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Targeted regeneration of spinal cord neurons across a complete injury restores walking

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par

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« In adult centers, the nerves are something fixed and immutable:
everything may die, nothing may be regenerated.
It is for the science of the future to change, if possible, this harsh decree. »

- Santiago Ramón y Cajal, 1928

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ABSTRACT

Spinal cord injury (SCI) interrupts axonal connections between the brain and the spinal cord, and is characterized by a spectrum of sensorimotor and autonomic impairments. While spontaneous recovery is limited, recent studies have shown that functional improvements can be greatly augmented via rehabilitative approaches based on targeted epidural electrical stimulation that recapitulates the natural pattern of spinal activation. Nevertheless, these strategies depend on the presence of spared axonal connections, and are consequently constrained in the extent of elicitable recovery, and inapplicable in the case of very severe or anatomically complete SCI. It is therefore agreed that future therapies for SCI will require strategies to repair the injured spinal cord by stimulating severed axons to regenerate across the tissue lesions.

Despite this need, axons from adult mammalian central nervous system (CNS) neurons are characterized by intrinsic incapacity of spontaneous regeneration. Research in the last several decades has uncovered multiple mechanisms underlying CNS regenerative failure, and a recent approach developed by our group has identified the requirements to induce experimental axon regrowth across anatomically complete SCI in rodents. Yet, while robust regeneration could be elicited with this and other strategies, restoring meaningful function after such injuries has been elusive.

In the work presented in this thesis, we sought to build on our previous approach by identifying, and then providing, requirements that are missing for recovery. Concretely, we sought to determine whether restoring neurological function requires regeneration of specific subpopulations of neurons directed to their natural target region.

To address these questions, we shifted our attention to a specific model of severe but incomplete SCI, following which natural reorganization of spinal circuits is associated with spontaneous recovery of walking. We performed projection-specific and comparative single-nucleus RNA sequencing to uncover the transcriptional phenotype and connectome of neuronal subpopulations involved in natural spinal cord repair, and identified a molecularly defined population of excitatory projection neurons in the thoracic spinal cord that extend axons to the lumbar spinal cord where walking execution centers reside.

We optimized our previous strategy to provide sustained chemoattraction to the identified neuronal subpopulation. We showed that regrowing axons from these neurons across anatomically complete SCI and guiding them to their appropriate target region in the lumbar spinal cord restores walking in mice, whereas regeneration of axons simply across the lesion has no effect. Selective loss-of-function experiments further revealed that recovery is largely dependent on regeneration of the characterized neuronal subtype.

These results demonstrate that mechanism-based repair strategies that recapitulate the natural topology of molecularly defined neuronal subpopulations can restore neurological functions following anatomically complete SCI.

Keywords: spinal cord injury, axon regeneration, functional recovery, locomotion, neuronal subpopulations, RNA-sequencing, repair, growth factors

RIASSUNTO

Le lesioni midollari interrompono le connessioni assonali tra il cervello e il midollo spinale, e sono caratterizzate da una gamma di deficit sensoriali e motori e disfunzioni autonomiche. Sebbene il recupero spontaneo sia modesto, studi recenti hanno dimostrato che significativi miglioramenti funzionali possono essere ottenuti tramite approcci riabilitativi basati su stimolazione elettrica epidurale mirata, volta a riprodurre i pattern naturali di attivazione midollare. Ciononostante, poiché tali strategie dipendono dalla presenza di connessioni neuronali residue risparmiate dalla lesione, il recupero funzionale rimane limitato, e l'applicazione di tali approcci non è possibile nel caso di lesioni particolarmente severe o anatomicamente complete. É pertanto opinione unanime nel settore che terapie future per le lesioni spinali richiederanno la produzione di nuovo substrato biologico, tramite la ricrescita attraverso la lesione degli assoni troncati.

Malgrado questa necessità clinica, gli assoni dei neuroni del sistema nervoso centrale (SNC) dei mammiferi adulti sono caratterizzati da un'intrinseca incapacità di rigenerazione spontanea. Il lavoro di ricerca degli ultimi decenni ha portato all'identificazione di molteplici meccanismi responsabili per l'inabilità rigenerativa del SNC, e un approccio recentemente sviluppato dal nostro gruppo ha definito i requisiti per indurre sperimentalmente la ricrescita assonale in topi e ratti attraverso una lesione midollare anatomicamente completa. Tuttavia, nonostante questa e altre strategie sperimentali producano robusta rigenerazione assonale, tale ricrescita non è associata ad alcun tipo di ripristino funzionale contestualmente a lesioni di questa severità.

Nel lavoro presentato in questa dissertazione, ci siamo proposti di sviluppare il nostro precedente approccio, identificando e ristabilendo i requisiti mancanti per il recupero funzionale. Concretamente, è stato nostro interesse determinare se il ripristino di funzioni neurologiche richieda o meno la rigenerazione di specifiche popolazioni di neuroni, e se tale rigenerazione debba essere mirata alle regioni con le quali tali popolazioni sono connesse naturalmente.

Per rispondere a questi quesiti, abbiamo rivolto la nostra attenzione a un modello specifico di lesione midollare severa ma incompleta, a seguito della quale avviene una naturale riorganizzazione dei circuiti neuronali associata ad un recupero spontaneo di capacità locomotoria. Abbiamo eseguito un'analisi di sequenziamento di RNA comparativa e regionalmente specifica, per identificare il fenotipo trascrizionale e l'insieme di connessioni delle popolazioni neuronali coinvolte in tale riparazione naturale; tale indagine ha portato all'individuazione di una popolazione, definita a livello molecolare, di neuroni eccitatori associativi con corpo cellulare nei segmenti spinali toracici, ed estendenti assoni nel midollo lombare dove risiedono i centri esecutivi della deambulazione.

Abbiamo pertanto ottimizzato il nostro precedente paradigma sperimentale per fornire chemioattrazione prolungata alla popolazione neuronale identificata. Mostriamo che, rigenerare assoni da tali neuroni, e guidarli alla loro appropriata regione target nel midollo lombare permette a topi paralizzati di recuperare la capacità di camminare a seguito di una lesione midollare anatomicamente completa; al contrario, rigenerazione assonale non mirata, semplicemente nel tessuto caudale alla lesione, non produce alcun effetto a livello funzionale. Ulteriori esperimenti genetici di perdita di funzione mostrano

che il recupero funzionale osservato dipende in misura essenziale dalla rigenerazione del sottotipo neuronale identificato.

Questi risultati dimostrano che strategie di riparazione biologica che ricapitolino la topologia naturale di popolazioni neuronali definite a livello molecolare hanno il potenziale per ristabilire funzioni neurologiche a seguito di una lesione spinale anatomicamente completa.

Parole chiave: lesione midollare, rigenerazione assonale, recupero funzionale, locomozione, popolazioni neuronali, sequenziamento di RNA, riparazione biologica, fattori di crescita

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1. INTRODUCTION AND CONTEXT

SPINAL CORD INJURY: THE PROBLEM

Clinical background: causes, symptoms, impact

Spinal cord injury (SCI) represents a devastating condition, that causes local nervous tissue death and severing of axonal connections between supraspinal or spinal centers above the lesion and circuits below it. As a result, patients experience an array of symptoms and permanent impairment of functions associated with the lost connections. These include disruption of motor and sensory functions below the injury level, with associated para-/tetra-plegia, spasticity, muscle atrophy, numbness and neuropathic pain[1]. In addition, autonomic functions are also deeply affected, including regulation of blood pressure, respiration, bowel control and micturition, and sexual functions[1]. Furthermore, maladaptive remodeling of spared neural tissue leads to emergence and exacerbation of other complications, as discussed in **Pathology of SCI**.
The severity of symptoms and functional impairment associated with SCI depend on multiple factors, and lesions can be classified according to scales along a range of functional and anatomical completeness (see **SCI classification**).

Trauma represent a primary cause of insults to the spinal cord, with falls and vehicle accidents being the most represented leading causes, and other relevant sources including sports activities, violence and surgical complications[2,3]. Additionally, particularly in cohorts of older patients, a vast proportion of spinal cord injuries presents a non-traumatic etiology, and can occur as a result of neurodegenerative diseases, tumors, and infections, among other causes[3,4].

SCI impacts tens of thousands of individuals around the world every year[1], often at an age at which people affected are in the prime of their lives, creating a significant social and economic burden for families of the patients and governments: several factors prevent affected subjects from fully participating in society - with an impact on occupational, marital and educational status[2] -, and lifetime costs – including patients' lost earnings – amount to millions of dollars per individual[1]. Moreover, the impairment of autonomic functions and the limited mobility predisposes patients to a higher risk of developing additional long-term complications (e.g. infections, cardiovascular failure), resulting in a significantly decreased life expectancy[1].

While SCI has long been considered a completely untreatable condition, increased knowledge on the injury mechanisms and on the importance of rapid intervention in the acute phases post-accident, as well as development of specialized care, have greatly improved patients' survival and outcome compared to the beginning of the last century[1,5]. Nevertheless, until recently, standard clinical approaches have been limited to acute management (including surgical decompression), administration of corticosteroids (the efficacy of which has been questioned) and rehabilitation[1]. This latter, crucial to prevent long term complications and to maximize patient independence, on its own only promotes a limited recovery of motor function that reaches a plateau after few months, following which patients return to their home with significant residual permanent disability[6].

In addition to this, research from our group and others has shown in the past years that strategies that engage the spared tissue can greatly augment the extent of functional recovery achieved with standard therapies: targeted electrical stimulation that mimics the spatial and temporal pattern of activation of the uninjured spinal cord, combined with intensive rehabilitation, allows substantial recovery of motor and autonomic functions in patients already acutely upon stimulation, with some subjects also regaining partial voluntary motor control chronically in the absence of stimulation[7,8] (the mechanisms underlying such recovery are explained in detail in **Recovery after incomplete injury: neuromodulation**).

Nonetheless, despite the significant recent advances, recovery achieved via such neuromodulation strategies is still limited compared to the clinical requirements[9]. Moreover, the impact of neuromodulation approaches is defined by the restricted resolution of electrical stimulation and, more importantly, is intrinsically dependent on the amount of axonal connections spared by the injury.
To overcome this limitation (particularly critical in severe or complete injuries, characterized by total or near-total lack of sparing), future therapies will need to combine electrical stimulation and rehabilitation with strategies capable of providing a new biological substrate compensating for the lost tissue, i.e. regrowing severed axonal fibers across the injury[10].
The aim of my PhD project was centered specifically on this idea, and we show a proof of principle of functional recovery in mice following a complete SCI lesion by specifically reconstituting relevant circuits for hindlimb locomotion.

SCI classification: incomplete vs complete

The degree of chronic impairment caused by a SCI is influenced by several factors besides level of the injury, including age, timeliness of intervention and comorbidities[11–13].

The level of the injury determines the functions that are affected, with supraspinal control of segments below the lesion being lost to variable extent depending on severity: lesions in the upper cervical segments are associated with tetraplegia, as well as with compromised respiratory function (artificial ventilation is needed for lesions at C3 or higher), and patients require complete assistance in most daily life activities; partial control of wrist and elbow extension is possible with lesions at lower cervical levels; patients with thoracic injuries are paraplegic and independent in most daily living activities including bladder-bowel care, although severe cardiovascular and autonomic disorders can greatly impact patients with upper thoracic or higher injuries[14,15]; lesions at lumbar levels or lower are usually associated with great independence and variable possibility to ambulate[6]. It is estimated that more than half of all SCI occur at cervical levels, with C4 to C6 being the most affected segments, and incomplete tetraplegia being the most frequent neurological category[16].

An important distinction with respect to completeness of the injury has to be done with respect to function and anatomy.

Functional completeness of SCI can be assessed clinically through several classification scales[17]. The most commonly used method is the International Standards for

Neurological Classification of Spinal Cord Injury (ISNCSCI) from the American Spinal Cord Injury Association (ASIA): based on the assessed degree of residual muscle and sensory function, a neurological level of the injury is determined, and the patient is assigned a grade on the ASIA Impairment Scale (AIS). Accordingly, depending on the AIS, injuries can be classified as:

- complete (AIS-A, i.e. complete loss of both motor and sensory function below the neurological level)

- sensory incomplete (AIS-B, complete loss of motor but partial preservation of sensory function below the neurological level)

- motor incomplete (AIS-C and AIS-D)

- normal (AIS-E, patients with prior deficits and full recovery)[18].

Functionally incomplete lesions comprise around 70% of traumatic SCI and almost the entirety of non-traumatic SCI[2,3].

Despite development of powerful imaging techniques which allow estimation of white matter tracts[19,20], standard practice for spinal cord injury imaging rely on radiography and magnetic resonance imaging (MRI), which have limitations in detecting abnormalities in spinal cord integrity[21]. This makes precise characterization of the anatomical extension and completeness of SCI lesions potentially challenging.

Anatomically complete lesions are defined as to span the whole breadth of the spinal segments interested, leaving no spared connection across the two sides of the lesion[22]. Nonetheless, functionally complete (AIS A) injuries are not necessarily associated with completeness in terms of anatomy[22], as "functionally dormant" residual connections across the injury are spared in case of severe but incomplete SCI but not sufficient to convey functional information[23,24]. This distinction has several therapeutic implications: anatomically incomplete injuries, as mentioned above, can be targeted via combinations of biomimetic electrical stimulation, pharmacological intervention and rehabilitation training to restore function[25], by leveraging the spared connections from supraspinal tracts or their projections on propriospinal neurons acting as a relay[26]; on the contrary, such interventions cannot produce any improvement of voluntary locomotion in the case of anatomically complete SCI if a biological intervention to provide de novo axonal substrate bridging the lesion is not performed first. Additionally, in severe but incomplete SCI the spared tissue is sufficient to produce functional improvements acutely upon electrical stimulation, but long-term neurological recovery in the absence of stimulation can be limited[7]; as discussed before, these types of injuries would therefore also greatly benefit from regenerative therapies augmenting the number of fibers that relay information from supralesional circuits[26].

Experimental models of spinal cord injury

Multiple strategies to generate experimental models of SCI exist, each associated with limitations and advantages; a review of the difference in terms of severity and mechanism is particularly important for the scope of this thesis, as the choice of the injury model is crucial to assess axon regeneration.

The three most used mechanisms of injury for experimental SCI models include[27]:

- Contusion, produced by means of a controlled weight drop via *impactor* devices, and resulting in an acute and brief collision with the spinal cord (**Figure 1.1a**). Contusion models represent a clinically relevant model for traumatic SCI including falls from height;
- Transection, performed usually by means of *microscalpel blades* or *scissors* (usually followed by redrawing of the lesion via a blade or needle to ensure proper severing), that generate a cut and physically separate the two lesion ends (**Figure 1.1b**). Ideal to interrogate function of specific axonal tracts (see below), transection models are, on the other hand, ill-suited to study specific aspects of secondary injury;
- Compression, that – in contrast to contusion – employs a prolonged (i.e. lasting few seconds) insult to the spinal tissue, and is performed typically by means of *forceps*, although other methods include compression via *clips*, *balloons* or *strapping*[28] (**Figure 1.1c**). Compression models recapitulate aspects of specific types of clinical SCI, including burst fracture injury.

Less common models involve generation of SCI via distraction, dislocation or chemical methods[28].

While specific parameters can be adjusted in order to influence injury severity in each of these models, anatomical completeness is typically only achieved via compression or transection.
For contusion models, adjustment of weight and height of the rod compared to the tissue surface, or of the controlled force of impact, can lead to more or less severe injury (e.g. midthoracic impacts of 70/95kdyn for mice, and 100/255kdyn for rats, result in mild/severe contusion without/with hindlimb paralysis respectively[23,29,30]), with the lesion expanding in the first weeks and typically resulting in cavity formation (**Figure 1.1a**); nonetheless, even more severe contusion injuries typically spare outer rims of white matter[23], therefore contusion models are considered to be virtually always anatomically incomplete.
Transection injuries are well suited to study function of specific axonal tracts: incomplete models can indeed be employed to sever only the specific cross-sectional regions of the cord where the pathways of interest lie; on the other hand, full transection of the cord can be performed to produce a model of anatomically complete injury (**Figure 1.1b**).
Severity of crush injuries can also be controlled: employing a spacer of different size, preventing full closure of the forceps, leads to incomplete injuries with variable sparing of the more external tissue (**Figure 1.1c**); on the other hand, compression using forceps with no sparing can be exploited to produce anatomically complete SCI; moreover, size of the tips of the forceps, or duration of compression, are also parameters that influence

severity of a crush injury, and even anatomically complete injuries can be more or less severe depending on such specifications {see also **Regeneration after complete SCI**}. As discussed also later in the thesis, incomplete SCI models are intrinsically ill-suited to study axon regeneration, considering that potentially labeled fibers cannot be fully discriminated as regenerating or spared axons. In particular, evaluation of axons regenerating specifically across – as opposed to around – the injury, which was part of the scope of this thesis, is also challenging.

For these reasons, the SCI model used in our experimental work to study regeneration is an anatomically complete injury model, specifically a complete crush (performed with forceps compressing the cord for 5s).

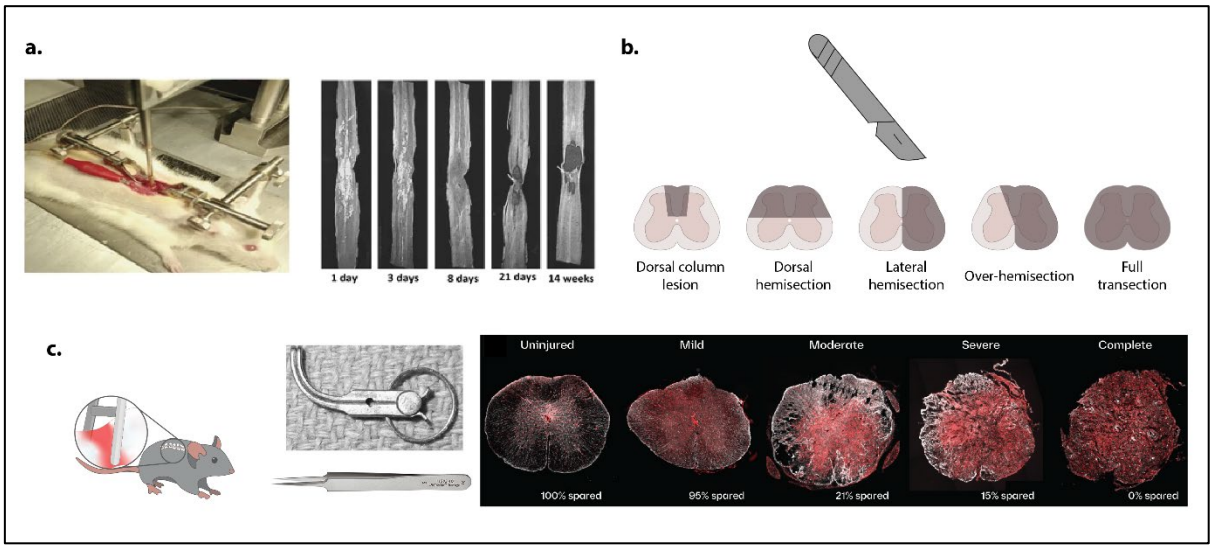

***Figure 1.1*. Main experimental models of spinal cord injury.** a. Contusion models. *Left: photograph of contusion SCI performed on a rat via an impactor device: after laminectomy, the spinal column is stabilized via clamping and controlled impact of the rod is used to perform an injury of desired severity. Right: Progressive histological changes seen at relevant timepoints after injury, with expansion of hemorrhage at day 1, and progressive extension of lesion area and cavity formation. Reproduced with permission from Cheriyan et al., 2014[28].* b. Transection models. *A scalpel blade or microscissors are used to sever the desired portion of the spinal cord. Incomplete injury models can be used to study regeneration of specific axonal tracts, e.g. sensory axons (dorsal column lesion) or corticospinal tract axons (dorsal hemisection), while full transection can be employed to produce an anatomically complete injury.* c. Compression models. *Left: Experimental compression of the spinal cord can be achieved via multiple methods including using forceps (Dumont™ #5 in the bottom picture) or clips (top picture, reproduced with permission from Cheriyan et al., 2014[28]). Right: example images of crush compression injury performed in rodents using forceps with variable spacers, resulting in drastically different amounts of tissue sparing (white: GFAP, red: DAPI).*

Pathology of SCI

Degenerative processes begin acutely immediately after insult to the spinal cord[31]. Subsequently, the response continues with cell proliferation few hours after, and inflammation starting 1 to 2 days post injury and peaking after weeks[31,32]. The remodeling process lasts for several months before stabilization into a chronic SCI lesion[31], and involves permanent changes at systemic level[33].

The pathological mechanisms occurring can be broadly classified into two phases of primary injury, directly related to the damage caused by the insult to the spinal cord, and secondary injury, involving inflammation and immune system recruitment[31] (**Figure 1.2**).

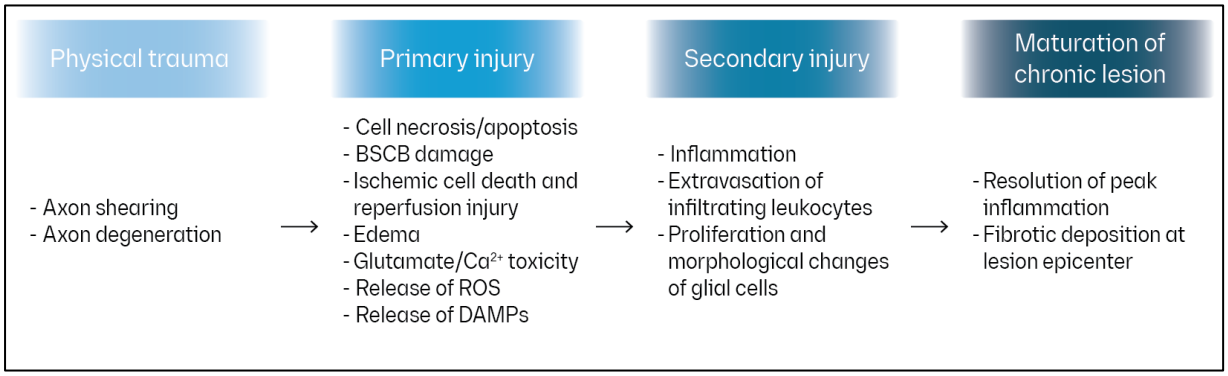

Figure 1.2. Overview of spinal cord injury pathophysiology**. After the pathological mechanisms occurring as a direct consequence of the physical trauma, the response to spinal cord injury can be divided in two phases of primary and secondary injury. Upon resolution of acute inflammation, a fibrotic scar is gradually deposited in the lesion core and the SCI assumes a chronic tripartite structure {described in* **Biology of SCI lesion}. Adapted from Tran et al., 2018[31].*

Primary injury

The first effects result directly from physical damage associated with the trauma: both glial and neuronal cells start undergoing necrotic processes that spread symmetrically from the impact point, and are characterized by unordered cell death and uncontrolled release of intracellular material[31]. The ejection of such molecules, as explained in detail below, additionally contributes to the recruitment of immune cells active during the secondary phase[34]. Necrosis persists along later phases of the injury and is further enhanced by other response mechanisms occurring during primary injury[31].

Apoptosis, on the contrary, defined as an ordered, molecularly-programmed process of cell dismantling[35], elicits a much more limited immune response and is confined to the earlier phase of the injury, particularly with neuronal apoptosis receding within the first 24 hours after injury[36].

Furthermore, as a direct consequence of the impact, axons passing through the lesion core are transected, leading to initiation of degenerative processes[31]. The proximal ends arrange in typical swollen structures symptomatic of regeneration failure, referred to as "retraction bulbs"; axonal dieback of such ends occurs in two steps, an earlier phase characterized by acute axon degeneration (AAD) and a more delayed second retraction phase mediated by macrophages infiltrated during secondary injury[37]. The distal stump

instead, separated from the cell body, undergoes stereotypical Wallerian degeneration (WD), characterized by mechanisms distinct from AAD[31,37].

Another main hallmark of the primary phase of the injury is the set of responses occurring because of the disruption of the blood supply system. Rupture of blood vessels, typically observed in capillaries rather than in larger vessels and arteries, which usually remain intact[38], results in ischemia and hematocyte extravasation[39]. Moreover, the tightly-arranged structure of capillary wall endothelium, pericytes and astrocyte endfeet constituting the blood-spinal cord barrier (BSCB), that selectively regulates entry of nutrients in the spinal cord under physiological conditions[40], starts to breakdown early after injury with significant permeability up to 5 days after[41]. The resulting uncontrolled increase in extracellular fluid and outflow of ions leads to edema formation; besides this vasogenic nature, edema after spinal cord injury is further enhanced by cytotoxic mechanisms of cellular swelling after ischemia[42]. This, in conjunction with the hemorrhaging, reported to be active for several days after injury[31], causes compression of the tissue and further expansion of the lesion area, with the appearance of intraparenchymal cavities at later timepoints[43,44].

