCI results in marked alterations in sensory, motor, and autonomic function. The quality of life of patients is thus severely affected. The interruption of ascending and descending neuronal tracts and the development of alternative neuronal circuits independent from supraspinal input that result from spontaneous sprouting of spared axons, give rise to compensatory but oftentimes erroneous plasticity that results in events such as pain or bladder dysfunction. This review addresses current knowledge on interactions of inhibitory molecules of axonal growth with their receptors and the downstream signaling pathways that lead to failure to regenerate and the state of the art on the application of this knowledge to the clinics.

Search Strategy

Literature search was performed on PubMed database on inhibitory molecules of axonal growth after SCI, their receptors and state of the art on the application of this knowledge to animal models and the clinics to treat spinal cord injuries. Original search was conducted between September and December 2022 to include progress in this field that spans at least the last 25 years.

Molecular Inhibitors of Axonal Growth

Myelin-associated inhibitors of axonal growth

Inhibition of axon outgrowth by CNS myelin has been demonstrated decades ago (Schwab and Thoenen, 1985; Caroni and Schwab, 1988a; Savio and Schwab, 1989) and early studies showed the presence of specific proteins in the myelin sheath with inhibitory properties for sprouting axons. While some remain to be identified, neurite outgrowth inhibitor A (Nogo-A), myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMgp) are now widely known as strong blockers of neurite outgrowth.

Nogo-A

Nogo is a member of the reticulon (Rtn)-family of membrane proteins, expressed in three isoforms: Nogo-A, -B, and -C. These isoforms are derived from the alternate promoter and splice usage but exhibit a common C-terminal domain related to the Rtn-family of proteins (GrandPré et al., 2000; Oertle and Schwab, 2003; Oertle et al., 2003). Nogo-A (*Rtn4A*) is expressed in oligodendrocytes that surround myelinated axons in the adult CNS (Caroni and Schwab, 1988a, b) and subpopulations of central and peripheral neurons (Chen et al., 2000). Nogo-A is expressed by the endoplasmic reticulum and cell membrane (Schwab, 2010) and encompasses two extracellular inhibitory

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domains: Nogo-66 and Nogo-Δ20. Nogo-66 interacts with Nogo receptor 1 (NgR1) (Fournier et al., 2001), but due to the lack of an intracellular domain, NgR1 forms a receptor complex with leucine-rich repeat and immunoglobulin-like domain-containing NgR interacting protein 1 (Lingo-1) (Mi et al., 2004) and p75 (McMahon et al., 1994) and/or TROY (*Tnfrs19*) (Park et al., 2005; Shao et al., 2005). Nogo-66 can also bind to the paired immunoglobulin-like receptor B (Pir-B) (Atwal et al., 2008). Nogo-Δ20 binds to membrane protein tetraspanin-3 (Thiede-Stan et al., 2015), which co-clusters with sphingosine-1 phosphate receptor 2 (Kempf et al., 2014; **Figure 1**).

In vivo studies using antibodies to block Nogo-A in rodents were initiated decades ago. Anti-Nogo-A antibody therapies induced the regeneration of damaged tracts (Schnell and Schwab, 1990; Brösamle et al., 2000) and improved sensory motor function after SCI (Schnell and Schwab, 1990, 1993; Bregman et al., 1995; Z'Graggen et al., 1998; Raineteau et al., 1999; Merkler et al., 2001; Liebscher et al., 2005; Freund et al., 2006, 2007). Anti-Nogo-A antibodies are also effective in restoring autonomous bladder voiding in SCI rats (Liebscher et al., 2005). More recently, work from Schwab's lab showed that the application of anti-Nogo-A antibodies to rats with incomplete SCI prevented the development of urinary dysfunction, in particular, detrusor-sphincter dyssynergia (Schneider et al., 2019; Sartori et al., 2020). The same effects, however, were not visible in animals with complete spinal cord transections, highlighting the importance of spared descending fibers in the reestablishment of lower urinary tract circuits. The effects of anti-Nogo-A therapy were also demonstrated in non-human primates (Fouad et al., 2004; Freund et al., 2007, 2009; Hoogewoud et al., 2013). Moreover, work from Ichiyama's lab showed a promising therapeutic approach for SCI treatment, the combination of anti-Nogo-A antibody administration with intense locomotor training. This leads to heightened recovery of locomotor functions, compared to single interventions (Chen et al., 2017). The clinical combined application of anti-Nogo-A antibodies and physical rehabilitation for SCI treatment is currently being investigated in a multicentric, European, randomized, double-blind, and placebo-controlled phase II trial (Sartori et al., 2020), and it will be discussed ahead.

MAG

MAG is a type-1 transmembrane protein and a member of the Siglec family of sialic acid binding proteins and the first myelin inhibitor to be identified (McKerracher et al., 1994; Mukhopadhyay et al., 1994). MAG comprises two isoforms: large (L)-MAG, mainly expressed in the peripheral nervous system, and small (S)-MAG, predominant in the CNS (Salzer et al., 1987; Miescher et al., 1997). MAG is expressed by myelinating glia, peripheral nervous system Schwann cells, and CNS oligodendrocytes (Quarles, 2007). It plays a dual role according to the developmental stage, as MAG regulates developmental axonal growth (Johnson et al., 1989; Turnley and Bartlett, 1998) but neurite outgrowth of adult neurons is inhibited by MAG (McKerracher et al., 1994; Mukhopadhyay et al., 1994; Shen et al., 1998). This switch is age- and neuron type-dependent (DeBellard et al., 1996; Shen et al., 1998) and makes MAG the only myelin inhibitor that promotes growth during development. MAG exerts its effect by binding independently to several receptors, including the GPI-anchored NgR1 and its homolog Nogo receptor 2 (NgR2) (Venkatesh et al., 2005). However, NgR receptors appear to play a minor role in outgrowth inhibition by MAG, as sensory neurons from *NgR1/2* double knockout mice exposed to MAG are still moderately inhibited (Wörter et al., 2009), indicating that MAG uses alternative receptors. These include neuronal gangliosides GD1a and GT1b (Yang et al., 1996; Vyas et al., 2002; Vinson et al., 2003), to which MAG binds independently, as well as Pir-B (Atwal et al., 2008), β1-integrin (Goh et al., 2008) and low-density lipoprotein receptor-related protein 1 (Stiles et al., 2013; **Figure 1**). After SCI, M*ag*-null mice show no enhancement of axonal growth (Bartsch, 1996), an observation later confirmed by Strittmatter's lab. They showed that Mag deletion does not affect axonal growth or locomotion (Cafferty et al., 2010). Zheng's lab also showed that even though *Mag* knockout mice exhibit sprouting of serotonergic fibers at the lumbar enlargement below the level of hemisection injury, deleting MAG in both hemisection and pyramidotomy models decreases corticospinal tract (CST) sprouting and does not promote its regeneration (Lee et al., 2010b). These findings suggest that MAG may play some sort of neuroprotective role after CNS injury (Lee et al., 2010b; Silver, 2010). However, the lack of published studies on the role of MAG in CNS regeneration precludes the true understanding of MAG function.

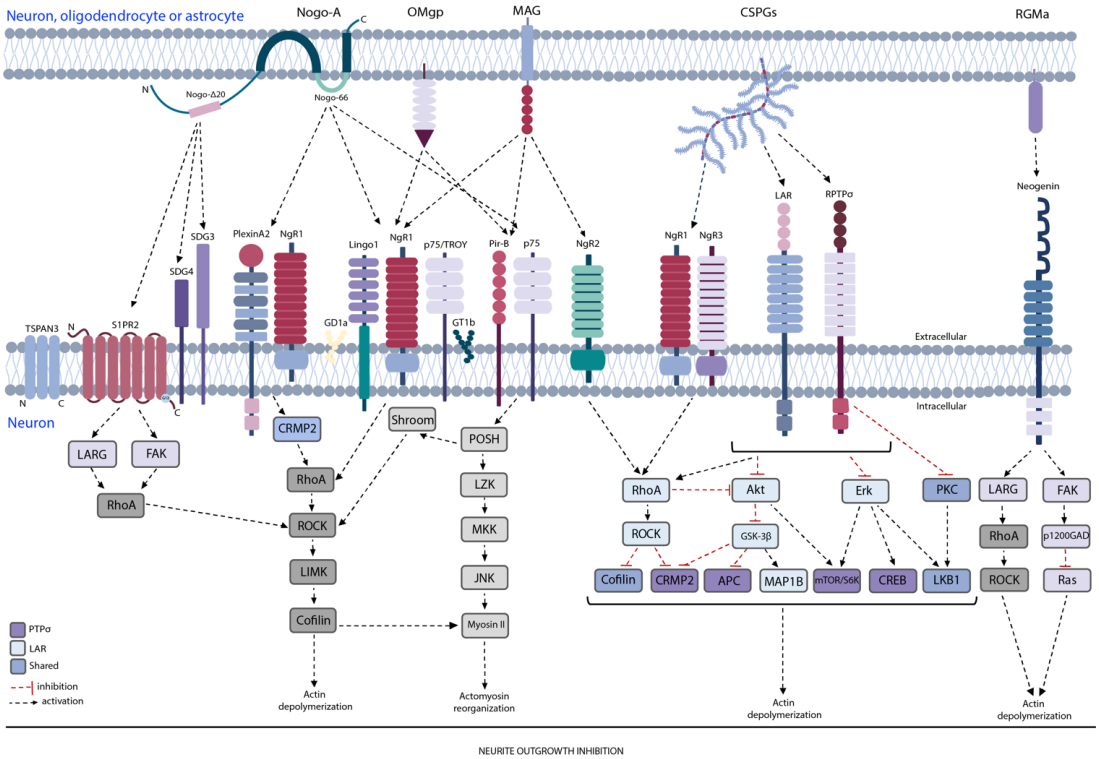

Figure 1 | **Molecular mechanisms of inhibitory and repulsive molecules signaling.**
Myelin-associated inhibitors neurite outgrowth inhibitor A (Nogo-A), through its Nogo-66 inhibitory domain, oligodendrocyte myelin glycoprotein (OMgp) and myelin-associated glycoprotein (MAG) collectively signal through the receptor complexes Nogo receptor 1 (NgR1), leucine-rich repeat and Ig domain containing 1 (Lingo-1) and p75 neurotrophin receptor/tumor necrosis factor receptor superfamily member 19 (p75/TROY), and through the NgR1 and paired immunoglobulin-like receptor B (Pir-B). MAG can also signal through the NgR1 homolog Nogo receptor 2 (NgR2) and via the gangliosides GD1a and GT1b. Additionally, Nogo-66 signals through the NgR1, PlexinA2, and Collapsin response mediator protein 2 (CRMP2). Nogo-Δ20, another Nogo-A inhibitory domain, signals through the receptor complex composed of Tetraspanin 3 (TSPAN3), Sphingosine-1-Phosphate Receptor 2 (S1PR2), and the syndecans SDG3 and SDG4, either via Leukemia-associated Rho guanine nucleotide exchange factor (LARG) or focal adhesion kinase (FAK) signaling, both converging to the Ras Homolog Family Member A/Rho-associated protein kinase (RhoA/ROCK) signaling pathway. NgR1, together with Lingo-1 and p75/Troy signals through RhoA/ROCK, LIM kinase (LIMK), and Cofilin, the same signaling pathway followed by PlexinA2, NgR1, and CRMP2 complex. NgR1 and Pir-B signaling can activate plenty of sarcoma (src) homology domain 3 (SH3) (POSH) through an unknown mechanism. POSH, in its turn, can activate the RhoA/ROCK signaling through Shroom or can activate a kinase cascade via leucine zipper kinase (LZK), mitogen-activated protein (MAP) kinase kinase (MKK), Jun N-terminal kinase (JNK) and Myosin II. LOTUS, an endogenous NgR1 inhibitor, can bind to this receptor and suppress axonal growth inhibition. Chondroitin sulfate proteoglycans (CSPGs) can induce growth inhibition by binding to NgR1 and NgR3 receptors, as well as receptor protein tyrosine phosphatase σ (RPTPσ), and leucocyte common antigen-related (LAR). When activated, these receptors activate the RhoA/ROCK signaling pathway and, in the case of RPTPσ and LAR, inactivate Akt and Erk pathways to inhibit axonal growth. When the repulsive molecule RGMa binds to its receptor neogenin, the RhoA/ROCK pathway can be activated through LARG activation; in parallel, RGMa/neogenin signaling can go inhibit FAK and p120GAP and also induce actin depolymerization and inhibition of neuronal outgrowth. Created with Adobe Illustrator 2021.

OMgp

OMgp is a member of the leucine-rich repeat protein family. It is a membrane-bound protein, connecting to the cell surface via a GPI-anchor. OMgp is expressed by oligodendrocytes, mature CNS neurons, in the glial-axonal border of myelinated axons in the brain and spinal cord (Vourc'h and Andres, 2004; Lee et al., 2009) and by astrocytes (Zhang et al., 2014). Like Nogo-A and MAG, OMgp binds to NgR1/Lingo-1/p75 and/or TROY receptor complex and Pir-B (Wang et al., 2002b; Atwal et al., 2008; **Figure 1**).

Much less is known about OMgp, but studies have demonstrated its strong inhibitory properties on axonal growth (Kottis et al., 2002; Wang et al., 2002b). Early evidence suggested in *OMgp*-null mice there is collateral sprouting at Ranvier nodes (Huang et al., 2005), highlighting the role of OMgp in the integrity of the myelin-axon interface. However, more recent studies reported contrary findings, evidencing the presence of OMgp at the surface of mature myelinated axons but excluded it from compact myelin and nodes (Chang et al., 2010). After SCI, OMgp is upregulated at the injury site (Guo et al., 2007) and involved in neurite outgrowth inhibition when it synergizes with MAG and Nogo-A (Ji et al., 2008; Cafferty et al., 2010). No alterations in OMgp expression are detected above and below the injury site (Guo et al., 2007; Chambel et al., 2022).

Scar-associated players of axonal growth

Following SCI, immune cells, microglia, and OPCs invade the injury site and recruited astrocytes become reactive and hypertrophic. The scar that subsequently forms has two main components: the lesion core with a fluid-filled cavity surrounded by fibroblasts, endothelial cells, and macrophages that give rise to a highly inhibitory environment for regeneration, the fibrotic scar; and an astrocytic border that seals the injury site, restricting migration of immune cells into the CNS and sparing non-damaged tissue, therefore allowing re-establishment of homeostasis (O'Shea et al., 2017; Zheng and Tuszynski, 2023). The true role of astrocytic borders is still not fully understood. In the past, astrocyte borders had been associated with axonal failure to regenerate after a CNS lesion, as reactive astrocytes secrete CSPGs, traditionally seen as important blockers of axonal regeneration (Silver and Miller, 2004). However, in the last few years, scar-forming astrocytes were shown to support post-SCI regeneration. When astrocytic scar formation is hindered, by preventing its formation, inhibiting scar-forming astrocytes or completely ablating chronic scars, transected axons in a mouse model of SCI are unable to regrow (Anderson et al., 2016), while the reverse occurs when growth factors are delivered (Anderson et al., 2016, 2018). The reason for this resides in the expression, by scar-forming astrocytes, of axon-growth supporting molecules, such as CSPG4 and CSPG5, previously thought to only have inhibitory properties (Anderson et al., 2016). Likewise, keratan sulfate proteoglycans, produced by macrophages and OPCs at the lesion site, and known to be highly inhibitory to regenerating axons (Jones et al., 2002), have recently been shown to have pro-regenerative properties. After complete spinal transection, the spiny mouse (*Acomys cahirinus*) exhibits both regeneration of several tracts and synapse formation due to the overexpression pro-regenerative keratan sulfate proteoglycans at the injury site, as well as lack of scar tissue at the lesion site (Nogueira-Rodrigues et al., 2022). Certain populations of neurons have also been demonstrated to express pro-regenerative genes in response to injury. Specifically, rodent spinocerebellar neurons express regeneration-associated genes (RAG), such as *Atf3*, *Sox11*, *Sprr1a*, and *Gap43* in the lumbar cord after thoracic SCI (Matson et al., 2022). These lumbar cord RAG^+ expressing neurons survive contusion, even increasing their numbers and evidencing branching and fiber reorganization in the thoracic cord (Matson et al., 2022). These findings break the widely accepted dogma that after a CNS injury, the CNS environment is exclusively inhibitory towards regrowth.

Guidance molecules, such as semaphorins, ephrins, and WNTs, also participate in post-SCI plasticity and become highly inhibitory, blocking regeneration. They are also important players during development, aiding growth cone navigation in embryonic environments by attracting or repelling growing axons. However, due to their complex roles and many potential interactions with both myelin and the extracellular matrix (Zheng and Tuszynski, 2023), their exact function is not fully understood. Here, we will only focus on CSPGs and RGMa. Further details on the role of guidance molecules can be found elsewhere (Giger et al., 2010; Hollis, 2016).

Chondroitin sulfate proteoglycans - CSPGs

Chondroitin sulfate proteoglycans are a diverse group of glycoproteins ubiquitously expressed in the extracellular matrix or cell surface (Maeda, 2015). CSPGs consist of core proteins to which one or more glycosaminoglycan chains are attached via serine residues. CSPGs are mainly synthesized by astrocytes and neurons, but also by OPCs and macrophages (Jones et al., 2002). CSPGs are named after their core proteins and are divided into lecticans (versican, neurocan, brevican, and aggrecan), phosphacan, neuron-glial antigen 2, and the small leucine-rich proteoglycans decorin and biglycan (Mironova and Giger, 2013; Bradbury and Burnside, 2019). In the normal CNS, CSPGs are expressed during development, regulating axon guidance, and in the adult, modulating synaptic connections and cell migration (Viapiano and Matthews, 2006). After CNS injury, CSPGs are upregulated and become abundant perilesionally and at distal spinal segments (Andrews et al., 2012; Chambel et al., 2022). They inhibit neurite outgrowth both *in vitro* (Jin et al., 2018) and *in vivo* conditions (Andrews et al., 2012), blocking axonal sprouting at the injury site and hindering the regeneration of damaged tracts. CSPGs inhibition of axonal growth requires activation of membrane-bound receptors including the receptor-type protein tyrosine phosphatase sigma (RPTPσ) (Shen et al., 2009), leukocyte common antigen-related phosphatase (LAR) (Fisher et al., 2011), NgR1 and Nogo receptor 3 (NgR3) (Dickendesher et al., 2012) (**Figure 1**).

The inhibitory nature of CSPGs in the CNS is highly variable, reflecting the type of injury, as well as the affected area. In traumatic lesions, CSPG expression can be observed at the injury sites as early as 24 hours and up to months post-injury. For example, in a rat model of cerebral cortex injury, OPCs and astrocyte-derived Versican and Neurocan are upregulated at 7 days-post injury (dpi) (Asher et al., 2000, 2002). After spinal contusion, at the lesion site, neurocan, brevican, and versican are elevated immediately after injury and peak at 14 dpi, with brevican remaining elevated for at least 2 months (Jones et al., 2003). Phosphacan expression in the lesion first decreases after injury to then slowly recover and peaking two months-post injury (Jones et al., 2003). The distribution of specific CSPGs around the lesion also varies. While neurocan and brevican are expressed near the lesion, phosphacan and versican are found further away (Jones et al., 2003). Upregulation of CSPGs at the lesion site has been well-documented and proven to hinder axonal regeneration, but alterations distant from the injury are also observed. An early work by Andrews et al. (2012) showed that after a T8 spinal contusion, aggrecan, neurocan, brevican, and neuron-glial antigen 2 were altered in distal lumbar and cervical enlargements. More recently, Chambel et al. (2022) demonstrated alterations in the expression of both phosphacan and neurocan at the lumbosacral cord in a rat model of thoracic spinal cord transection. Levels of both CSPGs increased 7 dpi and returned to baseline values 28 dpi. Despite the inhibitory properties of phosphacan and neurocan, sprouting of sensory bladder afferents still occurred within the lumbosacral cord, which has been linked to the emergence of bladder dysfunction.

Even though some evidence shows that CSPGs core proteins can inhibit neuronal growth (Ughrin et al., 2003), it is the accessory glycosaminoglycan chains the main responsibles for CSPG-induced blockade of axonal growth. Accordingly, degradation of glycosaminoglycan chains by chondroitinase ABC (ChABC), which exposes the protein core, promotes axonal sprouting and regain of function following SCI (Bradbury et al., 2002; Bartus et al., 2014; Muir et al., 2019; Rosenzweig et al., 2019; Day et al., 2020; Takiguchi et al., 2022). The beneficial effect of ChABC treatment has been firstly shown following *in vivo* administration (Bradbury et al., 2002), and further confirmed in neuronal cultures (Day et al., 2020) and several SCI models, such as contusion (Caggiano et al., 2005), compression (Novotna et al., 2011), crush (Barritt et al., 2006) and transection (Takiguchi et al., 2022) injuries. Its promising and safe use has also been tested in several species, such as mouse, rat, cat, a canine clinical model (Hu et al., 2018), and non-human primates (Rosenzweig et al., 2019). Approaches that combine ChABC with other experimental therapies are even more promising. In models of SCI, combining ChABC administration with neurotrophins (Tom et al., 2009; Lee et al., 2010a; García-Alías et al., 2011; Hunanyan et al., 2013; Kanno et al., 2014), growth factors (Karimi-Abdolrezaee et al., 2012; Lee et al., 2013; Tom et al., 2013; Zhang et al., 2013; Alluin et al., 2014; DePaul et al., 2017), rehabilitation (Wang et al., 2011a; Zhao et al., 2013; Alluin et al., 2014; Hu et al., 2018; Prager et al., 2021), stem cell therapy (Sarveazad et al., 2017; Suzuki et al., 2017; Buzoianu-Anguiano et al., 2020; Jevans et al., 2021), antibodies against Nogo-A (Zhang et al., 2013; Zhao et al., 2013), and various others (Bai et al., 2010; Mountney et al., 2013; Grosso et al., 2014; Janzadeh et al., 2017; Wu et al., 2017; Xia et al., 2017; Liu et al., 2018; Lu et al., 2020) shows enhanced axonal regeneration and functional recovery (locomotor or hand coordination), in comparison with single therapies.

In earlier works, ChABC was delivered intrathecally (Bradbury et al., 2002), but this enzyme was unstable at 37°C. Better results were obtained using other delivery strategies, including synthetic scaffolds or stabilized ChABC, with delivery using lentivirus and adeno-associated viral vectors currently being the most successful and used approach. These vectors can provide stable and long-term expression of ChABC in transduced cells at the injury site (Muir et al., 2019), as well as CSPG digestion over a large area of the cord (Bartus et al., 2014). Expression of ChABC transgenes by adeno-associated viral vectors begins 1 week after delivery and reaches maximal expression at 3 weeks; lentiviral vectors are able to start transgene expression 24-48 hours post-injury. These differences in expression of ChABC via viral vectors allow differential SCI treatment at different stages. In fact, timing and duration of ChABC administration seem to be detrimental for gain-of-function after SCI. Intrathecal administration of high doses of ChABC at the same time of SCI seems to produce nefarious effects (Cheng et al., 2015), including subarachnoid bleeding and animal death, not seen after sub-acute administrations. High-dosage intrathecal administration after spinal transection (Cheng et al., 2015; Janzadeh et al., 2017; Liu et al., 2018) or long-term viral vector delivery after spinal contusion (Bartus et al., 2014; James et al., 2015) result in axonal growth and functional recovery, making 48 hours–7 days following injury the ideal timeframe for ChABC intervention after SCI (Muir et al., 2019). For the optimal time of administration, it is necessary to consider an appropriate timeframe for tissue healing by scar formation and blood-brain barrier leakage (Rolls et al., 2009). Scar tissue, however, should not be fully consolidated. Studies in SCI dogs and monkeys (Hu et al., 2018; Rosenzweig et al., 2019), as well as in humans to treat disc herniation (Chiba et al., 2018) have proven that ChABC is effective and safe, without adverse effects and producing enhanced recovery in treated subjects, and thus evidencing the potential use of ChABC for SCI treatment.