On the other end, increased extracellular concentration of glutamate, discharged by necrotic neurons, causes abnormal recruitment of ionotropic receptors, resulting in aberrant activation of the target cells and further contribution to cell death in the area due to excitotoxicity and cascade Ca^{2+}-dependent neurotoxicity[45,46].

In a response mechanism to contain the bleeding, synthesis of vasoconstrictive molecules including endothelins and neuropeptides, as well as of platelet-derived factors, is increased[31,47]. Nonetheless, as a characteristic feature observed in several mechanisms after central nervous system (CNS) injury **(Table 1)**, this action has a double-edged effect, with the resulting vasospasm contributing to a further increase of ischemic damage in areas surrounding the injury site[48]. Similarly, restoration of oxygen and blood flow to ischemic areas leads to typical reperfusion injury: during ischemia, the prolonged oxygen deprivation causes cells to undergo a switch to anaerobic metabolism, with a cascade of events including mitochondrial damage, decrease of antioxidation mechanisms and formation of byproducts[46,49]; reperfusion of oxygen, as well as infiltration of leukocytes, results in generation of reactive oxygen species (ROS) from these metabolites; such free radicals then promote further damage and apoptosis both locally and in distant tissue through oxidation of proteins and membrane lipids[50,51].

Secondary injury

During the primary phase of the injury, the generalized damage and necrotic processes cause uncontrolled release of intracellular material from dying glial and neuronal cells[31]. Specifically, among the discharged products are damage-associated molecular patterns (DAMPs), which comprise a large variety of materials including cellular debris as well as endogenous alarmins, i.e. cell-bound molecules usually expressed at very low levels and only released after cell damage[34]. Alarmins, which range from nucleotide derivates such as ATP, to interleukins and chromatin-associated proteins, "sound the alarm" by recruiting the immune system and initiating the secondary phase of the injury, characterized by a sterile inflammatory response[34].

Cells of the innate immune system, as well as glial cells acting as immune regulators, express germline-encoded pattern-recognition receptors (PRRs)[52–54]. Among the subclasses of these receptors, traditionally regarded to respond to evolutionary-conserved molecules expressed by pathogens (pathogen-associated molecular patterns or PAMPs), specific families of PRRs are designed to bind and respond to DAMPs[55]. Activation of such receptors results in the stimulation of the respective cells, which are in turn prompted to release chemokines and mediators that amplify inflammation and injury response[34].
The initial stage of the inflammatory response (up to 2 days post injury) is characterized by the activation and/or mobilization of resident astrocytes and microglia to the lesion site, as well as recruitment of extravasated neutrophils. A later phase, beginning around 3 days after injury, involves infiltration of blood-borne monocyte-derived macrophages, as well as T and B cells (**Figure 1.3**)[39].
The role of the main cells involved in the process, including innate immune cells (monocytes/macrophages, neutrophils), glial cells (microglia, astrocytes) and adaptive immune cells (lymphocytes) is outlined more in detail below.

Neutrophils

Short-lived and continuously formed from stem cells in the bone marrow, neutrophils are a hallmark of acute inflammation, being the first blood-borne immune cells to infiltrate the injury site, where they start to be present after a few hours and peak at around one day post injury[34,39]. Typically recruited by chemokines including interferon (IFN)-γ, IL-8, CXCL1 and CXCL2[34,56], neutrophils have phagocytic/debris clearing activity, and exert a key role in initiation of the inflammatory cascade, by releasing cytokines to activate local glial cells and by promoting recruitment of additional peripheral immune cells to the injury site[39,57]. Neutrophils additionally release proteases and ROS that promote degradation of the extracellular matrix (ECM), and have therefore traditionally been regarded as detrimental in CNS injury and mediators of tissue damage[34,57]. Nonetheless, loss-of-function experiments have shown that neutrophil depletion results in worsened outcome of the injury[58]. Moreover, recent studies have begun to uncover mechanisms by which neutrophil chemotaxis to the injury site promotes CNS and peripheral nervous system (PNS) regeneration[59,60]. In spite of having both beneficial and detrimental effects after injury, it is now established that neutrophils are crucial mediators of inflammation at the initial stage of SCI that serve to direct the repair processes that occur at later timepoints[34,39].

Microglia and macrophages

After spinal cord injury, both microglia and macrophages densely populate the spinal cord. Indistinguishable morphologically and in terms of immunohistological markers upon activation[61,62], and both endowed with phagocytic activity, a discrimination needs to be made between the two cell populations. Notably, microglia can be defined as highly specialized tissue macrophage-like cells resident in the CNS since development, while peripheral macrophages differentiate from blood monocytes and are recruited to the spinal cord upon BSCB breakage as an additional source of phagocytic cells[31,63]. Nonetheless, microglia and monocytes originate from common embryonic progenitors[64]. While microglia, as resident immune cells, start being activated within a few hours post injury[65], macrophage influx into the cord and migration of activated microglia to the

lesion only peak at few days after the injury[39,62]; activated microglia and hematogenous macrophages occupy different locations in the injury site, with the first being confined to the perilesional area while the latter densely spread in the lesion core[39]. Proliferation is observed in both cell types, although more pronouncedly in macrophages[31,62].

Homeostatic microglia (defined in rodents by the marker P2RY12) is characterized by a typical morphology with elongated nucleus and ramified processes[61]; in physiological conditions, non-activated microglia serve a function of constant surveillance, by continuously rearranging their cellular processes to scan for potential danger[31].

Upon activation ($CD45^{high}$), both microglia and macrophages are characterized by retracted processes and ameboid shape[62], and have classically been subjected to classification into two phenotypes, although recent studies have challenged this net dichotomic separation[66]:

1) M1 ($CD16^+$, $CD32^+$, $IL\text{-}12^+$): shown to be induced by factors secreted by Th1 helper T cells as IFN-γ, they represent the classical activation state, characterized by a marked proinflammatory phenotype. Specifically, M1-like microglia/macrophages show diffuse expression of PRRs, and release several inflammatory cytokines (IL-12, IFNγ, TNFα, IL-6, IL-1β) and ROS, with also autocrine and paracrine self-induction effects[66]. Moreover, they additionally act as antigen-presenting cells by expressing MHCII and directly participate in the activation of other immune cells[39].
2) M2 ($CD206^+$, arginase I^+): they represent an alternative activation phenotype, shown to be induced by Th2 cytokines as IL-4, IL-10[39]. Although multiple subtypes can be identified[66], these all share an anti-inflammatory phenotype, associated with release of cytokines responsible for immune resolution (IL-10, IL-13, IL-4), secretion of ECM proteins for wound repair, and expression of receptors associated with phagocytosis/debris clearance[64,66].

Importantly, upon injury Th1 and Th2 cytokines are also produced by resident cells in the CNS, particularly astrocytes, which therefore have a critical role in microglia/macrophages activation and phenotype-biasing[39].

In particular, balanced proportion and timely shift between M1 and M2 phenotype of microglia/macrophages is critical as, while inflammation is crucial to promote cleaning and defense from pathogens in the affected area, sustained inflammation leads to further damage and cell death[66]. Prolonged presence in the lesion area of inflammatory cytokines and skewing towards M1-like phenotype might be a critical reason for the protracted damage after spinal cord injury[67]. On the other end, peripherally, excessive M2-like macrophage activity has also been shown to be detrimental, leading to fibroblasts activation and fibrosis[68].

Lastly, M1- and M2-like microglia/macrophages also exhibit opposite properties in terms of axon regeneration: the M1 phenotype is associated with in vitro expression of growth inhibitory molecules and reduced capacity for repair-promoting phagocytosis, and is thought to promote contact-mediated axon retraction in vivo[34,39]; on the contrary, M2-like microglia/macrophages have been shown to have pro-regenerative potential via release of cytokines, neurotrophins and proteases[34,39,67].

Therefore, due also to their complex heterogeneous nature, microglia and macrophages are responsible for both beneficial and detrimental effects after SCI.

Astrocytes

Their privileged positioning, with processes contacting on one end neuronal synapses and axons, and the endfeet wrapping blood vessels to form a main component of the blood-brain barrier (BBB)/BSCB, makes astrocytes critical actors in the CNS and in the orchestration of neural circuits[69]. Under non-pathological conditions, astrocytes are crucial in preserving homeostasis in the CNS: at the level of the synapses, they control fluid balance via surface water pumps and ion channels; moreover, they uptake and recycle excess neurotransmitters at the synaptic cleft, and in turn control synaptic transmission by actively releasing glio-transmitters; additionally, they contribute to CNS metabolism and are the main storage sites of glycogen in the CNS[69]. Moreover, astrocytes are crucial in synapse remodeling both in development, when they control synapse formation and pruning, and in the adult CNS, where they release cytokines to influence post-synaptic receptors scaling, and have key roles in memory function and cognition[69,70].
Following SCI, astrocytes become critical mediators of both innate and adaptive immune responses[39]. In response to DAMPs produced during CNS injury[34], astrocytes exhibit differential activation of multiple signaling pathways, leading to secretion of immune modulators already in the first hours post injury[65]: increased production of chemokines including CCL2, CXCL1 and CXCL2 causes chemotaxis of neutrophils and peripheral macrophages, while secretion of specific cytokines can bias microglia/macrophages towards M1 (TNF-α, IFN-γ, IL-12) or M2 (TGF-β, IL-10) phenotype[39], as well as causing autocrine activation[71].
Apart from their function in secondary injury, astrocytes have a crucial role in the formation of the border lining the fibrotic scar in chronic injuries, as discussed in **Biology of SCI lesion** and **CNS regeneration.**

Lymphocytes

Although recruited in lower numbers compared to innate immune cells, cells of the adaptive immune system also play a relevant role after SCI and CNS injury[34,56]. Lymphocytes progressively start to invade the lesion site around one week post injury, concomitantly to macrophages, and have been shown to be present chronically in rodent models[72]. SCI produces an autoimmune response in lymphocytes recruited in the CNS, which results in production of pro-inflammatory cytokines, direct toxicity on neuro-glial cells, promotion of M1-like phenotype in macrophages, differentiation of B cells to autoreactive plasma cells and generalized tissue damage[39]. Such trauma-induced autoimmunity (TIA), has been shown to be dependent on dysregulation of the control activity of CD4+CD25+ regulatory T cells on CD4+CD25- helper T cells, likely through the activation of PRRs present on T cell activators including dendritic cells[72,73]. B cells in their turn also produce pro-inflammatory cytokines that prevent resolution of the autoimmune reaction, by prolonging activation of CD4+ T cells[39].
In spite of the detrimental role in autoimmunity that can potentially provoke autoreactive effects also outside the spinal cord[39], lymphocytes might nonetheless show a key neuroprotective function after CNS trauma, as loss-of-function experiments reported worsened recovery and mechanisms of degeneration in rodent models lacking adaptive immune cells[34]. Therefore, while traditionally associated exclusively with negative functions, studies have begun to uncover a more complex role of adaptive immunity after SCI[34,74].

The interaction of lymphocytes with the injured nervous system can also exert a complex effect on regeneration, particularly in aging. Notably, CD8+ chemotaxis has been shown to be a critical mechanism in age-dependent decline of PNS regenerative capacity[75].

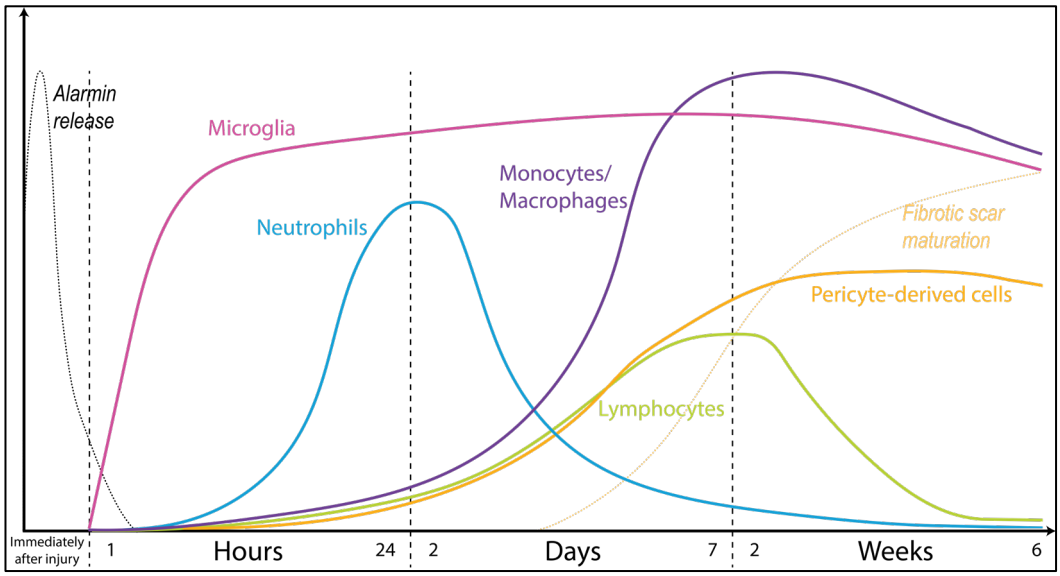

***Figure 1.3.* Global timeline of secondary injury response in rats.** *Right after injury, alarmins and DAMPs start to be released by necrotic cells. As a result, resident glial cells (astrocytes, microglia) become activated and are recruited to the injury site starting from few hours after injury. The first peripheral cells to be recruited are extravasated neutrophils, which peak around 1 day after injury. Later, blood-borne monocytes, differentiating into macrophages, and lymphocytes, start to invade the lesion, peaking several days post injury. Solid and dotted lines represent main cell populations migrating into the lesion and main mechanisms occurring during secondary injury, respectively. Adapted from Gadani et al.,2015[34], Hellenbrand et al.,2021[65], Neirinckx et al.,2014[56].*

The mechanisms of immune intervention after CNS injury have been extensively investigated, yet the complex interactions between the immune system and the nervous system, including crosstalk even under physiological conditions[34], remain to be fully characterized[34,76]. Single categories of cells might exhibit various functions and features in different areas of the lesion and at different timepoints after injury[77], and it is agreed that new lines of research could uncover contributions by additional cell types or even lead to the discovery of previously unidentified subpopulations at the interface between CNS and immune system[78]. Notably, recent evidence shows how additional systems including the microbiota also participate in the array of changes occurring after SCI and nervous system injury, along a gut-immune-neuroaxis[60,79]. Single-cell and spatial multi-omics technologies will be vital in further characterizing the role and the molecular signature of each of the abovementioned cell populations, as well as of other cell types (oligodendrocytes, pericytes, other immune cells, ...)[80].

As summarized in **Table 1.1**, multiple mechanisms of SCI pathology play mixed roles associated with both beneficial and detrimental contributions. In particular, it is more and more clear how the immunomodulation post SCI contributes to increased damage yet is vital to promote tissue repair and neurological recovery[76].
Accordingly, while strategies directly aiming at downregulation of systemic inflammation have been the main focus of clinical trials for pharmacological treatment

of SCI for more than 40 years, the results of these studies have been controversial[81,82]. In particular, administration of corticosteroids including high-dose intravenous methylprednisolone has long been included as standard care practice in treatment of acute SCI[34], but metanalysis studies have failed to display compelling evidence of neurological recovery, showing on the contrary emergence of serious adverse events[82]. These results seem to support the view of a double-edged nature of the inflammatory response.
Several other pharmacological agents targeting individual mechanisms have shown promising results in pre-clinical models over the years. The non-exhaustive list includes antibiotics (minocycline), channel blockers (riluzole), glycolipids (GM-1), molecules targeting claimed growth inhibitors (ChABC, Nogo-A antibodies), inhibitors of the Rho pathway (cethrin) and growth factors (G-CSF, FGF, neurotrophins)[12,81]. Nonetheless, to date none of these treatments has obtained approval as a therapy for SCI, with most drugs being stopped at intermediate phases of clinical trials or even not being investigated yet for clinical applications, and others with trials still undergoing[81].

It might be hypothesized that, due to the complexity of the response after SCI, with interactions and reciprocal feedback loops among the cells and mechanisms involved, no treatment targeting a single mechanism will be sufficient. Rather, it is increasingly clear that future therapies for SCI will have to rely on multimodal approaches, combining pharmacological treatment with other strategies including rehabilitation, electrical stimulation and biological repair[10].

Mechanism/cell population	Negative effects	Positive effects
Release of vasoconstrictive molecules[31,48]	Vasospasm	Bleeding containment
Temporary BSCB disruption/permeability[41]	Influx of inflammatory mediators and proteins usually restricted from CNS	Potential window of opportunity for drug delivery
Reperfusion[51]	Generation and diffusion of ROS (reperfusion injury)	Restoration of oxygen flow to ischemic areas
Macrophages/neutrophils[34]	- Contact-mediated axon dieback - Glutamate/ROS/protease release	- Debris clearance - Release of growth factors and protective cytokines
Lymphocytes[34]	Autoimmune response	Neuroprotective role
Fibrotic scar[83]	Prevention of axon regeneration	Maintenance of tissue integrity

Table 1.1. Double-edged response mechanisms after SCI. *Non-exhaustive list of biological mechanisms occurring after SCI or of cell populations that display both negative and positive contribution/potential towards recovery.*

Biology of SCI lesion

As described in **Pathology of SCI**, SCI causes catastrophic pathological changes in the lesion site and the surrounding areas, with an ensemble of cell damage mechanisms, immune activation and molecular responses lasting for several weeks after the injury before stabilization[31,32]. Chronically, a spinal cord injury lesion is characterized by three compartments[32] (**Figure 1.4**):

1) Fibrotic scar

Corresponding to the injury site, and accordingly referred to also as the non-neural lesion core or stromal scar, the fibrotic scar is the result of the response processes carried out by the organism during the secondary phase of the injury. Recruitment of immune cells causes local inflammation as well as general cleaning of the area, with removal of debris. On the other end, local fibroblasts and pericytes interact with blood-borne fibrocytes and begin to proliferate[22]. As a result, the original parenchyma is gradually substituted with extracellular matrix (ECM) molecules deposited by fibroblasts, including fibronectins, collagens, laminins and proteoglycans, and the immune cells progressively recede[22]. More specifically, work from Lee's and Frisén's groups has pointed to a subpopulation (type A) of pericytes as the main cells responsible for fibrotic scar formation, as opposed to meningeal fibroblasts infiltrating following tear of the dura[84]. Upon injury, type A pericytes, normally located mostly around larger blood vessels, detach from the vessels while adopting markers of collagen-producing fibroblasts and migrate in the lesion core, where they proliferate and persist for months[84,85]. Such migration has in its turn been suggested to be prompted via cytokine release by haematogenic macrophages extravasating to the injury site[86].

The emergency and pathological healing process associated with the deposition of the fibrotic scar allows to restore partial tissue integrity, and interfering with fibrotic scar formation by preventing proliferation of scar-forming cells results in failure of lesion closure[85]. Nonetheless, this process fails to compensate for the permanently lost functions of the original neural and glial tissue, behaving in addition as a hurdle for axon regeneration[83], as explained in **Regeneration after complete SCI**.

2) Astrocyte scar border

Traditionally broadly subdivided on the base of their morphology into gray matter-associated protoplasmic and white matter-associated fibrous subtypes, astrocytes represent a heterogeneous cell type with several subpopulations defined upon developmental patterning and local neuronal cues, and associated with diverse functions[69,87].

After SCI and more generally any type of CNS lesion, astrocytes undergo severe reactive astrogliosis, with pronounced cell hypertrophy and morphological reorganization[69], as a result of activation by cytokines, ROS, innate immunity mediators and several other molecules released during first and secondary injury[31].

Similarly to the heterogeneity observed under physiological conditions, reactive astrocytes also exhibit a variety of distinct phenotypes: after the first genomic studies at the beginning of the last decade, a binary categorization of reactive astrocytes in 'A1' neurotoxic and 'A2' neuroprotective profiles was proposed[88]; nonetheless, a large body of evidence is now suggesting that, as for M1/M2 microglia, this dichotomic classification is as a matter of fact outdated, and reactive astrocytes

rather constitute a continuum of several different molecular states depending on disease model and localization in the CNS[89].
Accordingly, the composition of the astrocyte scar border is also highly non-homogeneous: reactive astrocytes surrounding the lesion core are elongated and densely intertwined with each other, while reactive astrocytes further away maintain an hypertrophic but preserved stellate morphology, with cell density decreasing along a gradient with distance from the lesion[90]. The scar border is formed primarily by proliferating reactive astrocytes, while proliferation also tapers off with distance from the lesion: following insult to the spinal cord, reactive astrocytes adjacent to the lesion core start to undergo proliferation, and the newly formed elongated astrocytes arrange into a barrier-like structure that progressively encloses the lesion core and segregates inflammatory and fibrotic cells[90]. This process is crucially dependent on signal transducer and activator of transcription 3 (STAT3), as in mutant mice reactive elongated astrocytes fail to reorient and align to areas of tissue damage, but rather maintain perpendicular orientations which result in failure of restriction of inflammatory cells, and increased inflammation spread and neuronal death[90].
As explained more in detail in **CNS regeneration**, due to its physical appearance and to its production of potential growth inhibitors, the astrocyte scar border has long been regarded as a principal obstacle for axon regeneration in the CNS[22]; on the contrary, multiple studies have shown in the last ten years that not only scar-forming astrocytes do not prevent axon regrowth, but that different types of intervention are able to promote axon regeneration after CNS injury in spite of the presence of the astrocyte scar border[91], and that ablating the border or preventing its formation even results in more extensive lesions and decreased regeneration[92]. It is now established that scar-forming astrocytes have a crucial protective function, preventing spread of the inflammation by acting as a limitans border between the lesion core and the viable neural tissue around it[22].

3) Spared reactive neural tissue

Separated from the lesion core by the astrocyte scar border, the viable neural tissue around the injury site undergoes profound modifications for years after stabilization of the injury. Specifically, the input from sensory afferents becomes of crucial importance in areas below the lesion, guiding circuit reorganization that leads to both beneficial and detrimental effects[10].
Particularly for more severe injuries, neurons below the injury deprived of their physiological input start to receive increased aberrant projections from sprouting sensory afferents[93,94]. These maladaptive changes, involving also propriospinal connections, result in the development of typical SCI complications, including neuropathic pain and autonomic dysfunction[95,96].
On the other end, following incomplete injuries, the spinal cord is able to spontaneously rearrange to partially relay the information carried by the lost circuits via alternative connections; this reorganization relies on plasticity mechanisms of both supraspinal tracts and intraspinal interneurons, and leads to varying degrees of spontaneous but limited recovery of function[10].
Biomimetic electrical stimulation protocols can greatly augment functional recovery relative to plateau levels reached after chronic injury, and such improvement is observed already acutely after the start of stimulation[7]; appropriate recruitment of afferent fibers below the lesion has been shown to be a critical requirement in this

process[30,97]. Moreover, via repeated and active targeting of the relevant pathways through rehabilitation therapy, plasticity of the spared neural tissue can be exploited to trigger beneficial circuit reorganization that persists also in the long term and allows for restoration of function even in the absence of stimulation[7,98].
Therefore, knowing how to interrogate the spared neural tissue to prevent formation of detrimental circuits and to promote reinforcement of functional pathways is a fundamental need for successful SCI therapies.

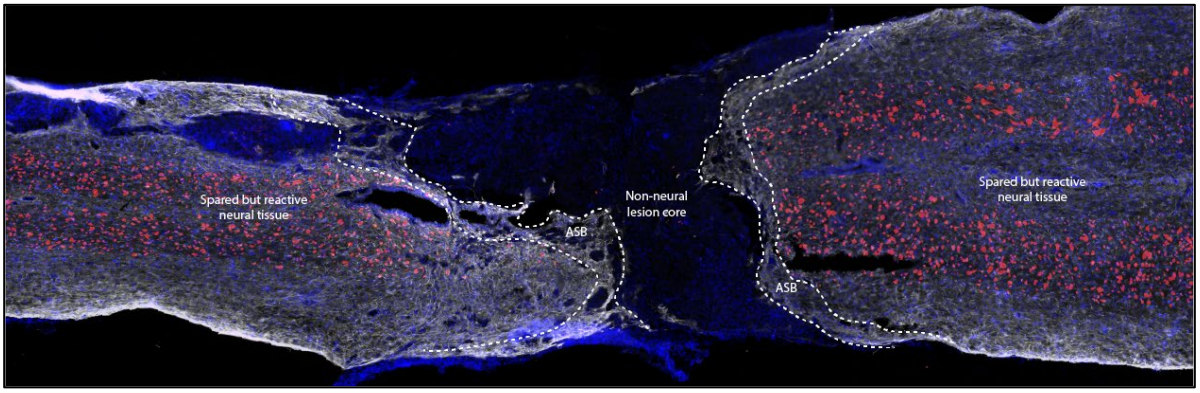

Figure 1.4. Compartments of mature SCI lesions. *Original survey image of mouse complete crush SCI (T12) showing the three lesion compartements: the astrocyte scar border (ASB), characterized by compacted and elongated reactive astrocytes that reorient parallel to the areas of tissue damage (Glial Fibrillary Acidic Protein (GFAP), white, overexpressed in reactive astrocytes), separates the spared but reactive neural tissue from the lesion core, which is devoid of neurons (as evident from the lack of NeuN signal - neuronal marker, red). Blue shows nuclear staining (DAPI).*

While the global tripartite structure is common to SCI in general, the morphology and microscopic characteristics of the lesion can vary substantially among species, even if closely related[99]. Notably, for instance, while injuries in rats, hamsters and primates are typically characterized by the presence of cystic cavities, mice lack cavitation at the lesion site and rather display remodeling mediated by deposition of matrix of connective tissue[99,100].

Accordingly, different mechanisms of injury also correspond to significantly diverse morphological properties and biological responses. Based on gross morphology, human SCIs can be classified as a solid cord injury, contusion/cavity, laceration and massive compression, with each category being characterized by variable extent of breaching of surface anatomy, presence of adherences to meninges, formation of cysts and fibrotic scarring[101]. Similarly, in preclinical species different lesion models promote distinct pathology, with contusion models resulting in more extensive demyelination and more robust macrophage infiltration compared to the focal, localized responses observed following transection SCI[102].

These and other variability aspects critically influence the biology of SCI lesions, and need to be taken into account when considering the translatability of potential therapies from preclinical to higher species[103].

RECOVERY AFTER INCOMPLETE INJURY: NEUROMODULATION STRATEGIES

As mentioned before, over the last 10 years our group and others have developed neuromodulation interventions that leverage targeted epidural electrical stimulation (EES) of the spinal cord, and that successfully promote restoration of motor and autonomic function after SCI, in rodents, non-human primates and patients.
In particular, I proceed here to discuss briefly the mechanisms and neural circuits that underlie restoration of voluntary locomotion after chronic paralysis SCI, highlighting why the same neuromodulation strategies are not suitable for use with anatomically complete injuries.

Central patterns generators and reflex circuits

Understanding of legged locomotion is complex, and the field has historically been focused on two main theories identifying distinct mechanisms of motor control (**Figure 1.5**): 1) feedforward control based on central pattern generators (CPGs) and 2) feedback control based on reflex circuits. Computational models generated for either theory have successfully recapitulated multiple aspects of locomotor control, and each of the two proposed neural circuit types are thought to underlie specific features of basic locomotion[104].

CPGs are defined as neural circuits embedded in the spinal cord capable of generating rhythmic, pre-programmed locomotion – referred to as "fictive locomotion" – autonomously via neural oscillators, i.e. in the absence of feedback (e.g. from sensory or supraspinal sources)[105]. Models based on reflex loops, on the contrary, hypothesize that sensory input – particularly proprioceptive – integrating in spinal reflex circuitry is on its own sufficient to produce locomotion without the need for an "engine" (i.e. the oscillators)[104].

Despite the concept being discussed already in the first half of the 20th century[106], demonstration of the existence of CPGs was only provided several decades later, when it was observed that the spinal cord of transected cats can be activated via L-DOPA to produce locomotion[105,107]. Since then, CPGs have been extensively studied in several other species including non-mammalian vertebrates (e.g. lamprey) and rodents[105]; on the other hand, presence of CPGs in humans has been questioned[108] and, while neonates and toddlers appear to possess conserved basic motor repertoires[109], the contribution of putative CPGs to the complex locomotor output in adults is presumably lower.

It is nonetheless established that the two theories are not antithetic, and that locomotion derives from a combination of both feedforward and feedback control[104,106], with sensory feedback from muscle proprioceptors modulating the output parameters of centrally-produced motor patterns[110]. Accordingly, computational models based on both mechanisms have been shown to outperform simulations based purely on either of the two control systems alone, as shown also in models of human locomotion[104,108].

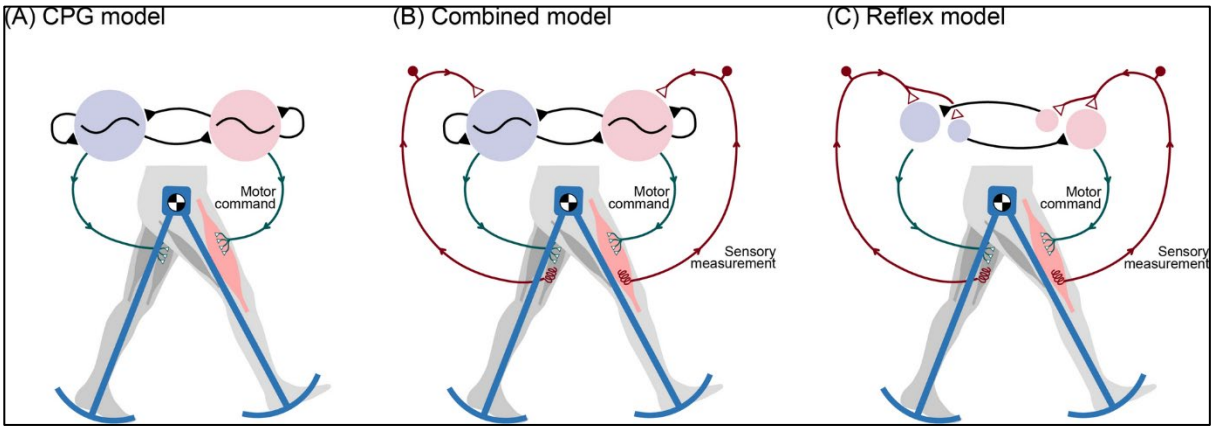

Figure 1.5. Models of motor control. *(A) The central pattern generator (CPG) comprises neural oscillators that can produce rhythmic motor commands, even in the absence of sensory feedback. Rhythm can be produced by mutually inhibiting neural half-center oscillators (shaded circles). (B) In normal animal locomotion, the CPG is thought to combine an intrinsic rhythm with sensory feedback, so that the periphery can influence the motor rhythm. (C) In principle, sensory feedback can also control and stabilize locomotion through reflexes, without need for neural oscillators. Any of these schemes could potentially produce the same nominal locomotion pattern, but some (B) combination of feedforward and feedback appears advantageous. Adapted from Ryu and Kuo, 2021[104] {licensed under Creative Commons Attribution 4.0}*

Importantly, the combination of sensory feedback and CPGs is on its own, in the absence of supraspinal control, sufficient to produce rhythmic, involuntary locomotion in lower species: in fully transected cats, for instance, bipedal stepping is observed on the treadmill upon training[111]. On the other hand, the scenario becomes more complex already in rodents, that require addition of EES and/or serotonergic agonists to exhibit walking patterns on the treadmill in case of complete injury[112,113].

Mechanisms of EES

As mentioned above, pharmacological agonists have been reported already in the 1960s to promote rhythmic locomotor activity from the spinal cord[107], and to facilitate stepping on the treadmill in rodent SCI models[114]; locomotor training was also shown to promote plasticity and partial recovery[115].
Similarly, EES has long been known to possess the potential to elicit responses from the spinal cord: stimulation applied to the lumbosacral spinal cord produces rhythmic hindlimb locomotion on treadmill in rodents with complete spinal cord injury[113], as well as promoting step-like patterns of electromyography (EMG) activity in motor complete SCI patients[116,117].

Nonetheless, none of these strategies alone supports robust recovery of weight-bearing[118]. Moreover, the end-goal of any therapy for SCI, and a crucial feature of the neuromodulation therapy developed by our group, is the *volitional* control of locomotor function. As discussed also below, activity-based rehabilitation is a fundamental requirement for the recovery of such capability[10].

Electrical stimulation of the lumbosacral spinal cord enables dormant circuits to become highly functional/responsive again by increasing the general excitability and

allowing sensory input to become a source of motor control[119,120]; such lowered threshold can then not only be exploited to generate involuntary sensory-driven locomotion (as mentioned above), but rather to promote restoration of voluntary locomotion with training. Our group showed that rodents with multiple types of severe (i.e. leading to chronic paralysis of hindlimbs) but incomplete SCI can regain voluntary hindlimb control over a range of motor tasks by means of a neuromodulation strategy combining continuous EES of the lumbosacral cord (40 Hz), monoaminergic agonists and weight-support – with the three mechanisms being all required[23,120]. Importantly, such protocols employing continuous, non-selective stimulation do not support instant recovery of voluntary locomotion in the case of such severe injuries, but voluntary stepping is rather developed after few weeks of intense locomotor training[23,120]: this activity-based training consists in repeated application of neuromodulation in two consecutive protocols – first the mouse is placed bipedally on a treadmill, thereby producing involuntary sensory-driven locomotion, and then is challenged to voluntarily move forward on a runway (absence of sensory input) in exchange of a reward; over time, the circuit plasticity deriving from the first protocol favors the emergence of similar beneficial processes from supraspinal centers leading to recovery of voluntary function.

On the other hand, the natural pattern of activation of the spinal cord requires recruitment of specific motor neuron pools at defined moments, and different motor tasks require distinct extent of activation of specific circuits. Recapitulation of such pattern of electrical activation via spatiotemporal EES, and via tuning of stimulation parameters (e.g. frequency is correlated with step height[121]), promotes recovery of locomotor function that significantly outperforms the results obtained with continuous EES[122]. Notably, spatiotemporal EES, contrarily to continuous EES, also allows instant recovery of locomotor function acutely[122].
The same technology, translated to humans, allowed immediate (within one week) regaining of function in patients with severe or complete paralysis, with substantial further improvement over the course of a rehabilitation period, partial neurological recovery even in the absence of stimulation, and increased autonomy in daily-life activities[7,98]. On the contrary, neuromodulation strategies based on continuous EES only allow limited recovery over the course of a much longer timescale (several months) and only when EES is applied[98,119].

Mechanistically, both experimental[123] and computational[124,125] evidence has demonstrated that EES, applied on the dorsal spinal aspects, does not stimulate the spinal cord directly, but rather enters it after recruiting dorsal roots afferents, primarily large-diameter proprioceptive afferents. EES therefore recruits muscle spindle feedback circuitry, at the same time also engaged by natural sensory inputs, and is integrated in the spinal cord together with supraspinal information from spared fibers to modulate motor patterns[124], as explained below.

Mechanisms of supraspinal control: systematic sparing of reticulospinal connections

As described above, neuromodulation strategies combined with locomotor training promote substantial recovery of voluntary locomotor function. Obvious but crucial point, in both patients and rodents, this is by definition only possible if the considered SCI lesion

is anatomically incomplete, i.e. spares either direct supraspinal projections or tissue through which relays of supraspinal information can extend; the consideration is also valid for subjects/animals regaining EES-mediated voluntary locomotion after chronic paralysis – see again **SCI: the problem** for distinction between anatomical and functional completeness.
Most human SCI lesions are as a matter of fact anatomically incomplete, and experimental models of contusion SCI are considered to recapitulate closely mechanisms of a large proportion of injuries in patients.

Contusion SCI is characterized by intrinsic variability, with varying pathway interruption and proportion of white matter sparing. Nonetheless, even in severe contusion models of rodent SCI, partial sparing of specific supraspinal pathways occurs, precisely of glutamatergic axons projecting from key brainstem motor nuclei – ventral gigantocellular reticular nuclei (vGi) and vestibular nuclei - and a subset of serotonergic axons[23]; on the contrary, the same injuries produce complete interruption of other crucial motor pathways, the corticospinal and rubrospinal tracts[23].
In particular, among the pathways with preserved connectivity, loss-of-function show that the main connections allowing rodents to recover voluntary control of locomotion with neurorehabilitation strategies are the reticulospinal fibers projecting from the vGi, that are systematically spared due to their distributed lateral and ventral location in the spinal cord white matter (**Figure 1.6**)[23].

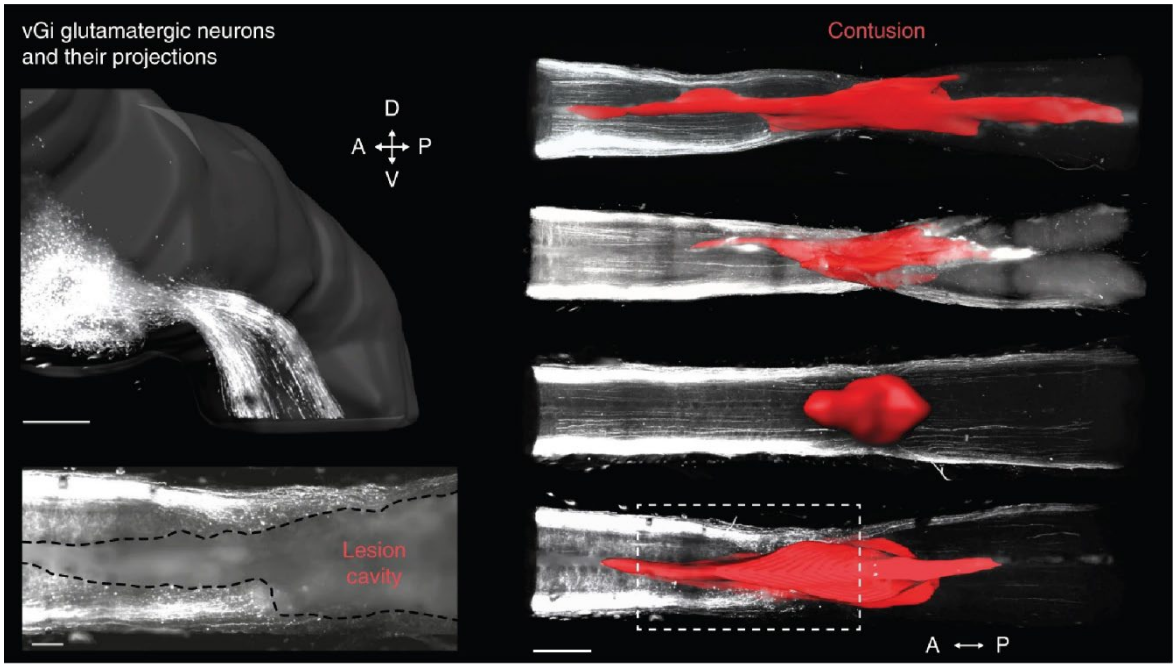

Figure 1.6. ***Clinically-relevant contusion SCI systematically spare a subset of reticulospinal projections from the vGi.*** *Representative images of CLARITY-processed spinal cord segments from mice receiving a severe contusion (90kdyn) at spinal level T12. Glutamatergic projections from the ventral gigantocellular nucleus (vGi) are visualized in white, as anterogradely labeled via injections of AAV1-DIO-tdTomato (Cre-dependent) in vGlut2-Cre mice. 3D reconstructions of the lesion cavity are shown in red. Labeling of spared reticulospinal tract fibers in the spinal tissue caudal to the injury site is systematically observed. A: anterior, P: posterior, D: dorsal, V: ventral. Reproduced with permission from Asboth, Friedli, Beauparlant et al., 2018[23].*

A similar anatomical distribution of the reticulospinal tract is observed in non-human primates, in which the role of the pathway for recovery after injury to other motor tracts has also been proved[126], and in humans, suggesting therefore that the same connections might be consistently spared in patients with contusion SCI and be responsible for the observed recovery of volitional control with neuromodulation[23].

Circuit integrators: local range Vsx2 neurons

In the previous two paragraphs we have described the two neuronal circuits that modulate spinal control networks from opposite ends to promote recovery with neuromodulation strategies. Nonetheless, in order for the system to function and produce locomotion, another component needs to integrate the multiple inputs and translate them into motor output.

A recent work from our group has revealed that such function is indeed exerted by a specific subpopulation of interneurons in the lumbar spinal cord, local range neurons expressing the gene Vsx2 and the marker for caudal spinal cord neurons Hoxa10 ($SC^{Vsx2::Hoxa10}$ neurons)[30]. $SC^{Vsx2::Hoxa10}$ neurons receive monosynaptic connections from both large diameter proprioceptive afferents and reticulospinal vGi axons; moreover, they project exclusively towards ventral laminae, where they establish synapses with glutamatergic and GABAergic neurons as well as cholinergic motor neurons[30] (**Figure 1.7**). Therefore, this subpopulation of neurons possesses the anatomical requirements to integrate all the relevant circuits necessary for production of locomotion via neuromodulation.

Accordingly, multiple loss-of-function experiments show that $SC^{Vsx2::Hoxa10}$ neurons are crucial in allowing locomotion mediated by neuromodulation, as well as to promote recovery over rehabilitation[30]; on the contrary, the same subpopulation is not required for basic walking in uninjured mice[30].

Importantly, Vsx2 (with the gene also referred to as Chx10[127]) interneurons derive from the developmentally-defined population of V2a neurons[128]. V2a neurons in the lumbar spinal cord have long been known in the field as key components of CPGs[128]: V2a neurons are excitatory, ipsilaterally-projecting neurons that establish synaptic contact with commisural V0 neurons, in their turn known to drive left-right alternation[129]. As such, disruption of V2a neurons leads to impairment of left-right coordination[129,130].

It is nonetheless crucial to specify that V2a neurons give rise to a variety of cells, including classes of neurons with low Chx10 expression[131], and that are a highly heterogeneous population[131]: V2a neurons have been described in the medulla (where they are required for breathing control in neonatal mice)[132], as well as in the reticular formation (where they are involved in abrupt stop of walking)[133] and in the cervical cord (where their ablation perturbs reaching tasks, and with this subpopulation projecting to brainstem nuclei)[134]; even V2a neurons described as part of lumbar CPGs are subdivided in subclasses with distinct firing properties[135,136].

The fact that specific subpopulations of V2a neurons possess distinct projectome and physiological properties therefore warrants caution in considering contribution to neurological functions made by one particular subclass.

Specifically, a clarification is important for the scope of this thesis: the class of Vsx2 neurons that we just described here, crucially involved in the integration of the signals that underlie recovery of walking mediated by neuromodulation therapy, is a subclass of the overall population expressing the marker Vsx2, specifically lumbar local-range Vsx2 neurons (expressing the gene Nfib). As such, it represents a distinct population of neurons compared to the ones that, as described in the experimental results of this thesis, emerge as responsible for spontaneous recovery of walking after severe but incomplete SCI, and that are the principal neuronal subpopulation responding to our regenerative intervention: the Vsx2 neurons that we characterized in the thesis are indeed thoracic long-projecting Vsx2 neurons (expressing the marker Zfhx3). In the thesis, long-projecting Vsx2 neurons are also referred to as ventral excitatory projecting Vsx2 (VEP^{Vsx2}), while local range Vsx2 neurons are indicated as ventral excitatory local Vsx2 (VEL^{Vsx2}).

While $SC^{Vsx2::Hoxa10}$ neurons have been described in mice, and while Vsx2 is known to be expressed in the human spinal cord[137], whether or not the same neuronal population also exists in humans has not been determined; nevertheless, plasticity mechanisms similar to the ones observed in rodents (reduction in the number of active spinal cord neurons after rehabilitation compared to acutely after SCI, with reinforcement of input on selected neurons including Vsx2) could be observed also in human patients via PET imaging (see also below)[30]. This suggests that the presence of analogous populations of neurons might underlie broadcasting of neuronal signals to direct recovery also in SCI patients.

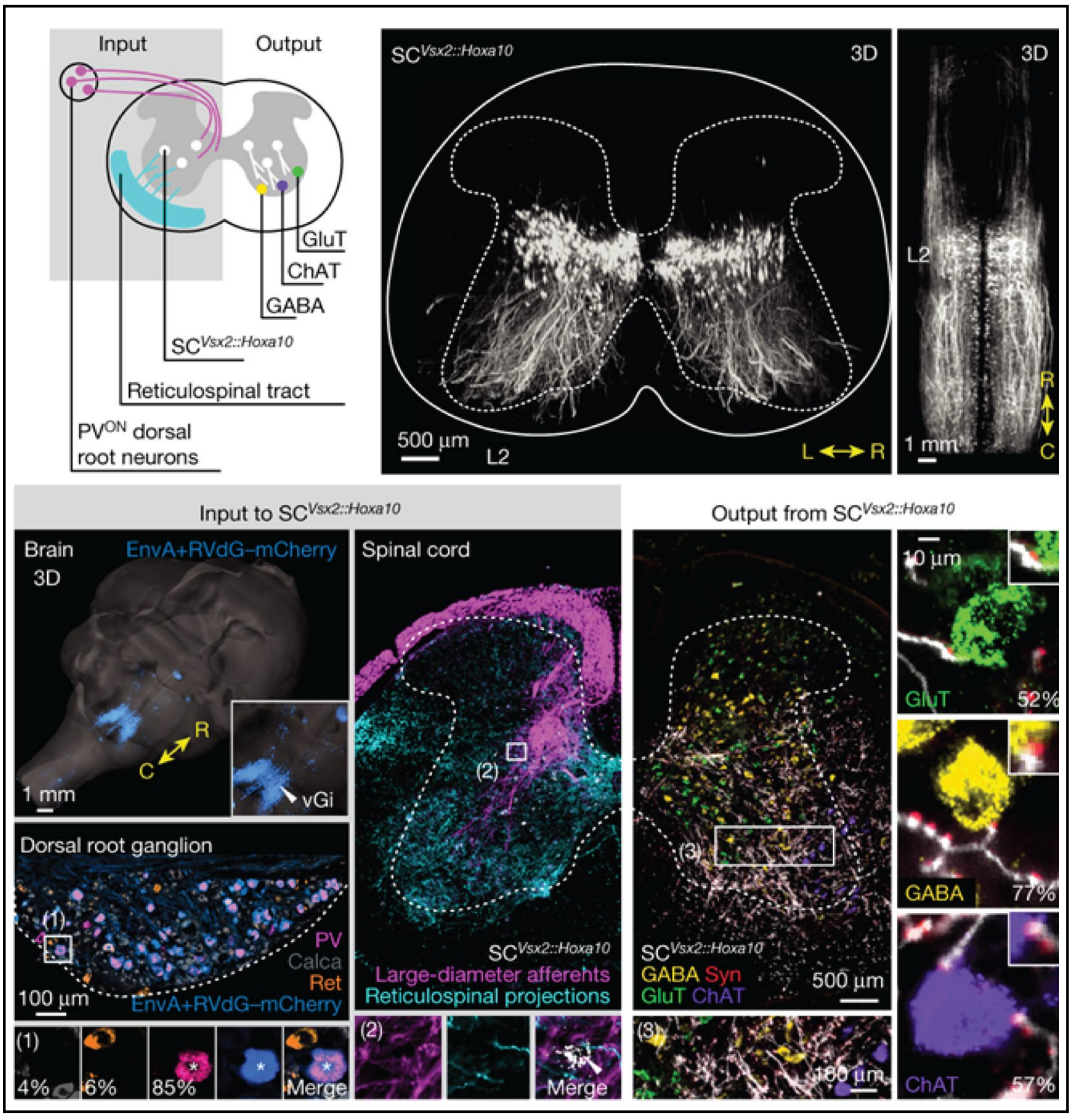

Figure 1.7. ***Local range Vsx2 (SC$^{Vsx2::Hoxa10}$) neurons integrate circuits underlying neuromodulation-based recovery of voluntary function.*** *Histological and CLARITY-based 3D reconstruction of input and output circuits to SC$^{Vsx2::Hoxa10}$ neurons.* Input: *Injection of monosynaptically-restricted pseudotyped tracing rabies virus (Env-A+RV-dG-mCherry) in vGi and dorsal root ganglion and of Cre-dependent TVA/G-protein in the lumbar cord in Vsx2-Cre mice reveals that SC$^{Vsx2::Hoxa10}$ receive extensive direct projections from reticulospinal axons and proprioceptive afferent fibers.* Output: *Anterograde tracing via injection of Cre-dependent AAV-DJ-hSyn-flex-mGFP-2A-synaptophysin-mRuby in the lumbar cord of Vsx2-Cre mice reveals synaptic projections of SC$^{Vsx2::Hoxa10}$ neurons on glutamatergic (GluT+), GABAergic (GABA+) and cholinergic (ChAT+) neurons, exclusively ventrally located (Syn: pre-synaptic marker Synaptophysin). L-R: left-right. R-C: rostral-caudal. Reproduced from Kathe, Skinnider, Hutson et al., 2021*[30] *{licensed under Creative Commons Attribution 4.0}.*

Activity-based rehabilitation and plasticity

A key principle of neuromodulation strategies is that, besides the functional effects observed acutely upon EES, rehabilitation further improves functional outcome on the long term, consistently with its known role in literature.