In addition to preventing axonal expansion necessary to repair injured axons, recent evidence highlighted a pro-inflammatory post-SCI role for CSPGs, exacerbating the immune response (Bartus et al., 2014; Didangelos et al., 2014; Dyck et al., 2018). Bradbury's lab showed the involvement of CSPGs in macroglia, macrophages, and T-lymphocytes modulation, preventing the conversion of immune cells at the injury site from a pro-inflammatory to a reparative phenotype. A pro-reparative phenotype can be achieved by lentiviral ChABC digestion, leading to attenuation of the pro-inflammatory environment and immune cell clearance (Francos-Quijorna et al., 2022). These findings highlight the dynamic role CSPGs play in both axon growth and inflammation following SCI and open the doors to immunomodulatory therapies that target both complications.

Repulsive guidance molecule A - RGMa

Repulsive guidance molecule A (RGMa) is part of the repulsive guidance molecule family. RGMa is present in myelin and accumulates in the glial scar after SCI (Schwab et al., 2005), where it is expressed in neurons, oligodendrocytes, astrocytes, activated microglia, and macrophages (Mothe et al., 2017). RGMa binds to its neuronal receptor, neogenin (Rajagopalan et al., 2004), inhibiting neurite outgrowth (Tassew et al., 2012; Yamashita, 2019; **Figure 1**). RGMa blockade promotes regeneration, plasticity, and recovery following SCI in rats (Hata et al., 2006; Mothe et al., 2017, 2020), mice (Nakanishi et al., 2019) and non-human primates (Nakagawa et al., 2019).

Receptors of Molecular Inhibitors of Axonal Outgrowth in Spinal Cord Injury

NgR1

Myelin inhibitors are known to induce growth cone collapse after SCI via activation of their surface receptors. This was demonstrated following the identification of NgR1 as a high-affinity receptor for both Nogo-A, MAG, and OMgp (Fournier et al., 2001; Domeniconi et al., 2002; Liu et al., 2002; Wang et al., 2002b), as dorsal root ganglion neurons from *NgR1*-null mice do not suffer growth cone collapse when acutely exposed *in vitro* to soluble Nogo-A, MAG, and OMgp (Kim et al., 2004; Chivatakarn et al., 2007). *In vivo*, *NgR1* deletion in SCI animals results in improved functional recovery and neuronal regeneration (Kim et al., 2004). Strittmatter's lab developed a strategy to block NgR1 by creating a soluble truncated NgR1 fusion protein- NgR1(310)ecto-Fc (Fournier et al., 2002). Administration of this decoy proved to be effective in treating rats acutely (Li et al., 2004; Wang et al., 2006) and chronically (Wang et al., 2011b) after SCI, increasing axonal growth and functional recovery. Pre-clinical studies in non-human primates further demonstrated the efficacy of NgR-Fc decoy protein and a clinical trial in chronic cervical SCI patients is currently undergoing (see below). However, targeting Ngr1 to treat SCI might not be sufficient, as this GPI-anchored receptor requires other co-receptors, such as p75 and TROY, to initiate signal transduction.

p75 and TROY

The function of p75 is complex and difficult to discern. When by itself or in association with Trk receptors, p75 can promote cell survival and nerve regeneration, but its activation has also been associated with cell death (Roux and Barker, 2002). Furthermore, p75 is known to co-localize with NgR1 and Pir-B, indicating its role in signal transduction of myelin inhibitors (Wang et al., 2002a; Wong et al., 2002; Fujita et al., 2011). In a mouse model of spinal contusion, p75 was shown to be essential for neuronal cell survival and locomotor recovery (Chu et al., 2007). However, genetic deletion or blockade with Fc-fusion protein promoted axonal regeneration after SCI in mice (Song et al., 2004), suggesting that p75 signaling could be compensated by other co-receptors. Another study used a different Fc-fusion protein, which also blocked p75. In the same model, this resulted in axonal regeneration, with decreased RhoA activation, and functional recovery (Wang et al., 2015). These conflicting results could be explained by different experimental time points for treatment delivery (2 *versus* 6 weeks post-injury) and might also reveal species-dependent differences. In contrast, the role of p75 signaling is rather clear in urinary dysfunction after SCI. Systemic administration to mice of LM11A-31, an agent that blocks activation of p75 by nerve growth factor precursor, reduced bladder pressure and hyperreflexia, increased bladder capacity, and led to earlier development of automatic micturition (Ryu et al., 2018). LM11A-31 also prevented urothelial hyperplasia and detrusor hypertrophy (Ryu et al., 2018). Similar results were obtained by Zabbarova et al. (2018). In this case, LM11A-31 ameliorated neurogenic detrusor overactivity and detrusor-sphincter dyssynergia, leading to greater voided volumes, which was also accompanied by preservation of the urothelial layer.

TROY is a functional homolog of p75 and was independently identified by two groups (Park et al., 2005; Shao et al., 2005). Neurons from *Troy*-null mice are less sensitive to myelin inhibitors (Shao et al., 2005) and TROY negatively modulates CNS remyelination. When OPCs submitted to *Troy* knockdown were transplanted into the spinal cord of SCI rats, increased remyelination and recovery were observed (Sun et al., 2014), confirming the role of TROY in blocking axonal growth.

Lingo-1

Lingo-1 is an element of the NgR1/p75 and/or TROY receptor complex (Mi et al., 2004). Lingo-1 activation appears to be critical for CNS myelination, inhibiting oligodendrocyte differentiation and myelination in mice (Jepson et al., 2012) and zebrafish (Yin and Hu, 2014). Lingo-1 blockade leads to increased myelination competence (Mi et al., 2005) while its blockade with a Lingo-1-Fc protein improved neuronal and oligodendrocyte survival, and axonal sprouting in SCI (Ji et al., 2006). Interestingly, after thoracic SCI in the rat, *Lingo-1* levels are downregulated in lumbosacral sensory neurons that undergo intense sprouting into the superficial layers of the spinal cord, possibly in response to increased nerve growth factor levels (Chambel et al., 2022), which concurs with previous reports (Lee et al., 2007).

CRMP2 and PlexinA2

Recently, a novel NgR1 molecular partner has been identified. Collapsin response mediator protein 2 (CRMP2) is a CNS ubiquitously expressed microtubule-associated protein, expressed at pre- and post-synaptic sites. CRMP proteins mediate Semaphorin signaling through Plexin-containing receptors (Schmidt and Strittmatter, 2007). The Strittmatter lab has shown that CRMP2 physically associates with NgR1 and PlexinA2 into a signaling complex, a process mediated by Nogo-A (Sekine et al., 2019). This work demonstrated that the NgR1/PlexinA2/CRMP pathway is required to mediate Nogo-A induced inhibition of cortical neuron regeneration and CST sprouting. Accordingly, after SCI, CRMP2 knock-in (CRMP2KI) mice display improved SCI pathophysiology, exhibiting microtubule stabilization in neurons (Sugeno et al., 2021).

Pir-B

Pir-B is a receptor for Nogo-A, MAG, and OMgp (Atwal et al., 2008). Genetic ablation or pharmacological blockage of Pir-B *in vitro* promotes neurite outgrowth on substrates rich in myelin inhibitors or CNS myelin (Atwal et al., 2008). After SCI, Pir-B is overexpressed in dorsal root ganglion neurons, sciatic nerves, and spinal cord segments (Peng et al., 2015). Like NgR1, Pir-B can associate with p75 and mediate inhibition of axon growth (Fujita et al., 2011).

NgR3, LAR, and RPTPσ

NgR3, LAR, and RPTPσ are the exclusive high-affinity receptors for CSPGs. Several studies using genetic ablation of these receptors demonstrated their role in regulating axonal growth. Dickendesher et al. (2012) reported that CSPGs are able to bind to NgR1 and NgR3 with high affinity, resulting in inhibition of axonal growth. After genetic deletion of *NgR3* and *NgR1*, there is no inhibitory effect of CSPGs and the optic nerve regenerates after crush injury in mice. Moreover, these authors also showed enhancement of regeneration of optic nerves genetic inactivation of RPTPσ in *NgR1*- and *NgR3*-null mice (Dickendesher et al., 2012), further demonstrating the role of RPTPσ in CSPG-mediated inhibition.

The importance of RPTPσ and LAR in CSPG-mediated axonal inhibition has also been demonstrated in SCI models. Mice in which RPTPσ was genetically eliminated exhibit increased regrowth of sensory and CST axons into the scar and caudal spinal cord, respectively (Shen et al., 2009; Fry et al., 2010). *Lar*-null mice showed improved locomotor function and sprouting of serotonergic and CST axons after SCI (Xu et al., 2015). Deleting genes encoding RPTPσ and LAR in adult neuronal cultures produces synergistic enhancements of axon growth (Ohtake et al., 2016). Furthermore, pharmacological inhibition of RPTPσ and LAR signaling has been shown to improve functional outcomes and sprouting of serotonergic fibers in spinal cord injured rats (Xie et al., 2006; Fisher et al., 2011; Lang et al., 2015) and to promote recovery of diaphragm function after cervical SCI (Urban et al., 2020; Cheng et al., 2021).

Downstream Signaling Pathways in Spinal Cord Injury

Following SCI, when growth cones contact with inhibitory molecules, their cognate receptors are activated. These interactions initiate downstream signaling pathways involved in cytoskeletal dynamics, the most well studied of which is the RhoA/Rho kinase (ROCK) pathway. When activated, RhoA/ROCK promotes F-actin disassembly in growth cones and hinders axon elongation, by preventing microtubule recruitment, which ultimately results in growth cone collapse (Maekawa et al., 1999; Wu et al., 2005). When active, Rho-GTP activates its downstream effector, ROCK. In turn, activated ROCK phosphorylates several downstream effectors, such as LIM kinase, CRMP2, adducin, and myosin light chain, among others, eventually leading to growth cone collapse and neurite outgrowth inhibition (Boghdadi et al., 2018; Kimura et al., 2021). NgR1-mediated signaling pathway includes RhoA, ROCK, LIM kinase, and Cofilin, ultimately resulting in actin depolymerization (Hsieh et al., 2006). Pir-B signals through different but interconnected downstream effectors that are able to crosstalk. These include plenty of sarcoma homology domain 3, that either converges with the ROCK pathway; or activates the leucine zipper kinase, mitogen-activated protein kinase kinase, Jun N-terminal kinase and Myosin II, eventually resulting in inhibition of axonal growth (Taylor et al., 2008; Gou et al., 2014; Boghdadi et al., 2018; **Figure 1**). CSPG activation of RPTPσ and LAR results in downstream activation of RhoA/ROCK and blockade of Akt and ERK cascades (Ohtake et al., 2016), as described in **Figure 1**.

Myelin inhibitors and CSPGs share several common downstream effectors that can potentially crosstalk and compensate for one another, hence presenting as potential therapeutic targets for SCI treatment. RhoA inhibition has attracted considerable attention and C3 transferase, obtained from *Clostridium botulinum*, has been shown to selectively inhibit RhoA. Tested in several pre-clinical studies, RhoA inactivation results in axonal sprouting and recovery of locomotor function after SCI (Dergham et al., 2002; Dubreuil et al., 2003), by reversing Rho activation and preventing p75-dependent cell death. The clinical application of C3 transferase, cethrin, is discussed ahead.

Therapeutic Modulation of Inhibitory Molecules for Spinal Cord Injury Treatment

Administration of anti-Nogo-A antibody in animal models of SCI has been shown to induce neural repair in rodents (Liebscher et al., 2005; Craveiro et al., 2013) and non-human primates (Fouad et al., 2004; Freund et al., 2007, 2009; Hoogewoud et al., 2013). These results supported a phase I, open-label, multicenter study in human patients with severe, acute SCI, initiated in 2006 and concluded in 2011 (ClinicalTrials.gov; Identifier: NCT00406016). In this trial, intrathecal administration of Nogo-A antibody in the sub-acute phase improved functional deficits of patients (Kucher et al., 2018). In 2019, the NISCI (Nogo inhibition in SCI study, a multicenter, European, randomized, double-blind, and placebo-controlled phase II trial was launched to investigate the efficacy of early treatment initiation (within 28 days post-injury) via repeated injections of anti-Nogo-A antibodies in participants with acute, cervical spinal injuries (ClinicalTrials.gov; Identifier: NCT03935321). In addition to anti-Nogo-A therapy, patients received rehabilitation training for up to six months after injury. The NISCI study was closed in 2022, with more than 110 patients enrolled (nisci-2020.eu) and results are currently under assessment.

In 2019, the RESET (ReNetX safety efficacy and tolerability of AXER-204 for chronic SCI) study, a two-part clinical trial to assess safety, tolerability, pharmacokinetics, and efficacy of AXER-204, a human NgR-derived fusion protein was initiated. This compound acts as a soluble decoy/trap for Nogo-A, MAG and OMgp and is delivered by intrathecal lumbar infusion to patients with chronic (≥ 1 year) cervical spinal cord injuries (Clinical Trials.gov; Identifier: NCT03989440). In pre-clinical studies, Strittmatter's group has demonstrated that AXER-204 promotes functional recovery and enhances axonal growth in rats with spinal contusion (Wang et al., 2014) and recovery of forelimb and corticospinal regeneration in non-human primates with hemisection injuries (Wang et al., 2020). The RESET trial is currently recruiting patients.

The ELASCI, a phase II randomized, double-blind, placebo-controlled proof of concept study was initiated in 2020 and aims to assess the safety and efficacy of systemic intravenous administration of elezanumab, a human anti-RGMa monoclonal antibody, in acute traumatic cervical spinal cord injuries (ClinicalTrials.gov; Identifier: NCT0495538). Pre-clinical studies using anti-RGMa antibody in rats with contusion/compression spinal injuries showed neuronal sparing and neuroplasticity, as well as improvements in bladder function (Mothe et al., 2017, 2020). In non-human primates, elezanumab administration in an acute stage promoted neuroprotection, neuroplasticity, and recovery of neuromotor function after a hemicompression thoracic spinal injury (Jacobson et al., 2021). The ELASCI trial is currently in the recruitment stage.

Another promising drug to treat subjects with acute traumatic cervical spinal cord injuries that reached a phase 2b/3, double-blind, randomized, placebo controlled, multicenter study, was VX-210, (previously known as BA-210 and cethrin), a Rho-GTPase antagonist (ClinicalTrials.gov; Identifier: NCT02669849) (Fehlings et al., 2018). VX-210 proved to improve locomotor function and decrease lesion extension in rodents after spinal contusion (Lord-Fontaine et al., 2008). Even though VX-210 was well-tolerated by human subjects, the study ended prematurely when it failed to meet the primary efficacy endpoint. The study's final analysis revealed no statistically significant differences between the VX-210 and placebo groups (Fehlings et al., 2021).

Conclusion

The adult mammalian CNS is unable to regenerate after injury. Consensus on axonal regeneration following SCI seems to lay on three major requirements: activation of neural intrinsic growth, to allow neurons to regenerate their axons following injury; modulation of neuron-extrinsic factors, such as myelin- and scar-derived inhibitors, creating both a permissive and supportive environment for axonal regrowth; and availability of specific chemoatractive growth factors, for neuronal survival and axon regeneration (Courtine and Sofroniew, 2019; Zheng and Tuszynski, 2023).

Pre-clinical and clinical investigation focused, for many years, on extrinsic factors, with the development of numerous pharmacological approaches to "inhibit the inhibitors" and promote repair. Targeting of myelin inhibitors in animal models and, more recently, in clinical trials, generates good results, with gain-of-function outcomes reported. However, these approaches mostly just induce plasticity and axonal sprouting rather than proper repair of the injured tracts and returning to pre-lesion conditions (Geoffroy and Zheng, 2014). While sprouting, defined as extension of new processes along the shaft on a spared axon, of uninjured distal axons occurs spontaneously after injury and allows new connections to be formed, neuronal circuits are usually not restored by these strategies and only replaced by new alternative pathways. This maladaptive sprouting leads to the emergence of unwanted, bothersome and dangerous conditions that emerge after SCI, as is the case of chronic central neuropathic pain, with nociceptive processes being activated in the absence of stimulation and/or amplified in the presence of a noxious stimulus (Deumens et al., 2008; Kang et al., 2020). Abnormal sprouting of peptidergic sensory afferents into the spinal cord, dependent on nerve growth factor, is also critically involved in development of autonomic dysreflexia (Cameron et al., 2006; Hou et al., 2008). Finally, nerve growth factor and brain-derived neurotrophic factor also regulate sprouting of bladder afferents, leading to urinary impairment (Yoshimura et al., 2006; Frias et al., 2015; Wada et al., 2018).

One of the issues of current clinical trials to treat SCI is the variable degree of success reported. These differences could be mitigated by back-translational approaches to fine-tune the animal models used in pre-clinical studies and their translational value in the clinics. For example, the use of rodents versus more clinically relevant models, such as non-human primates, should be explored. Many obstacles are still in the way of wider use of primates in research, mainly due to ethical, logistic, and cost-associated reasons. Advantages brought on by their use in pre-clinical studies could greatly improve the cost and outcomes in the clinic which would, ultimately, contribute to the advancement of SCI therapies. The type of injury used in pre-clinical studies should also be considered. Transection injuries are reproducible and usually accompanied by modest recovery; however, contusion models are more clinically relevant, but with severity of injury and functional outcomes being highly variable. Over the last few years, research has also shown that pre-clinical studies should aim to apply combinatorial therapies in the treatment of SCI. Extensive pre-clinical and clinical pharmacological approaches that target inhibitory molecules are delivering promising results and have the potential to produce breakthroughs in the treatment of CNS injuries. These treatments, however, would probably benefit from joint application with other supportive therapies, such as growth promoting or neurotrophic factors, stem-cell-based therapies and physical rehabilitation. Furthermore, neuroengineering therapies have proven, with great success, to produce extraordinary recovery outcomes after SCI (Capogrosso et al., 2016; Asboth et al., 2018; Bonizzato et al., 2018). Their synergistic use with already tested and hereafter established biological therapies should be the next great approach in the treatment of spinal cord injuries.

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Publication II

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Development of Neurogenic Detrusor Overactivity after Thoracic Spinal Cord Injury Is Accompanied by Time-Dependent Changes in Lumbosacral Expression of Axonal Growth Regulators

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Abstract: Thoracic spinal cord injury (SCI) results in urinary dysfunction, which majorly affects the quality of life of SCI patients. Abnormal sprouting of lumbosacral bladder afferents plays a crucial role in this condition. Underlying mechanisms may include changes in expression of regulators of axonal growth, including chondroitin sulphate proteoglycans (CSPGs), myelin-associated inhibitors (MAIs) and repulsive guidance molecules, known to be upregulated at the injury site post SCI. Here, we confirmed lumbosacral upregulation of the growth-associated protein GAP43 in SCI animals with bladder dysfunction, indicating the occurrence of axonal sprouting. Neurocan and Phosphacan (CSPGs), as well as Nogo-A (MAI), at the same spinal segments were upregulated 7 days post injury (dpi) but returned to baseline values 28 dpi. In turn, qPCR analysis of the mRNA levels for receptors of those repulsive molecules in dorsal root ganglia (DRG) neurons showed a time-dependent decrease in receptor expression. In vitro assays with DRG neurons from SCI rats demonstrated that exposure to high levels of NGF downregulated the expression of some, but not all, receptors for those regulators of axonal growth. The present results, therefore, show significant molecular changes at the lumbosacral cord and DRGs after thoracic lesion, likely critically involved in neuroplastic events leading to urinary impairment.

Keywords: spinal cord injury; repulsive molecules; axonal growth; bladder dysfunction; neurogenic detrusor overactivity

1. Introduction

Normal body function requires an intact spinal cord, functioning as a relay and processing centre for peripherally generated input and conveying supraspinal commands to effector organs. The spinal cord is complex and comprises neurons, glial cells, and ascending and descending nerve fibres. Spaces between cells and fibres are occupied by extracellular matrix (ECM), a scaffold composed of secreted complex proteins and sugars, required to support cellular activity and survival [1]. Traumatic spinal cord injury (SCI)

significantly alters the dynamics and composition of ECM at the lesion site as a consequence of neuronal and glial cell death, activation of surviving cells and immigration of peripheral inflammatory cells.

Post-SCI changes in ECM composition at the peri-lesion site reflect accumulation of inhibitory cues that participate in axon guidance and regulate axonal growth. These molecules are normally present at low levels, but some are overproduced in response to injury, creating a highly repulsive environment at the lesion site and impairing repair of disrupted neuronal tracts [2,3]. Sources of these inhibitory molecules include astrocytes, oligodendrocytes and the myelin sheath. Binding of these proteins to their receptors, present in neurons, causes actin depolymerization, growth cone collapse and blockade of regenerative growth after SCI [4]. The most well-studied repulsive molecules fall into three major categories: chondroitin sulphate proteoglycans (CSPGs), myelin-associated inhibitory proteins (MAIs) and repulsive guidance molecules.

Astrocyte-derived chondroitin sulphate proteoglycans (CSPGs) are glycoproteins that contribute to the structural scaffold of the spinal ECM and have been associated with regulating synaptic stability, reduced plasticity and inhibition of axonal elongation [5,6]. Examples of CSPGs include Neurocan and Phosphacan [7]. CSPG-mediated axonal growth cone collapse is linked with the activation of receptor-type protein tyrosine phosphatase sigma (RPTPσ) [8], leukocyte common antigen-related phosphatase (LAR) [9], Nogo receptor (NgR1) and Nogo receptor 3 (NgR3) [10].

Myelin-associated inhibitory proteins (MAIs) include Nogo-A [11], myelin-associated glycoprotein (MAG) [12] and oligodendrocyte-myelin glycoprotein (OMgp) [13,14]. Nogo-A is the most well-described MAI. It is produced mostly by oligodendrocytes [15,16], but also by certain subpopulations of central and peripheral neurons [11,17]. Nogo-A signals with two binding regions through two receptor components, NgR1 [18], forming a receptor complex with leucine rich repeat and immunoglobulin-like domain-containing NgR-interacting protein 1 (LINGO-1) [19] and p75 [20], and/or TROY [21,22], and sphingosine 1-phosphate receptor 2 (S1PR2) [23]. Nogo-A has been associated with regulation of neuroplasticity, learning and memory [24–26]. MAG is involved in maintenance of myelin sheaths. It is expressed at high levels by oligodendrocytes in the central nervous system (CNS) while Schwann cells, in the peripheral nervous system, may also produce MAG [27]. MAG can signal through the NgR1/LINGO1/p75 and/or TROY receptor complex [28]. OMgp is present in the cell membrane of oligodendrocytes and the glial–axonal interface of myelinated axons both in the brain and spinal cord [29,30]. OMgp is involved in the inhibition of neurite outgrowth following injury [31,32] and acts via NgR1/LINGO1/p75 and/or TROY receptor complex [13,28].

Guidance molecules, besides playing an important role in neural circuit assembly during development, are also involved in CNS response after injury [33]. Ephrins and Semaphorins are involved in the guidance of ascending sensory and descending motor pathways, but after SCI, exert a highly repulsive effect towards axons [34]. Repulsive Guidance Molecule A (RGMa) is present in myelin and accumulates in the glial scar following a spinal cord injury [35]. After SCI, RGMa is expressed in neurons, oligodendrocytes, astrocytes, activated microglia and macrophages [36], and binds to its neuronal receptor, Neogenin 1 (Neo1), [37], inhibiting neurite outgrowth [38,39].

Changes in peri-lesional ECM have been well studied and linked to impaired recovery of sensorimotor function in SCI patients [4]. However, albeit less studied, there is indication that changes in the ECM composition may also occur in spinal areas distant from the lesion site [40], where they likely contribute to maladaptive plasticity. For example, development of SCI-induced urinary impairment, one of the major degraders of quality of life for SCI patients [41], is associated with abnormal sprouting at the lumbosacral spinal cord of the central processes of sensory peptidergic afferents [42–44]. While the fine molecular mechanisms regulating this aberrant axonal growth are still mostly unresolved, it may reflect changes in lumbosacral spinal ECM, making it more permissive to expansion of the central processes of sensory afferents. These ECM modifications remain poorly investigated.

Moreover, it is not well understood if sprouting sensory neurons regulate the expression of the receptors for these centrally expressed repulsive molecules. Therefore, this study investigated whether levels of repulsive molecules, including CSPGs (Phosphacan and Neurocan), MAIs (Nogo-A, OMgp and MAG) and RGMa, are altered in the lumbosacral cord of SCI animals submitted to thoracic spinal transection. The expression of receptors for these repulsive cues in sensory neurons was also analysed.

2. Results

2.1. Spinal Cord Transection Induces Urinary Dysfunction

At the end of each time point, cystometries under urethane anaesthesia were performed to evaluate urinary function. Spinal intact animals (control) exhibited a normal pattern of bladder reflex activity (Figure 1A), showing regular contractile activity. The frequency of bladder contractions was 0.7 ± 0.3 contractions/min (Figure 1D). The amplitude of bladder contractions was 25.7 ± 2.5 cm H_2O (Figure 1E), while the peak pressure was 37.4 ± 3.2 cm H_2O (Figure 1F) and the basal pressure was 11.7 ± 3.7 cm H_2O (Figure 1G).

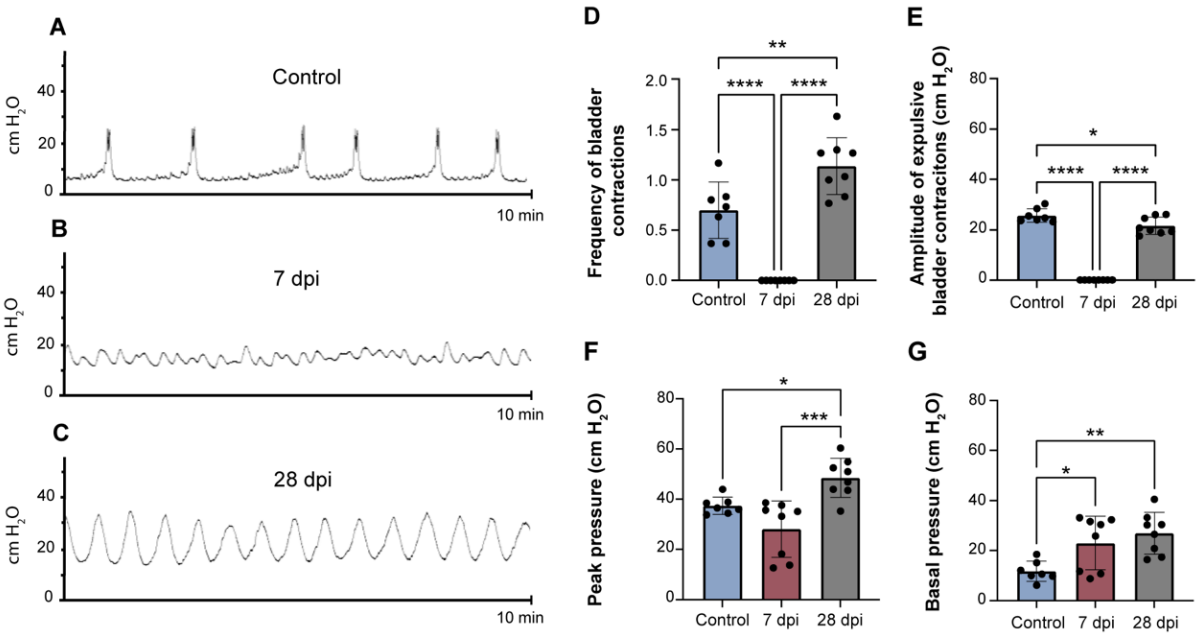

Figure 1. Representative cystometrograms depicting the voiding pattern of animals with intact spines (control) and after spinal cord injury. Control animals exhibited a normal pattern of bladder reflex activity (**A**). SCT animals exhibited hallmarks of bladder dysfunction. At 7 days post-injury (7 dpi), bladder contractions with an amplitude higher than 5 cm H_2O were nonexistent (**B**), while at 28 days post-injury (28 dpi) a typical pattern of NDO was well established (**C**), evidencing signs of strong, frequent and involuntary bladder contractions. Analysis of urodynamics parameters, such as frequency of bladder contractions (**D**), amplitude of expulsive bladder contractions (**E**) and peak (**F**) and basal pressures (**G**) are also detailed. Graphs represent mean ± SD and $p < 0.05$ was considered statistically significant. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$; one-way ANOVA followed by Tukey's multiple comparison test.

Thoracic spinal transection caused marked alterations in bladder function, observable both at 7 and 28 days post-injury (dpi). While at 7 dpi, bladder contractions were abolished (Figure 1B–E; $p < 0.001$ vs. control animals), at 28 dpi there were evident signs of bladder overactivity (Figure 1C). In this experimental group, the frequency of bladder contractions was 1.1 ± 0.3 contractions/min (Figure 1D). The amplitude of contractions was 21.6 ± 3.4 cm H_2O (Figure 1E), while peak pressure was 48.5 ± 7.8 cm H_2O (Figure 1F) and basal pressure was 26.9 ± 8.3 cm H_2O (Figure 1G). For all parameters, values were

significantly different from those observed in control animals and indicate the development of SCI-induced urinary dysfunction.

2.2. SCT-Induced Urinary Dysfunction Is Accompanied by Increased Spinal Expression of GAP43

Previous studies assessing animals submitted to complete spinal cord transection have demonstrated that post-SCI urinary impairment courses with axonal sprouting of sensory afferents at the lumbosacral spinal cord [42,45]. To confirm whether the SCI-induced bladder dysfunction observed here was also linked to sprouting of the central processes of sensory afferents, levels of GAP43, an established marker of axonal sprouting [46], were evaluated. GAP43 expression was mostly found in laminae I-II of the lumbosacral spinal cord (L5-S1) (Figure 2), with some processes present in other regions such as the dorsal commissure, corticospinal tract and dorsolateral funiculus in spinal intact animals (not showed). While in control animals, GAP43 immunoreactivity was low (Figure 2A,D), GAP43 expression levels significantly increased 7 dpi (Figure 2B,D; $p < 0.0001$ vs. control animals). At twenty-eight dpi, GAP43 levels decreased (Figure 2C,D; $p < 0.05$ vs. 7 dpi animals) but remained elevated compared to control animals ($p < 0.01$). In the DRG, GAP43 was also significantly elevated at 7 dpi and slightly decreased at 28 dpi (Figure 2E; $p < 0.05$ versus control). These observations confirm that development of urinary impairment coincides with lumbosacral axonal sprouting.

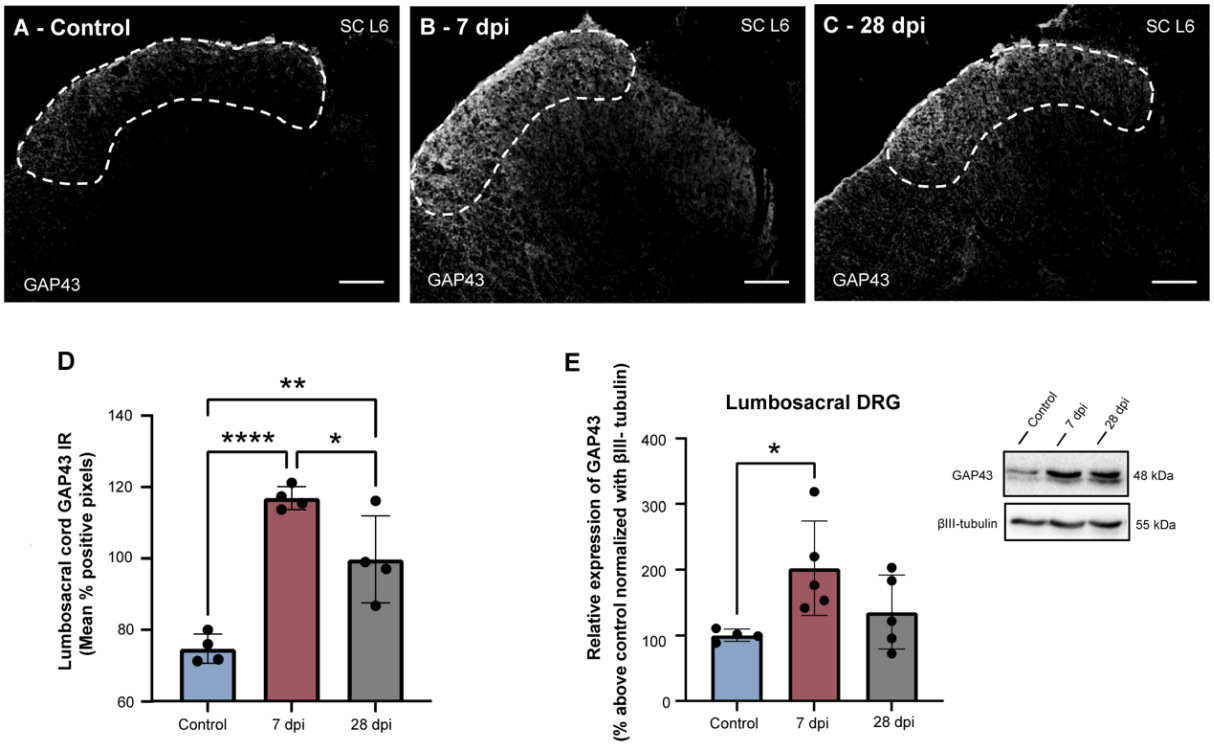

Figure 2. Time-dependent alterations in GAP43 levels at the lumbosacral spinal cord and DRG after spinal cord injury. Distribution of GAP43 in the L6 segment of the lumbosacral spinal cord (**A**–**C**). GAP43 was detected via fluorescent immunohistochemistry in the L5, L6 and S1 spinal segments of control and SCT animals. GAP43 expression was predominantly observed in the superficial dorsal horn (laminae I and II). Levels of spinal GAP43 expression increased significantly 7 dpi (**D**). Twenty-eight days post-injury, GAP43 levels at the lumbosacral spinal cord remained elevated compared to control animals (**D**). In lumbosacral DRG neurons, Western blotting analysis showed a significant increase in GAP43 expression 7 dpi, compared to control animals (**E**). Scale bar: 100 µm. Graphs represent mean ± SD and $p < 0.05$ was considered statistically significant. * $p \leq 0.05$; ** $p \leq 0.01$; **** $p \leq 0.0001$; one-way ANOVA followed by Tukey's multiple comparison test.

2.3. Thoracic SCT Induces Changes in Lumbosacral Expression of Axonal Growth Regulators

While axonal sprouting is very limited in the adult central nervous system [47], spinal lumbosacral GAP43 expression upregulation suggests changes in the extracellular environment, which could have become less repulsive, allowing the expansion of neuronal processes after a thoracic spinal injury. To explore this hypothesis, the expression of proteins known to control axonal growth was analysed.

Total Neurocan levels detected by Western blot were not significantly changed by SCT (Figure 3A), but changes in levels of specific isoforms were found. Neurocan-260 significantly increased 7 dpi ($p < 0.05$ vs. control animals) and Neurocan-160 significantly increased 28 dpi ($p < 0.05$ vs. control animals) (Figure 3A). Total Phosphacan expression in the lumbosacral cord, as well as Phosphacan-250 kDa and Phosphacan-190 kDa, showed a significant three-fold increase at 7 dpi (Figure 3B). Immunohistochemical analysis of Phosphacan expression at the lumbosacral cord showed similar results (Supplementary Figure S2). Importantly, while Phosphacan and GAP43 were present in the same spinal areas, there was no overlap of immunofluorescence.

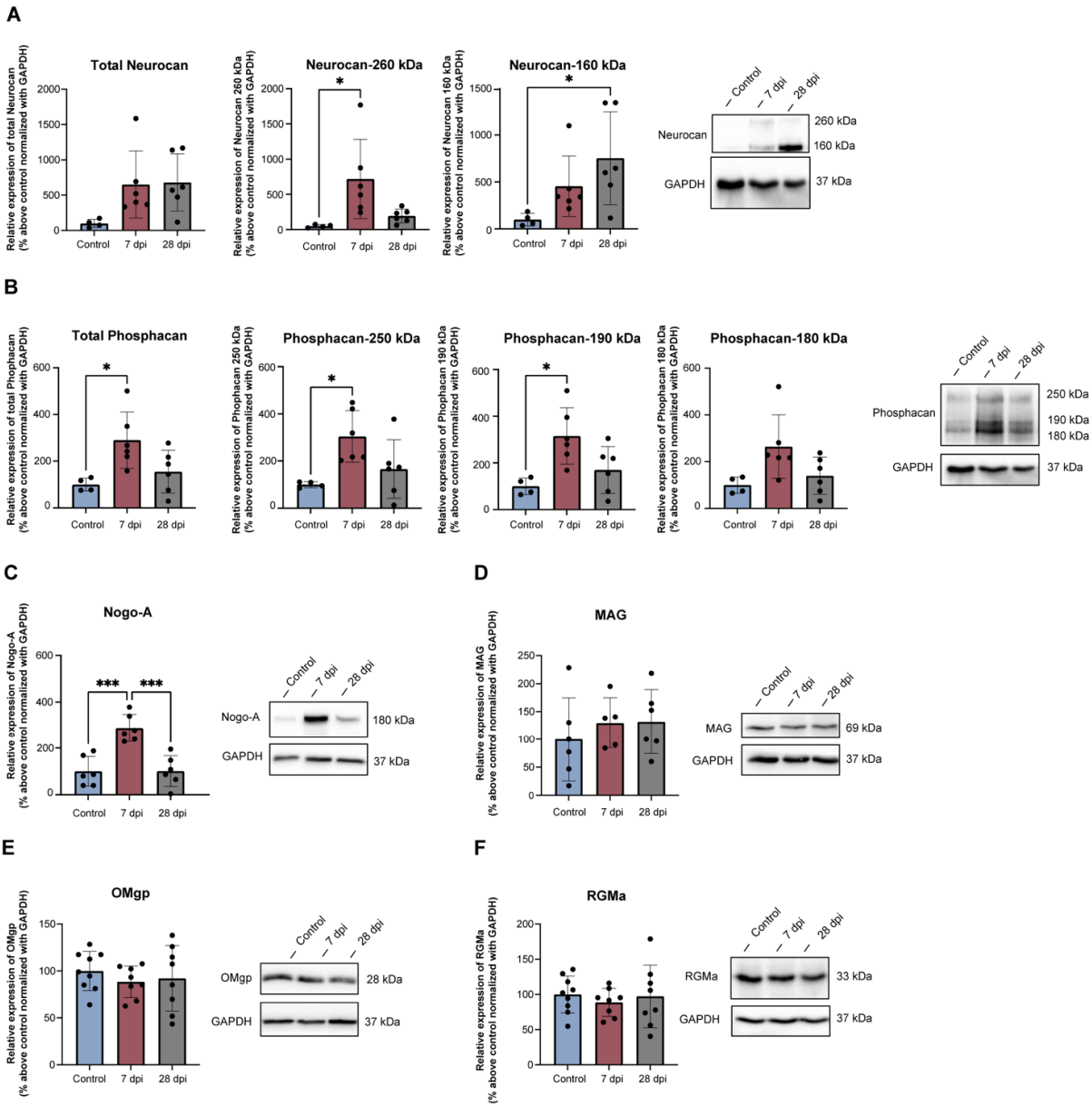

Figure 3. Time-dependent changes in repulsive proteins expression after thoracic spinal cord injury. Western blots of Neurocan (**A**), Phosphacan (**B**), Nogo-A (**C**), MAG (**D**), OMgp (**E**) and RGMa (**F**) and

respective ratio against the endogenous protein GAPDH at the lumbosacral spinal cord in spinal intact animals (control) and after SCT. Total Neurocan (A) expression did not show any significant differences between controls and SCT animals, but Neurocan-250 significantly increased 7 dpi and Neurocan-160 increased significantly 28 dpi. Total Phosphacan (**B**) and Nogo-A (**C**) expression significantly increased 7 dpi (days post-injury), decreasing at 28 dpi. Expression of MAG (**D**), OMgp (**E**) and RGMa (**F**) was not changed by the thoracic lesion. Graphs represent mean ± SD and $p < 0.05$ was considered statistically significant. * $p \leq 0.05$; *** $p \leq 0.001$; one-way ANOVA followed by Tukey's multiple comparison test.

The expression of MAIs was also analysed. Lumbosacral expression of Nogo-A was low in spinal intact rats but had a significant three-fold increase 7 dpi ($p < 0.001$ vs. control animals), returning to baseline 28 dpi ($p < 0.001$ vs. 7 dpi animals) (Figure 3C). Like Phosphacan, immunodetection of Nogo-A showed a similar variation (Supplementary Figure S3), with Nogo-A and GAP43 present in the same spinal laminae but without overlap.

For other MAIs, also studied here, no changes were found in levels of MAG (Figure 3D) and OMgp (Figure 3E). Expression of RMGa was also unaltered (Figure 3F). These results demonstrate the occurrence of dynamic patterns of expression of regulators of axonal growth in the lumbosacral cord, restricted to some inhibitory cues.

2.4. Alterations in Lumbosacral Expression of Axonal Growth Regulators Is Accompanied by DRG Changes in Levels of Specific Receptors

As GAP43 upregulation in the superficial laminae of the lumbosacral cord, where visceral afferents are known to project [48,49], was inconsistent with changes in CSPGs and MAIs in the lumbosacral cord, we hypothesized that the expression of their specific receptors was altered in lumbosacral sensory afferents, making them insensitive to inhibitory cues and allowing axonal elongation. To investigate this, mRNA levels of receptor complexes were analysed by RT-qPCR for MAIs: *NgR1/Lingo1/p75* and/or *Troy;* CSPGs: *NgR1/NgR3*, *Rptpσ/Lar;* and RGMa: *Neo1*. Levels of mRNA encoding for MAI complex receptor *NgR1/Lingo1/p75* and/or *Troy* (Figure 4A–D) significantly decreased in a time-dependent manner. *NgR1*, *Lingo1* and *Troy* significantly decreased at 28 dpi compared to control animals, while *p75* mRNA levels did not reach statistical significance, supporting the duality of this receptor complex (Figure 4A, $p < 0.001$ vs. control; Figure 4B,D, $p < 0.05$ vs. control).

Likewise, mRNA expression of *NgR3* (Figure 4G), *Rptpσ* (Figure 4F) and *Lar* (Figure 4E), part of the CSPG receptor complexes *NgR1/NgR3* and *Rptpσ/Lar*, was also time-dependently reduced at 28 dpi (*NgR3*: Figure 4B, $p < 0.05$ 7 dpi vs. 28 dpi animals; *Rptpσ/Lar*: Figure 4E,F, $p < 0.05$ control vs. 28 dpi animals). In contrast, mRNA levels of the RGMa receptor *Neo1* were similar between experimental groups (Figure 4H). These results show DRG neurons express receptors for spinal inhibitory cues, the levels of which vary with disease progression.

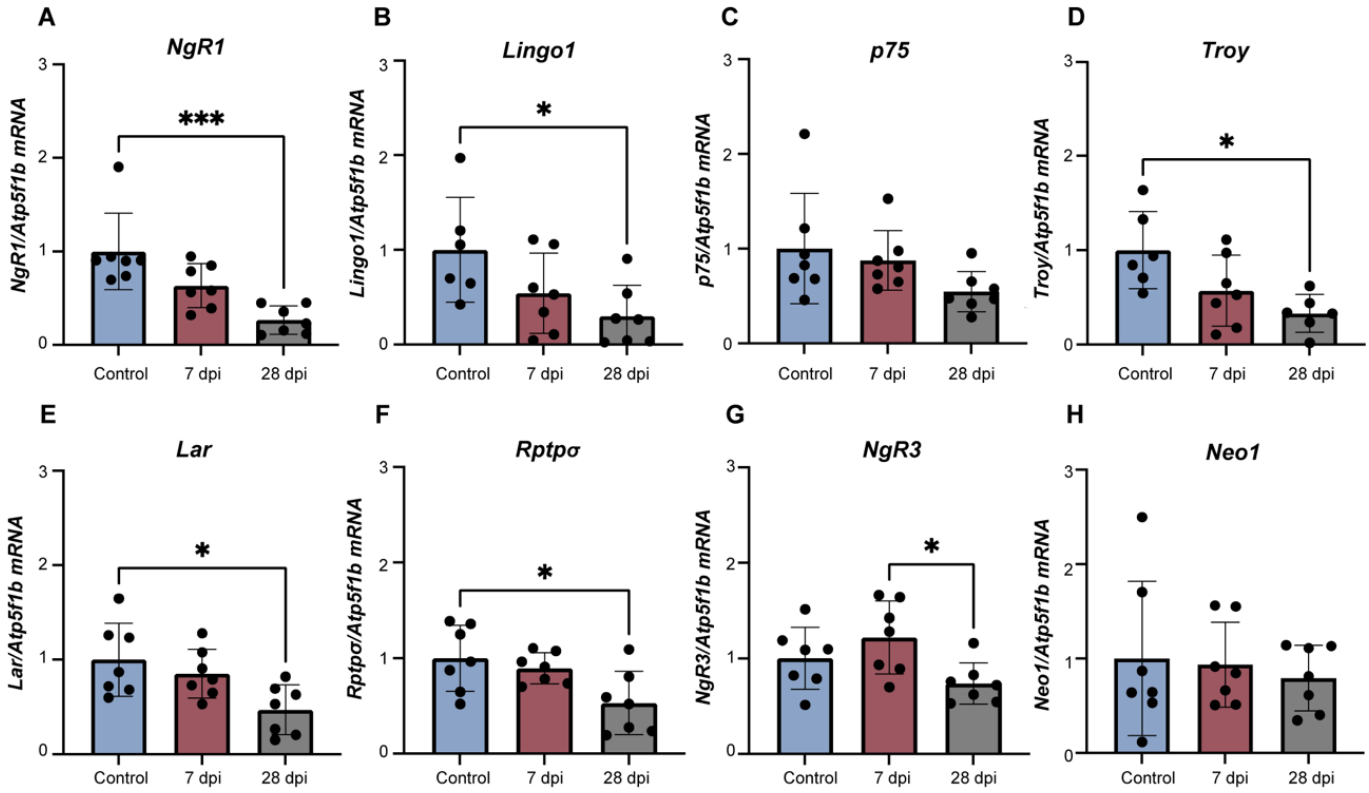

Figure 4. Levels of mRNA of myelin-associated inhibitors (MAI) receptor complex *NgR1* (**A**), *Lingo1* (**B**), *p75* (**C**) and *Troy* (**D**), CSPGs receptors *Lar* (**E**), *Rptpσ* (**F**) and *NgR3* (**G**); and RGMa receptor *Neo1* (**H**) in lumbosacral (L5-S1) DRG of spinal intact (control) and SCT animals. After thoracic spinal cord transection, mRNA levels of receptor complex *NgR1*/*Lingo1*/*p75* (**A**–**C**) and/or *Troy* (**D**) tended to significantly decrease 28 dpi (days post-injury). *Lar*/*Rptpσ*/*NgR3* (**E**–**G**) also followed the same trend. RGMa receptor *Neo1* mRNA levels (**H**) were not changed by the thoracic spinal cord lesion. Graphs represent mean ± SD and $p < 0.05$ was considered statistically significant. * $p \leq 0.05$; *** $p \leq 0.001$; one-way ANOVA followed by Tukey's multiple comparison test.

2.5. Rhoa/Rock Module Is Not Altered in Lumbosacral Drg Neurons following Thoracic Spinal Transection

Binding of MAIs, CSPGs and repulsive guidance molecules to their specific receptors causes collapse of the axonal cone via activation of RhoA and inactivation of protein kinase B (Akt) signalling pathways downstream of the receptors [50]. A reduction in the expression of those receptors would result in lack of activation of RhoA and its effector ROCK, which is responsible for mediating rearrangements of the actomyosin cytoskeleton, consistent with the ongoing axonal sprouting and increased GAP43 expression observed here (Figure 2). Since ROCK is also responsible for the phosphorylation of myosin phosphatase target subunit 1 (MYPT1) at Thr696, inactivating it and leading to neurite retraction and neurite outgrowth inhibition [51], the expression of MYPT1 and phosphorylated MYPT1 (pMYPT1) at Thr696 was resolved by Western blotting in lumbosacral (L5-S1) DRG samples. No changes were found between control and SCT groups in the expression of pMYPT1 (Figure 5A) or MYPT1 (Figure 5B). Ratio between pMYPT1/MYPT1 (Figure 5C) followed the same pattern, indicating lack of activation of the RhoA/ROCk signalling pathway, consistent with ongoing axonal growth.