As mentioned above, fully-transected rats are able to perform rhythmic locomotion – i.e. in the absence of supraspinal commands – upon EES combined with delivery of serotonergic agonists and weight-support[10], and this occurs as early as one week after

injury. Importantly, such automatic tasks become more and more proficient if the rats undergo the same procedure repeatedly, implying that the spinal cord undergoes sub-lesional plastic changes upon sustained and consistent recruitment of locomotor circuits[118].

The same principle applies to recovery of *volitional* function in incomplete injuries: the immediate restoration of function observed acutely upon EES in animal models and patients, even motor complete, is greatly augmented with rehabilitation[7,23,98], and human subjects with milder injury can in the long term, upon consistent and repeated neuromodulation over periods of intensive training, also regain voluntary function when EES is not turned on[7,98].
This process is also dependent upon beneficial plasticity mechanisms, in this case occurring not only sub-lesionally, but also on supraspinal projections[23,120]: upon training, reticulospinal fibers from the vGi significantly increase the number of projections to the lumbar spinal cord below the lesion, and augmented corticobulbar connectivity is also observed[23]; likewise, the density of projections of reticulospinal and proprioceptive afferent fibers on local range Vsx2 neurons also increases[30]. More in general, while the injured spinal cord is characterized by extensive yet unorganized neuronal activity, often resulting from maladaptive projections, repeated recruitment of functionally-meaningful circuits via EES- and activity-based rehabilitation allows the spinal cord to select specific favorable connections[30]: as a result, both rodent and human subjects present a reduction in overall spinal cord neuronal activity after weeks of neuromodulation training, symptomatic of beneficial plasticity mechanisms that potentiate meaningful connections while discarding aberrant projections[30]; in mice, local range Vsx2 neurons represent one of the main neuron subpopulations favored by these plasticity mechanisms, and the decreased overall neuronal activation is compensated by increased Vsx2 transcriptional activity[30].

The role of voluntary engagement of the subject in such beneficial plasticity mechanisms is, once more, crucial, as treadmill-based training – not engaging cortical circuits – fails to promote such remodeling of descending pathways[23,120].

Inapplicability with complete SCI

We have here discussed how neuromodulation therapies coupled with rehabilitation promote remodeling of supraspinal projection pathways that results in functional recovery of locomotion, even in motor complete patients.

Despite the impressive results, which represent an extraordinary improvement for the patient, such strategies are ill-suited to application with complete or near-complete SCI, with recovery being correlated to the severity of the injury, and ASIA A patients exhibiting limited or no recovery in the absence of stimulation[7].

Moreover, in the theoretical case of complete absence of spared connections, corresponding to the experimental model of anatomically complete SCI, application of neuromodulation approaches leveraging surviving descending projections is intrinsically impracticable. Biological repair and promotion of regrowth from severed axons, which is the scope of this thesis, is therefore a requirement in more severe injuries[9].

On the other hand, it is envisioned that pro-regenerative and neuromodulation strategies could be used in combination, as one intervention could potentially positively steer the effect of the other[10], as discussed in **Conclusions**.

RECOVERY AFTER INCOMPLETE INJURY: NATURAL REPAIR

Evidence from imaging and clinical assessments shows that most SCI are anatomically incomplete, with a variable proportion of sparing; nonetheless, only about 50% of patients spontaneously recover some extent of lower limb locomotion at chronic timepoints[138].
Accordingly, among the different injury models available {**Experimental models of SCI**}, some paradigms for incomplete SCI result in substantial recovery of locomotor function whereas more severe models including the clinically relevant severe contusions produce permanent flaccid paralysis.

As mentioned before in the thesis, after SCI the spared neural tissue retains extensive plasticity capabilities and undergoes remodelling to adapt to the post-injury environment and to the altered inputs. Such processes can be leveraged and augmented to direct beneficial plasticity via neuromodulation strategies (as seen in the previous chapter), but also result in development of maladaptive connections that are associated with emergence of complications.
On the other hand, spontaneous plasticity also leads to varying degree of recovery of autonomic, motor and sensory function[139].

Recovery is thought to often be dependent on spared descending supraspinal axons that directly convey the signal caudal to the injury. Nonetheless, importantly, spontaneous recovery of function can also occur in the absence of sparing of direct supraspinal connections, by means of spinal cord (i.e. propriospinal) interneurons that relay the signal from the brain around and past the lesion: this has been shown specifically also for recovery of *voluntary* motor function, as rodents deprived of all direct supraspinal inputs at the thoracic level are able to recover hindlimb locomotion, in the absence of any intervention (see below).
As already discussed before, an essential condition for restoration of volitional control is, by definition, that the lesion be not anatomically complete, i.e. a bridge of intact tissue is required to allow for spared supraspinal axons or relay interneurons to pass on the other side of the injury. Accordingly, the extent of recovery achievable as a result of spontaneous plasticity is also limited by injury severity: specific incomplete models such as severe contusion SCI lead to chronic paralysis that is not reversible in the absence of neuromodulation – see **previous chapter.**

Staggered delayed double hemisection model

Among the experimental models of incomplete SCI, one specific rodent model that allows for spontaneous recovery of hindlimb locomotion in the absence of direct descending projections is the staggered delayed double hemisection model, i.e. a combination of two lateral hemisection SCI on opposite hemicords performed at different spinal levels and spaced by few weeks (**Figure 1.8**).

Despite not being a clinically relevant model, as even a single unilateral injury – corresponding to Brown-Séquard syndrome* – represents a rare (4% of all traumatic SCI) form of SCI, this experimental paradigm is particularly insightful in providing information on the mechanisms allowing for spontaneous improvement.
After a single lateral hemisection SCI performed at a given thoracic level, rodents experience immediate unilateral paralysis of the ipsilateral hindlimb due to complete interruption of all direct supraspinal inputs; 10-14 days after, the animal starts to recover stepping ability and, over the course of few weeks, weight-bearing basic locomotion on the ipsilateral hindlimb is spontaneously restored, without need of rehabilitation[143]; this has been observed in mice[143], rats[144] and monkeys[145], and is consistent with the recovery observed in Brown-Séquard syndrome patients.
In the staggered delayed double hemisection model, a second lateral hemisection is then performed, after recovery from the first injury, at a different thoracic segment (either caudally or rostrally) on the side contralateral to the first lesion, resulting in acute paralysis of both hindlimbs (partial for the hindlimb ipsilateral to the originally lesioned side, complete for the hindlimb ipsilateral to the newly injured side)[143]; by four weeks after the second injury, nonetheless, the animal is again able to recover locomotor control of both hindlimbs[143].
On the contrary, no recovery is observed if the two spatially-separated lesions are performed simultaneously, resulting in permanent bilateral hindlimb paralysis[143]; this suggests a potential time-dependence of the beneficial plasticity mechanisms[143], or that compensatory sprouting might be required specifically from uninjured axons, rather than branching from injured axons.

Locomotor function, particularly after double- but also following single-hemisection, is not completely restored and overall gait patterns after recovery are significantly distinct to those of uninjured animals (see also experimental work for the thesis), with the recovering mice being unable to perform precision motor tasks[146]. Nonetheless, substantial spontaneous improvement of basic locomotion is observed, with plantar stepping and weight-bearing capacity[143,146].

Mechanisms of recovery: spinal cord interneurons and plasticity of supraspinal pathways

The double hemisection paradigm proves that, in spite of absence of all direct supraspinal circuits, the spinal cord is able to undergo natural repair processes that promote spontaneous recovery of function. Mechanistically, as already mentioned, such recovery is dependent on the formation of detour circuits of propriospinal neurons that act as a relay connection; such relay is on its own sufficient to transmit functional signals from supraspinal pathways to the lumbar spinal cord, as supported by anatomical findings: over the course of weeks following the hemisection injury, key descending motor pathways, including reticulospinal tract fibers, display preserved projections in

* Brown-Séquard syndrome – i.e. the clinical manifestations of one single lateral SCI –, is characterized, given the anatomical placement of the various axonal tracts, by acute loss of motor function, mechanoception and proprioception ipsilaterally, whereas pain and temperature sensation is lost on the controlateral side[140,141]. Importantly, patients recover over time significant control of crude motor function in the leg ipsilateral to the injury, and are able to walk again with limited need of assistance[142].

the tissue around the lesion[144] and direct connectivity on spinal cord interneurons [experimental data of this thesis]; the same propriospinal interneurons, in their turn, exhibit increased projections in the lumbar spinal cord upon recovery compared to acute timepoints[143,146] (**Figure 1.8**). Moreover, after recovery, extensive remodelling of connectivity is also observed the brain[141,147].

Importantly, the functional relevance of spinal cord interneurons that act as a relay after hemisection differs significantly between the uninjured and the lesioned CNS: ablation of mid-thoracic spinal cord neurons in uninjured mice only results in minor impact on hindlimb kinematics, with no change in overall locomotor score[143,148]; on the contrary, in the staggered double hemisection model, ablating neurons in the same region, corresponding to the spinal levels comprised between the two lesions, results in complete abolishment of the recovered hindlimb function[143].

Notably, a very limited yet non-null amount of direct connections from supraspinal axons is detected in the lumbar spinal cord at chronic timepoints after delayed double hemisection[143] – likely as a result of spontaneous regrowth around the two staggered injuries –; nonetheless, the observation that recovered function is completely lost upon ablation of thoracic interneurons conclusively identifies such relay neurons as the principal mediators of spontaneous recovery after natural repair.

In the work presented in this thesis, we identified the molecular signature and the specific neuronal subtype of these recovery-organizing interneurons.

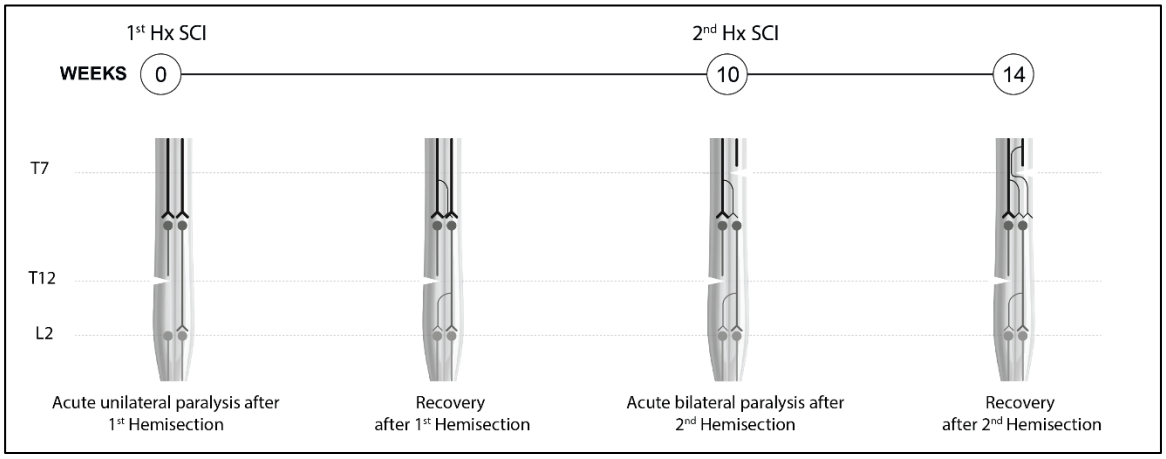

Figure 1.8. ***Natural repair mechanisms promote spontaneous recovery after staggered delayed double hemisection SCI.*** *Schematics of experimental paradigm of staggered double hemisection SCI In mice, as presented in Courtine et al., 2008[143]. A lateral hemisection is performed on the left side at spinal level T12, resulting in acute paralysis of the left hindlimb; over the course of 10 weeks, mice spontaneously recover locomotor function on the paralyzed limb; a second lateral hemisection SCI is then placed on the right side at spinal level T7, resulting in acute bilateral paralysis; by 4 weeks after the second injury, mice spontaneously recover weight-bearing stepping on both hindlimbs. Natural repair occurs by sprouting/synaptic potentiation of injured supraspinal pathways on thoracic propriospinal interneurons and of these latter in the lumbar spinal cord. Double-midline crossing interneurons not depicted.*

Mechanisms of recovery: sensory feedback

While we have discussed the crucial role of propriospinal interneurons for natural repair, another critically important component for spontaneous recovery of function is sensory feedback. In particular, specific loss-of-function studies in mouse models of lateral hemisection SCI show that muscle-spindle feedback from proprioceptive afferent fibers in the lumbar spinal cord is fundamental for spontaneous recovery to occur: mice lacking muscle-spindle feedback remain severely impaired (exhibiting hindlimb dragging) and are unable to perform even basic locomotor tasks[146,149]; administration of monoamine agonists in the acute phase does not exert any compensatory effect on the lack of recovery either[146]. Moreover, delayed – after recovery has already occurred – disruption of proprioceptive feedback also results in rapid abolishment of restored function, showing that proprioceptive input below the lesion is similarly necessary to maintain functional improvement[149].

Mechanistically, proprioceptive afferents are thought be critically important in natural repair by directing Hebbian plasticity: repeated volitional involvement of supraspinal centers (explainable with an "intention" of the rodent to walk) promotes reorganization and local axon growth from thoracic spinal cord interneurons naturally projecting to the lumbar cord (in the case of delayed double hemisection) or also from spared descending supraspinal projections (in case of single hemisection); on the other end, task-specific proprioceptive information from hindlimb muscles – potentially originating from the fact that the rodents move freely around the cage using the spared forelimbs – enters the lumbar spinal cord and might strengthen the sprouting connections from such axons[146]; accordingly, proprioceptive afferents below the lesion also undergo extensive connectivity changes[149]. Lack of sensory feedback might on the contrary result in absence of muscle-specific information and insufficient support to the newly-formed connections, with consequent Hebbian pruning.
This is also potentially consistent with observations of functional improvement with rehabilitation in patients, as repetitive movement during locomotor training repeatedly engages sensory feedback[146].
The combined information from relay circuits and proprioceptive fibers might then integrate in the local lumbar circuitry[146] and result in motor output, consistent with the knowledge of lumbar interneurons (Vsx2) receiving direct anatomical projections from both afferents and supraspinal tracts and connecting to motor neurons (see **previous chapter).** I hypothesize that embedding of lumbar neurons in CPG-like circuitry predisposed to generate *basic* locomotor patterns might also explain why only recover of unskilled motor tasks occurs.

Finally, the capacity of sustained proprioceptive feedback to mediate beneficial plasticity after injury is also confirmed by the observation that environmental enrichment in the cage (providing increased sensory stimuli compared to standard housing) prior to an incomplete SCI in mice is on its own sufficient to promote regeneration of a cohort of sensory – mainly proprioceptive – fibers inside and across the lesion[150] (see also **Regenerative competence: PNS vs CNS**).

Role of spinal cord interneurons in injuries lacking natural repair

As discussed, propriospinal interneurons exhibit remarkable plasticity after injury capable of promoting recovery of meaningful function.
Remarkably, such properties can also be leveraged in more severe but incomplete lesions that do not support any spontaneous recovery of function. The evidence comes from a study in a model of *simultaneous* staggered double hemisection, which results in chronic paralysis: Chen et al. showed that, in the case of such severe injuries, the lack of spontaneous recovery is associated with an imbalance of excitatory/inhibitory input in the thoracic spinal cord, particularly associated with over-activation of local (i.e. projecting on other neurons in the same segments) inhibitory neurons; pharmacological rebalancing of activity of such inhibitory neurons, indeed, promotes recovery of substantial stepping ability[25]. Therefore, in such severe injuries, dysregulated activity of local inhibitory neurons renders functionally dormant the circuitry in the perilesional area; such circuitry also includes the excitatory spinal cord interneurons projecting to the lumbar spinal cord that underlie natural repair: re-establishing proper balance allows these excitatory projection neurons to become functional again, and to successfully relay supraspinal signals to the lumbar centers to promote recovery[25]. Therapies aiming at restoring inhibition/excitation balance have therefore the potential to promote natural repair also after severe incomplete SCI.

Finally, the capacity of spinal cord interneurons to relay functional signals is also supported by other observations in incomplete SCI models that are characterized by permanent paralysis: as we discussed in the previous chapter, neuromodulation strategies combined with rehabilitation support recovery via spared reticulospinal tract fibers originating in the vGi[23]; while reticulospinal axons significantly increase *direct* connectivity to the lumbar spinal cord, the same axonal tract also exhibits increased sprouting on thoracic interneurons below the injury projecting to the lumbar cord (i.e. functioning as a monosynaptic relay)[23]. This suggests that thoracic interneurons might potentially also have a partial role in the recovery observed with neuromodulation strategies, by relaying signals via an indirect vGi-thoraco-lumbar connection.

How to mimic natural repair in more severe injuries?

In conclusion, in this chapter and in the previous, we have discussed how recovery of function is correlated with formation of detour circuits, either from spared supraspinal projections or spinal cord interneurons. While proper functionality of circuits below the injury, including sensory feedback, is a requirement, the nature of the specific tract constituting the relay signal does not seem to be crucial to promote restoration of basic movements[149].

On the other hand, the findings presented in this chapter underscore that propriospinal interneurons represent a particularly promising target for potential SCI therapies, as they possess extraordinary capacity to mediate recovery of function after incomplete injury, forming relay circuits that can transmit supraspinal motor signals to the lumbar spinal cord even in the absence of direct descending projections.
Depending on injury severity, repeated recruitment of supraspinal circuits together with the sensory feedback might be sufficient to trigger spontaneous recovering-promoting

plasticity via propriospinal fibers, or might need to be supplemented with other interventions such as rebalancing of local inhibition.

While anatomically complete injuries do not preserve the spared tissue bridge necessary for natural repair to occur, it is conceivable that providing a de-novo biological bridge for neural signals to be relayed across the lesion could lead to similar "spontaneous" recovery.

Accordingly, the main aim of this thesis was to mimic the natural repair occurring after severe but incomplete SCI by regenerating the same populations of spinal cord interneurons underlying such recovery.

REGENERATIVE COMPETENCE: PNS vs CNS

In the previous chapters, we have extensively discussed how biological repair and regrowth of severed axons represent crucial needs to improve functional recovery after SCI compared to currently available approaches, particularly in the case of severe or complete injuries. Nonetheless, contrarily to what is observed after lesions of the peripheral nervous system, injured central nervous systems axons are characterized by an intrinsic inability to spontaneously initiate a regenerative response. We here proceed to describe the mechanisms underlying such difference.

The different regenerative competence of the peripheral nervous system (PNS) and central nervous system (CNS) was already evident since the dawn of modern neuroscience. In 1914 Ramón y Cajal in his *Studies on Degeneration and Regeneration of the Nervous System* wrote «It is well known that, when one cuts a nervous cord {i.e. nerve} in a young animal, the peripheral extremity thus severed from its central portion degenerates and dies immediately, resorbing its remnants; months later [...] the peripheral nervous stump shows numerous newly-formed fibres; in the end, totally or partially, the sensibility and motility of the paralyzed member are re-established»[151]; at the same time, on the CNS he noted that «[recent investigations] [...] have confirmed the old concept of the essential impossibility of regeneration, showing that, after a more or less considerable period of progress, the restoration is paralyzed, giving place to a process of atrophy and definitive breakdown of the nerve sprouts. [Re]generative acts occur only in a small number of fibres which are usually of moderate and small diameter. The immense majority of the conductors of the white matter, and especially those of large calibre, undergo degeneration and atrophy exclusively»[152].

More than a century later, after enormous research advances and with the evolution of technologies, most of these observations have been confirmed and further characterized.
Injuries to peripheral nerves result in a variable yet robust degree of spontaneous axon regeneration and associated functional recovery[153]. This regenerative process is far from perfect, as functional recovery is in most cases limited[154], and improperly-guided regeneration of the nerve fibers can result in mistargeting, contributing to injury-associated symptoms as involuntary muscle contractions[155]. Moreover, axon regeneration in the PNS is characterized by a relatively slow rate of ~1-4mm/day[154,156]: as a consequence, the distance of the denervated target from the injury site is an additional important variable on recovery outcome, as more proximal injuries require longer times for reinnervation to occur, often resulting in irreversible atrophy of target organs[154]; accordingly, the limited timespan before which atrophic changes occur makes prompt intervention a critical determinant of successful recovery[156]. The extent of PNS regeneration and restoration of function is also influenced by the injury mechanisms: transections and lesions implying a physical separation between the stumps are characterized by a more severe outcome compared to continuity-preserving crush injuries, with the use of autografts or scaffolds bridging the lesion partially improving functionality and target reinnervation[157–159]. Finally, regenerative competence of the PNS drastically declines with age[160]: this decrease is not dependent on neuron-intrinsic factors, as aged neurons can be prompted to regrow into grafts of young tissue[161];

identified responsible mechanisms include instead impairment of repair response in aged Schwann cells, as well as aging-dependent increase in T cell signaling and recruitment to the dorsal root ganglia (DRGs)[75,161].

Despite such limitations, the imperfect spontaneous regenerative response observed in peripheral nerves strikingly contrasts with the near-complete lack of regeneration that characterizes the adult CNS. As from Cajal's observations above, after CNS injury in few instances branching from severed fibers and sprouting from uninjured axons are observed; nevertheless, these mechanisms fail to result in fiber elongation of more than few millimeters, and are soon aborted[162,163]. Injured CNS axons respond by retracting away from the injury site, with fibers belonging to different axonal tracts exhibiting a variable degree of degeneration: some axonal systems including the corticospinal tract (CST) display a profound dieback that can amount to several millimeters from the original lesion area[164], while others including serotonergic fibers persist close to the lesion edge[92,165]. Similarly, CNS axonal systems are characterized by differential regenerative abilities, with the CST notoriously being among the most refractory tracts, and different tracts respond to varying extent to growth-promoting interventions[166]: for instance, delivery of the growth factor NT-3 via cell grafts does not result in regeneration of CST axons into the graft[167,168], whereas it promotes graft penetration of sensory fibers[167,169].
Individual subpopulations of CNS neurons also exhibit drastically different regenerative ability and survival following injury, as discussed in **CNS regeneration**.

Mechanisms underlying the differential regenerative capacity

The principles driving the differential regenerative competence of PNS and CNS axons are complex and still not entirely uncovered. We here highlight some of the main mechanisms that contribute to explain such differences between the two districts of the nervous system.

Morphology

Morphologically, after injury, growth-competent PNS neurons and growth-incompetent CNS neurons exhibit contrasting responses at the end of the severed axon: the first display a motile tip that functionally and structurally resembles the growth cones observed in extending axons during embryonic development; on the contrary, CNS axons fail to rearrange into a growth cone, and instead draw back into swollen structures referred to as retraction bulbs[170]. Growth cones are hand-like shaped assemblies of constant size characterized by structured compartments of filamentous actin (F-actin) and dynamic tyrosinated microtubules, in which ordered reorganization of the cytoskeleton and trafficking of mitochondria and vesicles promotes axon extension[170,171]; retraction bulbs instead, are characterized by disorganized accumulation of mitochondria and vesicles, size increase over time, and dispersed unstable microtubules which prevent axon elongation[170,172] (**Figure 1.9a**). In particular, while microtubules that extend from the axon shaft to the central region of growth cones are characterized by typical arrangement in tubulin bundles, retraction bulbs possess dispersed and highly disorganized tubulin networks[172] (**Figure 1.9b**). Work from Bradke's group and others has

shown that promoting microtubule bundling via pharmacological agents including taxol and epothilone B reduces growth cone collapse and retraction in CNS axons, and stimulates regeneration of sensory and serotonergic fibers[173,174]; vice versa, microtubule destabilization via the drug nocodazole causes PNS growth cones to collapse into bulbs[172].