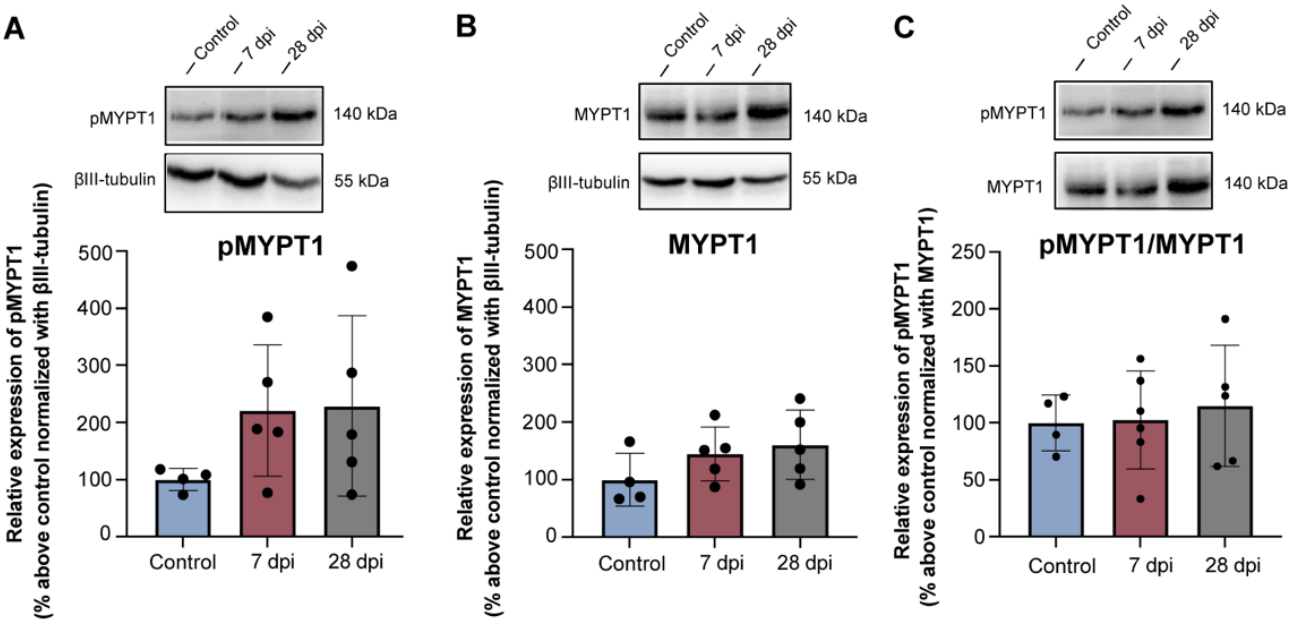

Figure 5. Time-dependent expression of MYPT1 and pMYPT1 in lumbosacral (L5-S1) DRG neurons of spinal intact (control) and SCT (7 and 28 dpi (days post-injury)) animals, determined by Western blotting. pMYPT1 (**A**) and MYPT1 (**B**) were normalized against βIII-tubulin and levels of pMYPT1 were also normalized against endogenous levels of MYPT1 (**C**). In all cases, no statistical differences were found between experimental groups. Graphs represent mean ± SD. One-way ANOVA followed by Tukey's multiple comparison test.

2.6. NGF Regulates the Expression of Receptors for Axonal Guidance Cues in DRG Neurons

To investigate the mechanisms governing the peripheral downregulation of MAIs, CSPGs and repulsive guidance-molecules-specific receptors, L5-S1 DRG neurons were collected from control and SCT animals (7 and 28 days post-injury) and cultured for 22 h in different concentrations of NGF—0, 50 and 100 ng/mL of NGF. NGF was chosen, as it has been shown to be upregulated following SCT in rostral and caudal spinal cord (in relation to the injury site), in the cell body of DRG neurons and in peripheral organs such as the bladder [52–54]. NGF is also known as an inducer of axonal elongation [55,56] and its increase has been linked to SCT-induced urinary dysfunction [57,58].

Neurons adhered well to the substrate and emitted long neurites, easily discernible with βIII-tubulin immunostaining (Figure 6A). These neurites were particularly evident and long in DRG neurons obtained from SCT animals, even in the absence of NGF. Neurite length analysis showed that the presence of NGF in the medium induced significantly longer neurites in SCT animals (Figure 6B). Ramification of neurites was also prominent. Analysis of growing cells showed that branching was significantly and dose-dependently upregulated in cells obtained from SCT animals treated with NGF (Figure 6C–E). Altogether, these results show that DRG neurons obtained from SCT animals can emit longer and more ramified neurites than neurons from control animals. The length and ramification were positively correlated with NGF concentration in the medium, indicating this neurotrophin potentiates the SCT-induced activation of a growth program.

To investigate whether NGF would potentiate axonal growth by interfering with the expression of CSPGs, MAIs and repulsive guidance molecules receptors, RNA was extracted from cultured DRG cells from control and SCT animals (7 and 28 days post-injury) and reverse transcribed, and mRNA levels of *NgR1*, *Lingo1*, *p75*, *Troy*, *NgR3*, *Rptpσ*, *Lar* and *Neo1* were determined. In cultured neurons from SCT animals, exposure to NGF resulted in significant dose-dependent downregulation of the levels of *NgR1* and *Lingo1* (Figure 7A,B). Levels of the remaining receptors (Figure 7C–H) were not altered by SCT, nor by NGF exposure. It is important to mention that we were unable to determine expression by qPCR of some SCT samples (represented by grey triangles in Figure 7) for some of the studied receptors (*NgR1, Lingo1, Troy* and *Neo1*), although levels of the housekeeping gene *Ywhaz* were detected. As samples were run in triplicate and on two separate runs, it is feasible to assume that the lack of detection was not due to technical issues but rather reflects a lack of expression.

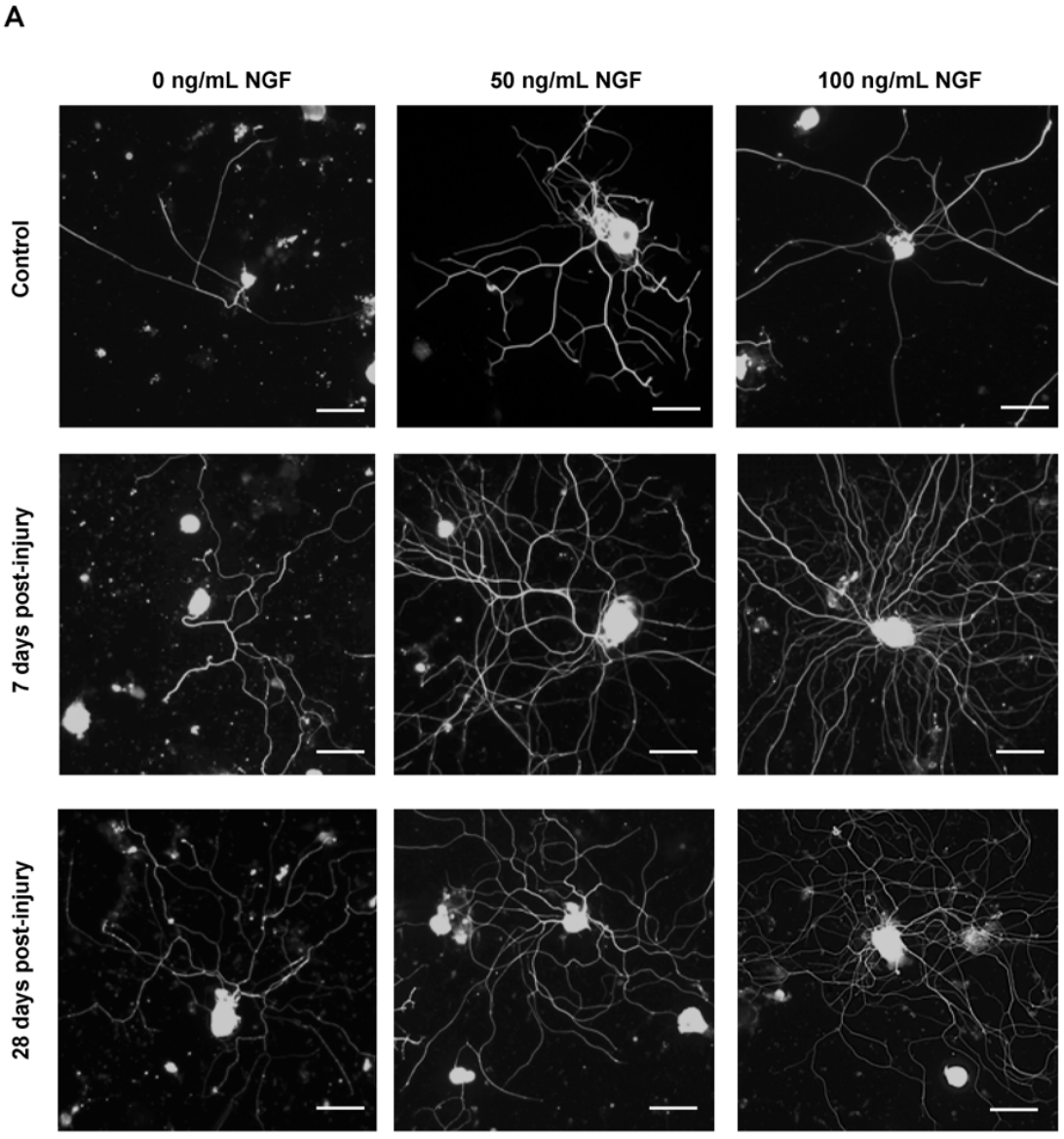

Figure 6. *Cont.*

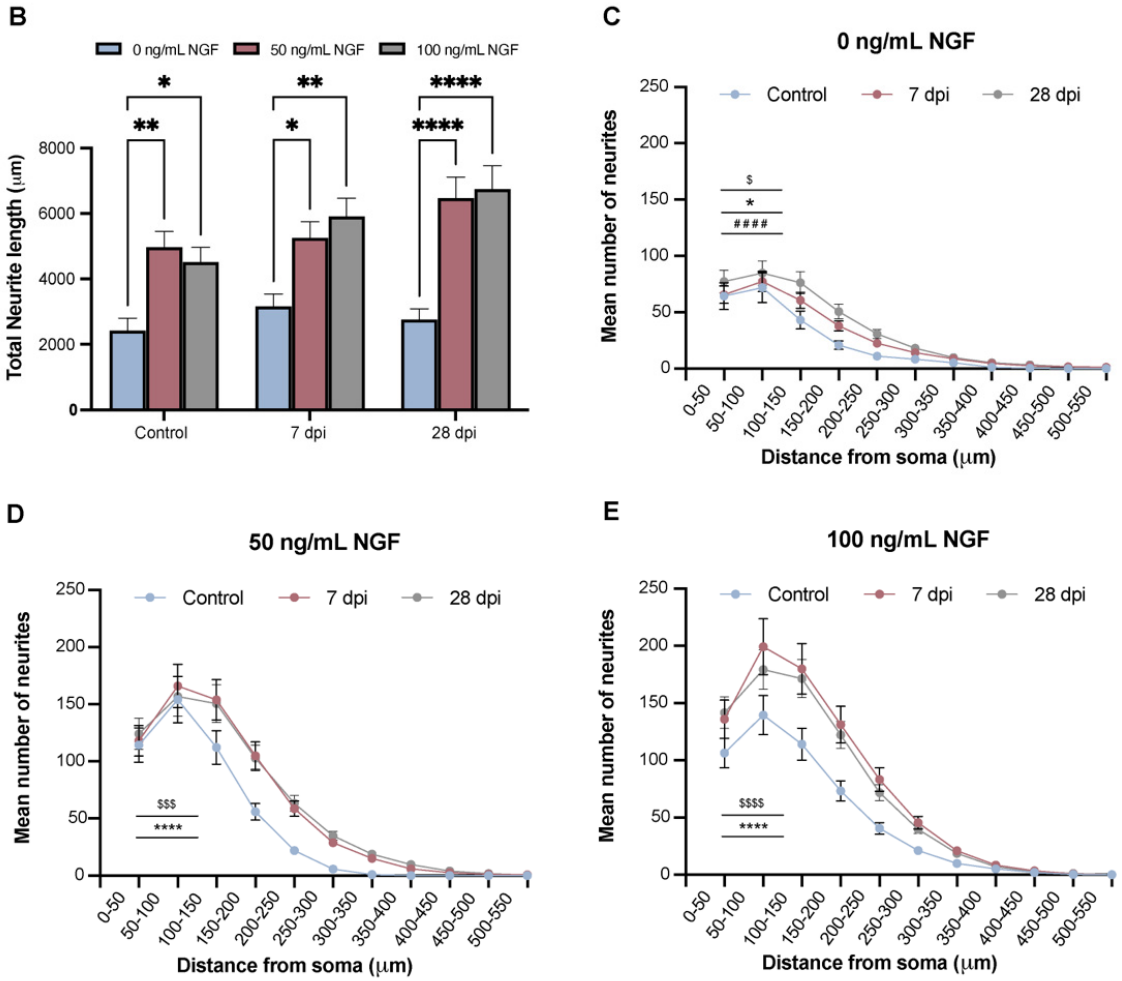

Figure 6. In vitro experiments using lumbosacral (L5-S1) DRG neurons from spinal intact (control) and SCT animals (7 and 28 dpi (days post-injury)) cultured for 22 h in distinct concentrations of NGF (0, 50 and 100 ng/mL). DRG neurons from control and SCT animals were immunostained against βIII- tubulin, and analysis showed that neurons emitted long and ramified neurites, which were more prominent in cells from SCT animals (**A**). Axonal growth was exacerbated by the presence of NGF in the culture medium. Scale bar: 100 μm. Total neurite length (**B**) of cultured DRG cells significantly increased in all experimental groups treated with either 50 ng/mL or 100 ng/mL of NGF, compared to animals treated with 0 ng/mL of NGF. Graphs represent mean ± SEM and $p < 0.05$ was considered statistically significant. * $p \leq 0.05$; ** $p \leq 0.01$; **** $p \leq 0.0001$; two-way ANOVA followed by Newman–Keuls multiple comparison test. Correlation between mean number of branches and distance from the soma (**C**–**E**) indicated that branching was significantly upregulated by NGF dosage in SCT animals, compared to controls. Graphs represent mean ± SEM and $p < 0.05$ was considered statistically significant. (**C**) \$ $p \leq 0.05$ 7 dpi vs. control animals; * $p \leq 0.05$ 28 dpi vs. control animals; #### $p \leq 0.0001$ 7 dpi vs. 28 dpi animals. (**D**) \$\$\$ $p \leq 0.001$ 7 dpi vs. control animals; **** $p \leq 0.0001$ 28 dpi vs. control animals. (**E**) \$\$\$\$ $p \leq 0.0001$ 7 dpi vs. control animals; **** $p \leq 0.0001$ 28 dpi vs. control animals; two-way ANOVA followed by Tukey's multiple comparison test.

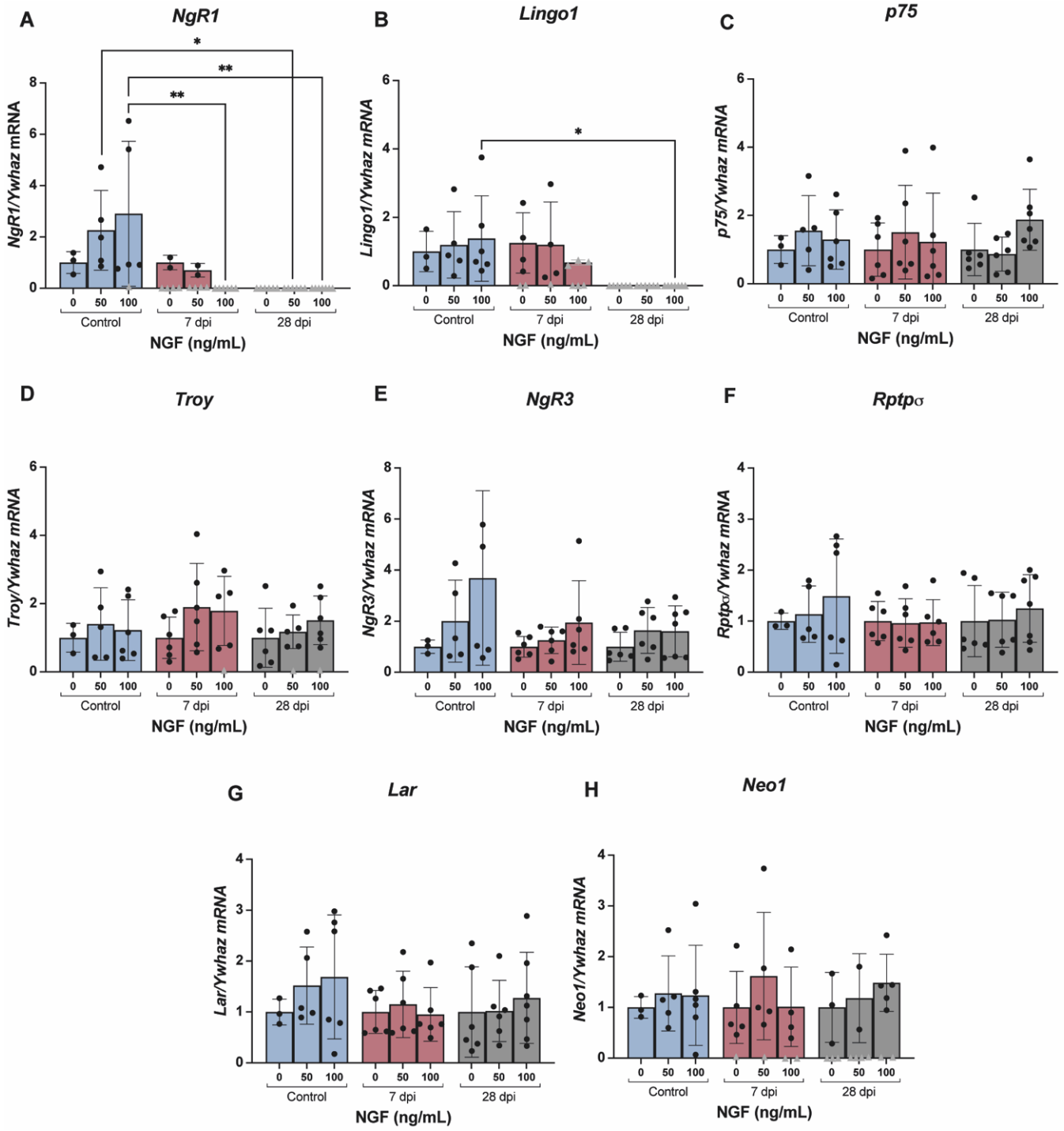

Figure 7. Levels of mRNA of myelin-associated inhibitors (MAIs) receptors *NgR1* (**A**), *Lingo1* (**B**), *p75* (**C**) and *Troy* (**D**), CSPGs receptors *NgR3* (**E**), *Rptpσ* (**F**) and *Lar* (**G**) and RGMa receptor *Neo1* (**H**) in cultured lumbosacral (L5-S1) DRG cells of spinal intact (control) and SCT animals. Graphs represent mean ± SD and $p < 0.05$ was considered statistically significant. mRNA levels of *NgR1* and *Lingo1* significantly decrease 28 dpi (days post-injury), independently of NGF concentration, as amplification was nondetected (represented in the graphs by grey triangles) in these groups. mRNA levels of the other receptors remained unaltered. * $p \leq 0.05$; ** $p \leq 0.01$; two-way ANOVA followed by Newman–Keuls multiple comparison test.

3. Discussion

Traumatic SCI is known to alter the composition of the spinal ECM at the injury site, where inhibitory cues that preclude axonal growth and regeneration of damaged tracts become accumulated. Although poorly investigated, there is also evidence that the ECM composition may be altered in segments distant from the injury location, and one can hypothesize that such changes likely contribute to maladaptive neuroplasticity, a matter investigated here.

In the present study, we used a model of complete spinal cord transection, which causes urinary dysfunction, a major concern for SCI patients [41,59]. SCI-induced urinary impairment is hypothesized as resulting from maladaptive reorganization of spinal circuits at the lumbosacral cord [60]. The chosen model has been previously validated [45] and, as before, we found that development of urinary dysfunction was accompanied by upregulation of GAP43 expression below the lesion. This protein is a marker of axonal sprouting [46] and was observed in lumbosacral DRG neurons and in the superficial laminae of the lumbosacral cord, where the central processes of bladder afferents terminate [48,49]. In SCT animals, these terminals have been previously shown to be peptidergic in nature, consistent with their bladder origin [42].

Axonal sprouting at the lumbosacral cord suggests changes in the spinal extracellular matrix, particularly in the superficial layers of the cord. To investigate this, spinal cord samples were analysed by Western blotting for expression of Phosphacan and Neurocan (CSPGs), Nogo-A, MAG and OMgp (MAIs) and RGMa. Surprisingly, we found an upregulation at 7 dpi of Neurocan, Phosphacan and Nogo-A, which coincided with increased GAP43 expression, with all proteins declining at 28 dpi. This is a puzzling observation, as those inhibitory proteins should have repressed axonal growth. However, the patterns of labelled structures did not coincide: immunolabelling showed that expression of GAP43 and Phosphacan and Nogo-A did not overlap, but rather coursed in parallel (Supplementary Figure S2G–I and Supplementary Figure S3G–I, respectively). Therefore, one could speculate that, as seen during development [61,62], the repulsive proteins act by directing the growing axons. It is well recognized that sprouting of peripheral afferents triggers the rearrangement of synaptic contacts at the lumbosacral cord and the final formation of new neuronal circuits. Hence, one can speculate that these circuits are restricted to the lumbosacral cord and operate without supraspinal input, leading to loss of voluntary control over bladder function and urinary incontinence [60,63]. Importantly, not all investigated inhibitory proteins were altered at the lumbosacral cord in response to thoracic spinal injury, suggesting that these dynamic changes are specific to undergoing neuroplastic rearrangements and not a generalized response.

The concurrent expression of GAP43 with Phosphacan, Neurocan and Nogo-A at the same time points and similar locations, but without colocalization, suggests that sensory afferents express receptors for those centrally produced inhibitory cues. To investigate this, the expression of key receptor components for CSPGs, MAIs and RGMa was analysed in lumbosacral DRG neurons collected from control and SCT animals. While mRNA levels of the RGMa receptor were not altered, in tandem with spinal protein levels, we found a time-dependent decrease in mRNA levels of CSPG- and MAI-receptors, which reached statistical significance at 28 dpi. Activation of these receptors with CSPGs or MAIs results in downstream intracellular activation of the RhoA/ROCK signalling pathway. When activated, ROCK catalyses actin depolymerization, growth cone collapse and arrest of axonal growth [64] via phosphorylation of several downstream targets, including MYPT1 [65]. In the present study, we found signs of axonal growth, as shown by upregulation of GAP43 expression and reduction in the expression of receptors for growth inhibitors in the DRG neurons. In line with these changes, activation of RhoA/ROCK, as evaluated by the levels of phosphorylated MYPT1, was minimal and not different between experimental groups. However, in addition to RhoA, regulation of neuronal growth by CSPGs, myelin-derived growth inhibitors and RGMa might also occur due to the activation of alternative pathways, e.g., inactivation of the pro-growth signalling cascade via Akt and glycogen synthase

kinase-3 (GSK-3). When GSK-3 is blocked in rodent models of spinal cord transection and contusion, enhanced axonal growth of descending corticospinal and raphespinal tracts, as well as locomotor functional recovery was observed [66]. Accordingly, using three genetic mouse models with high, intermediate or no GSK3β activity in neurons, Liz and co-workers demonstrated that reduced levels or complete elimination of this enzyme in SCI animals promoted axon regeneration [67].

While our results indicate a time-dependent decrease in the peripheral expression of receptors for CSPGs and MAIs, associated with enhanced central axonal sprouting of sensory afferents, the underlying mechanisms remain to be elucidated. A likely candidate involved in these mechanisms is Nerve Growth Factor (NGF). NGF is a neurotrophin, essential for growth and survival of sensory and sympathetic neurons, playing a crucial role in both CNS recovery and dysfunction following SCI. NGF is upregulated following SCI [52–54] and, while in perilesional locations' NGF increase could help to promote some regeneration and protection of surviving neuronal circuits [68–70], it has also been associated with severe SCI outcomes, such as autonomic dysreflexia, urinary dysfunction and pain [54,58,71–73], all of which have been linked to abnormal axonal sprouting [44,71]. Here, and in other studies [42,45], GAP43 upregulation has been found in the lumbosacral cord and DRG. As NGF regulates GAP43 [74], one can assume that in the present study, NGF levels could also be increased and would play a role in regulating the expression of receptors for CSPGs and MAIs. An indication in this direction was provided by our in vitro studies: lumbosacral DRG neurons were collected from control and SCT animals and cultured with different concentrations of NGF. The neurite outgrowth elicited by NGF was longer and more ramified in DRG neurons obtained from SCT animals, suggesting that these cells were primed by spinal trauma and their sprouting potential was enhanced by NGF exposure. Analysis of the expression of CSPG-, MAI- and RGMa-receptors in these cells showed a dose-dependent decrease in the levels of *NgR1* and *Lingo1*. As those receptors are part of multi-subunit complexes, reduction in the levels of a single subunit could compromise the activation of the whole receptor, blocking the inhibitory effects on axonal growth of Nogo-A, and possibly CSPGs. This is a novel mechanistic concept, as NGF is typically seen as a growth promoter by inducing the activation of pro-survival/pro-growth genes [75], rather than by reducing the expression of genes linked to inhibition of axonal growth.

Interestingly, the effects of NGF seemed to be restricted to *NgR1* and *Lingo1*, as mRNA levels of the other studied receptors remained unaltered. The reasons for this are only speculative at present. However, it should be recalled that, as NGF overcomes the inhibitory effect of CSPGs in conditioned lesioned neurons via integrin-linked kinase (ILK) signalling [76], this might explain the lack of differences in mRNA levels of receptors associated with CSPGs (*NgR3*, *Lar*, *Rptpσ)* when NGF concentration in the medium increased. Moreover, while the importance of NGF in regulating axonal growth is indisputable, other potential molecules could also contribute. Several studies indicate that various growth factors co-participate in regulating axonal growth. Gavazzi et al. demonstrated that both NGF and Glial Cell line Derived Neurotrophic Factor (GDNF) are necessary for growth of sensory neurons [77]. Brain Derived Neurotrophic Factor (BDNF) and Neurotrophin 3 (NT-3) also participate in this process [42,78]. Neurite growth in vitro follows NGF-independent and NGF-dependent mechanisms, but is also influenced by other growth factors [79] and inflammatory mediators, including interleukin (IL)-1β and IL-6, the levels of which are upregulated at 7 dpi at spinal cord injury sites [80]. The present results are consistent with a major role played by NGF in the abnormal lumbosacral expansion of sensory bladder afferents. Several studies have shown that NGF manipulation, initiated at chronic stages after SCI, can improve bladder function in SCI rodents [57,58]. Those studies suggested that in SCI animals, NGF contributes to bladder dysfunction by upregulating the bladder expression of the ion channels P2X3, TRPA1 and TRPV1, and inducing hyper-excitability of capsaicin sensitive bladder afferents. Our results indicate that, in addition to published reports [57,58], the beneficial effects of NGF manipulation on bladder function

could also include changes in the expression of genes involved in axon guidance, likely further contributing to harnessing maladaptive neuroplasticity.

4. Material and Methods

4.1. Animals

In-house bred adult female Wistar Han rats, derived from Charles River Laboratories (France) and weighing approximately 200–290 g, were maintained under a 12 h dark/light cycle, in a temperature and humidity-controlled environment with free access to food and water. Experimental procedures were carried out according to the European Communities Council Directive 2010/63/EU and local regulations (protocol ORBEA_56_2017/1218; 31 January 2019). All efforts were made to reduce the number of animals used, as well as animal stress and suffering.

4.2. Chemicals and Reagents

Spinal cord transection surgeries were performed under deep anaesthesia, induced by intraperitoneal injection of ketamine (75 mg/kg) and medetomidine (0.5 mg/kg) and reversed with atipamezole (1 mg/kg). For cystometries, animals received a subcutaneous injection of urethane (1.2 g/kg). For terminal procedures, rats were administered a lethal dose of sodium pentobarbital (65 mg/kg).

Primary and secondary antibodies used in Western blotting, immunohistochemistry and immunocytochemistry assays, as well as sources and dilutions, are listed in Tables 1 and 2, respectively. For antibodies suitable for immunohistochemistry and Western blotting, the specificity was confirmed by immunolabelling of the injury site (Supplementary Figure S1) prior to Western blotting.

Table 1. Primary antibodies used in Western Blotting, Immunohistochemistry and Immunocitochemistry assays.

Primary Antibody Name	Catalogue #	Company	MW	Source	Dilution for WB	Dilution for IHC/ICC
Nogo-A	11C7	Kindly gifted by Prof. Martin Schwab	180 kDa	Mouse	1:1000	1:1000
Phosphacan	MAB5210	Merck Millipore	180, 190 and 250 kDa	Mouse	1:1000	1:1000
Neurocan	MAB5212	Merck Millipore	245, 160, 130 and 90 kDa	Mouse	1:1000	1:1000
Omgp	ab109746	Abcam	28 kDa	Rabbit	1:5000	-
Mag	ab203060	Abcam	69 kDa	Rabbit	1:1000	-
Rgma	28045	IBL Japan	28 kDa	Rabbit	1:500	-
GAP43	ab16053	Abcam	43–48 kDa	Rabbit	1:2000	1:2000
GAPDH	ab8245	Abcam	37 kDa	Mouse	1:10,000	-
βIII tubulin	302302	Synaptic Systems	55 kDa	Rabbit	1:1000	1:1000
MYPT1	#2634	Cell Signalling	140 kDa	Rabbit	1:1000	-
Phospho-MYPT1 (Thr696)	#5163	Cell Signalling	140 kDa	Rabbit	1:1000	-

Table 2. Secondary antibodies used in Western Blotting (WB), Immunohistochemistry (IHC) and Immunocitochemistry (ICC) assays.

Secondary Antibody Name	Catalogue #	Company	Source	Dilution for WB	Dilution for IHC/ICC
Alexa Fluor™ 568 Donkey Anti-Rabbit IgG	A10042	Invitrogen	Donkey	-	1:1000
Alexa Fluor™ 488 Donkey Anti-Rabbit IgG	A21206	Invitrogen	Donkey	-	1:1000
Alexa Fluor™ 568 Donkey Anti-Mouse IgG	A10037	Invitrogen	Donkey	-	1:1000
Goat Anti-Rabbit IgG (HRP)	ab6721	Abcam	Goat	1:10,000	1:1000
Goat Anti-Mouse IgG (HRP)	ab205719	Abcam	Goat	1:10,000	-

Chondroitinase ABC from *Proteus vulgaris* (C3667), Collagenase IV-S from *Clostridium histolyticum* (C1889) and Laminin from Engelbreth–Holm–Swarm murine sarcoma basement membrane (L2020) were purchased from Sigma-Aldrich, USA. DMEM (1X) + GlutaMAX™-I (31966-021), Fetal Bovine Serum (10082147) and Penicillin Streptomycin (15070-063) were purchased from Thermo Fisher Scientific, Oeiras, Portugal. Poly-L-Lysine (0413-SC) was bought from Quimigen, Alverca, Portugal. Nerve growth factor (mNGF, 2.5S) (G5141) was purchased from Promega. PowerUp™ SYBR™ Green Master Mix (A25777) was purchased from Alfagene, Carcavelos, Portugal. Primers were synthetized by STABvida, Caparica, Portugal.

4.3. Spinal Cord Transection

A complete spinal cord transection (SCT) at the level of the low thoracic spinal segments T8/T9 was used [45]. In SCT groups, T7 to T10 vertebrae were exposed and animals underwent a laminectomy at T8/T9 level. The spinal cord was sectioned with a scalpel and a sterile absorbable haemostatic sponge was placed between the sectioned ends of the cord. Muscle and skin were sutured in layers. SCT animals submitted to spinal transection were left to recover for 7 days or 28 days. Control animals were left spinal intact. To compensate for blood loss and poor oral water intake following SCT surgery, animals received a 1 mL subcutaneous injection of 5% saline–glucose. For discomfort and pain management, animals received oral tramadol (1 mL/kg) for an average period of 5 days post-surgery. Rats also received an oral suspension of enrofloxacin (5 mg/kg) for 7 days post-surgery. In SCT animals, bladders were manually emptied daily by abdominal compression and until automatic micturition developed, to avoid urinary retention and kidney damage.

4.4. Cystometries

At each time point, all animals received a subcutaneous bolus of urethane to induce deep anaesthesia. Bladders were exposed through a low midline abdominal incision and a 21-gauge needle connected to an infusion pump and to a pressure transducer was inserted into the bladder dome for saline infusion. Animals were placed on a heating pad and body temperature was kept at 37 °C. Fifteen minutes after needle insertion, saline infusion was initiated at a constant rate of 6 mL/h. Intraluminal pressure was recorded for 1 h and the urethra remained unobstructed so that infused saline could easily be expelled by bladder contractions. After cystometries, animals were euthanized with pentobarbital and the lesion site (T8/T9), lumbosacral spinal cord (L5-S1) and respective dorsal root ganglia (DRG) were collected for protein analysis (n = 6/group), qPCR (n = 7/group), immunohistochemistry

(n = 4/group) and cell culture (n = 3–7/group). Cystometrograms were analysed and frequency, amplitude and peak and basal pressure of bladder reflex contractions were determined using LabScribe Software v4 (iWorx, World Precision Instruments, Friedberg, Germany). When amplitude of bladder contractions was less than 10 cm H_2O, amplitude and frequency of bladder contractions was considered zero.

4.5. Western Blotting Analysis

For protein analysis, tissue was freshly collected and frozen at −80 °C. Lumbosacral spinal cord segments and lumbosacral DRG were homogenized using a MagNa Lyser Instrument (Roche Diagnostics, Mannheim, Germany) and 1.4 mm ceramic Beads (Qiagen, Hilden, Germany) in lysis buffer (Tris 50 nM, NaCl 150 nM, Triton X-100 0.5% (*v/v*), EDTA 1nM, pH 7.6) supplemented with cOmplete™ Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany) and PhosSTOP Phosphatase Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany). Homogenates were sonicated and protein concentration was determined using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA). For CSPGs detection, 30 µg of spinal tissue homogenates was treated with 0.3 U/mL chondroitinase ABC (ChABC) for 3 h at 37 °C [40]. Ch ABC activity was stopped by boiling the samples at 95°C for 15 min in 1 X gel-loading buffer (GLB). Proteins were electrophoresed on 8% SDS-polyacrylamide gels and transferred onto PVDF membranes using a Trans-Blot Turbo transfer system (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked in 5% (*w/w*) milk in Tris buffer saline with 0.1% (*w/w*) Tween-20 (TBS-T) and incubated overnight at 4 °C with specific primary antibodies (Table 1), diluted in 5% (*w/w*) bovine serum albumin (BSA) in TBST. Membranes were then incubated with HRP-conjugated secondary antibodies (Table 2) for 1 h at room temperature. Immunoblots were developed via chemiluminescence (SuperSignal™ West Pico PLUS Chemiluminescent Substrate, Thermo Fisher Scientific, Rockford, IL, USA). Digital images were obtained in a Chemidoc MP System Imager using an ImageLab 5.1 software (Bio-Rad Laboratories, Hercules, CA, USA). Protein levels were quantified by densitometry and normalized against GAPDH levels using Fiji software for Mac OS X.

4.6. Perfusion and Tissue Processing

For immunohistochemical tissue analysis, after cystometry, animals were submitted to immediate transcardiac fixative perfusion with calcium-free Tyrode's solution (0.12 M NaCl, 5.4 mM KCl, 1.6 mM $MgCl_2 \cdot H_2O$, 0.4 mM $MgSO_4 \cdot H_2O$, 1.2 mM $NaH_2PO_4 \cdot H_2O$, 5.5 mM glucose and 26.2 mM $NaHCO_3$) followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). L5-S1 spinal segments and the spinal segment approximately 5 mm caudal and rostral to the injury site were collected, post-fixed overnight (ON) in 4% PFA, followed by immersion in 30% sucrose in 0.1 M PB with 0.1% sodium azide. Next, 20 µm of transverse serial sections of lumbosacral spinal cord tissue and 12 µm longitudinal sections of the injury site were obtained in a Leica cryostat, collected in Superfrost Plus slides and stored at −20 °C until further processing.

4.7. Immunohistochemistry Assays

Alternate slides were thawed and washed with phosphate-buffered saline (PBS) and PBS containing 0.3% Triton X-100 (PBST). Sections were blocked in PBST containing 10% normal horse serum (NHS) for 1 h. Primary antibodies (Table 1) were diluted in in PBST containing 2% NHS and sections incubated for 48 h at 4 °C. Sections were then washed with PBST and subsequently incubated for 1 h at room temperature with species-specific Alexa™ fluorochrome-labelled secondary antibodies (Table 3). After several washes with PBST, sections were slide covered and mounted with SlowFade Gold antifade mounting medium (Thermo Fisher Scientific, Rockford, IL, USA).

Table 3. Primer (forward and reverse) sequences, annealing temperatures and NCBI accession numbers.

Gene Name	Gene Bank Accession Number	Primer Sequence (5′→3′)	Annealing Temperature (°C)
Atp5f1b	NM_134364.1	**Forward:** TGC TAT TGC TGA GTT GGG CA **Reverse:** GGA GAT GGT CAT AGT CAC CTGC	60
Gap43	NM_017195.3	**Forward:** ACC ACT GAT AAC TCG CCG TC **Reverse:** TGG CTT CAT CTA CAG CTT CTT TCT	60
Lar (Ptprf)	NM_019249.1	**Forward:** CTC CCA GCG CTT TGA GGT AA **Reverse:** GAG CCT TCT CCA CCA CCT TC	59
Lingo1	NM_001100722.1	**Forward:** CTC CTG AGC TCC TGC CCT A **Reverse:** CTC TCG CTA CCT GCA TCT C	60
Neo1	XM_008766432.1	**Forward:** GTT GCT AGG GAG CGT GTT GA **Reverse:** GTA GGC CAC CAC TCG GAA AC	60
NgR1 (Rtn4r)	NM_053613.1	**Forward:** CGA AGA TGA AGA GGG CGT CC **Reverse:** TAG TTG CTC CAG GAG GGT CA	60
NgR3 (Rtn4rl1)	NM_181377.3	**Forward:** GGG ATT TGA ATC TGG ACC CCA **Reverse:** CAA TTC CAC ACA GCA CCC TTT G	59
P75^{NTR} (Ngfr)	NM_012610.2	**Forward:** TGA CTC TCC ATT TGG GAC TTT **Reverse:** TAC GGG TGC GGT TCT TTC	60
RPTPRσ (Ptprs)	NM_019140.2	**Forward:** CAG ACG CCA GGT ATG CTC AG **Reverse:** ATA GGC GAT GAC ATT GGC GT	60
Troy (Tnfrsf19)	NM_001044229.2	**Forward:** TCT GCA AAC AGT GTG GAC CT **Reverse:** TCT TCC GGT AAA ATC CTG GCA	58
Ywhaz	NM_013011.4	**Forward:** ACC CAC TCC GGA CAC AGA ATA **Reverse:** TCG AGA CGA CCC TCC AAG AT	60

Representative images were obtained using a Zeiss microscope (Axioimager Z1, Zeiss Z1 from Zeiss, Oberkochen, Germany) using the Axiovision 4.8 software. For spinal GAP43 quantification, L5-S1 dorsal horns were captured at 10x using the same exposure settings for all sections. GAP43 expression in lamina I and II was quantified in at least 6 sections per segment. Briefly, the mean level and standard deviation of background immunofluorescence was obtained in the region of interest without visible GAP43 staining for each section analysed using ROI analysis (Fiji software for Mac OS X). The threshold level for GAP43 positive pixels was set at a value of 5 standard deviations above the mean background level. The mean percentage of GAP43 staining was then calculated by delimiting lamina I and II in each section and using ROI analysis [81].

4.8. RNA Extraction and cDNA Synthesis

Lumbosacral DRG neurons (L5-S1) were homogenized using a MagNa Lyser Instrument (Roche Diagnostics, Mannheim, Germany) and 1.4 mm ceramic beads (Qiagen, Hilden, Germany)). Total RNA was extracted from L5-S1 DRG tissue using the RNAqueous®-Micro kit (Applied Biosystems, Bedford, MA, USA). An aliquot of each RNA sample was run on a denaturing agarose gel to assess integrity. Intact RNA was then quantified by NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA), followed by treatment with DNase. DNase-treated RNA (500 ng) was reverse transcribed using the NZY M-MuLV First-Strand cDNA Synthesis Kit (NZYtech, Lisbon, Portugal), according to the manufacturer's instructions. cDNA was diluted and stored at −20 °C for later use.

4.9. Quantitative Real-Time PCR (qPCR)

Relative gene expression levels were measured by real-time qPCR using the StepOnePlus Real-time PCR system (Applied Biosystems, Bedford, MA, USA). Reaction mixes were prepared using 7 µL of PowerUp™ SYBR™ Green Master Mix (Applied Biosystems,

Bedford, MA, USA), 1 µL of primer mix (forward + reverse primers in a final concentration of 10 µM), 5 µL of Milli-Q H_2O and 1 µL of cDNA sample, to a final volume of 14 µL. Each sample was run in triplicate. The amplification protocol consisted of forty cycles with denaturation at 95 °C for 15 s, followed by annealing at predetermined temperatures that varied from 58–60 °C, for 30 s, and extension at 72 °C for 30 s.

Target gene expression was normalized against the expression of the endogenous ATP Synthase F1 Subunit Beta (*Atp5f1b*) gene in in vivo samples and against Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta (*Ywhaz*) in in vitro samples. Primer sequences and respective Tm are listed in Table 3.

4.10. Primary DRG Cell Culture

L5-S1 DRG were collected from control and SCT animals (7 and 28 days following injury) (n = 3–7 per group) and placed in Dulbecco's modified Eagle's medium (DMEM)-Glutamax with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin. Nodules underwent enzymatic digestion with 0.125% collagenase IV-S for 2 h at 37 °C, followed by mechanical dissociation with glass Pasteur pipettes of increasingly small diameters. The single-cell solution was purified and resuspended in DMEM-Glutamax with 1% penicillin/streptomycin, 2% B27 and 0, 50 or 100 ng/mL of NGF. Cells were plated onto poly-L-lysine and laminin-coated coverslips or wells for immunocytochemistry and qPCR assays, respectively. Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere for 22 h. For immunocytochemistry assays, coverslips were washed with ice-cold PBS and fixed with 4% PFA for 15 min. After several washes with PBST for cell membrane permeabilization, cells were blocked with 5% NHS in PBST for 1 h and then incubated overnight with anti-βIII-tubulin in 2% NHS in PBST (Table 1), at 4° C. Cells were then washed several times and incubated for 1 h at room temperature with a species-specific Alexa Fluorochrome-labelled antibody (Table 2) in 2% NHS in PBST. After washing, coverslips were mounted with SlowFade Gold antifade mounting medium (Thermo Fisher Scientific, USA). Quantification of neurite branching (Scholl analysis) and total neurite length analysis were performed with SYnapse Detector (SynD) [82], using MATLAB R2021a.

From other coated wells, total RNA was extracted from DRG neurons cultured using RNAqueous-Micro kit (Applied Biosystems). The same procedure was followed as previously described for in vivo RNA extraction from DRG.

4.11. Data Analysis

Data from in vivo Western blotting, immunohistochemistry and in vivo qPCR assays were statistically analysed using one-way ANOVA followed by Tukey's multiple comparison post hoc test using GraphPad Prism 9 software. Results are represented as mean ± SD and $p < 0.05$ was considered statistically significant. Cell culture data to obtain Scholl analysis and neurite length were analysed in MATLAB using SYnapse Detector (SynD) [82], a synapse and neurite detection software program. SynD can quantify neurite branching by measuring the number of cellular processes crossing concentric circles placed around the cell body and measure neurite lengths. Statistical analysis was performed in GraphPad Prism 9 software. For total length of neurite extension, neurite branching and for each distance interval from the nucleus, as well as in vitro qPCR analysis, two-way ANOVA followed by the Student–Newman–Keuls Method post hoc test was used. In these instances, values are presented as mean ± SEM. $p < 0.05$ was considered statistically significant.

5. Conclusions

Traumatic SCI is a catastrophic event, with life-changing consequences arising from the lack of regeneration of neuronal tracts. At the lesion site, the extracellular environment becomes highly repulsive, which averts neuronal rewiring. The present study showed that a similar phenomenon occurred in spinal segments distant from the injury site. Such changes could lead to maladaptive neuroplasticity, resulting in typical complications such as urinary dysfunction. We showed that urinary dysfunction arising after complete thoracic

spinal cord transection was accompanied by central sprouting of sensory afferents at the lumbosacral cord, in parallel to upregulation of inhibitory cues (Phosphacan, Neurocan, Nogo-A). Expression at the DRG of receptors for Phosphacan, Neurocan and Nogo-A was time-dependently downregulated, thus enhancing afferent fibre sprouting. Such changes could, at least partially, be mediated by peripheral NGF, as NGF downregulated inhibitory factor receptor subunits in cultured DRG neurons. Altogether, our results confirm the occurrence and importance of molecular mechanisms operating far from the injury site than can be accounted for maladaptive neuroplastic events. Moreover, data also support that NGF manipulation should be further explored as a potential therapeutic tool to harness neuroplasticity and control SCI-induced urinary impairment.

Supplementary Materials: The supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms23158667/s1.