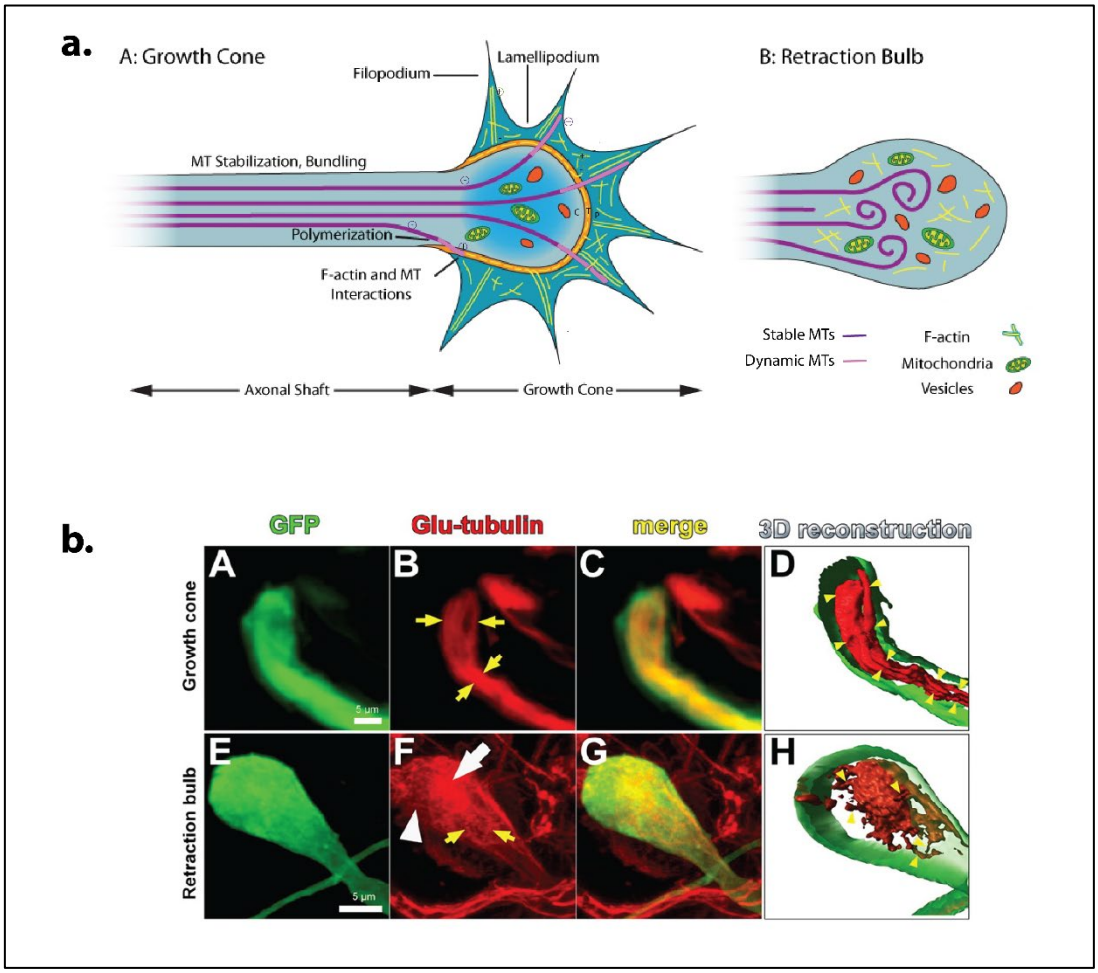

Figure 1.9. ***Cytoskeletal structure of growth-competent and -incompetent axons.*** a. *Graphical representation of structural organization of growth cones and retraction bulbs. [A] Regenerating axons exhibit growth cone structures: bundles of stable microtubules (MTs) are restricted in the shaft or central (C) domain, while the peripheral (P) domain contains dynamic MTs and filamentous actin (F-actin) – here branched F-actin forms a network in lamellipodia, while bundles of F-actin form filopodia and guide polymerizing individual MTs. This dynamicity is necessary for axon elongation. The transition (T) zone is a thin region between the C and P domains. Abbreviations: +, plus end; –, minus end. [B] Retraction bulbs form at the tip of regenerative-incompetent axons and prevent regeneration: in the bulb, the separate domains are lost, and the MTs are either depolymerized or disorganized. Reproduced and adapted from Cytoskeleton Inc., 2018[175] and Curcio and Bradke, 2018[170].*
b. *Histological detail of microtubule organization: A-D. Growth cones possess tightly bundled microtubules parallel to the axonal axis (arrows in B and arrowheads in D). E–H. Retraction bulbs have highly dispersed and disorganized microtubules (yellow arrows in F and arrowheads in H indicate dispersed microtubules that are highly deviated; in F the white arrow indicates regions in which microtubules are densely accumulated, and the white arrowhead indicates regions without microtubules). Immunostaining of GFP transgenically-labeled neurons with antibody against Glu-tubulin (marker for detyrosinated α-tubulin). Adapted from Ertürk et al, 2007[172] (Copyright 2007 Society for Neuroscience).*

Nonetheless, while microtubule stability, i.e. physical bundling of tubulin, is required for growth cone structuring, functional growth cones also need to be dynamic, with such properties being largely influenced by post-transcriptional modifications of tubulin[176,177]:

in this respect, PNS injury causes enrichment at the axonal end of tyrosinated tubulin[172,177], potentially involved in recruitment of trafficking proteins[176]; moreover, growth-competent axons after PNS injury are characterized by deacetylation of tubulin near the injury site that is not observed in CNS injured axons[177], and restriction of tubulin deacetylation impairs axon regeneration both *in vitro* and *in vivo*[177] {see also below in **Epigenetic regulation**}. Excessive growth cone dynamics, on the other hand, also cause impaired regeneration[177], suggesting that mechanisms influencing microtubule stability and dynamics require tight regulation and balancing.

Microenvironment

Historically, two main factors have been considered to underlie the observed difference in regenerative ability between central and peripheral nervous system: a differential ability to express regeneration-associated genes (discussed below) and diversity of the injury environment[178]. The role of inhibitory factors in CNS regenerative inability has been extensively considered since the observation, in the seminal work by David and Aguayo, that CNS axons can grow extensively inside PNS transplants[179]; since then, the field of CNS regeneration has intensely focused on identifying potential growth-inhibitory molecules, particularly myelin-associated, in the CNS lesion environment and testing multiple strategies to target these molecules to overcome regeneration failure.

We extensively discuss about interventions on myelin-associated and other molecules regarded as inhibitory in **CNS regeneration**; nonetheless, it is worth to briefly mention other features of the PNS microenvironment that have been studied as potential explanation for its regenerative competence.

In particular, after adult PNS injury, a robust increase in the synthesis of neurotrophic factors and expression of their receptors by neurons, satellite glial cells and Schwann cells is observed[180,181]. Schwann cells, specifically, known to be able to influence both PNS and CNS regeneration, extensively secrete trophic factors and produce cell adhesion and ECM molecules capable of positively regulating axonal growth[182]. Neurotrophic factors, in particular neurotrophins (NGF, BDNF, NT-3 and NT-4/5), are crucial during development, acting as survival factors and modulators of growth and differentiation for embryonic neurons and contributing to shape the developing nervous system[183]. After PNS injury, neurotrophins secreted in the distal side of the nerve are thought to act as chemoattractants for regenerating neurons[184]; in addition, neurotrophins retrogradely transported to the soma of DRG neurons are thought to drive downstream molecular changes that promote activation of regenerative pathways[184].

Based on these observations, delivery of neurotrophins has been extensively used over the years to target CNS regeneration (as further discussed in **CNS regeneration**). In vitro studies, for instance, showed that exposing cultured neurons to neurotrophins before placing them on myelin-associated molecules blocks neurite outgrowth inhibition[185]. In vivo, multiple approaches based on neurotrophins delivery have successfully resulted in increased neuronal survival and decreased atrophy[186,187]; when used as chemoattractants, exogenous neurotrophins have also been successfully used to promote CNS regeneration, but typically only when coupling neurotrophins delivery with other strategies including grafts and pre-conditioning[188]; this last observation, as highlighted elsewhere in the thesis, points to the importance of approaches targeting multiple mechanisms at the same time (e.g. induction of molecular growth programs,

supply of a growth-permissive substrate, chemoattraction) for successful CNS regeneration.

Biologically active neurotrophins are known to also be expressed in the adult CNS[189], where they regulate adult neurogenesis[190]. Neurotrophins are also expressed after CNS injury, by immune cells invading the lesion site, where they are thought to exert both autocrine and paracrine effects[191]. The extent to which differences in the expression of neurotrophic factors after PNS and CNS injury by the various cellular components involved might underlie discrepancy in growth ability remains to be characterized.

Injury-driven signaling and Regeneration-associated genes

Biological perturbations in general, and catastrophic ones as injuries more so, require surviving neurons to adapt to the new environment and therefore drive profound transcriptional changes[192]. In this respect, a main pointed cause for the differential regenerative response in PNS and CNS lies in the expression of so-called regeneration-associated genes (RAGs): while PNS injury drives a coordinated response that leads to RAG activation, CNS injury fails to do so[193].

DRGs and conditioning lesion

Dorsal root ganglia (DRGs) neurons represent the model system par excellence to investigate differential regenerative ability of CNS and PNS, owing to their unique position at the interface between the two districts: DRG neurons possess a pseudo-unipolar shape, with the soma lying in the DRG and two axons, i.e. a peripheral branch carrying information from the receptor towards the soma and a central-projecting branch relaying the information from the cell body into the spinal cord and towards supraspinal centers (**Figure 1.10**). Notably, in spite of the fact that the two branches belong to the same cell body, lesion to the peripheral branch leads to spontaneous regeneration, whereas the central branch displays the typical CNS regenerative incompetence observed in axons from neurons that have the soma in the brain or in the spinal cord[170].

The observation of such discrepancy led to the hypothesis that injury to the peripheral branch causes molecular changes in the soma that are not triggered by lesion to the central branch: studies from the 1950-60s pointed to an anabolic response pattern consisting in increases in RNA and protein synthesis in the soma following axotomy in the peripheral neurons, and not observed in central neurons[194–196]. In the next two decades, specific proteins upregulated after injury in regenerative-competent PNS neurons but not in regenerative-incompetent CNS neurons started to be identified, including the developmentally-expressed growth associated protein 43 (GAP-43), a now established growth cone marker[197,198].

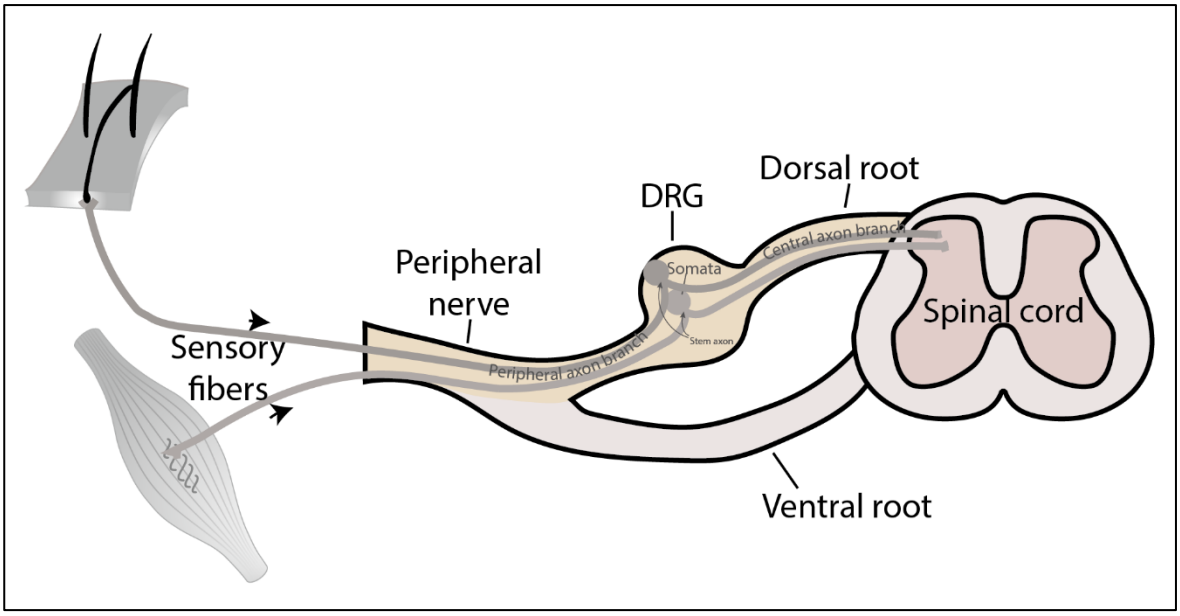

Figure 1.10. Anatomy of DRG neurons. *Adult primary sensory neurons possess a pseudo-unipolar shape, with the cell body lying in the dorsal root ganglion (DRG) and typically one single stem axon that bifurcates into two branches[199]: a peripheral axon branch that transmits sensory information from receptors in the periphery (e.g. muscle spindles, cutaneous receptors) and travels back towards the DRG via the peripheral nerve, and a central axon branch that relays information to the spinal cord and supraspinal centers via the dorsal root. Note that peripheral nerves also contain motor fibers (not depicted here) that exit the spinal cord via the ventral root and convey motor signal to the periphery.*

The idea of molecular changes driving regenerative competency was then further confirmed by the finding that the central-projecting branch of the DRG can gain regenerative ability if the neuron is subjected to the well-known *conditioning lesion* paradigm: seminal studies by Richardson and Issa first, and Neumann and Woolf after, showed that performing a peripheral lesion days prior to an injury of the central-projecting axon of the same neuron dramatically increases regeneration of spinal axons of DRGs in either peripheral grafts or CNS tissue, by prompting a change that "prepares" the neuron into a growth-competent state[200,201]. Later work by Filbin and others exposed signaling via the nucleotide cAMP as a main mechanism to prompt such molecular conditioning (although shown to recapitulate it only partially[202]): as a result of Ca^{2+} influx in the axotomized axon, cAMP generation is increased and cAMP retrogradely signals through PKA to drive RAG expression in the soma[203,204]; cAMP basal levels are correlated to regenerative capacitive, being higher in regenerative-competent PNS axons[205], and exposing CNS neurons to cAMP or analogs recapitulates the effect of a conditioning lesion by promoting CNS axon outgrowth on inhibitory substrates in vitro[206] and regeneration, albeit modest, in vivo[207].

Injury signals driving RAG transcription

Besides cAMP-mediated signaling, several other mechanisms responsible for backpropagating the injury signal from the lesioned peripheral axon to the soma to drive RAG expression have then been exposed in the following years[204]. These factors, existing or newly synthesized after injury, include mainly:

- Kinases, e.g. Dual leucine zipper kinase (DLK), a kinase part of the mitogen-activated protein kinase (MAPK) cascade that signals into the c-Jun N-terminal Kinase (JNK) and p38 MAPK pathways, and found to regulate neuronal apoptosis and degeneration after injury[208]. DLK has been shown to be a main signaling hub controlling regenerative capacity after injury: deletion of DLK in mice abolishes, both in vitro and in vivo, the conditioning lesion-triggered increase in axon outgrowth as well as the activation of pro-regenerative transcription factors STAT3 and cJun in the soma of DRG neurons[209]; moreover, DLK is directly required for retrograde transport of phosphorylated STAT3 to the cell body[209]. Likewise, terminal kinases of the MAPK cascade including JNK3 have also been shown to be retrogradely transported from the injured axon end to the soma upon peripheral nerve injury[210] .

  Another relevant example is Ca^{2+}/calmodulin-dependent protein kinase II α (CaMKIIα): activated by Ca2+ influx in the injured axon[211], CAMKIIα has been shown, after PNS but not CNS injury, to promote degradation of AMPK, in its turn known to negatively regulate - via downstream signaling cascades – activation of pro-regenerative pathways in the nucleus; this signaling mechanism therefore also underlies the regenerative response observed after PNS injury[212].
- Transcription factors, that can also themselves act as injury signals directly translocating to the nucleus to control synthesis of regeneration-associated proteins. An example is STAT3, locally translated in in the injured peripheral nerve and retrogradely transported to influence neuronal responses[209,213].
- Oxidases involved in reactive oxygen species (ROS) signaling. While ROS, of which oxidases represent one of the main mechanisms of production, contribute to tissue damage and cell death after injury (as described in **Physiology of spinal cord injury**), ROS signaling has also been shown to play a critical role for PNS regeneration[214]. An example of oxidases involved in such role are NADPH oxidases (NOXs): upon nerve injury, NOXs are released by chemoattracted inflammatory macrophages and endocytosed in the lesioned axon, from which they are retrogradely transported to the nucleus where they drive activation of pro-regenerative pathways via oxidation of molecular targets[214].

This backpropagation of injury signals in the injured peripheral nerve is at least partially dependent on binding of transport molecules – including importins – to motor proteins as dyneins and kinesins, that carry the cargo molecules to the nucleus along microtubules[210,214,215].

In addition, interruption of supply of trophic support from target regions has also been classically regarded as a factor contributing to transmission of injury information to the soma; this interruption in backpropagation of trophic molecules is referred to as "negative signaling", as opposed to the "positive", i.e. active transport of signals described above[216].

While the backpropagation mechanisms have been extensively studied in peripheral nerves, the extent to which such ability to generate and retrogradely transport injury signals is present in CNS neurons, and whether or not a difference could partially explain the CNS regenerative incompetence, remains elusive[217]. Studies in DRGs point at potential differences in structural components of the cytoskeleton of PNS and CNS axons, particularly microtubule density[199]. Nonetheless, experiments on the optic nerve or the corticospinal tract have shown that deletion of DLK or of DLK together with its homolog Leucine Zipper Kinase (LZK), respectively, hampers the effect of CNS pro-

regenerative interventions such as mTOR activation {see **CNS regeneration**} without affecting mTOR signaling, pointing to an at least partial conservation of the backpropagation machinery between CNS and PNS[218,219].

RAGs and necessity vs sufficiency for regeneration

Over the course of the last decades, dozens of genes have been identified as candidate RAGs. Several of these consist in transcription factors - including c-Jun, CREB, ATF3, STAT3, SOX11, KLF family members and others - that in turn drive expression of other genes associated with a positive role on regeneration[204]. Accordingly, other RAGs include molecules involved in the promotion of growth cone formation, such as cytoskeletal components and growth-associated proteins (including the abovementioned GAP-43), as well as extracellular matrix components, neuropeptides and molecules that can influence neuronal survival or drive specific injury-response, such as neurotrophic factors and cytokines[204,220].

Technological advances have now enabled to perform systematical comparisons between regenerative-competent (e.g. axoplasm of DRG peripheral branch or soma of DRG with sciatic nerve injury) and regenerative-incompetent (e.g. axoplasm of DRG central branch or soma of DRG with dorsal column axotomy) experimental models, therefore allowing to unbiasedly extract information about differential multi-ome signatures; this includes data from RNA-sequencing, proteomics, assay for transposase-accessible chromatin sequencing (ATAC-seq), chromatin immunoprecipitation sequencing (ChIP-Seq) and metabolomics[60,212,221,222].

It is to be noted, nonetheless, that while PNS and CNS injury drive different transcriptional changes, not all genes exhibiting a differential transcriptional perturbation in the two conditions - particularly overexpression after PNS injury – are necessarily associated with promotion of regeneration[204]. On the contrary, multiple genes found to be overexpressed after PNS injury compared to CNS injury are associated with a detrimental role towards regeneration: a notable example is SOCS3, that is found to be overexpressed after peripheral injury[204,221], but is known to be detrimental for axon regeneration in the CNS[223,224] {see also **CNS regeneration**}. Likewise, expression of thousands of genes is perturbed after injury, and differentially expressed genes might not have any functional implication in regenerative capacity[204]. In addition, regeneration-associated genes might not display any difference in terms of overall transcript expression among pro- and anti-regenerative conditions, but might still be able to exert stronger effect and promote regeneration in the first conditions by means of expression changes of other RAGs[204].

Therefore, while development of profiling technologies advances and allows for more and more precise characterization of multi-ome expression, careful analysis of the results, and focus on biological questions other than pure differential expression inferences, are necessary.

On the other hand, gene expression is orchestrated in complex regulatory networks that interact with each other, and the same applies to pathways associated with regeneration, as a smaller number of hub regeneration-associated genes – typically transcription factors – coordinates expression of several downstream RAGs[193]. Consequently, regenerative interventions targeted towards "terminal" RAGs might only

recapitulate individual specific mechanisms of PNS injury and therefore not drive the coordinated response required for successful regrowth[204]: a relevant example in this sense is again GAP-43 that, in spite of being specifically associated with axon terminals of regeneration-competent neurons, is not sufficient to promote regenerative growth when delivered alone after CNS injury[225]; likewise, the abovementioned limited regeneration achieved after SCI with c-AMP analogs delivery alone[207] is another instance of restricted recapitulation of pro-regenerative requirements by targeting single mechanisms. In contrast, targeting of multiple RAGs or pathways can promote more optimal activation of downstream RAGs and regenerative response[204]: delivery of db-cAMP in conjunction with constitutive activation of its transcriptional mediator and established RAG cAMP-responsive Element-binding Protein (CREB) significantly enhances in vitro neurite outgrowth compared to either approach alone[226]; on the same line, interventions that activate the pro-regenerative JAK-STAT and m-TOR pathways at the same time have been shown to trigger, in multiple CNS injury models, a significantly higher axon regrowth than activation of either pathway alone[224,227] {see also **CNS regeneration**}. Such pathways can interact synergistically with each other and also share common targets but at the same time retain independent actions[227–229].

Therefore, while studies have traditionally focused on *necessity* of individual RAGs in driving regeneration, identifying RAGs with *sufficiency* towards CNS regeneration remains challenging[204]. This shifts the view towards targeting upstream hub RAGs that can elicit a broader response, capable of recapitulating multiple PNS injury mechanisms to drive significant CNS regeneration[193], while bearing in mind that activation of master regulator genes such as hub RAGs can potentially activate undesired biological mechanisms concomitantly to their pro-regenerative effects.

Transcription-independent actions of injury signals

Making the scenario even more complex, molecules involved in injury signaling might be able to exert a pro-regenerative action also via transcriptional-independent ways, i.e. without causing RAG transcription. As an example, the MAP3K JNK mediates transcriptional responses by activating downstream transcription factors, but isoforms can also regulate axon growth by modulating microtubule organization[230]. Likewise, cAMP is also known to inactivate the small GTPase RhoA[231], which in its turn, when active, influences cytoskeletal organization by stimulating actin polymerization[232]; similarly to what is observed with tubulin destabilization, actin depolymerization has been shown to promote axon elongation[233], and the fact that RhoA is inactivated by cAMP and activated by several extracellular axon growth inhibitors that are present in the CNS but not in the PNS points to this as another potential transcriptional-independent mechanism that promotes PNS but not CNS regenerative competence[170]. Overall, this suggests that interventions aiming at more completely recapitulating the conditioning lesion-dependent regenerative response might need to combine RAG transcription with e.g. activation of second messengers (cAMP/Ca^{2+}).

To summarize, identifying the transcriptional scheme that underlies the regenerative difference observed between PNS and CNS after injury remains to date a challenging and unfinished task. Apart from intricate and not-fully characterized interactions

among several RAG transcriptional networks, the scheme is made more complex by the multifaceted spectrum of signaling mechanisms and extracellular triggers, and by differences even inside the individual neuron (e.g. cytoskeletal rearrangements or specific protein synthesis mechanisms being localized to axon or nucleus). Interventions aimed at influencing regeneration by acting on transcription will need to balance these factors, as well as to gauge necessity and sufficiency of the networks being targeted.

Epigenetic regulation

As mentioned above, signaling cascades activated by injury-induced mechanisms can influence regeneration by driving binding of transcription factors to promoter regions of RAGs, or by directly increasing expression of RAGs coding for transcription factors. Nonetheless, several lines of research point to epigenetic mechanisms as another more indirect yet crucial way of influencing regenerative capacity[234].

Histone/DNA acetylation and methylation

Gene expression can be regulated via multiple epigenetic mechanisms: acetylation of histones, promoted by histone acetyl transferases (HATs) and negatively regulated by histone deacetylases (HDACs), increases gene transcription, as the placement of acetyl groups increases accessibility of chromatin regions comprised between the histones facilitating transcription factor binding[235]; histone methylation, balanced by histone methyltransferases and demethylases[236], also influences transcription by leading to chromatin remodeling or promoting binding of transcriptional repressors, with different methylation motifs placed at specific histone locations resulting in either transcriptional repression (e.g. di-methylation of the 9-th lysine residue of histone protein H3, H3K9me2) or activation (e.g. tri-methylation of the 4-th lysine residue of H3, H3K4me3)[237,238]; other modifications can occur directly on DNA instead of histone proteins, including methylation[239].

Presence of epigenetic mechanisms exclusive to the injured PNS might contribute to explain the contrast in growth ability with the CNS: peripheral axotomy, for instance, has been shown to induce acetylation of histones at promoter sites of specific genes including RAGs, while such acetylation pattern is not observed after central injury[221]; likewise, increased DNA demethylation is observed in regenerative-competent neurons after sciatic nerve injury, with demethylation mediators driving downstream RAG expression and being both required for functional regeneration in this PNS injury model and involved in successful CNS regenerative interventions such as PTEN deletion[240]. Mechanistically, multiple processes triggered by PNS injury are responsible for the observed epigenetic modifications, often by backpropagating the injury signal from the axon to the soma; in the case of histone acetylation these include, for instance, kinase-mediated removal of HDACs (e.g. HDAC5) from the nucleus via injury-induced calcium waves[241] or phosphorylation of HATs (e.g. PCAF) via retrograde MAPK signaling[242].

Therefore, an "epigenetic barrier" might be present in injured CNS neurons, that prevents RAG expression by making transcription enhancer regions inaccessible to their

cognate transcription factors[178]. Targeting this roadblock is an attractive avenue to translate PNS regenerative competence to the CNS[240].

In addition, epigenetic regulators can also influence regenerative ability by acting on non-histone proteins. For instance, the HATs CBP/p300 also act as transcriptional co-activators, and after PNS injury acetylate the transcription factor p53 leading to the formation of a transcriptional complex that binds to the promoter of the RAG GAP-43[243].

Transcription-independent action of epigenetic regulators

Furthermore, histone regulators can act via transcriptional-independent modalities. As an example, the above-mentioned HDAC5, once exported from the nucleus after PNS injury, is translocated into the axon[241], where it influences cytoskeletal stability[177]: as discussed in ***Morphology***, in order to be regeneration-competent, growing axons need to have a microtubule organization that is stably arranged (organized in bundles) but also dynamic and ready to respond, and part of this function is regulated by means of tubulin acetylation – nuclear-exported HDAC5 can bind and deacetylate tubulin, therefore promoting growth-cone dynamics[244]; this mechanism is injury-dependent, relying on PKC-mediated activation of HDAC5 following Ca^{2+} influx, as HDAC5 does not affect tubulin acetylation under basal conditions[244]. On the contrary another deacetylase, HDAC6, regulates also basal tubulin acetylation[244,245], and experimental findings seem to suggest an opposite role on regeneration compared to HDAC5, as in vitro assays show that HDAC6 knockout promotes regeneration of cultured DRG neurons[245] – such role is nonetheless more complex as this regrowth improvement is only observed on inhibitory substrates[244,245]. This last observation suggests that transcription-independent epigenetic regulation might contribute to account for the limited CNS regeneration not only because specific pro-regenerative mechanisms are exclusive to the injured PNS, but also as a consequence of active growth-repressive control systems that might be present in the CNS[246].

miRNAs

Although studied less extensively than the abovementioned mechanisms, other epigenetic modulators seem to play a role in axon regeneration, including microRNAs (miRNAs)[204]. Unlike acetylation and methylation modifications, microRNA do not exert their action either on histones or DNA, but rather directly attach onto messenger RNA (mRNA), either causing mRNA degradation or limiting ribosome binding and therefore preventing translation[247].

miRNAs, that together with small interfering RNAs (siRNAs) form the basic components of the gene regulatory process known as RNA interference (RNAi), are small non-coding RNA molecules that are endogenously originated from RNA-coding genes[247,248]; generation of miRNA requires a multi-step maturation process starting from a more complex transcript (pri-miRNA) and involving several proteins including the enzyme Dicer[249].

Evidence shows that SCI causes changes in miRNAs profiles, and that specific miRNAs are crucial in mechanisms that follow CNS injury, including gliosis[250]. Moreover, miRNAs might also directly influence molecular pathways that determine intrinsic growth

capacity, by reducing expressions of proteins that negatively regulate such pathways[250]. More in general, preventing miRNA processing via in vivo Dicer knockout negatively affects axon regeneration after PNS injury[251]; the same study nonetheless shows that regeneration and functional recovery occur also in the knockout group, although delayed, to a lower extent and with significant impairment compared to the control group[251]. This seems to suggest that, while miRNA-mRNA interactions have an influence on post-injury events, other mechanisms might be more crucial in determining regenerative competence.

It is known that different miRNAs can play both protective and detrimental roles after nervous system injury, and that both PNS and CNS injury cause altered miRNA expression[252,253]; miRNAs nonetheless operate in highly complex networks that have not been completely uncovered yet, pointing to the need for further research on the subject[254]. Moreover, miRNAs rely on coordinated action, as single miRNAs alone are not sufficient to exert an effect on an entire biological pathway; as with RAGs, potential therapies manipulating single miRNAs after injury are therefore unlikely to be effective[254].

Factors influencing epigenetic signatures

Epigenetic signatures represent a biological embedding of the life experience of an organism[255], being influenced by interactions with the environment particularly during the perinatal period but also during adulthood[256]. Several factors constantly reshape chromatin accessibility and transcription via epigenetic alterations, including dieting regimes, exposure to chemicals and psychosocial stress among others[256–258], and as a consequence, differentially predispose towards various biological processes such as development of cancer[258]. This applies also to functions specific to the nervous system, as multiple mechanisms have begun to be uncovered that negatively or positively prime PNS and CNS regeneration competence in view of potential injury[234].

In particular, environmental enrichment has emerged as a positive mediator of regenerative capacity[150]: axon regeneration after PNS injury is significantly enhanced in mice housed prior to the injury in cages with enriched environment (i.e. larger cages, more cagemates, multiple enrichment items) compared to mice housed in a standard cage; mechanistically, the increased environmental stimuli intensify neuronal activity of proprioceptive DRG neurons, causing increased activation of the HAT Cbp, and ultimately triggering transcriptional-dependent plasticity processes via Cbp-mediated histone acetylation[150]; activation of Cbp acetyltransferase activity via pharmacological treatment also promotes regeneration after CNS injury[150]. Therefore, an enriched environment and the correspondent increased activity of sensory afferents seem to predispose neurons towards a more regenerative-competent state via increased histone acetylation, likely enhancing accessibility of RAG genes.

Likewise, age-dependent decrease in CNS regenerative ability, with near-complete absence of regeneration within days after birth (see below in **Spectrum of CNS regeneration**), is also dependent on epigenetic mechanisms. In particular, aging processes in general are associated with accumulation of specific DNA methylation patterns, and DNA aging clocks based on methylation have been developed in the last

years[259]. Age based on DNA clocks correlates with biological age of the organism[260], and is greatly increased as a consequence of biological perturbations, including cancer and spinal cord injury[259,260]. Recent work showed that in vivo reprogramming via delivery of Yamanaka transcription factors in adult mice results in axon regeneration after CNS injury by reverting methylation age to pre-injury levels[260]; such regeneration is observed also in older mice, expectedly to a weaker extent reflecting the higher pre-injury methylation age[260]; delivery of the same factors in uninjured old mice also rescues natural ageing-dependent vision loss, restoring visual function to levels comparable to young mice, by reverting methylation patterns to more youthful ones[260]. These results therefore also point to a role of age as predetermining factor towards regenerative competence via epigenetic mechanisms.

Spectrum of CNS regeneration

Inter-species differences

The experimental work discussed in this thesis was entirely conducted on mouse models, and other work from our laboratory also involves rats, non-human primates, as well as clinical research on human patients. While, as discussed above, in each of these species the adult CNS is characterized by an intrinsic regenerative incapacity after injury, it is worth to briefly mention – although outside the scope of this report – other instances in nature where this does not hold true.

Indeed, in contrast to the abovementioned cases, spontaneous regeneration after CNS injury during adulthood is observed in several animal species, including lower vertebrates and some mammals[178,261]:

- Fish. Several fish species exhibit remarkable CNS axon regenerative capacity which is conserved in adulthood, including non-bony species such as lampreys, historically one of the most studied species in spinal cord research[262]. Nonetheless, one of the best characterized models is zebrafish (*Danio rerio*): while paralysis is observed acutely after spinal cord injury, transected zebrafish are able to spontaneous recovery swimming functionality within weeks[263]. Such recovery is accompanied by, and critically dependent on, regrowth of transected axons across the injury site[263]. Mechanistically, both extrinsic and intrinsic factors contribute towards adult CNS regenerative competence in zebrafish: a critical difference compared to mammalian injury is that, in spite of glial and immune cellular players having a similar role {see **Physiology of SCI**}, deposition of a scar is not observed, therefore allowing sealing of the lesion via formation of a permissive neural bridge[263]; likewise, intrinsic mechanisms are also likely to play a role, including differential transmission of injury signals resulting in activation of a regenerative response[263].

- Amphibians. Other lower vertebrate species including amphibians also exhibit significantly higher CNS regenerative capacity compared to mammals. In particular, amphibians of the order of *Urodela* such as salamanders, newts and axolotl, are capable of regenerating lesioned spinal cord axons at any stage of

their life including adulthood, a unique instance among tetrapod vertebrates[264]. The remarkable regenerative capacities of urodeles are not restricted to the CNS, as these animals are for instance also capable of regenerating limbs even after repeated amputation[265]; such properties seem to at least partially rely on the presence of adult progenitor cells: after limb amputation, the formation of a typical "blastema", a mass of undifferentiated cells driving regeneration, is observed[265], while ependymoglial cells, acting as amphibian neural stem cells, underlie spinal cord regeneration via formation of a permissive lesion bridge[266,267]. Other amphibians such as those of the order of *Anura* (frogs) also exhibit CNS regenerative capacity in the post-embryonic stage, but this is limited to the larval stage, and is lost after metamorphosis into a froglet[268]; the only exception is the optic nerve, that instead retains regeneration capabilities throughout the entire life of the animal[268].

- *Acomys.* In spite of the common name of "spiny mouse", owing to the stiff spine-like hairs that make up their fur, Acomys belong to a different genus than the common mouse (Mus). These rodents have been known for their wound healing properties, with regeneration of full skin tissue including hairs, adipous and muscle tissue, glands and dermis following a full-thickness skin injury that instead causes fibrosis in Mus[269]; this regenerative behavior also extends to other tissue including skeletal muscle, and myocardial infarction results in significantly reduced negative outcome, with recovery of microvasculature and heart functionality[270]. Only more recently, it has emerged that Acomys are also capable of adult CNS regenerative capacity, representing an exclusive exception among the mammals species studied to date[261,271]: even after a complete SCI, regeneration of multiple tracts - including those regarded as traditionally most refractory as the CST – is observed, with regenerating axons being able to functionally propagate electrical signals across the lesion and – contrarily to what observed in mice after regenerative intervention (see **CNS regeneration**) – being partially myelinated[261]; regeneration is also associated with spontaneous recovery of bladder control and motor function as early as one and two weeks post injury, respectively[261]. Mechanistically, the regenerative behavior observed in Acomys is accompanied by differential gene induction compared to Mus, with lower activation of inflammatory-related genes and higher activation of molecular mechanisms associated with neurogenesis and synthesis of specific proteoglycans – including some traditionally regarded as inhibitory to axon growth (see **CNS regeneration**)[261,271]; reduced fibrosis (with lower total collagen deposition) and weakened, more localized astrocyte reactivity are observed, resulting in the formation of a scar-less tissue bridge at the injury site[261,271].

While the abovementioned species all exhibit to different extent post-embryonic CNS regeneration capacity after spinal cord lesions, it has also been shown that such regeneration only recapitulates the pre-injury spinal cord properties to a limited extent: as an example, even if restoration of sensory function is observed, several of these species do not display regeneration of ascending sensory fibers, with functional recovery therefore likely being dependent on interneurons[266].

Other species instead are still characterized by a dichotomy in axon regeneration competence between PNS and CNS, but over the years have largely been used, owing to their simplicity, as model organisms to study the nervous system and principles underlying axon regeneration. These include mainly invertebrates such as the nematode *Caenorhabditis elegans*, one of the simplest species endowed with a nervous system: the whole nervous system is indeed comprised of 302 neurons, and the transparency of the worm's cuticle, facilitating study via fluorescent markers, allowed modelling of synaptic-resolution connectomes as early as in 1984[272]. The simplicity of the organism also made it possible to perform studies involving single-axon axotomy via laser surgery, uncovering how multiple types of neurons possess spontaneous regeneration properties, as well as to perform genetic screening to identify candidate molecules involved in regeneration[273]. Nonetheless, as mentioned above, in spite of its relevance as an easy model to study nervous system properties, C.elegans also displays a dichotomy in regenerative-incompetent and regenerative-competent neurons, as neurons located in the nerve ring, equivalent to a CNS-like region, display regenerative incompetence after injury[274].

The same applies to the fruit fly *Drosophila melanogaster*, endowed with a significantly more complex nervous system (3016 neurons) than C.elegans and to date the most complex species whose nervous system connectome has been completely modeled[275]. D. melanogaster is also a convenient model system for nervous system research due to the transparency of the larval body wall, that allows easy manipulation and live imaging, and to the existence of targeted genetic tools[276]. While the CNS/PNS dichotomy in axon regeneration might also exist in this animal model[277], the fruit fly has nonetheless retained relevant interest in the field as it has been extensively used particularly to uncover mechanisms of axon degeneration following injury[278].

Age-dependence

With the exclusion of the abovementioned Acomys and other potentially yet unstudied species, the adult mammalian CNS is characterized by intrinsic regenerative incapacity. Nonetheless, evidence suggests that mammals could retain CNS growth ability in the very early stages of postnatal life: in mice, two and ten weeks after a complete SCI performed at postnatal day 2 (P2), serotonergic and CST axons, respectively, are shown to cross the injury and to densely innervate the caudal tissue, a behavior that is already absent at the next examined timepoint in the study (P7)[279]. P2 injury is accompanied by the formation of a scar-free lesion site, with low deposition of extracellular matrix (ECM) molecules, restored vasculature and presence of a bridge with continuous GFAP signal, which contrasts with the adult lesion characterized by extensive deposition of ECM components forming a fibrotic scar in the core and GFAP-positive reactive astrocytes lining the scar in the typical dense border[279]. Mechanistically, the scar-free neonatal lesion is critically dependent on time-restricted activation of microglia: in P2 mice, activated microglia reverts to homeostatic phenotype by 2 weeks post injury and is responsible for deposition of a transient bridge of fibronectin in the lesion that allows scar-free healing and disappears by 7 days post injury; on the contrary, in adult SCI microglia remains in the lesion with an activated phenotype, and fibronectin also persists in the fibrotic core along with other basal components[279]. Neonatal injuries seem therefore to be characterized by an inflammatory response that, contrarily to the sustained cascade observed in adults, is transient and quickly-resolving: this hypothesis

is further supported by the finding that transplanting in adult lesions either neonatal microglia or adult microglia treated with protease inhibitors results in improved lesion healing and axon regeneration[279]. Moreover, besides microglia, other cell populations might underlie healing in neonatal injuries, including ependymal-like cells with stem cell properties[280].

The scar-free healing observed in neonatal mice, with formation of a bridge between the two stumps, is reminiscent of the permissive bridges of glial and meningeal cells observed in abovementioned regenerative-competent species including amphibians and fish, highlighting lesion healing/remodeling as a critical requirement for regeneration. As a note, and as discussed further in **Regeneration after complete SCI**, this potentially points even more to the importance of taking into account potential presence of glial sparing when comparing experimental SCI lesion models.

As emphasized by the authors themselves, a limitation of the abovementioned study on P2 mice is that, at such stage of neonatal life, it is not possible to fully characterize whether the axons observed below the injury few weeks after are actually regenerated: indeed, in rodents, the corticospinal tract elongates in the spinal cord fully after birth within the first 9 days of life[281], therefore not allowing to fully exclude that part of the axons observed caudally might be late-arriving developing axons rather than regenerating.
Nonetheless, regardless of actual regeneration being present or not in early life stages, CNS *regenerative capacity* does negatively correlate with age in mammals, as reported by several other studies. In vitro regeneration assays (laser-mediated axotomy of fibers from individual rat embryonic neurons) show a progressive loss of intrinsic CNS regenerative ability with maturation in culture[282]. In vivo, CNS growth-promoting manipulations (PTEN deletion) performed at a fixed age (neonatally) in mice result in regeneration in mice injured both at younger (2 months) and older (5 months) age, but regeneration seems to be more extensive in mice injured at younger age[283]; likewise, the same manipulations, when initiated at the same time before injury (4-6 weeks before injury) but at different mouse ages, promote more extensive regeneration if initiated at early age rather than when the mouse is older[284]; in these latter study, in spite of the age-dependent decrease in regeneration, no significant difference in activation of the signaling pathways (mTOR) targeted by the manipulation is observed, pointing at neuron-extrinsic mechanisms, including more pronounced inflammation with age, as potential contributors to the decreased regeneration[284].
Recent findings point to additional mechanisms potentially responsible for age-dependent regenerative capacity, including existence of enhancers that are selectively activated after injury (to drive RAG expression) and for which their cognate binding transcription factors might be only present at earlier age[285,286].

Similarly, without aiming the attention to regeneration, plasticity and potential recovery of function are also generally considered to be higher in younger compared to older mammals[287]. This is consistent with human reports of significant functional recovery achieved in children, with some pediatric patients suffering initially functionally complete SCI being able to recover ambulation[288,289].
Analogous observations are also described in species with adult CNS regenerative competence: for instance in goldfish, complete functional recovery is observed at all

ages, but it requires more time and is associated with more limited regeneration as the age of the animal at injury gets higher[290].

In conclusion, as observed in the PNS, age seems to play a role in regenerative capacity also in the CNS, although such regenerative competence in the CNS likely presents a much larger drop in the early post-natal phases, leading to absence of spontaneous regrowth in the adult.

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In conclusion, efforts in characterizing factors that underlie not only differential growth ability in CNS and PNS, but also CNS regenerative competence in the abovementioned species and in neonatal rodent models will be of paramount importance for translational purposes and might be leveraged for the development of potential therapies in human patients.

CNS REGENERATION

In the previous chapter we aimed at describing a list of biological factors that are thought to underlie the differential response to injury, particularly in terms of axon regrowth, between the regeneration-competent peripheral nervous system and the regeneration-incompetent central nervous system. Here, we proceed to summarize specifically the state of the art of the experimental approaches that have been attempted over the last fifty years to revert CNS regenerative failure after injury.

We here focus on models of CNS axonal injury that comprise injury to the spinal cord or at relevant site of passage of supraspinal pathways (e.g. pyramidotomy, i.e. interruption of the corticospinal tract in the medullary pyramids, before decussation and entrance in the spinal cord). In addition, models of injury to the optic nerve are also included, as such structure is considered to be part of the CNS rather than the PNS: being originated embryonically from the diencephalon rather than from neural crest cells, the optic nerve is accordingly myelinated by oligodendrocytes instead of Schwann cells[291], and is characterized by lack of spontaneous regeneration typical of CNS structures[292].

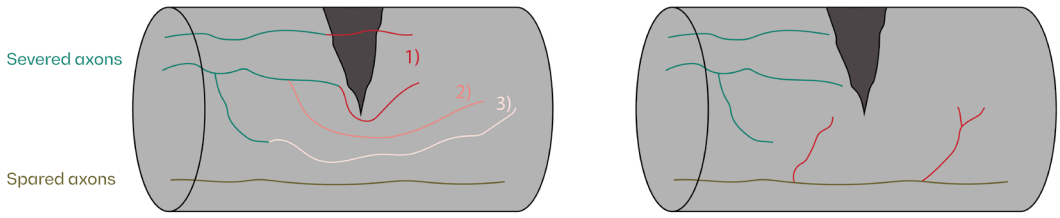
For what concerns SCI, models encompassing both complete and incomplete injuries have been used in the field {see **Experimental models of SCI** for more details on SCI experimental models}, although anatomically complete injuries are unarguably most suited for regeneration studies, as they ensure complete interruption of axonal pathways and therefore minimize potential ambiguity in axonal labeling between actually regenerating and spared fibers. A summary of approaches specifically used for regeneration after anatomically complete SCI is presented in **Regeneration after complete SCI**, whereas we proceed to describe more extensively CNS regenerative strategies below.

Regeneration vs sprouting: definitions

Multiple forms of axon growth after injury have been described over the decades in the field, often using non-univocal and error-prone terminology[293]. Definition of the differences is important for the scope of this thesis, as eliciting specific types of axonal repair compared to others can be more challenging, and this is associated with distinct implications for clinical translation potential. Here and elsewhere in the thesis, we refer to the definitions described in Geoffroy and Zheng, 2014 [ref. [293]].

Regeneration, in particular, is defined as growth from *injured* axons. This can occur 1) directly from the cut axonal end, 2) as de-novo branching from the axonal shaft or 3) as elongation from pre-existing branches. Importantly, regenerating axons can reach the opposite end of the lesion not only by growing across it, but also extending around the injury site (in case of incomplete SCI).

Sprouting, on the contrary, is defined as growth from *uninjured* axons, that can occur on either side of the lesion.



(Adapted from Geoffroy and Zheng, 2014)

Such distinction is critically important to assess the impact of regenerative interventions. Firstly, plasticity mechanisms from uninjured, sprouting axons can promote variable extent of functional recovery that, while being of important clinical interest, substantially bias the evaluation of the contribution of actual regenerating axons to recovery. In addition, anatomical analyses of regeneration in the case of incomplete SCI are particularly challenging, as axonal fibers labeled on the lesion side opposite to tracer injection could belong to either spared, potentially sprouting axons or to authentically regenerating neurons; qualitative assessments can be made on the basis of morphology (regenerating axons typically follow more tortuous, irregular trajectories compared to linearly-projecting spared fibers), but cannot fully resolve the ambiguity.
While sprouting and regeneration can both be potentially beneficial in supporting functional improvements, discerning the extent to which a specific manipulation promotes one or the other is crucial for understanding of biological mechanisms.

Therefore, specific considerations need to be taken into account in the selection of a suitable injury model, depending on whether regeneration or sprouting is the main mechanism of axon growth that the investigators intend to study. Anatomically complete injuries represent the best choice for studies of regeneration, in particular in the case of axonal populations (e.g. spinal cord interneurons) that are not confined to a specific region in the transverse plane. On the other hand, sprouting is often conveniently studied as compensatory growth of uninjured axons in response to injury of other neurons, therefore incomplete injuries that only sever one side leaving the contralateral intact are often used, such as unilateral pyramidotomy or lateral hemisection.

As already discussed elsewhere, the scope of the experimental work of this thesis was to promote specifically regeneration of transected fibers from spinal cord interneurons across the injury, i.e. regeneration from axonal ends, and a complete crush SCI model was chosen accordingly.

Anti-growth inhibitors

In spite of theories dating back to Ramon y Cajal pointing to a lack of production of chemotropic factors as a main reason for adult CNS regeneration failure, already in the first half on the 20th century alternative theories on physical barriers in the injured CNS being the principal factor preventing axon regrowth started to develop[22]. This idea

stemmed from the correlative observation of transected axons stopping in correspondence of the lesion scar[22,294], and multiple pharmacological treatments were attempted in the effort to attenuate such barrier[22]: tested molecules comprised for instance Piromen, i.e. a fever-producing bacterial polysaccharide[295], immunosuppressants such as cyclophosphamide[296], and digestive enzymes including trypsin[297], with studies reporting axon regeneration and even functional recovery following treatment[298]. Such results failed to be reproduced[299] and suggestions of experimental faults in some of the studies were also raised[300], yet the knowledge of such astroglial scar constituting a barrier to regrowth stayed[22].

As described in the previous chapter, in 1981 one of the landmark studies in the field, by David and Aguayo, could prove for the first time, thanks to the development of tract-tracing techniques, that CNS axons can grow across a bridge of peripheral nerve tissue, and that such axons stop after a short distance when re-entering the CNS tissue on the opposite end[179]. This observation led to the exaggerated – and now we know, erroneous – conclusion that the CNS is not intrinsically growth-incapable and that an inhibitory nature of the CNS microenvironment is the sole mechanism responsible for the lack of regeneration observed[22], sparking a second wave of studies attempting to tackle the CNS inhibitory environment[301].