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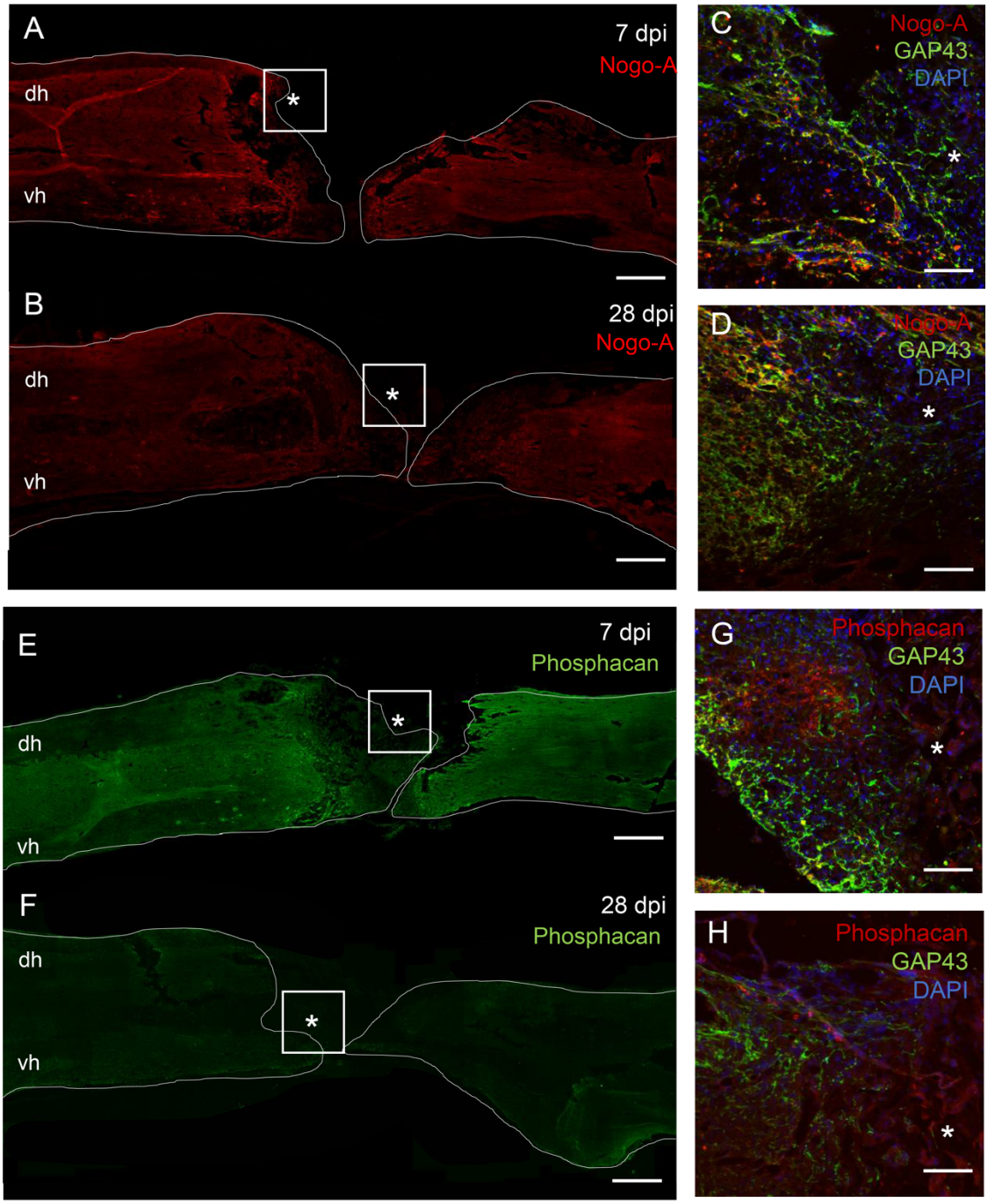
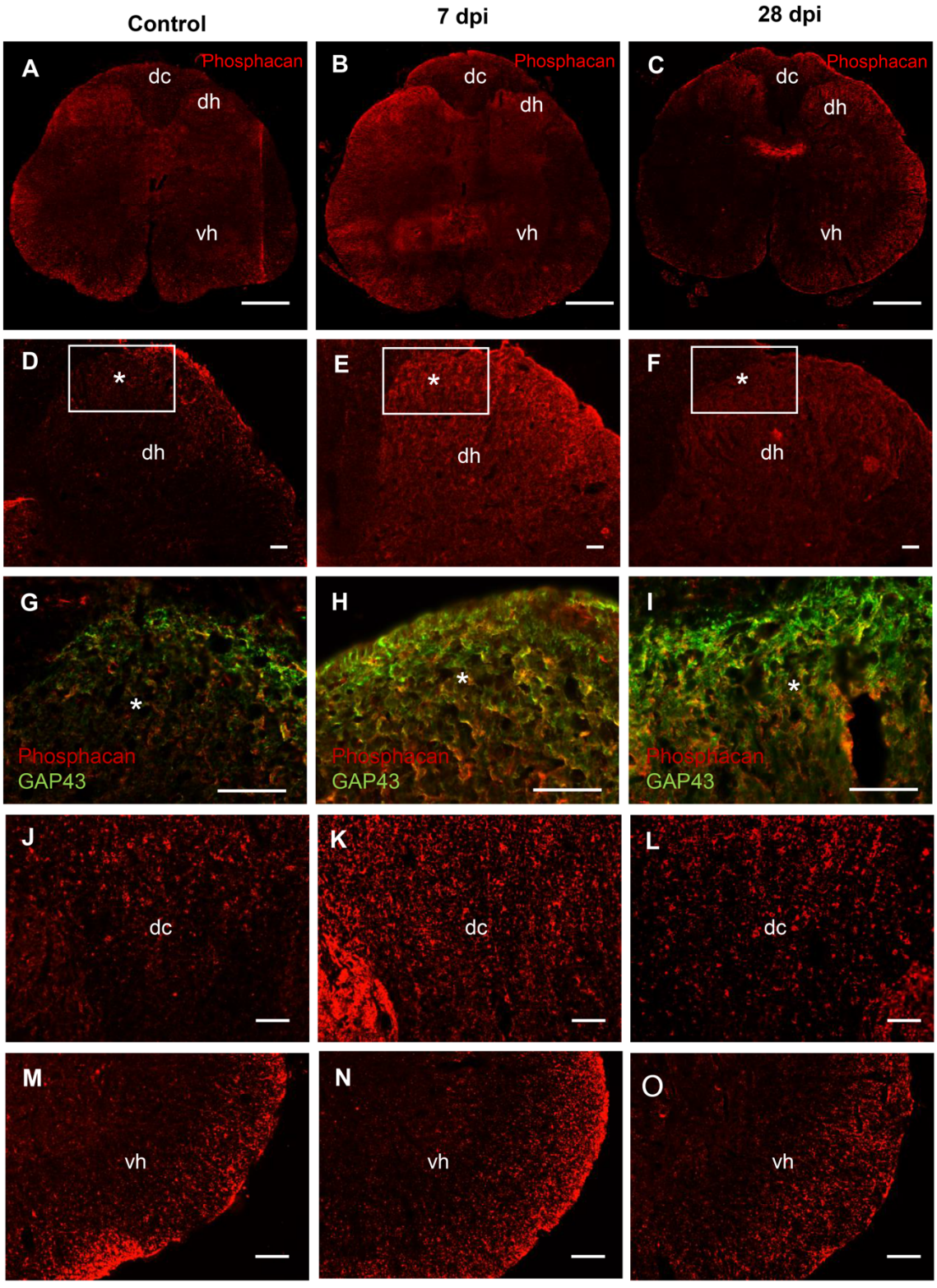
Supplementary material in *Publication II*

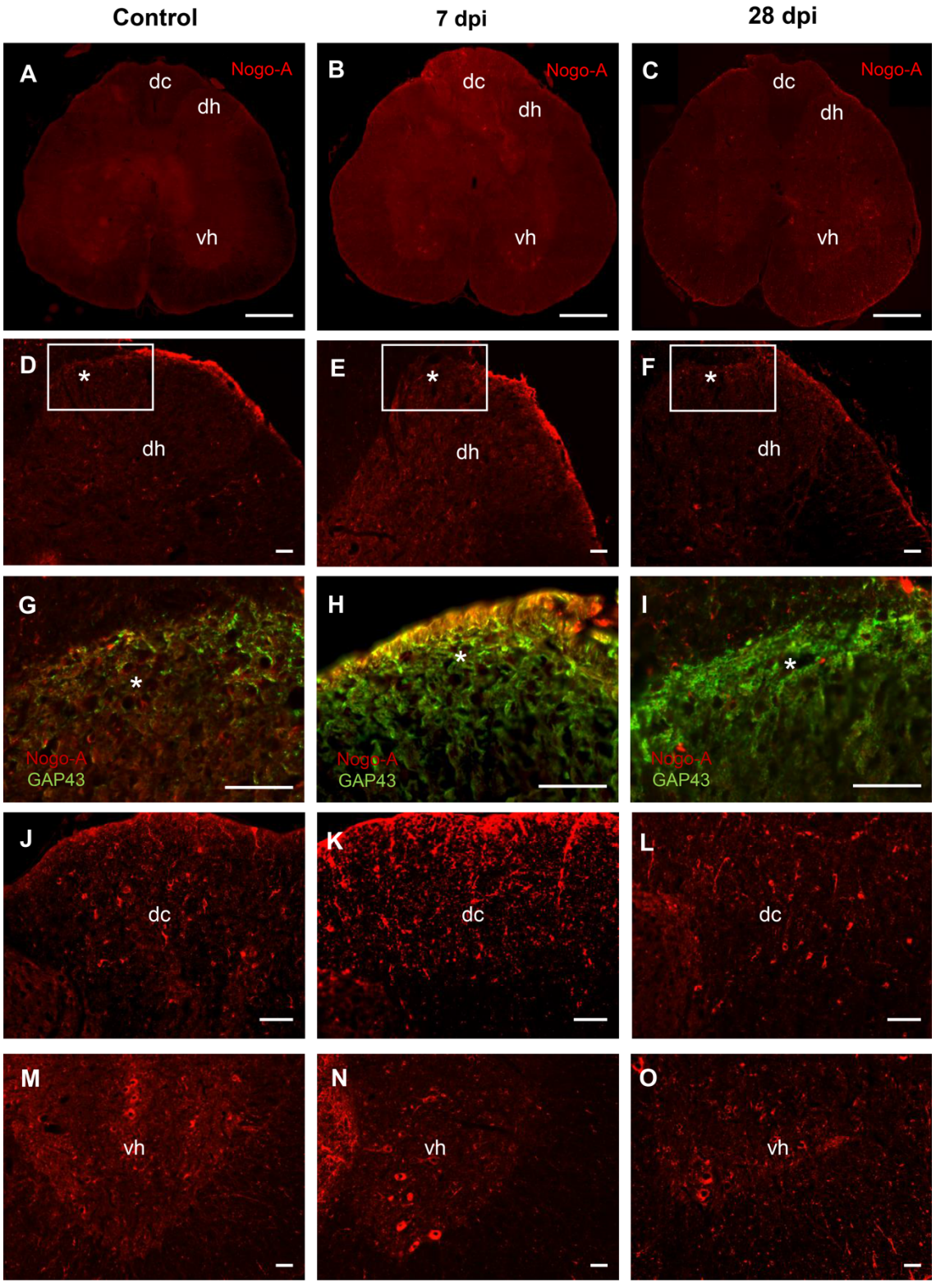


Supplementary Figure 1. Immunodetection of time-dependent expression of growth inhibitory molecules Nogo-A (A-D) and Phosphacan (E-H), as well as neural sprouting marker GAP43, in transverse sections of the lesion site of thoracic spinal segments T8/T9 (±1 cm from the lesion epicenter) of SCT (7 and 28 dpi (days post-injury)) animals. Fluorescent immunohistochemistry images show the extent of the injuries after thoracic spinal cord transection 7 and 28 dpi. The expression of repulsive molecules in the periphery of the injury epicenter is evident (A, B, E, F). The boxed areas in * are enlarged on panels (C,D and G, H). Scale A, B, E and F = 500 µm; Scale C, D, G, H = 100 µm.



Supplementary Figure 2. Time-dependent expression of Phosphacan in lumbosacral spinal cord segments of spinal intact (control) and SCT animals (7 and 28 dpi (days post-injury)). Panoramic view of L6 spinal cord segments immunoreacted for Phosphacan in control (A), 7 dpi (B) and 28 dpi (C) animals. Phosphacan immunoreaction in the dorsal horn (dh) of L6 spinal segments of control (D) and 7 dpi (E) and 28 dpi (F) shows an increase in Phosphacan expression 7 dpi throughout the horn, especially in the superficial laminae. Magnification of the superficial lamina of the dorsal horn (represented by boxed in *) in control (G), 7 dpi (H) and 28 dpi (I) shows some co-localization with the sprouting marker GAP43, especially at 7

dpi. Phosphacan expression in the dorsal commissure (dc) (J-L) and the ventral horn (vh) (M-O) of lumbosacral segments also show an increase in Phosphacan immunofluorescence in these areas. Scale A, B and C = 200 µm; Scale D-O = 50 µm.



Supplementary Figure 3. Time-dependent expression of Nogo-A in lumbosacral spinal cord segments of spinal intact (control) and SCT animals (7 and 28 dpi (days post-injury)). Panoramic view of L6 spinal cord

segments immunoreacted for Nogo-A in control (A), 7 dpi (B) and 28 dpi (C) animals. Nogo-A immunoreaction in the dorsal horn (dh) of L6 spinal segments of control (D) and 7 dpi (E) and 28 dpi (F) shows a decrease in Nogo-A expression 28 dpi throughout the horn. Magnification of the superficial lamina of the dorsal horn (represented by boxed in *) in control (G), 7 dpi (H) and 28 dpi (I) shows some co-localization with sprouting marker GAP43 at 7 dpi, disappearing 28 dpi. Nogo-A expression in the dorsal commissure (dc) (J-L) and the ventral horn (vh) (M-O) of lumbosacral segments also show an increase in Nogo-A immunofluorescence in these areas, especially in motoneurons of the ventral horn. Scale A, B and C = 200 µm; Scale D-O = 50 µm.

Publication III

Chambel SS, Ferreira A, Charrua A, Reguenga C, Cruz CD (2023) *Effects of tropomyosin-related kinase A inhibition to treat neurogenic detrusor overactivity in rats with a traumatic spinal cord injury* - ***Submitted.***

Effects of tropomyosin-related kinase A inhibition to treat neurogenic detrusor overactivity in rats with a traumatic spinal cord injury

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Abstract

Nerve growth factor (NGF) is one of the major players in lower urinary tract dysfunction after spinal cord injury (SCI). Neurogenic detrusor overactivity (NDO) emergence courses with increased NGF levels in the bladder and in neuronal pathways involved in bladder function where the NGF high affinity receptor tropomyosin-related kinase A (TrkA) is expressed. While NGF blockade has been previously shown to improve bladder function in chronic SCI rodents when urinary impairment is already installed, this study aimed to investigate the effects of early TrkA inhibition after SCI on urinary dysfunction.

Female Wistar rats underwent spinal cord transection (SCT) and were orally treated with vehicle or a selective TrkA inhibitor, GW441756, for 7 or 28 days. Twenty-eight days post-SCT, GW441756-treated animals displayed improved bladder function, characterized by increased amplitude of bladder contractions and decreased frequency of expulsive bladder contractions, hallmarks of improved voiding efficiency. Moreover, GW441756-treated animals showed significantly decreased levels of Calcitonin Gene Related Peptide (CGRP) and Growth Associated Protein 43 (GAP43) in the bladder following TrkA inhibition. This was accompanied by significant overexpression of the

Nogo-A and Neurocan in the lumbosacral spinal cord, where these molecules are known to modulate axonal growth.

Our results show that TrkA inhibition via GW441756, initiated immediately after SCI, resulted in protection of bladder function, via modulation of peripheral and central neuroplasticity. These observations further support the need to introduce therapeutic measures at early stages of disease progression to preserve bladder function.

1. Introduction

Nerve growth factor (NGF) is a key neurotrophic factor essential for survival, maintenance and growth of sympathetic and small-diameter, primary sensory neurons during development (Crowley et al., 1994), but not essential for cell survival in the adult. Like other neurotrophins, NGF is produced not only by neurons but also by peripheral tissues, including the bladder smooth muscle (Steers et al., 1991; Steers and Tuttle, 2006), particularly in pathological conditions such spinal cord injury (SCI) (Yoshizawa et al., 2014; Kadekawa et al., 2017a). NGF exerts most of its effects upon binding to its high affinity receptor, tropomyosin-related kinase A (TrkA), triggering the activation of downstream signalling pathways (Mizumura and Murase, 2015; Denk et al., 2017). About half of sensory afferents express TrkA and 92% of the TrkA-expressing afferents co-express calcitonin gene related peptide (CGRP) (Averill et al., 1995), abundantly expressed by bladder afferents (Avelino et al., 2002).

The involvement of NGF in lower urinary tract (LUT) function has been widely discussed (Ochodnicky et al., 2012; Coelho et al., 2019), particularly in SCI-induced bladder impairment. Traumatic SCI is typically followed by a period of little of no bladder reflex activity, known as spinal shock (Bywater et al., 2018; Anderson et al., 2023), followed by

the emergence of neurogenic detrusor overactivity (NDO), often concurrent with detrusor-sphincter-dyssynergia (DSD) (Wada et al., 2022). NDO and DSD development course with increased levels of NGF in the bladder and in the neuronal pathways regulating micturition (Yoshimura et al., 2006; Kadekawa et al., 2017a; Wada et al., 2018). Emergence of SCI-induced urinary impairment occurs simultaneously with abnormal sprouting of sensory afferents in the bladder, as measured by GAP43 expression (Vizzard, 1999; Oliveira et al., 2019). At the spinal cord level, this NGF-dependent expansion of axonal processes also occurs after SCI (Zinck et al., 2007; Frias et al., 2015; Chambel et al., 2022), together with time-dependent changes in lumbosacral expression of axon guidance molecules, including myelin-associated molecules, such as Nogo-A, and chondroitin sulphate proteoglycans (CSPGs), as Neurocan (Chambel et al., 2022).

Many studies have pinpointed NGF as a critical player in development of SCI-induced urinary impairment and its modulation has attracted therapeutic interest. In SCI animals, NGF neutralization resulted in improved of bladder function (Seki et al., 2002; Kadekawa et al., 2017a; Wada et al., 2018), but treatment was only initiated at late post-SCI timepoints, the earliest timepoint reported being two weeks post-SCI (Seki et al., 2002; Shimizu et al., 2018; Wada et al., 2018; Ni et al., 2022). However, there is evidence supporting the need for early intervention to prevent NDO development (Sievert et al., 2010; Biardeau et al., 2017; Oliveira et al., 2019). Therefore, the present study investigated the effects of TrkA inhibition, initiated immediately after spinal lesion, on bladder function, by administration of an oral selective inhibitor of the NGF receptor TrkA. The effects of TrkA inhibition on the mechanisms regulating NGF-dependent abnormal axonal growth were also investigated.

2. Material and Methods

2.1 Animals and surgical procedures

In-house bred female Wistar Han rats (200-250g; n=5/6 per experimental group) were kept under an inverted 12h dark/light cycle, in a temperature and humidity-controlled environment, with free access to food, water and environmental enrichment. Procedures were carried out according to the European Communities Council Directive 2010/63/EU as well as institutional regulations (ORBEA_56_2017/1218). Every effort was made to reduce the number of animals used as well as their stress and suffering. Prior to SCI model induction, animals were acclimated to the manipulator and to being syringe fed strawberry jam, the vehicle for oral drug administration, for at least 5 days.

Complete spinal cord transection (SCT) was performed under deep anaesthesia (ketamine (75 mg/kg)/medetomidine (0,5 mg/kg)). Briefly, thoracic vertebrae were exposed, and a laminectomy was performed at T8/T9, exposing the dura mater. After complete spinal transection, a sterile haemostatic sponge was placed between the sectioned ends of the cord and the surgical wound sutured. As part of surgery aftercare, animals received 5% glycosylated saline subcutaneously, to compensate for blood loss and reduced water intake. Additionally, enrofloxacin (5 mg/kg) was administered for 7-10 days after surgery, as well as buprenorphine (0,1 mg/kg) for at least 5 days following surgery and for as long as animals exhibited pain-like behaviours. Animals underwent daily abdominal compression until automatic micturition emerged.

2.2 TrkA administration

GW441756, a selective TrkA inhibitor, was purchased from Tocris (Cat. No. 2238) and its administration was initiated on surgery day. GW441756 was reconstituted in

dimethyl sulfoxide (DMSO), as per the manufacturer's recommendations, and orally delivered at a concentration of 58 µg/kg/day in 1% DMSO (Dias et al., 2019). Daily dosages were kept at -80°C until administration, and then dissolved in saline and mixed with jam. Vehicle-treated SCT animals received 1% DMSO diluted in saline and mixed in jam. Another group of SCT animals received jam alone. SCT animals received jam, DMSO or GW441756+DMSO for 7 or 28 days. A group of spinal intact (control) animals was also studied.

2.3 Assessment of general health

Animals were daily weighed and evaluated according to a pre-defined score that evaluated bladder volume, estimated by abdominal palpation, and the volume of urine expelled upon abdominal compression. The score translates as follows:

1 - Very small bladder, with urine volumes between 0 mL – 1.5 mL; difficult to perform abdominal compression.

2 - Small bladder, with volumes between 1.6 mL – 3.9 mL; easy to feel and to perform abdominal compression.

3 - Medium-sized bladder, with volumes between 4 mL – 5.5 mL; easily noticeable bladders and compliable to compression.

4 - Large-sized bladder, with volumes between 5.6 mL and 8.9 mL; very large bladders and vesical compression is difficult to perform.

5 - Very large-sized bladder, with volumes between 9 mL – 12.5 mL; bladders present very evident turgency, resulting in high pressures and difficulty to perform vesical compressions.

2.4 Cystometries

All animals underwent cystometries under urethane anaesthesia (1,2 g/kg; subcutaneous injection) at the end of each timepoint. Once anaesthetized, bladders were exposed through a low midline abdominal incision and a 21-gauge needle, connected to an infusion pump and a pressure transducer, was inserted in the bladder dome. Body temperature was maintained at 37°C. Saline infusion (6 mL/h) was initiated 15 minutes after needle insertion to allow for bladder stabilization and maintained for 1 hour. Cystometrograms were analysed with LabScribe Software v4 (iWorx, World Precision Instruments) and frequency, basal and peak bladder pressures and amplitude of bladder reflex contractions were evaluated. Bladder contractions were disregarded if the amplitude was less than 5 cm H_2O. After cystometries, animals were euthanized with sodium pentobarbital (65 mg/kg). The bladder and lumbosacral spinal cord (L5-S1) were collected and immediately frozen at -80°C for subsequent analysis.

2.5 Western blotting analysis

The bladder and lumbosacral spinal cord segments were homogenized using a MagNa Lyser Instrument (Roche Life Science) with 1.4 mm ceramic Beads (Qiagen) in lysis buffer (Tris 50 nM, NaCl 150 nM, Triton X-100 0.5% (v/v), EDTA 1nM, pH 7.6) supplemented with cOmplete™ Protease Inhibitor Cocktail (Roche, Germany) and PhosSTOP Phosphatase Inhibitor Cocktail (Roche, Germany). Protein concentration was determined using the Pierce™ BCA Protein Assay kit (Thermo Scientific, USA). To detect the presence of CSPGs, spinal cord homogenates (30 µg) were treated with 0.3U/ml chondroitinase ABC (ChABC) for 3 h at 37°C. Enzyme inactivation was performed at 95°C in 5X SDS-PAGE sample loading buffer (NZYtech, Portugal) for 10 minutes. To detect the

presence of the remaining proteins studied, either in spinal cord or bladder homogenates, 30 µg of samples were boiled a 95°C for 10 minutes in 5X SDS-PAGE sample loading buffer. Samples were then electrophoresed in either 7,5% or 12% SDS-polyacrylamide gels and transferred to 0,45 µm or 0,2 µm, respectively, PVDF membranes using a Trans-Blot Turbo transfer system (BioRad, Portugal). Membranes were blocked in 5% (w/w) milk in Tris buffer saline with 0.1% (w/w) Tween-20 (TBST) and incubated in primary antibodies (**Table 1**) in 5% (w/w) bovine serum albumin (BSA) in TBST, at 4°C overnight. On the following day, membranes were incubated with HRP-linked secondary antibodies (**Table 1**) at room temperature for 1 hour. Immunoblots were developed by chemiluminescence (SuperSignal™ West Pico PLUS Chemiluminescent Substrate, Thermo Fisher Scientific, USA). Digital images were obtained in a Chemidoc MP System Imager using the ImageLab 5.1 software (Biorad, California, USA). Protein levels were quantified by densitometric analysis using the lane profile tool in ImageLab and normalized against endogenous GAPDH protein levels.

Table 1. Primary and secondary antibodies used in Western Blotting assays.

Primary Antibody	**Catalog #**	**Company**	**MW**	**Source**	**Dilution**
Nogo-A	11C7	Kindly gifted by Prof. Martin Schwab	180 kDa	Mouse	1:1000
Neurocan	MAB5212	Merck Millipore	245, 160, 130 kDa	Mouse	1:1000
GAP43	ab16053	Abcam	43-48 kDa	Rabbit	1:2000
CGRP	#14959	Cell Signalling	25 kDa	Rabit	1:1000
GAPDH	ab8245	Abcam	37 kDa	Mouse	1:10 000
Secondary antibodies	**Catalog #**	**Company**	**MW**	**Source**	**Dilution**
Goat Anti-Rabbit IgG (HRP)	ab6721	Abcam	-	Goat	1:10 000
Goat Anti-Mouse IgG (HRP)	ab205719	Abcam	-	Goat	1:10 000

2.6 Data analysis.

All data was statistically analysed using GraphPad Prism 9 software. Cystometric and Western blotting data were analysed using one-way ANOVA followed by Tukey's multiple comparison test. Body weight variation and bladder score were analysed using two-way ANOVA (repeated measures) followed by the Tukey's multiple comparison test. Data are represented as mean ± SD and $p<0.05$ was considered statistically significant.

3. Results

3.1 General health status

Prior to handling and surgery, animals were randomly allocated to 7 experimental groups: control animals (spinal intact and untreated; n=5); SCT animals, left to recover for 7 days post-SCT surgery, and receiving oral jam with either saline (n=5), DMSO (n=5) or GW441756 in DMSO (n=5); and SCT animals, left to recover for 28 days, and receiving either oral jam with saline (n=5), DMSO (n=6) or GW441756 in DMSO (n=5) (**Figure 1A-B**). To evaluate the general health status of the animals, body weight was registered daily throughout the experimental protocol. While after SCT surgery, all animals, irrespective of treatment, had a decrease in body weight (**Figure 1C**), GW441756-treated animals recovered faster from the surgery, becoming more alert and beginning to eat, drink and nest 3-5 days before saline and DMSO-treated counterparts, despite lack of statistical significance. Among all groups, DMSO-treated animals reached the end of the protocol having gained the highest percentage of weight, compared to the initial weight registered before surgery (**Figure 1C**).

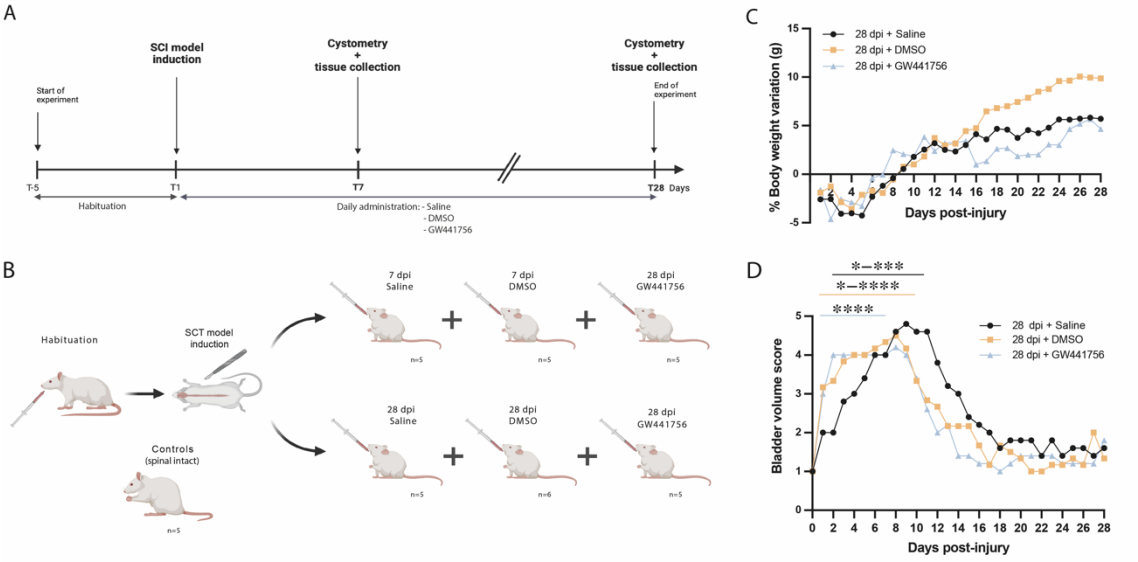

Figure 1. Experimental design and assessment of general health. (**A-B)** Timeline and schematic representation of the progression of the experiment. Prior to handling and surgery, animals were

randomly allocated to 7 experimental groups: control animals (spinal intact and untreated; n=5); SCT animals, left to recover for 7 days post-SCT surgery, and receiving oral jam with either saline (n=5), DMSO (n=5) or GW441756 in DMSO (n=5); and SCT animals, left to recover for 28 days, and receiving either oral jam with saline (n=5), DMSO (n=6) or GW441756 in DMSO (n=5). Rats were trained to eat strawberry jam from a syringe for easier oral drug (or vehicle) administration. After the habituation period (from T-5 to T1), animals underwent a thoracic laminectomy (T1), followed by a complete spinal cord transection of the T8/T9 spinal cord. Animals were left to recover for 7 days (T7), the hallmark of spinal shock, or for 28 days (T28), when neurogenic detrusor overactivity (NDO) is already observable. During this time, animals either received, mixed with strawberry jam, saline, dimethyl sulfoxide (DMSO) or GW441756, a selective tropomyosin receptor A (TrkA) inhibitor. Spinal intact animals (controls) were also used in these experiments. At the end of each timepoint, animals underwent cystometries under urethane anesthesia, followed by euthanasia and tissue collection (bladders and lumbosacral (L5-S1) spinal cords). **(C)** Effects of saline, DMSO and GW441756 administration on body weight variation following spinal cord transection. Animals were daily weighted throughout the experimental protocol after abdominal compression for urine removal. The percentage of weight variation was calculated against the initial body weight (prior to surgery) of each animal. All animals presented a decrease in body weight in the early days after spinal lesion. SCT animals treated with GW441756 returned to their initial body weight first, compared to saline and DMSO-treated animals. DMSO-treated animals, however, reached the end of the experimental protocol having gained the highest percentage of weight, compared to the averaged initial weight recorded. Still, no statistical differences were found between groups. **(D)** Effects of saline, DMSO and GW441756 administration on bladder score following spinal cord transection. In animals receiving saline, bladder score increased significantly until day 11 post-SCT ($p<0.0001$ vs. day 2 of lesion; saline), decreasing from day 12 onwards and plateauing from day 18 until cystometries. In animals receiving oral DMSO, the bladder score was significantly upregulated until day 9 post-SCT ($p<0.0001$ vs. day 0; DMSO). It then decreased until day 18 post-SCT and remained at similar values until euthanasia. In animals treated with GW441756, the bladder score significantly increased until day 7 ($p<0.001$ vs. day 0; GW441756) and steadily reduced until day 15 post-SCT, remaining constant until the end of the experimental period (**Figure 1D**). We found no statistically significant differences between groups, but bladder score of animals receiving saline never returned to a score observed at day 0, in contrast with animals receiving GW441756 (day 14) and rats treated with oral DMSO (day 21) (**Figure 1D**). Graphs represent mean ± SD and $p<0.05$ was considered statistically significant. *$p<0.05$; ***$p<0.001$; ****$p<0.0001$ vs. initial bladder score. Two-way ANOVA (repeated measures) followed by Tukey's pos-hoc multiple comparison test.

3.2 Assessment of bladder function: bladder score and cystometries

A bladder score, according to a previously defined urine volume scale and bladder size determined by palpation, was daily recorded throughout the experimental protocol (**Figure 1D**). In saline-treated animals, statistical differences in bladder score were detected the day immediately after SCT surgery and up until the 11 days that followed ($p<0.0001$ vs. day 2 of lesion). DMSO decreased these differences in bladder score and animals took 9 days following SCT to reach a bladder score that was not statistically

different from the initial score recorded before surgery ($p<0.0001$ vs. day 0) (**Figure 1D**). GW441756-treated animals had the shortest recorded timeframe in which the bladder score was statistically different from the initial score, only taking, on average, 7 days to return to a score similar to that of pre-surgery ($p<0.001$ vs. day 0) (**Figure 1D**).

Table 2. Cystometric parameters from spinal intact and SCT animals treated with saline, vehicle or GW441756.

	Frequency of voiding contractions (contractions/min)	**Peak pressure of voiding contractions (cm H_2O)**	**Basal pressure of voiding contractions (cm H_2O)**	**Amplitude of voiding contractions (cm H_2O)**
Spinal intact (Control)	0.58 ± 0.20	35.80 ± 1.82	9.68 ± 2.18	26.58 ± 2.71
7 dpi + saline	0 ± 0	0 ± 0	30.62 ± 2.86	0 ± 0
7 dpi + DMSO	0 ± 0	0 ± 0	18.81 ± 7.69	0 ± 0
7 dpi + GW441756	0 ± 0	0 ± 0	21.97 ± 8.90	0 ± 0
28 dpi + saline	1.24 ± 0.25	48.60 ± 9.47	30.21 ± 7.37	19.27 ± 1.18
28 dpi + DMSO	0.98 ± 0.16	31.63 ± 5.63	20.72 ± 8.01	10.91 ± 3.62
28 dpi + GW441756	0.79 ± 0.25	41.20 ± 4.18	13.68 ± 2.59	27.52 ± 7.72

Abbreviations: 7 dpi, 7 days-post injury; 28 dpi, 28 days-pos injury; data presented as mean ± standard deviation.

Spinal intact animals (control), as well as SCT animals, seven (7 dpi) and twenty-eight (28 dpi) days post-injury, were deeply anaesthetized with urethane and submitted to cystometric evaluation. Urodynamic recordings obtained from control animals were indicative of normal bladder function (**Figure 2A-E**; **Table 2**). At 7 days-post injury, bladder contractions were absent, consistent with the spinal shock stage. Since the amplitude of bladder contractions was less than 5 cm H_2O, peak pressure and frequency of bladder contractions were considered zero. Despite this, 7 days post-SCI, saline-

treated animals showed a significant increase in basal bladder pressure, compared to controls (**Figure 2B** and **2F**; p<0.001 vs. control animals). On the other hand, 7 dpi, DMSO and GW441756-treated animals showed no significant differences in bladder pressure compared to control animals (**Figure 2B** and **2G-H; Table 2**).

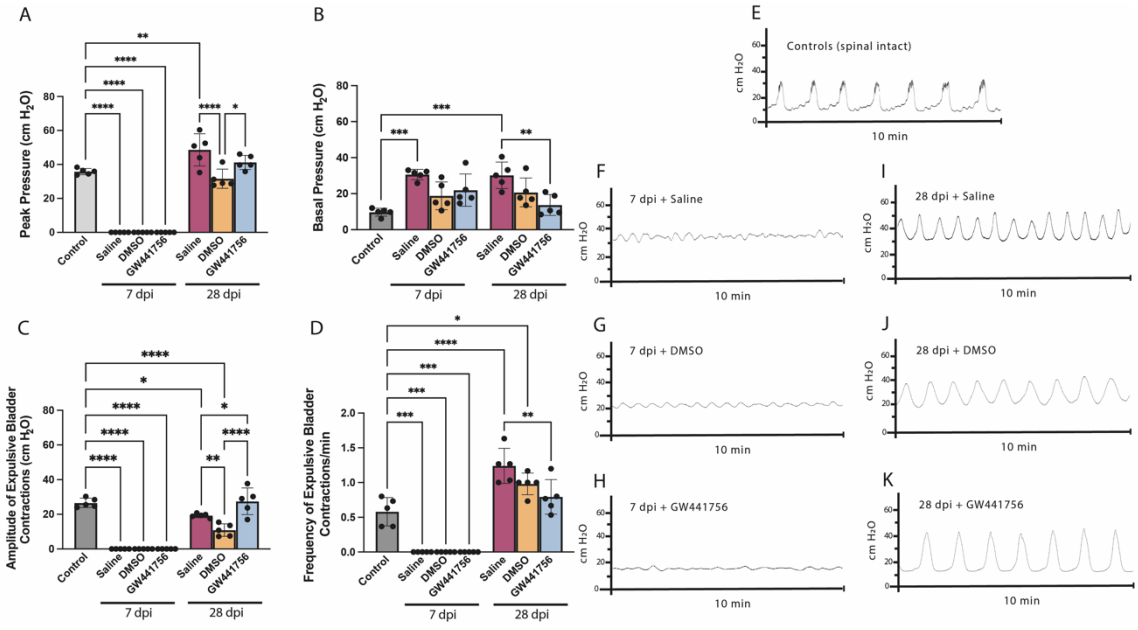

Figure 2. Effects of saline, dimethyl sulfoxide (DMSO) and GW441756 on voiding patterns of spinal cord transected animals. (A-D) Analysis of urodynamic parameters, including **(A)** Peak pressure, **(B)** Basal pressure, **(C)** Amplitude of expulsive bladder contractions and **(D)** Frequency of expulsive bladder contractions and **(E-K)** representative cystometrograms depicting the voiding pattern of spinal intact, 7 days post-injury (7 dpi) and 28 days post-injury (28 dpi) animals that received daily administration of either saline, dimethyl sulfoxide (DMSO) or GW441756. Spinal intact animals had a normal pattern of bladder reflex activity (**A-D; E**), while SCT animals showed clear signs of bladder dysfunction. At 7 dpi, independently from the treatment, bladders were acontractile (**A-D; F-H**), while at 28 dpi, saline treated animals displayed clear signs of bladder overactivity, evident by significant increases in peak and basal pressures, as well as in the frequency of bladder contractions, and decreases in the amplitude of bladder contractions (**A-D; I**). GW441756 administration partly reverted bladder dysfunction, as 28 days of treatment lead to decreases in basal pressures and in the frequency of bladder contractions, as well as an increase in the amplitude of bladder contractions (**A-D; K**). DMSO, the vehicle for GW441756 administration, when administered by itself, led to significant decreases in peak pressure and in the amplitude of bladder contractions (**A-D; J**). When the amplitude of bladder contractions was less than 5 cm H_2O, peak pressure and frequency of bladder contractions were considered zero. Graphs represent mean ± SD and p<0.05 was considered statistically significant. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. One-way ANOVA followed by Tukey's pos-hoc multiple comparison test.

Twenty-eight days post-injury, clear signs of bladder overactivity were visible in saline-treated animals, compared to controls. Bladder dysfunction in these animals was evidenced by significant increases in frequency ($p<0.0001$ vs. controls), peak pressure ($p<0.01$ vs. controls) and basal pressure ($p<0.001$ vs. controls), as well as a decrease in the amplitude of bladder contractions ($p<0.05$ vs. controls) (**Figure 2A-C** and **2I-K**). Compared to saline-treated animals, GW441756-treated rats exhibited significant lower frequency of bladder contractions ($p<0.01$ vs. 28 dpi + saline) and basal pressure ($p<0.01$ vs. 28 dpi + saline). A significant increase in peak pressure ($p<0.05$ vs. 28 dpi + DMSO) and in the amplitude of bladder contractions ($p<0.001$ vs. 28 dpi + DMSO; $p<0.05$ vs. 28 dpi + saline) was also observed in GW441756-treated animals, consistent with increased bladder efficiency (**Table 2**). Surprisingly, DMSO treatment by itself produced significant but less pronounced decreases in peak pressure ($p<0.0001$ vs. 28 dpi + saline) and in the amplitude of bladder contractions ($p<0.01$ vs. 28 dpi + saline).

3.3 Assessment of bladder innervation

Sprouting of bladder nerve fibres was evaluated by examining GAP43 expression, a known marker of sprouting axons (Benowitz and Routtenberg, 1997). GAP43 levels significantly increased at 7 dpi ($p<0.0001$ vs. spinal intact) and decreased at 28 dpi ($p<0.05$ vs. 7 dpi + saline) in saline treated animals. Treatment with DMSO and particularly GW441756 significantly reduced GAP43 overexpression at 7 dpi ($p<0.05$ vs. 7dpi + saline for DMSO; $p<0.0001$ vs. 7 dpi + saline for GW441756). At 28 dpi, no differences were found (**Figure 3A**).

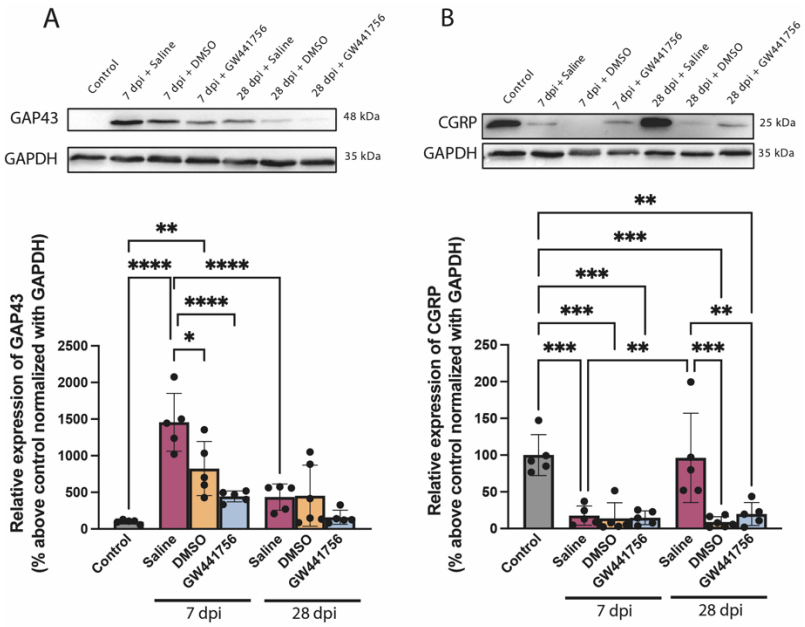

Figure 3. **Effects of saline, dimethyl sulfoxide (DMSO) and GW441756 on bladder innervation after spinal cord transection in the bladder.** Western blots of **(A)** GAP43 and **(B)** CGRP in bladder tissue of control (spinal intact), 7 days-post injury (7 dpi) and 28 days-post injury (28 dpi) animals, with daily administration of either saline, DMSO or GW441756. **(A)** GAP43 expression was significantly increased at 7 dpi, in saline-treated animals, compared to controls. Both DMSO and GW441756 administration led to significant decreases in GAP43 expression, compared to 7 dpi, saline-treated animals. At 28 dpi, saline-treated animals showed a significant decrease in GAP43 expression, compared to saline-treated 7 dpi animals, but neither DMSO nor GW441756 produced any significant differences in GAP43 expression in the bladders of treated animals. **(B)** CGRP expression did not show any significant differences at 7 dpi, independently of treatment. At 28 dpi, on the other hand, both DMSO and GW441756 administration significantly decreased the expression of CGRP in the bladder, compared to saline-treated animals. Values were rationed against the expression of the endogenous GAPDH and normalized against values from control (spinal intact) animals. Graphs represent mean ± SD and $p<0.05$ was considered statistically significant. *$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$. One-way ANOVA followed by Tukey's pos-hoc multiple comparison test.

Effects in sensory bladder fibres were also investigated by examining the CGRP levels, present in bladder afferents (Avelino et al., 2002). Bladder CRGP levels at 7 dpi + saline significantly decreased compared to spinal intact animals ($p<0.001$ dpi vs. controls). This pattern remained unaltered despite the administration of DMSO ($p<0.001$ vs. controls) or GW441756 ($p<0.001$ vs. controls) in this experimental timepoint (**Figure 3B**). Twenty-eight days post-injury, on the other hand, CGRP expression increased to spinal intact levels in saline-treated animals ($p<0.01$ vs 7 dpi + saline) but in DMSO- ($p<0.001$ vs. 28

dpi + saline) and GW441756-treated animals ($p<0.01$ vs. 28 dpi + saline), CGRP expression remained low.

3.4 Expression of axonal inhibitory molecules at the lumbosacral spinal cord

The expression of two inhibitory molecules of axonal growth in the lumbosacral spinal cord, Neurocan, a CSPG, and Nogo-A, a myelin-associated inhibitor, was studied. At 7 dpi, Neurocan-260, Neurocan-160 and total Neurocan expression levels were increased in the lumbosacral spinal cord following oral treatment. Neurocan-260 significantly increased in GW441756-treated animals ($p<0.01$ vs. 7 dpi + saline) and Neurocan-160 and total Neurocan exhibited significantly increased expressions in both DMSO- ($p<0.05$ vs. 7 dpi + saline) and particularly in GW441756-treated animals ($p<0.01$ vs. 7dpi + saline) (**Figure 4A-C**), compared to saline-treated animals. At 28 dpi, Neurocan expression in the lumbosacral cord was still significantly elevated by DMSO and GW441756 treatments. Neurocan-160 and total Neurocan expression were significantly elevated, compared to saline-treated animals of the same timepoint, both when animals were either treated with DMSO alone ($p<0.05$ vs. 28 dpi + saline for Neurocan-160; $p<0.01$ vs. 28 dpi + saline for total Neurocan) or GW441756 ($p<0.05$ vs. 28 dpi + saline, for Neurocan-160 and total Neurocan) (**Figure 4A-C**).

Nogo-A expression followed a similar trend. At 7 days post-SCT, Nogo-A expression significantly increased in the lumbosacral spinal cord when animals were treated with DMSO alone ($p<0.01$ vs. 7 dpi + saline). Concomitantly, Nogo-A expression in L5-S1 segments significantly increased when rats were either administered DMSO alone ($p<0.001$ vs 28 dpi + saline) or GW441756 ($p<0.001$ vs 28 dpi + saline) for 28 days (**Figure 4D**).

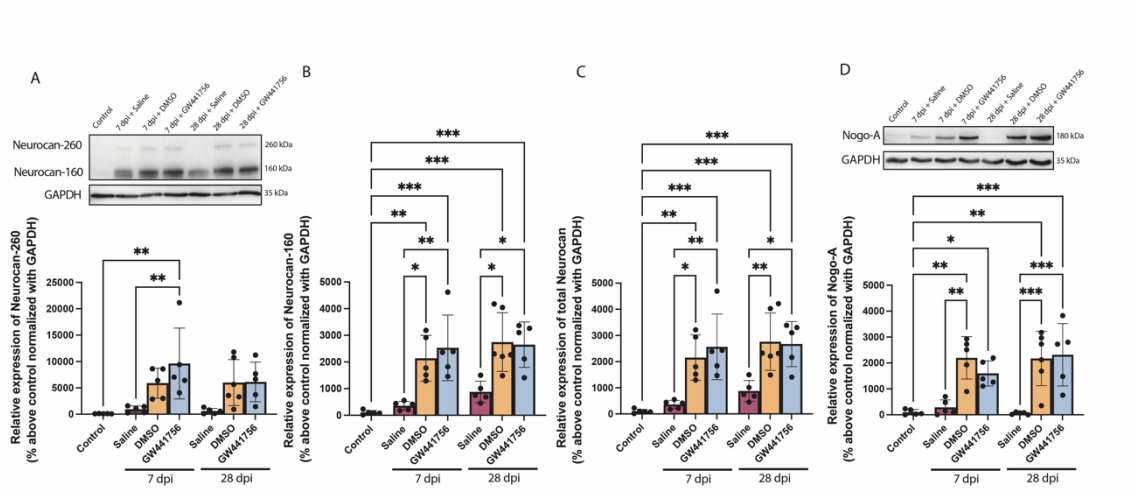

Figure 4. Effects of saline, dimethyl sulfoxide (DMSO) and GW441756 on the expression of axonal inhibitory molecules after spinal cord transection in the lumbosacral spinal cord. Western blots of **(A-C)** Neurocan and **(D)** Nogo-A in lumbosacral (L5-S1) spinal cord tissue of control (spinal intact), 7 days-post injury (7 dpi) and 28 days-post injury (28 dpi) animals, with daily administration of either saline, DMSO or GW441756. **(A)** Neurocan-260 expression was significantly increased at 7 dpi, in GW441756-treated animals, compared to saline-treated counterparts, but no significant differences were seen at 28 dpi. **(B)** Neurocan-160 and **(C)** total Neurocan expression followed the same trend seen in Neurocan-260, significantly increasing at 7 dpi, but in both DMSO- and GW441756-treated animals, compared to saline-treated counterparts. This tendency was similarly seen at 28 dpi, where the expression of Neurocan-160 and total Neurocan were significantly increased when animals were administered DMSO or GW441756, compared to saline-treated animals. **(D)** Nogo-A expression in the lumbosacral cord followed a similar trend to Neurocan. At 7 dpi, DMSO-treated animals had significantly higher Nogo-A expression, compared to saline-treated counterparts and this same significantly increased expression was still seen 28 dpi, in both DMSO- and GW441756-treated animals, compared to saline-treated counterparts. Values were rationed against the expression of the endogenous GAPDH and normalized against values from control (spinal intact) animals. Graphs represent mean ± SD and $p<0.05$ was considered statistically significant. *$p<0.05$; **$p<0.01$; ***$p<0.001$. One-way ANOVA followed by Tukey's pos-hoc multiple comparison test.

4. Discussion

In the present study, an oral, easily administered TrkA inhibitor, GW441756, was given to SCT rats for 7 or 28 days, the temporal hallmarks in rodents of spinal shock and detrusor overactivity, respectively, to study the effects of NGF in bladder function and in the expression of inhibitory molecules associated post-SCI neuroplasticity. Unlike other studies (Shimizu et al., 2018; Wada et al., 2018), treatment was initiated immediately after spinal lesion to prevent degradation of bladder function.