Myelin-associated inhibitors

In a quest for molecules underlying the growth inhibitory CNS nature, the attention quickly shifted on CNS myelin: early observations by Martin Schwab and colleagues in the late 80s showed in particular that mature oligodendrocytes, as well as myelin isolated from adult spinal cords, are inhibitory to neuron adhesion and outgrowth in vitro, and the research group isolated specific myelin protein fractions that were pointed to as responsible for the inhibition[302,303]. Monoclonal antibodies against one of these fractions were developed[304], and particularly one antibody (referred to as IN-1) started to be extensively tested in vivo by Schwab's group and others: when delivered via hybridomas – to ensure continuous supply –, IN-1 promoted regeneration of CST axons around and caudal to incomplete SCI sites[305]; co-delivery with fetal transplants or neurotrophins was reported to increase the observed axon elongation around and caudal to the injury, as well as sprouting rostrally[306,307], and promoted regeneration in other models of CNS injury[308,309]; increased sprouting of serotonergic fibers below the injury was also described and, importantly, at longer experimental timepoints, a positive impact of IN-1 supply on functional recovery started to be claimed[310]. Similar reports of CST regeneration were also later made when, to investigate more clinically translatable approaches, delivery of a "humanized" IN-1 was performed intrathecally via osmotic pumps[311].

It was only in 2000 that the putative antigen recognized by IN-1 was discovered and identified as Nogo, a protein of the Reticulon family expressed by oligodendrocytes but not by Schwann cells, with the gene *nogo* producing three isoforms (Nogo-A, -B and -C) depending on splicing and recruited promoters[312,313]; nonetheless, IN-1 is potentially not specific to Nogo and might bind additional molecules[314].

In parallel to these studies, other molecules including two myelin-associated proteins, myelin-associated glycoprotein (MAG) and oligodendrocyte-myelin glycoprotein (OMgp), were discovered and, based on in-vitro studies, started to being referred to as

"potent inhibitors" of axon growth[315,316]. Nogo, MAG and OMgp are thought to be expressed directly on oligodendrocytes and CNS myelin debris, and act via a common receptor complex expressed on neuronal surfaces, NgR/p75/TROY/LINGO-1, through which they signal intracellularly on the small GTPase RhoA - which regulates actin polymerization {see also **Regenerative competence: PNS vs CNS**} –[316]; the three myelin-associated proteins are therefore thought to inhibit axon elongation via Rho-dependent stabilization of cytoskeletal components (**Figure 1.13**)[316].

The evolution of genetic engineering technologies and the development of transgenic lines gave further impulse to the field, and studies on knockouts of the claimed myelin-associated growth inhibitors started to be produced. Instead of confirming the results observed with IN-1 delivery, nonetheless, such investigations started to unearth complex interactions among the different molecular players, as well as to call for caution on the previous reports[315].
Knock-out studies on MAG and OMgp, for instance, failed to show compelling evidence of restriction of axon regeneration, with OMgp-mutant mice supporting regeneration of serotonergic fibers – and potentially dependent on genetic background – but completely lacking CST regeneration[317], and MAG-mutants showing lack of regeneration after optic nerve injury[318].
More surprisingly, in 2003 three studies published on the same issue of *Neuron* and run independently by three groups showed drastically different results when mouse lines of Nogo knock-out, although generated with different genetic techniques, were examined: Strittmatter's group (knockout of Nogo-A and -B via gene trapping) reported significantly increased sprouting of CST fibers rostral to an dorsal hemisection SCI (incomplete injury that interrupts almost entirely the rodent CST), extensive regeneration around and caudal to the injury, and functional recovery compared to wild-type mice[319]; for a comparable injury model, Schwab's group (knockout of Nogo-A but unwanted increased expression of Nogo-B) reported a trend for increased axon labeling caudal to injury, although non-significant, and even reduced sprouting rostrally, with no information on function[320]; finally, Zheng's group (knockout of Nogo -A and -B) found no significant change in sprouting rostral to the injury, no change in functional recovery, and no observation of fibers caudal to the injury[314].
The controversy recurred when again, in 2010, contrasting results were presented about mouse lines with simultaneous knockout of Nogo, MAG and OMgp. Strittmatter's laboratory claimed a synergistic inhibitory effect of the three proteins, with CST sprouting rostrally and regeneration around and caudal to an incomplete injury being increased (compared to control mice) in single mutants of Nogo- but not of MAG- and OMgp-; regeneration and sprouting were maximal in the triple mutant line, with associated functional recovery[321]. Zheng's group, on the contrary, presented results showing a lack of synergy among the three proteins, with no regeneration and functional recovery in any group; CST sprouting above the lesion was increased in Nogo-single mutants and instead decreased in MAG-single mutants, and the triple-mutant line showed lack of significant change in sprouting compared to controls, consistent with a compensatory role of Nogo and MAG[322]. In this second study, in particular, the Nogo knockout was even more comprehensive, as it eliminated all isoforms of Nogo (A-, B- and C-, compared to A- and B- only in Strittmatter's study), yet no effect on regeneration could be observed.

Ruled out changes in production of the lesion[314], multiple explanations were attempted to explain the contrasting results observed in the two cohorts of knockout studies: the different strategy through which the mutations were generated was for instance pointed as a potential source of confound, with neighboring genes potentially being activated as a result of the particular knockout strategy[314,323]; a role for genetic background was also highlighted, as each line was derived from a variable combination of two mouse strains with different regenerative responses[317,323,324]. Other reports even pointed to the observed regenerative phenotype in Strittmatter's mutants as simply deriving from mislabeling of non-CST axons via systematic tracer leakage via the CSF[325,326] or from potential rescuing of partially-axotomized neurons at the edge of the incomplete injury site[315].

While it is difficult to reconcile the discrepancy among the different reports, we believe that specific aspects of Zheng's studies make these investigations stronger: for instance, a role for the difference in production of the lesion can still not be completely excluded, as Zheng's studies employed a double cut hemisection (i.e. redrawing a blade in the injury site right after performing the transection, to ensure for completeness), while no mention of such measure was made by the other group, potentially highlighting the possibility of a milder injury and with higher risk of fiber sparing; the 2010 study from Zheng's group specifically also appears more rigorous than Strittmatter's, with more measures for behavioral assessments being considered, and multiple lesion models being investigated for regeneration.
The discrepancy between the regenerative effects shown in the IN-1 experiments and the lack of results in the knockout studies, also remains not completely understood: besides the problems of potential sparing and injury variability always associated with incomplete injuries for regeneration studies (including a systematic sparing of the minor ventromedial part of the CST with dorsal hemisection injuries[327]), other factors include the difference in the species used (rats in the IN-1 studies, transgenic mice in the knockout experiments)[314] or in the method to inactivate the inhibitory molecules (blockage via antibody-complexing acutely after injury with IN-1 delivery, elimination of the gene even before birth in the genetic knockout). Another hypothesis is that potential unexplored inhibitors other than Nogo, MAG and OMgp might be targeted by IN-1, as the antibody was shown to not be exclusively specific for Nogo[314]; in this respect, later studies performed by Schwab's group with antibodies more specific for Nogo showed more limited regeneration compared to the previous reports[328], and claims of regeneration and increased functional recovery when such therapy was translated to monkeys[329] received criticisms of important statistical and experimental flaws[330,331].

What undoubtedly emerges from all the above-mentioned studies, nonetheless, is that the potential roles of supposed growth inhibitors on regeneration *in vivo* are more complex than what initially hypothesized in the studies from the 90s.
In particular, it appears clear that therapies directed against myelin-associated inhibitors are not *sufficient* for regeneration of fibers *across* a complete injury, which is the scope of the experimental work presented in this thesis: in both Schwab's and Strittmatter's studies, fibers claimed as regenerating penetrate only rarely the injury site, and never grow across it, but rather pass the lesion area by extending around the core to invade the caudal tissue; the authors themselves also repeatedly report that animals with slightly more severe injuries and reduced sparing do not exhibit axon regrowth[311] and are therefore even excluded from statistical analyses[307,310]. Moreover, most of the original studies showing IN-1 mediated regeneration consisted in co-delivery

of such antibody with other factors known to potentially positively influence axon regeneration {see also below}, including grafts[306], neurotrophins[308,309] or growth factors[309].
The lack of *necessity* for successful regeneration of therapies directed against myelin-associated proteins has also been proven in several instances: ours and other groups have presented different extent of regeneration of CNS axons across either complete[332] or incomplete injuries[212,227] without directly targeting any of the abovementioned growth inhibitors {see also below and in the next Chapter}; similar inputs come from the observation that Nogo and other myelin-associated proteins are extensively expressed in the adult injured CNS of regenerative-competent species, including zebrafish and axolotl[333].
It is possible that, nonetheless, while not being sufficient per se to produce regeneration across the lesion, Nogo might be able to promote increased elongation from axons already regenerating in response to other interventions: a later study by Zheng's group, for instance, showed that codeletion of PTEN (with PTEN deletion being a known intervention resulting in axon regeneration {see also below in **Intrinsic growth capacity**}) and Nogo does not result in increased CST sprouting compared to PTEN deletion only, but instead unexpectedly increases regeneration[334]; this could, potentially, retrospectively explain at least in part the regenerative phenotype observed with codelivery of IN-1 and pro-regenerative neurotrophic factors.

Regardless of their limited effect on regeneration, it appears established in the field that myelin-associated inhibitors can instead, on their own, modulate compensatory sprouting of uninjured axons, as confirmed by the triple knockout experiments mentioned above and other specific studies[335,336]. This could therefore potentially still translate in a clinically relevant therapy in the case of incomplete injuries, if the observed sprouting produced enhanced recovery of function; multiple clinical trials targeting myelin inhibitors are currently under way[337].

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CSPGs
In addition to degenerating myelin, by the late 90s another class of growth-inhibitory molecules started to be identified and proposed as a major hurdle for regeneration of CNS injured axons: proteoglycans associated with the "glial scar", including chondroitin sulfate-proteoglycans (CSPGs) and keratan sulfate-proteoglycans (KSPGs)[22,338]. Proteoglycans (PGs) are heavily glycosylated proteins, composed of a core protein and several branching sulfated carbohydrate chains referred to as glycosaminoglycans (GAGs); GAGs are in their turn composed of repeats of disaccharide units, and the specific disaccharide unit gives the name to both the GAG and the PG – e.g. CSPGs are PGs with branches of chondroitin sulfate(CS)-GAGs, with N-acetylgalactosamine (GalNAc) and glucuronic acid (GlcA) being the repeating unit of CS[339] (**Figure 1.11**). The pattern of sulfation can vary significantly among different GAGs and, specifically in CS-GAGs, particularly important motives are addition of a single sulfate group at the oxygen at either the 4th or 6th position of GalNAc, referred to as 4-O-sulfation (CS4) and 6-O-sulfation (CS6); notably, depending on the proportion of CS4 and CS6, CS-GAGs (and CSPGs) have drastically different properties, which can influence their role in plasticity and axon growth mechanisms (**Figure 1.11**) {see below}[340].

CSPGs in the uninjured CNS are also a crucial component of perineuronal nets (PNNs), special ECM components present on the surface of specific CNS neurons and neurites, composed of multiple CSPGs attached to a mesh of another GAG, hyaluronan, and held together by tenascins and link proteins (**Figure 1.12**)[340]. PNNs have an extensive role in control of synaptic morphology, are expressed since the last period of embryonic development - coinciding with the closure of the critical period for plasticity - and are thought to influence synaptic activity via multiple mechanisms including modulation of receptor mobility and scaffolding of repulsive molecules[340]; therefore, targeting of CSPGs (and other components) in PNNs has been largely investigated to promote axonal sprouting and to modulate synaptic structure in the uninjured CNS and in multiple CNS disorders[22,340].

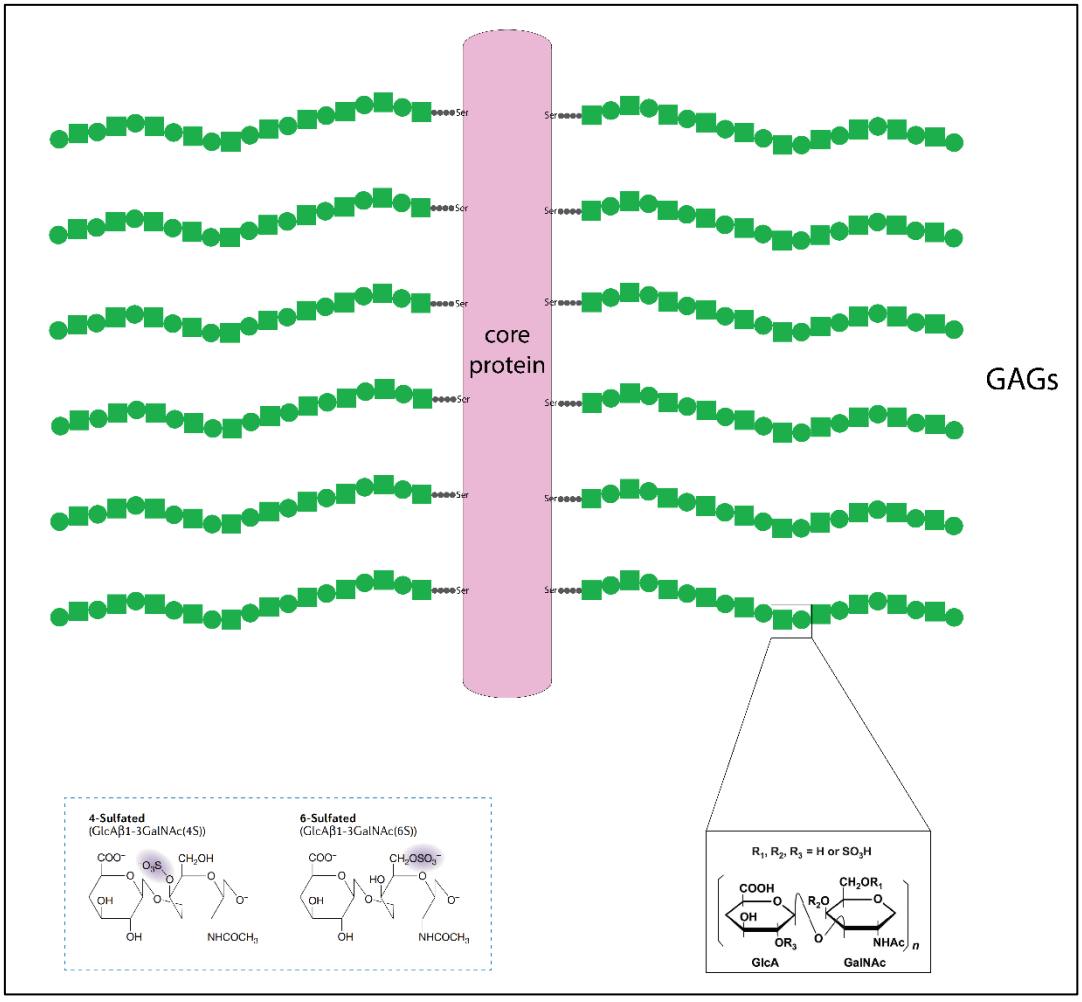

Figure 1.11. Proteoglycans. *Simplified representation of the structure of a proteoglycan (PG), consisting of a core protein with one or multiple GAG chains. GAGs are linear polysaccharide chains made of repeats of disaccharide units, and are typically covalently linked to Serine (Ser) residues on the core protein via a tetra-saccharide bridge (grey circles). Inset in solid line: disaccharide unit specific for CS-GAGs, made of repeat of GlcA and GalNAc (Reproduced from Hussein et al., 2020[341] {licensed under Creative Commons Attribution (CC BY)}). Inset in dashed-line: 4-O-sulfation (CS4) and 6-O-sulfation (CS6) on GalNAc, two of the most important sulfation sites for CS-GAG (Reproduced with permission from Fawcett, Oohashi and Pizzorusso, 2019[340]).*

Accordingly, CSPGs are not expressed only on astrocytes, but also on neurons as part of PNNs – although CSPGs in PNNs only account for 2% of total CSPG content in the CNS[340] –, as well as other glial cells[92,340].

CSPGs started to be investigated as potential *in vivo* growth inhibitors following in vitro studies in the 90s showing their detrimental role in axon outgrowth of multiple cultured neuron populations[342]. In particular, the theory of CSPGs specifically expressed by astrocytes in the scar border being a major hurdle to CNS regeneration represented a dominant theory in the field until recently, and particularly started to gain impulse following a study by Silver's group in 1999: the scientists transplanted DRG neurons (competent of regeneration in sciatic nerve lesions) in the spinal cord of adult rats via a specific technique allowing minimal production of tissue damage and scarring at the transplant site; the axons successfully managed to extend into CNS white matter for several spinal segments (in contrast to the theory of myelin-related inhibition), but they then stopped when reaching the edge of a SCI site, in correspondence of the astrocyte scar border, where high density of CSPGs was detected immunohistochemically[343]. Moreover, it was later found that multiple CSPGs are differentially regulated in SCI sites[342].

In view of this, in 2002 a pivotal study by Fawcett's and McMahon's groups investigated the effect of applying to the injured spinal cord Chondroitinase ABC (ChABC), a bacterial enzyme isolated several decades before and known to cleave and digest CS-GAGs from CSPGs[344]; intrathecal delivery of ChABC to a site of a dorsal column crush SCI (incomplete injury) successfully resulted in degradation of GAG chains from CSPGs close to the injury, and promoted significantly higher functional recovery compared to control lesioned rats[345]. Importantly, the study also showed labeling of few CST axons caudal to the lesion in treated animals, which was interpreted as signs of regeneration, although limited[345].

As observed with myelin-derived inhibitors, combinatory approaches based on co-delivery of ChABC with other interventions resulted in an increased impact on regeneration: studies from Silver's group for instance combined ChABC with peripheral grafts placed outside the spinal cord and relaying the rostral end of an incomplete SCI site to the spinal tissue caudal to it, showing regeneration across the graft and associated functional recovery[346,347]; importantly, the same group also achieved regeneration of a cohort of brainstem and propriospinal neurons across an anatomically complete SCI, by combining ChABC with a peripheral graft and fibrin glue embedding FGF[348]. This latter study, although with the limitations associated with peripheral grafts {as explained in the section below} shows at the same time the importance of combinatory approaches to promote regeneration in the injured CNS.

Alternative strategies also focused on modulating CSPGs on the astrocyte scar border via non-enzymatic interventions, including genetic targeting of specific receptors expressed on CSPGs pointed to be responsible for the inhibitory effect[349]. Among the various molecules exposed on CSPGs or with which CSPGs are known to interact are also found Nogo receptors, including NgR1 and NgR3 (**Figure 1.13**), highlighting crosstalk mechanisms between the two groups of molecules classically considered as growth inhibitors[350]. At the same time, actions of CSPGs and Nogo seem to be also partially complementary, as deletion of Nogo-A combined with ChABC treatment promotes a higher extent of sprouting/regeneration around a partial SCI site, with associated increased recovery, compared to either treatment alone, and fibers of different diameter seem to be preferentially targeted by each of the two treatments[351].

Similarly to what we discussed above for therapies targeting myelin-derived inhibitors, modulation of CSPGs in the injured CNS has now been established to exert its action

mostly on sprouting and synapse remodeling, with results from multiple studies being replicated consistently[22,293,337].

On the contrary, *sufficiency* of CPSGs digestion for axon regeneration has failed to hold up to the test of replication and, as mentioned above, the most successful studies showing axon regeneration with ChABC relied on combinations with other interventions.

Likewise, *necessity* of CSPGs digestion for regeneration, and the presumed role of reactive astrocytes in the scar border as inhibitory for axon growth, have been challenged by more recent studies. Multiple groups have shown successful regeneration across an SCI site without influencing CSPG composition, as mentioned above when referring to literature of regenerative interventions in which addressing myelin-inhibitors was not necessary.
Moreover, in 2016, Sofroniew's group conducted a systematic knockout study on scar-forming astrocytes, and showed that either preventing scar formation (by selectively killing proliferating astrocytes) or ablating astrocytes in chronic scar borders fails to promote regeneration across an SCI site, resulting instead in increased axon dieback[92]; on the other hand, consistent with the beneficial role of the astrocyte scar border in restricting inflammation, the lesion sites were larger in groups in which formation of the border was prevented[92]. The same study also showed that, in knockout groups, the total CSPG content of the lesion was not decreased[92]; moreover, comparison of bulk RNA-sequencing data between a portion specifically precipitated from astrocytes ribosomes and the flow-through showed that multiple inhibitors including CSPGs are extensively expressed by non-astrocyte cells in the lesion, and that astrocytes on the contrary also express a variety of growth-promoting molecules[92].

As an important note, among the multiple existing CSPGs not all are associated with a growth-inhibitory in vitro effect: putatively inhibitory CSPGs include aggrecan (CSPG1), versican (CSPG2), neurocan (CSPG3), brevican (CSPG7) and phosphacan; on the contrary, others including NG2 (CSPG4) and neuroglycan C (CSPG5) have been shown to be supportive for axon growth[92]. The same considerations apply directly to CS-GAG chains, with the C4S/C6S content being a critical factor in determining CSPG properties: 4-sulfated CS-GAGs are thought to be suppressors of plasticity and growth inhibitors, whereas 6-sulfated CS-GAGs are growth-permissive and promote plasticity[340]. Importantly, the abovementioned study by Sofroniew's group showed that aggrecan, that is the prototypical inhibitory CSPG, is not even detectably expressed by scar-forming astrocytes, and that these latter express a higher proportion of growth-permissive CSPGs than inhibitory CSPGs[92].

These results struck a decisive blow to the long-standing theory of CSPGs associated with scar-forming astrocytes being a major hurdle for axon regeneration. In support of this, as observed for myelin-related inhibitors, we know now that regenerative-competent species also extensively express inhibitory CSPGs in the lesion site, including newts[267].
Nonetheless, modulation of CSPGs in populations other than astrocytes might still be a beneficial approach in the injured CNS: blocking PNN signaling after SCI can for instance positively regulate reorganization of synapses and neuronal circuits[22], and ChABC-mediated digestion of PNN CSPGs mediates plasticity mechanisms that can be exploited to promote enhanced functional recovery when task-specific rehabilitation is performed[352].

Finally, astrocyte-produced CSPGs are known to form feedback interactions with the immune system, as CSPGs are thought to promote a pro-inflammatory phenotype in microglia/macrophages, with the increased inflammation in its turn inducing further deposition of CSPGs via astrocyte activation[350]. Therefore, while modulation of CSPGs might not represent a primary requirement for the regeneration of injured CNS axons, completely dissecting the role of such molecules after SCI remains a difficult task.