Analysis of bladder scores showed improvements in GW441756-treated rats, which took less time to return to basal levels than animals receiving saline or DMSO. This is concurrent with improved health condition of GW441756-treated animals after surgery, recovering their initial weight faster, even if statistical significance was not reached. Cystometric evaluation showed improvements in bladder reflex activity, consistent with improvement in voiding efficiency. This was accompanied by harnessing of neuroplasticity, as indicated by a reduction of sprouting and CGRP expression in the bladder and high levels of axonal inhibitory cues at the lumbosacral cord. Our data supports that that NGF modulation by early administration of the TrkA inhibitor, GW441756, may avert full development of NDO and presents potential as a pharmacological approach in the prevention of post-SCT NDO.

The importance of NGF in the context of SCI-induced urinary impairment has been under investigation for many years. NGF blockade with monoclonal antibodies was able to improve LUT function in chronic SCI mice (Shimizu et al., 2018; Wada et al., 2018) but it was only given when LUT dysfunction was installed. Here, an oral TrkA inhibitor was given but treatment was initiated immediately after spinal lesion. While GW441756-treated animals still had signs of abnormal bladder function, NDO was less pronounced.

This indicates that early NGF modulation has a protective effect on bladder function and adds to the growing body of evidence supporting early interventions to prevent SCI-induced development of urinary impairment (Biardeau et al., 2017; Oliveira et al., 2019).

Protection of bladder function by TrkA inhibition likely resulted from harnessing of plasticity in the bladder and neuronal pathways. Indeed, in animals receiving GW441756, GAP43 levels in the bladder remained low, suggesting that NGF modulation was able to influence peripheral axonal growth. This coursed with low levels of bladder CGRP, in line with other studies (Wada et al., 2018). In the bladder, most sensory afferents express CGRP (Avelino et al., 2002) and they are critical for NDO development and maintenance (Kadekawa et al., 2017b). Therefore, one can hypothesize that the beneficial effects of GW441756 on bladder function may result from an effect on peptidergic fibres.

Neuroplastic events also take place in the lumbosacral cord. During NDO development, CGRP-positive afferents extend their central processes into the lumbosacral cord (Zinck et al., 2007; Frias et al., 2015), where the majority of bladder afferents terminate (Morgan et al., 1981). This abnormal growth reflects changes in the levels of inhibitory molecules at the lumbosacral cord, where there is an early upregulation of Neurocan and Nogo-A followed by a reduction at 28 dpi (Chambel et al., 2022). Here, oral GW441756 induced an upregulation of both Neurocan and Nogo-A at both early and late stages of disease progression, suggesting that the spinal environment is less permissive for axonal expansion and likely repressing the maladaptive neuroplasticity leading to urinary impairment. However, this should be interpreted with care as our results show that DMSO, the vehicle for GW441576, was not devoid of effects. A similar upregulation of Neurocan and Nogo-A spinal levels was

seen after DMSO, which is able to cross the blood-brain barrier (Broadwell et al., 1982). While DMSO has been described as a potent anti-inflammatory agent in early SCI studies (de la Torre et al., 1975; Gelderd et al., 1983), the molecular mechanisms of action of DMSO are not fully understood. Positive results have also been described after traumatic brain injury (Bulama et al., 2022). However, care should be taken as DMSO may produce toxic effects, even at low concentrations (Galvao et al., 2014), not seen here as DMSO concentration used was very low (1%). In the bladder, cystometric evaluation also showed some improvement of bladder reflex activity, accompanied by a reduction of bladder CGRP and GAP43, after DMSO. However, improvements of bladder function and changes in bladder GAP43 and CGRP were more noticeable in animals receiving oral TrkA inhibitor, suggesting that the site of action of GW441756 was likely restricted to the periphery, with less interference from DMSO.

5. Conclusions

In the present study, we tested the effects of prolonged TrkA inhibition, initiated immediately after SCI. The oral administration of GW441756, a selective TrkA inhibitor, improved general health and attenuated the degradation of bladder function, by modulating neuroplasticity in the bladder and lumbosacral spinal cord. Future studies should consider the use of alternative vehicle solutions. NGF modulation, initiated immediately after SCI, protected bladder function and supports the need for early interventions to preserve quality of life.

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Final Considerations

Spinal cord injuries (SCI) are devastating events that impact not only sensory, motor, and autonomic functions, but leave everlasting complications throughout the remainder of a patient's life. Even though life expectancy has greatly improved over the last few decades, still no specific and efficient treatment has been approved to regenerate damaged tracts and fully regain function. Even if not life-threatening once proper care of the upper urinary tract is assured, urinary dysfunction that emerges after a spinal cord injury rostral to the lumbosacral level of the cord is amongst one of the most life-altering for SCI patients. Unlike systems that maintain some level of function despite elimination of neural input, the lower urinary tract (LUT) is innervated by the somatic and autonomous nervous system and relies on voluntary, supraspinal control to coordinate micturition (Fowler et al., 2008). After loss of supraspinal input due to SCI, the central branches of sensory afferents greatly expand into the lumbosacral spinal cord, slowly creating a new, automatic neural circuitry that eventually gives rise to neurogenic detrusor overactivity (NDO). While current pre-clinical and clinical investigation has been mainly focusing on re-establishing motor and/or sensory functions (Rosenzweig et al., 2019; Wang et al., 2020; Milton et al., 2023), the full understanding of the molecular mechanisms that give rise to SCI comorbidities, such as urinary impairment, is still to take place. This is critical to fast-forward the design of new therapeutic approaches that can be used at early stages of disease progression, to prevent deleterious conditions, and, at later stages, to alleviate chronic bladder impairment. Therefore, this thesis focused on understanding the fine molecular mechanisms regulating neuroplastic rearrangements operating at the lumbosacral cord that lead to NDO. Specifically, we investigated changes in the extracellular environment of the lumbosacral spinal cord, evaluating the expression of axonal inhibitory molecules at 7 and 28 days post injury,

considered to be, respectively, early and late stages of post-SCI bladder impairment. The expression of the receptors of those inhibitory molecules by sprouting sensory afferents was also evaluated *in vivo* and *in vitro*, in primary dorsal root ganglia (DRG) neurons grown in different nerve growth factor (NGF) concentrations. Finally, as NGF is a key mediator in SCI-induced bladder dysfunction (Ochodnicky et al., 2012), we tested the effects of its early modulation on bladder function and spinal neuroplasticity.

1. Abnormal sprouting of bladder sensory afferents occurs within the inhibitory milieu of the lumbosacral spinal cord

The complete spinal cord transection rat model of spinal cord injury was used throughout this thesis. This animal model has been widely used in our group (Frias et al., 2015; Oliveira et al., 2019) and, even if less translational than the contusion or hemisection rodent models, it allows for homogenous lesions and patterns of urinary dysfunction between animals to be obtained, thus decreasing the number of animals used per study. Nevertheless, considering its translational value and possible different molecular mechanisms governing urinary dysfunction, it would be important to reproduce these studies in a contusion animal model. Moreover, only female rats were used in this thesis, to simplify manual urine expulsion until automatic micturition developed after SCI. However, the majority of SCI cases are amongst the male population (Raguindin et al., 2021), highlightening the need to use both female and male animals in these studies, as evidence of sex disparities in injury pathogenesis and neurological recovery after SCI exist (Sipski et al., 2004).

The selected experimental timepoints used in ***Publications II and III*** recapitulate the hallmarks or urinary dysfunction after spinal cord injury: 7 days post-injury represents the spinal shock stage, when there is little or no bladder reflex activity (Bywater et al., 2018; Anderson et al., 2023), and 28 days post-injury, when NDO in rats is usually already established (Frias et al., 2015; Oliveira et al., 2019).

Urinary dysfunction in SCI animals was accompanied by upregulation of growth associated protein 43 (GAP43). GAP43, a neuron-specific protein, binds pre-synaptically to multiple molecules involved in plasticity, neurite formation and regeneration, serving as an accurate marker of axonal sprouting (Holahan, 2017). Significant expression of GAP43 was observed in the superficial laminae of the lumbosacral dorsal horn (***Publication II***), where bladder afferents are known to terminate (Vizzard, 1999), as well as in lumbosacral DRG neurons (***Publication II***) and in the urinary bladder (***Publication III***) of SCI animals already at 7 days post-lesion, evidencing sprouting of sensory afferents involved in LUT innervation. This suggested that the extracellular environment at the lumbosacral cord was permissive for axonal growth. However, results show that GAP43 upregulation was not accompanied by a decrease in the lumbosacral cord expression of inhibitory molecules of axonal growth (***Publication II***). In fact, the expression of Phosphacan and Neurocan, two chondroitin sulphate proteoglycans (CSPGs), and Nogo-A, a myelin-associated inhibitor of axonal growth, was upregulated at 7 days post-injury, a timepoint when $GAP43^{+}$ fibres were significantly upregulated. Immunohistochemical analysis of lumbosacral spinal cord segments revealed that $GAP43^{+}$ fibres never overlapped with Phosphacan and Nogo-A immunostained areas (***Publication II, Supplementary data***). Similarly to what happens during development, these inhibitory molecules could be directing sprouting fibres to areas where new synapse formation is

needed, to compensate for the lack of supraspinal input to regulate LUT function. Interestingly, the upregulation of these inhibitory molecules did not occur as a generalized response. In fact, while the expression of other axon guidance cues was evaluated (***Publication II***), only Phosphacan, Neurocan and Nogo-A exhibited time-dependent changes after SCI, possibly indicating that the mechanism that governs the emergence of the new micturition reflex within the lumbosacral cord is a dynamic one, where only specific molecular responders are recruited. It is also important to note that, in the context of urinary dysfunction, only two timepoints after injury were studied. We cannot discard the possibility that the time scale at which each individual player or molecular pathway is active during the emergence of this micturition reflex might simply not have been studied. Finally, other molecular players, such as axon guidance molecules belonging to the semaphorin, ephrin and netrin families may also be participating in the emergence of this new reflex mechanism in the lumbosacral cord (Yiu and He, 2006), even if the only guidance molecule studied – repulsive guidance molecule A (RGMa) – did not exhibit any regulation in the lumbosacral cord after SCI.

The high levels of axon guidance cues at the lumbosacral spinal cord after thoracic spinal cord injury coursed with GAP43 upregulation in the superficial dorsal horn, where bladder afferents project (Morgan et al., 1981; Nadelhaft and Booth, 1984). This inconsistency suggested that sensory afferents express receptors for those inhibitory proteins and the expression of such receptors changes after SCI. To assess this, the mRNA levels of these receptors in lumbosacral DRG neurons from spinal intact and SCI animals (7 and 28 days post-injury) were evaluated. As supraspinal projection neurons (Barrette et al., 2007), DRG neurons also express CSPG- and myelin-associated receptors. However, unlike what has been described in the brain, in which the

expression of some of these receptors is not affected by SCI (Barrette et al., 2007), their mRNA levels in DRG neurons were time-dependently downregulated after SCI (***Publication II***).

To try to understand how these extracellular inhibitory players could be factoring in the maladaptive sprouting of bladder afferents, intracellular mechanisms were also studied. CSPG- and myelin-associated molecules converge onto the small GTPase ras homolog gene family member A (RhoA), inducing growth cone collapse (Sami et al., 2020). In our study, we found that the downregulation of CSPG- and myelin-associated receptors was not accompanied by changes in the RhoA signalling pathway. This led us to hypothesize that bladder afferents were able to grow in the repulsive environment of the lumbosacral cord because receptors for these inhibitory molecules were downregulated in DRG neurons, thus not activating downstream signalling pathways associated with growth cone collapse. However, it should be taken into consideration that only two intervenients in this signalling cascade were studied: myosin phosphatase target subunit 1 (MYPT1) and its phosphorylated form, pMYPT1, which are involved in the regulation of the interaction of actin and myosin downstream of RhoA. Moreover, the expression of these downstream effectors was only studied in DRG neurons, but recent evidence has highlighted how RhoA plays cell type-specific opposing roles during CNS regeneration, as neuronal RhoA activation prevents axon regeneration, while astrocytic RhoA controls injury-induced astrogliosis and CSPG production. (Stern et al., 2021). Hence, the role of RhoA in the abnormal sprouting of sensory bladder afferents after SCI is still undefined and should be further explored in future studies. Finally, putative intervenients in this pro-growth process can also include enzymes such as glycogen synthase kinase-3 (GSK-3). Although not evaluated in the present work,

inactivation of this kinase can be induced by NGF, leading to robust axonal elongation (Zhou et al., 2004; Zhou et al., 2006).

The importance of NGF in the context of SCI-induced urinary dysfunction has been under intense scrutiny for many years (Seki et al., 2002; Seki et al., 2004; Yoshimura et al., 2006; Shimizu et al., 2018; Wada et al., 2018). NGF is essential for maintenance and growth of sensory neurons. Evidence also points to NGF upregulation after SCI, both at the lumbosacral spinal cord, lumbosacral DRG neurons and urinary bladder (Vizzard, 2000; Murakami et al., 2002; Yoshimura et al., 2006; Brown et al., 2007). In SCI animals, excessive NGF-mediated sprouting of peptidergic afferents (Zinck and Downie, 2006; Zinck et al., 2007) has been well documented and linked to development of urinary impairment (Yoshimura et al., 2006) and autonomic dysreflexia (Yoshizawa et al., 2014), while NGF blockade with antibodies has been demonstrated to reduce sprouting and attenuate these SCI-related conditions (Cameron et al., 2006; Wada et al., 2018). At the molecular level, NGF seems to potentiate axonal elongation via stabilization of GAP43 (Perrone-Bizzozero et al., 1993; Irwin et al., 2002). Accordingly, GAP43 transfection in PC12 cells can enhance their NGF-dependent responses, further suggesting that NGF interacts with presynaptic GAP43 to modulate neurite outgrowth (Neve et al., 1991). Therefore, we hypothesized that in our SCI animals NGF could also be involved in abnormal axonal sprouting by regulating the expression of receptors of inhibitory molecules in DRG neurons.

2. Receptors of inhibitory molecules are downregulated in dorsal root ganglia neurons after spinal cord injury in an NGF-dependent manner

To study the role of NGF in the expression of receptors of inhibitory molecules in DRG neurons, axotomized and dissociated lumbosacral DRG neurons collected from spinal intact and SCT animals (7 and 28 days post-injury) were placed in culture under increasing NGF concentrations. Neurons primed by the spinal cord injury displayed additional vigorous neurite growth, both in length and ramification of neurites. This growth pattern was enhanced by the increasing concentration of NGF in the media (***Publication II***). When the expression of receptors of molecular inhibitors was evaluated in these cultured neurons, an NGF-dependent downregulation in *NgR1* and *Lingo1* mRNA levels, particularly 7 days-post lesion, was found (***Publication II***), in agreement with previous reports (Lee et al., 2007). Interestingly, the mRNA levels of other receptors, particularly ones involved in CSPG signalling, were not altered either by the lesion or concentration of NGF in the culture media. A possible explanation for this observation could reside in the downstream signalling pathways that are activated by exposure to NGF and CSPGs (or, in the case of our study, neurons primed by high CSPGs levels *in vivo* and cultured in increasing concentrations of NGF). It has been demonstrated that preconditioned lesioned DRG neurons are able to bypass the inhibitory effect of a CSPG in culture by activating an alternative NGF-dependent signalling pathway (PI3K/ERK), resulting in robust axonal growth, despite the inhibitory environment (Zhou et al., 2006). However, the same is not true when Nogo-A is present in culture, as another signalling pathway (ECM-integrin) is activated, inhibiting axonal growth (Zhou et al., 2006). To further clarify these interactions, including how receptors of inhibitory molecules and their downstream signalling pathways are regulated by NGF,

in vitro microfluidic assays should be employed in future studies. As protein phosphorylation is one of the most important mechanisms of cellular regulation and an important matter not addressed in this work, phosphoproteomics studies of growth cones of these sensory neurons primed by SCI should be considered. Finally, other molecular players and growth factors could be involved in the regulation of this abnormal growth of bladder sensory afferents, such as Neurotrophin-3 (NT-3) (Fornaro et al., 2020).

The results presented in ***Publication II*** were consistent with a regulatory role of NGF in the molecular mechanisms involved in sprouting of bladder afferents and the emergence of NDO after SCI. Several studies have also highlighted how NGF manipulation by application of specific antibodies is effective in improving urinary dysfunction after SCI (Seki et al., 2002; Seki et al., 2004; Wada et al., 2018). However, these studies focus on improving urinary impairment once it is already a chronic condition, with little chance of reverting. Since the molecular alterations seen in ***Publication II*** were detected early on after SCI, in ***Publication III*** we investigated the effects of early NGF manipulation by means of inhibition of its high-affinity receptor, tropomyosin receptor kinase A (TrkA), on urinary dysfunction and the molecular mechanisms associated with NDO emergence.

3. Early and prolonged TrkA inhibition protects bladder function after spinal cord injury

In ***Publication III***, to understand the role early NGF manipulation might play in the emergence of NDO and in the neuroplastic events that occur within the lumbosacral cord after SCI, animals were acutely treated with a selective TrkA inhibitor, the high

affinity receptor of NGF. We found that early NGF modulation had a positive impact on the development of NDO. While animals still showed signs or urinary dysfunction, especially at early stages of treatment, prolonged TrkA inhibition led to improvements in the bladder score and all cystometric parameters evaluated, resulting in increased bladder efficiency (***Publication III***). These improvements in bladder function after TrkA inhibition were concurrent with low levels of GAP43 and calcitonin gene-related peptide (CGRP) proteins in the bladder (***Publication III***), evidencing how NGF modulation was able to influence peripheral plasticity, particularly in peptidergic fibres, in line with other studies (Wada et al., 2018). NGF is responsible for growth, development and maintenance of sympathetic and sensory neurons. About half of sensory afferents express TrkA and approximately 90% of TrkA-expressing afferents co-express CGRP (Averill et al., 1995), making CGRP a good indicator of the NGF-responsive sensory population. Sympathetic fibres are also NGF-dependent, as they express TrkA (Fagan et al., 1996), but, due to technicalities, we were not able to assess the expression of tyrosine hydroxylase (TH) in the bladder.

4. Early and prolonged TrkA inhibition altered the inhibitory environment in the lumbosacral spinal cord with interference from its vehicle, DMSO

In ***Publication III***, we also evaluated the expression of Neurocan and Nogo-A at the lumbosacral spinal cord, the inhibitory molecules of axonal growth found to be altered by SCI on ***Publication II***. NGF modulation greatly increased the expression of these inhibitors of axonal growth in a time-independent manner. In the adult, these molecules are inhibitory for growing axons (Zheng and Tuszynski, 2023) but, during development, CSPGs in particular are known to participate in axon guidance processes (Viapiano and

Matthews, 2006). Nogo-A and Neurocan spinal overexpression in TrkA inhibitor-treated groups could represent an attempt to guide the growth of the remaining fibres in the dorsal horn. This hypothesis should be explored in future work by assessing the sprouting of sensory afferents in the lumbosacral cord. However, a similar upregulation of these inhibitory molecules was also seen in dimethyl sulfoxide (DMSO)-treated animals, the vehicle for TrkA inhibitor administration. These were confounding results: even if beneficial effects of DMSO administration after spinal cord injury had been previously reported in very early studies (de la Torre et al., 1975; Gelderd et al., 1983), and more recently in other CNS injury models (Bulama et al., 2022), the concentration of DMSO used in ***Publication III*** was very low and not expected to induce molecular alterations. However, since DMSO is able to cross the blood-brain barrier, while this specific TrkA inhibitor is not, due to its elevated molecular weight, this might justify the differences here observed.

An important issue that needs discussion in ***Publication III*** refers to lack of data about how the expression of receptors of inhibitory molecules might have been affected by NGF modulation, an important link between the possible modulatory effect of NGF on the expression of these receptors hypothesized in ***Publication II***. These experiments were performed but results were puzzling and not included in ***Publication III***. When animals were treated with TrkA inhibitor or its vehicle DMSO, independently from the timepoint, mRNA levels were undetectable for all receptors analysed, including *NgR1*, *NgR2*, *Ngr3*, *Lingo-1*, *p75*, *TROY*, *Ptprs* and *Lar*, as well as for endogenous control genes such as *Gapdh*, *Hprt1*, *Tbp* and *Ywhaz* (*data not shown*). The same did not happen to naïve (control) and SCT animals left untreated, and these animals recapitulated the results obtained in ***Publication II***, thus excluding technical problems during RNA

extraction or cDNA synthesis. Moreover, when the expression of genes associated with regeneration, such as *Atf3*, was analyzed, not only were mRNA levels detected, but showed significant increases in animals treated with TrkA inhibitor, compared to controls (*data not shown*). These puzzling observations prevented us from including these findings in the manuscript, but opened new research possibilities. Since the inability to detect mRNA levels in lumbosacral DRG neurons was restricted to some genes while others were easily amplified, we hypothesized that the mRNA processing in these cells could be altered after treatment, compromising, for example, mRNA stability, splicing or translation. RNA methylation, specifically the N6-methyladenosine (m6A) post-transcriptional modification, is one of the most prevalent and well-studied reversible RNA modifications in eukaryotic cells (Liu et al., 2022). m6A methylation is related to the development of pathological processes, such as spinal cord injury. Recent studies have established that the overall m6A level in the lesion site is increased following SCI, as well as the content of related regulatory factors, such as methyltransferase 3 (METTL3) and methyltransferase 14 (METTL14) (Wang et al., 2021; Xing et al., 2021; Gao et al., 2022). These regulators of methylation can also influence cell survival after SCI by regulating m6A levels (Wang et al., 2021). Research on m6A methylation after spinal cord injury is still in its early stages, and mainly focuses on central mechanisms involved in these epitranscriptomic events. No data is currently available on putative peripheral alterations or affecting other CNS areas following a central injury. Nonetheless, m6A regulation in DRG neurons has been described following mice sciatic nerve injury and was found to be responsible for promoting injury-induced protein synthesis and axon regeneration (Weng et al., 2018).

If epitranscriptomic m6A methylation is the reason why gene expression detection by qPCR was not feasible in these experiments is unclear, but methylation is a strong plausible cause, being the main obstacle that interferes with transcriptional elongation and leads to non-effective cDNA conversion and qPCR efficiency. If the presence of methylation in the mRNA of lumbosacral DRG neurons of treated animals was confirmed, for example, by using an RNA demethylase to efficiently perform the qPCR assays, future work could focus on characterizing these novel mechanisms of RNA methylation in the emergence of urinary dysfunction after spinal cord injury.

Conclusions

In conclusion, data collected in this dissertation uncovered some of the molecular mechanisms behind the emergence of neurogenic detrusor overactivity in a rat model of after spinal cord injury.

Publication II evaluated how the molecular milieu within the lumbosacral cord after spinal cord injury exhibited, much like at the injury site, an upregulation of inhibitory molecules for axonal growth. However, this apparent repulsive environment still harboured the sprouting of bladder sensory afferents, which was concomitant with the emergence of neurogenic detrusor overactivity. A time-dependent downregulation in the expression of the receptors of these inhibitory molecules in lumbosacral DRG neurons was also demonstrated. This suggested that these sensory afferent fibres were able to expand their processes despite the inhibitory environment of the lumbosacral spinal cord, as they were perhaps less sensitive to these cues. When we studied these DRG neurons *in vitro*, NGF emerged as the putative molecular modulator for the downregulation of the expression of these receptors of inhibitory molecules. These results showed that the consequences of a thoracic spinal injury are widespread, both within the cord, but also in peripheral structures, such as DRG neurons.

Publication III aimed to further address the putative role of NGF in the molecular mechanisms of NDO emergence after spinal cord injury. Early and continued NGF modulation after SCI, via the inhibition of its high-affinity receptor TrkA, was able to attenuate the pattern of urinary dysfunction at 28 days following SCI, which was accompanied by a decrease in peripheral plasticity, particularly in peptidergic afferent fibres. However, an array of technical difficulties, such as the vehicle for TrkA inhibitor

administration and the inability to evaluate the expression of receptors for inhibitory molecules, kept us from further developing this work. Future work should address these issues to try to further uncover the role of NGF in the modulation of the molecular intervenients of NDO emergence. Additionally, future studies should focus of early, preventive interventions to mitigate the development of urinary dysfunction after spinal cord injury.

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