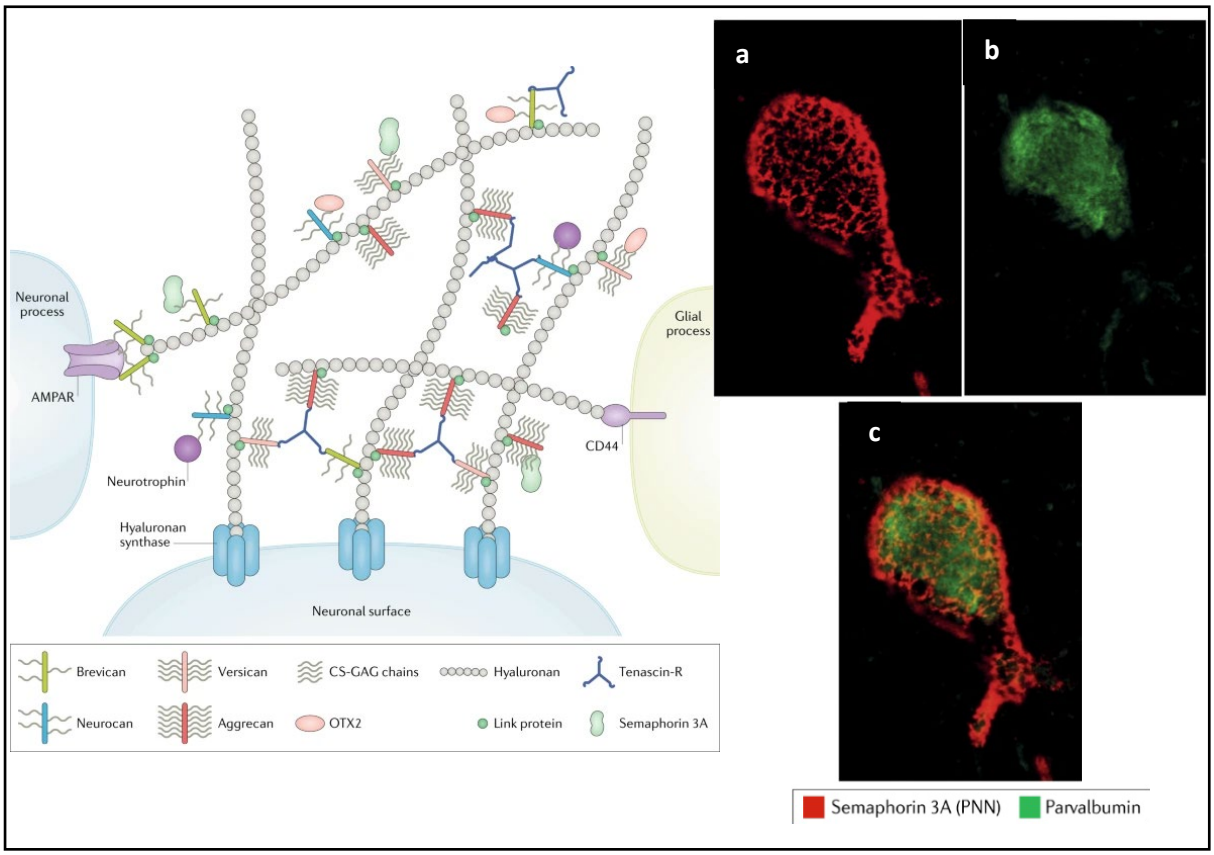

***Figure 1.12*. Perineuronal nets and associated CSPGs.** Left. *Schematic representation of the structure of perineuronal nets (PPNs): PNNs have a ternary structure not unlike that of cartilage, consisting of a backbone of hyaluronan (produced by the neuronal cell surface enzyme hyaluronan synthase present in the membrane of PNN-bearing neurons) to which CSPGs bind via their link domain - aggrecan is a major constituent of all PNNs, and three other CSPGs (neurocan, versican and brevican) are present in many PNNs to varying degree. The extracellular matrix (ECM) glycoprotein tenascin-R links the CSPGs to each other. Link proteins (including hyaluronan and proteoglycan link protein 1 (HAPLN1) and HAPLN4) are necessary to stabilize CSPG–hyaluronan binding. In addition, many molecules (including neurotrophins, semaphorin 3A and the transcription factor OTX2) bind to the CS-GAG chains, allowing them to be presented to synapses and other processes in the surrounding CNS and to associate with ion channels.*
Right. *a–c | A parvalbumin-expressing interneuron from the rat cortex surrounded by a PNN that has been stained using an antibody for semaphorin 3A, showing the net-like structure of the PNN with holes that are occupied by synapses. Reproduced and adapted with permission from Fawcett, Oohashi and Pizzorusso, 2019[340] and Vo et al., 2013[353].*

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In conclusion, while molecules traditionally regarded as growth inhibitors seem to play a relevant role in modulating plasticity after CNS injury, it also appears evident today that their contribution to CNS regeneration failure is much more limited than what studies from 30 years ago attempted to prove. Strategies targeting other mechanisms, including intrinsic growth capacity {see below}, enable on the contrary robust effects on axon regeneration and are therefore likely to be more necessary for successful SCI interventions.

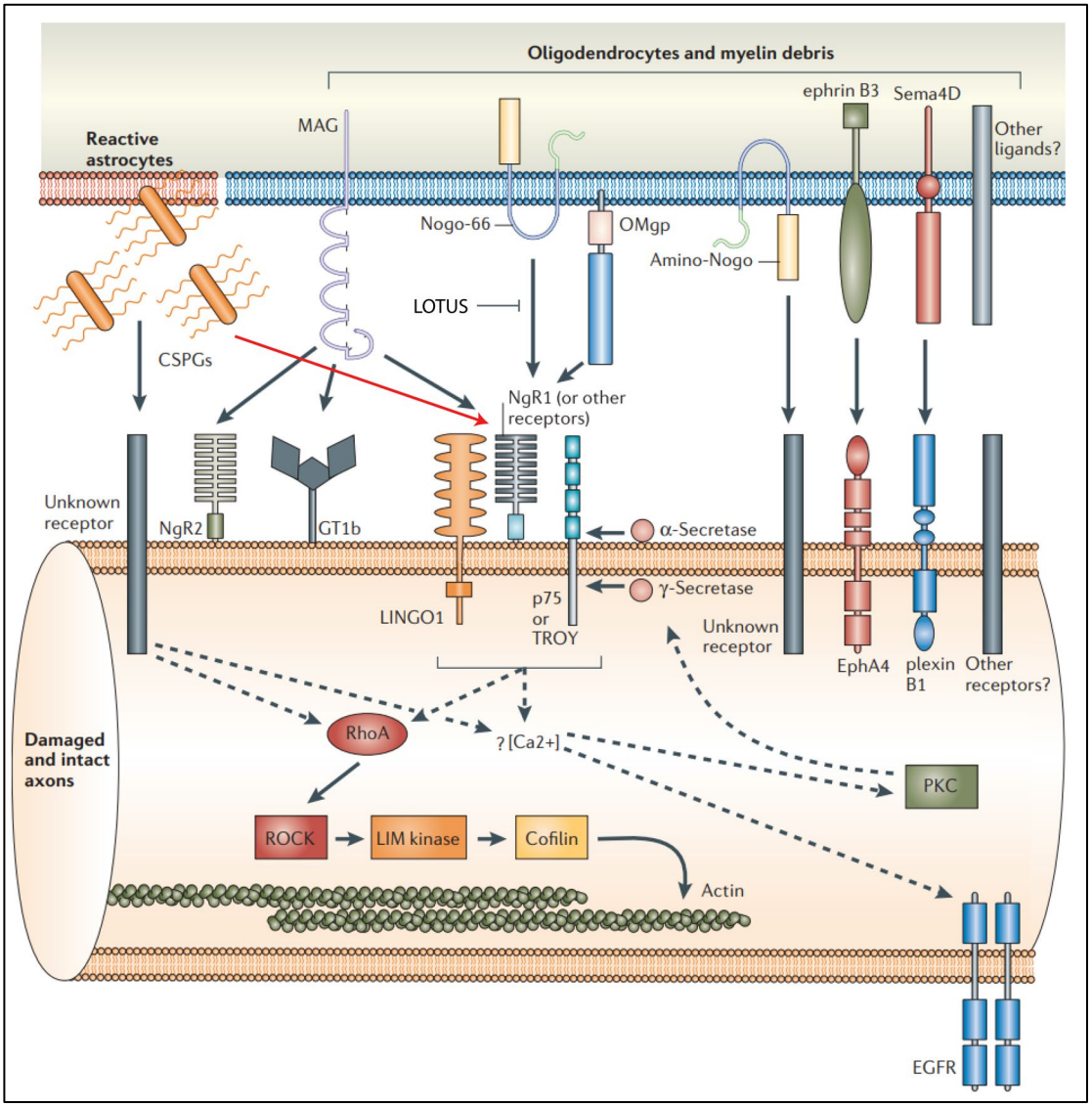

***Figure 1.13*. Putative growth inhibitors and associated intracellular signaling.** *Overview of the signaling mechanisms of the main molecules identified over the years as putative inhibitors of adult CNS regeneration, including CSPGs associated with reactive astrocytes – but also with other cell populations, as discussed above – and myelin-associated inhibitors from oligodendrocytes and myelin debris (Nogo, MAG, OMgp, as well as ephrin B3 and semaphorin 4D (Sema4D)). Although the topology of Nogo-A remains unclear, both the 66 amino acid loop (Nogo-66) and the amino-terminal domain (amino-Nogo) have been described as inhibitory to axon outgrowth, with Nogo-66 signaling being antagonized by the LOTUS protein. CSPG signaling can also occur via Nogo receptors, including NgR1 and NgR3 (red arrow).*
Among the signaling components that are common to both CSPGs and myelin-associated inhibitors are the activation of RhoA and the rise in intracellular calcium. Whereas the signals downstream of RhoA that lead to the actin cytoskeleton are well characterized (solid grey arrows), the relationship between components upstream of RhoA and the role of calcium influx are still ambiguous (dashed arrows). Reproduced with permission from Yiu and He, 2006[316] with adaptation from Schwab and Strittmatter, 2014[354].

Cellular transplants

The field of spinal cord injury has extensively focused on transplantation of grafts or suspensions of multiple types of cells, with the aims of bridging lesion ends, replacing lost neural/glial tissue and providing a supportive environment for axon regrowth[355]. We here proceed at summarizing the main cell-based approaches used to target CNS regeneration.

Peripheral grafts

In the original abovementioned study by David and Aguayo from 1981, a peripheral (sciatic nerve) bridge was used to relay two CNS regions, the medulla and the thoracic spinal cord, by suturing the two ends on the target regions through laminectomies and having the graft running outside the vertebral column, subcutaneously[179]. The goal of the study was to test specifically the hypothesis that CNS axons growth inability was «more dependent on the environment in which these axons are located than upon intrinsic properties of neurons»; therefore, given the specific experimental model, although local CNS damage was produced at the graft insertion sites, the authors themselves could not determine whether the fibers observed in the graft were regenerating damaged CNS fibers or sprouting uninjured axons, or both[179]. Nonetheless, work on PNS transplants specifically in CNS injuries had been carried out since several years before, with systematic studies in the 1940s[356] and early neuroscientist Jorge Francisco Tello investigating on the matter in as early as 1907[178].

However, it was not until the diffusion of modern neuronal tracing techniques such as horseradish peroxidase (HRP) labeling that groups including Aguayo and colleagues could test the effect on axon regeneration in SCI models[357]: PNS transplants connecting the stumps of a complete SCI lesion promoted regeneration of spinal interneurons and DRG neurons inside the graft, but the longest-regenerating axons seemed to stop at the interface between the graft and the distal stump, i.e. when they re-encountered CNS tissue[357,358]. This, together with the observation that limited regrowth was detected when grafting CNS (optic nerve) segments in a PNS lesion – with few PNS axons extending in the graft and to its distal end[357,359]-, led to a further reinforcement of the idea of a more inhibitory nature of the CNS: again Aguayo suggested that «CNS transplants are less receptive to the regenerating peripheral axons than are peripheral nerve grafts or the distal stumps of transected peripheral nerves»[357].

Presence of Schwann cells in the graft was found to be a fundamental element for the observed regrowth of CNS axons in PNS grafts, and particularly work from Bunge's group in the early 90s focused on delivery of Schwann cells in multiple CNS injury models: some extent of regeneration was found to be promoted, and Schwann cells were shown to both promote remyelination of the host spinal cord and provide trophic support[360]; nonetheless, regeneration was typically limited to a small number of axons and particularly failed to produce an effect on descending supraspinal tracts[182,361,362].

More complex interventions have also been tested in the following years by other groups: in a highly cited study, Olson and colleagues, for instance, presented a strategy to bridge spinal cord segments across a complete transection by suturing multiple small nerve grafts between the two ends; suturing was performed in a cumbersome organization

that both allowed regrowing axons to avoid white matter-associated myelin and recapitulated anatomy of the uninjured spinal cord – i.e. connecting proximal white matter to distal gray matter laminae to promote restoration of original topology of descending tracts and redirecting distal white matter to proximal gray matter for ascending pathways[363]. The study showed extensive axon regeneration including CST axons penetrating the graft and reaching the lumbar cord; moreover, recovery of hindlimb function was also claimed[363].

The approach developed in the specific study accompanied PNS grafting with stabilization via fibrin-based glue containing the growth factor aFGF[363]; similarly, several other studies have proposed interventions based on combinatorial delivery of PNS grafts with other molecules, including neurotrophins, other growth factors and ChABC[364]. In general, compared to graft delivery alone, addition of these molecules contributed, to different extents, to improve regeneration of CNS axons inside or even past the PNS graft[364].

Nonetheless, in spite of decades of research, to date all strategies relying on PNS grafts in combination with other treatments have failed to translate into clinically-applicable therapies[355]; particularly, concerning the study by Olson and colleagues, attempted clinical trials have failed to produce any improvement in complete-injury patients[365], and even studies in non-human primates models of incomplete injury have only partially confirmed results in terms of regeneration – although limited only to penetration in the graft, and no regeneration past it – without detecting any recovery of voluntary locomotor function[366].

Relevant to the scope of this thesis, it is important to include a specific additional note regarding Olson's study: the field now generally agrees that, while regeneration-based recovery of function relies on the requirement of restoring transmission of neuronal signals between originally-connected regions, reinstatement of connections via the original anatomical pathways might not necessary[22]; on the contrary, relaying supraspinal signals originally monosynaptically transmitted to target regions via polysynaptic interneuronal connections might be sufficient to achieve recovery[22,143]. Therefore, I argue that strategies attempting at recapitulating gross anatomy of uninjured axonal tracts as the one presented in the abovementioned study might not only not necessarily provide any additional benefit, but on the contrary also require excessively complex surgical procedures at the expense of clinical applicability.

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Fetal grafts

In parallel to PNS grafts-based approaches, from the late 70s until the early 2000s other groups focused extensively on grafting central nervous system tissue from embryos into SCI sites[367].

For instance, results from experiments employing grafting of fetal (embryonic day 14 (E14)) spinal cord tissue into lesion sites seemed to point to small yet increased functional recovery after incomplete SCI in rats: Bregman's group showed that rats receiving an over-hemisection SCI at birth and a graft in the lesion displayed significantly reduced impairment compared to lesioned-only animals when tested at 8 weeks of age, and approached performance of uninjured rats[368]; Reier and colleagues tested a similar approach in adult rats receiving a contusion SCI, and showed

improvement of some fine motor parameters but none in generalized motor tasks[369]. Mechanistically, nonetheless, while in neonatally-injured animals host axons – potentially late-developing - were observed to innervate also the caudal spinal cord[370], regenerating axons in the adult typically only penetrated the first portion of the fetal graft failing to regenerate across it[355,371,372]; the authors themselves pointed to the observed recovery in adult rats as non-primarily dependent on regeneration of host axons, but rather on the graft itself[372], with graft neurons integrating into the host tissue, building a relay circuit to transmit neuronal signals across the lesion[368,371], and the graft promoting host axon sparing and myelination[369].

As observed with PNS grafts, combinatorial strategies delivering embryonic spinal cord tissue together with neurotrophic factors significantly increased regeneration of host axons: Bregman and colleagues showed that co-delivery, acutely after injury, of E14 spinal cord tissue and a gel containing any of the neurotrophins NT-3, BDNF and NT-4 in the site of an adult incomplete SCI (lateral overhemisection) promoted – to different extent – increased length and density of regeneration of supraspinal axons in the graft[373]. Another study by the same group showed that the same strategy, if graft transplant was delayed 2-4 weeks after injury and NT-3 or BDNF were delivered continuously in the graft via an osmotic pump, could promote regeneration of supraspinal tracts inside and even past the graft also after a complete (transection) injury[374]; importantly, the authors claimed that such strategy promoted recovery of hindlimb weight-bearing locomotor function after such anatomically complete SCI model[374].

Similarly to PNS grafts, approaches based on direct delivery of fetal tissue have, to the best of our knowledge, also failed to result in successful clinical translation to date, partially owing to risk-benefit considerations for patients[375]; moreover, the difficulty in supply of fresh embryonic tissue unarguably represents a priori an important hurdle towards human applications[168].

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Stem cell approaches

As abovementioned, direct grafts of PNS tissue or its cell derivatives (Schwann cells) have failed to translate to clinics; transplant of whole CNS tissue of embryonic origin, on the other hand, in addition of being intrinsically limited by supply and ethical considerations, also are potentially associated to a higher risk of producing further damage to the spared host tissue compared to injectable cells[364]. Therefore, significant effort has and is actively being done towards regenerative treatments based on delivery of isolated (i.e. not whole tissue) embryonic or undifferentiated cells. In this respect, progress on the discovery of neural stem cells (NSCs) and progenitors cells (NPCs) found in the developing spinal cord, as well as on the techniques to culture such cell populations, was also crucial in promoting the shift from whole fetal grafts to approaches based on delivery of only NSCs/NPCs found in such grafts[376].

The field of cell transplantation for SCI is extremely vast, and detailed description of all approaches is of limited interest for the scope of this thesis, since our experimental work is based on manipulation of intrinsic neural tissue, and does not rely on use of any cellular graft. While we refer the reader to more extensive reviews[377], we here provide a summary of the most important studies, specifically focusing on stem cells or progenitor cells.

Critical to all types of cell grafts, including the whole-tissue CNS fetal grafts mentioned above, are survival of the cells and integration with host tissue; in addition, in the case of non-lineage restricted cells, differentiation is also a major challenge[355]. Cells can be delivered as suspensions in buffers[378] but, given the lower survival - as in the case of NSCs[379] –, they are often deployed in the SCI lesion after being seeded on a supportive matrix.

Multiple types of nervous system-associated cells have been employed in cell-based therapies including NSCs/NPCs, fate-restricted neural and glial precursors, and precursors derived from embryonic stem cells or from inducible pluripotent stem cells (iPSCs)[377,380]; in addition, terminally differentiated glial cells have also been considered, such as Schwann cells (as mentioned above from PNS grafts, or and also derived in vitro from progenitors[360]) and olfactory ensheating cells (OECs)[377].
Each of these cell populations presents advantages and contraindications: for instance, particular fetal- or embryonic-derived cells are associated with the same ethical and supply considerations as fetal CNS grafts[364]. On the contrary, iPSCs not only do not present this issue but, if autologously derived, also do not require immunosuppressive therapy[381]; nonetheless, because of their pluripotency, they are associated with a multitude of other risks such as potential tumorigenesis[364].
Moreover, additional populations of progenitor cells not directly related to neuroglial development have been used for SCI transplants. The most notably example is mesenchymal stem cells (MSCs): so-named because of their ability to differentiate, both in vitro and in vivo, in cells that developmentally originate from the mesenchyme (i.e. bone, fat cells and cartilage), MSCs are as a matter of fact *adult* multipotent progenitor cells found in the stroma and are therefore also referred to as "mesenchymal stromal cells"[382]. First isolated from bone marrow stroma, MSCs have later been isolated from connective tissue of umbilical cord[383] and virtually any organ of the body[384], although their properties of stem cells (i.e. self-renewal and differentiation) when derived from alternative tissue is disputed[382,385]. Importantly, MSCs can differentiate also in cells of non-mesodermal origin, including neuroglial cells, *in vitro*[382].

Besides being used in the attempt to promote host axon regeneration or more in general host-graft connectivity (e.g. by forming relays between host axons and graft-derived neurons), cell-based approaches have been employed for, and can result in promotion of, a variety of other mechanisms. These include[377]:

- Neuroprotection - many of the used cells are thought to dampen the effect of secondary damage that follows injury;
- Trophic support – multiple cell types can secrete trophic factors[386] and other molecules in vitro and enhance their presence when transplanted;
- Immunomodulation – Transplant of NSCs/NPCs and MSCs can alter cytokine levels and is associated with phenotype changes of inflammatory cells, although actual interactions of transplanted cells with immune cells have not yet been characterized;
- Angiogenesis – Alterations induced by transplanted cells, including released factors and metabolic changes, could potentially improve angiogenesis;
- Bridge formation – The graft itself constitutes a substrate that fills the lesion cavity bridging the lesion ends. Moreover, cells can deposit ECM molecules – e.g. laminin - that facilitate axon growth inside the lesion, and the effect can be enhanced by codelivery with growth factors and/or biomaterials;

- Remyelination – As mentioned above for the case of Schwann cells, or e.g. in the case of oligodendrocytes differentiated from the transplanted cells, the graft might positively influence myelination of host axons;
- Modulation of glial response – Cell transplants can for instance influence gliosis of host astrocytes;
- Active delivery of molecules – Graft cells can be modified before implantation to act as cargos for sustained delivery of molecules, including for instance neurotrophic factors {as described below}.

For what concerns specifically regeneration of host axons, effect of cells when transplanted in suspension in the absence of other factors is generally limited: for instance, grafting of MSCs as suspension in contusion SCI sites does not promote ingrowth of host axons in the graft[387]; transplant of embryonic stem cells (ESCs) as suspensions of embryoid bodies in contusion SCI injuries produces some limited functional recovery but survival of the graft is comparatively low[388,389]; delivery of a suspension of clonal NSCs in a dorsal hemisection SCI model promotes partial ingrowth of host axons in the lesion[386], but suspension of embryonic-derived NSCs in complete transection SCI sites even fails to result in filling of the lesion, and graft survival is limited[379]. Cell delivery on its own is particularly limited by the fact that the cells tend to be washed away, e.g. by the cerebrospinal fluid (CSF)[390], and therefore greatly benefits from embedding in gel matrices[364].
Moreover, as observed with whole tissue-based grafts mentioned above, also in the case of cell-based strategies co-delivery with trophic or growth factors enhances impact of the approach: neurotrophic factors promote survival of the grafted cells[388] – and a potential increased survival could underlie also the improvement observed in whole tissue-grafts when co-delivered with such molecules; in addition, the selection of molecules and their dose also steers differentiation of the grafted cells towards specific cell types (e.g. neurons, astrocytes, oligodendrocytes)[391]. On the other hand, additional delivery of trophic factors with grafted cells alone does not necessarily increase regeneration of host axons into the lesion: for instance, supplementing MSCs with NT-3 does not significantly increase regeneration of sensory axons in a dorsal column transection SCI site compared to MSCs alone, but regeneration rather requires provision of additional factors (conditioning lesion, chemoattraction)[392].
In general, combinatorial strategies greatly overcome single-component strategies in the case of cell-based approaches[364]; moreover, as discussed below and multiple times in this thesis, they are particularly crucial for successful regeneration of host axons across the lesion (and this is valid both for cell-based and non-cell-based regenerative approaches).

NSCs/NPCs

Particularly successful in terms of functional outcome and translational potential have been strategies employing NSCs/NPCs. In particular, Tuszynski's group developed an approach based on delivery of embryonic-derived NSCs dispersed in a fibrin matrix filled with a cocktail of multiple growth factors including neurotrophic factors: application, 2 weeks post injury, of such graft in a site of a complete transection thoracic SCI resulted in successful bridging of the lesion, via survival and integration of the graft[379]; partial recovery of hindlimb locomotion – after such complete injury – was

observed, although with lack of weight support[379]. Grafted cells differentiate extensively in glial cells and – contrarily to the more limited differentiation achieved with other strategies[389] – also in a significant proportion of neuronal cells (~27.5% of total surviving cells)[379]. Importantly, this strategy does promote regeneration of host axons in the graft, including propriospinal and serotonergic fibers[379] and even CST neurons[168], but host axons stop mostly within the most rostral portion of the graft and do not grow across it and in the caudal tissue except in the case of milder focal lesions[168,379]; on the contrary, graft-derived neurons extend extensively for several centimeters invading the host tissue both caudal and rostral to the lesion along several spinal levels (**Figure 1.14**), being myelinated by host oligodendrocytes and forming synapses with host neurons[379].
Host axons integrate within the graft tissue relaying functional signals and recruiting graft neurons in a pattern that resembles the anatomical organization and activation pattern observed in the uninjured spinal cord[393], a finding that is not trivial as other stem-cell based approaches showed potentially negative effects of regenerated fibers on function[394].
The approach was repeated with similar results also with stem cells of human origin, including embryo-isolated stem cells, stem cell lines and skin-derived iPSCs directed towards neural fate[168,379]; importantly, cells need to be either derived from spinal cord tissue or directed specifically towards caudal fate, as cell driven towards more rostral fate or isolated from embryonic brain tissue fail to produce comparable regeneration[168].

Finally, the strategy was adapted and successfully translated also to non-human primate models, resulting in predominant differentiation of grafted cells to neurons, regeneration of hundreds of thousands of axons for up to 5 cm from the graft including in host white matter and indications of improvement of forelimb function when implanted in a cervical hemisection SCI site[390].

We would like to draw the attention once more on the fact that the (limited) recovery of hindlimb locomotory function observed with the abovementioned approach relies on elongation of *graft-derived* axons, and on the formation of a relay circuit between host neural tissue regions rostral and caudal to the lesion via the outgrowing graft neurons. Graft-derived axonal outgrowth had been reported also in some of the whole tissue-grafts mentioned above[371], although the strategy developed by Tuszynski's group undoubtedly resulted in unprecedented degree of graft-derived axon growth, and the circuit reorganization allowing for graft-to-host and host-to-graft connectivity was in this case – contrarily to the other studies – systematically characterized. We nonetheless feel that this clarification is important, since the abovementioned approach does not result in regeneration of host axons, particularly from propriospinal interneurons, across an anatomically complete SCI, which was the scope of the experimental work presented in this thesis.

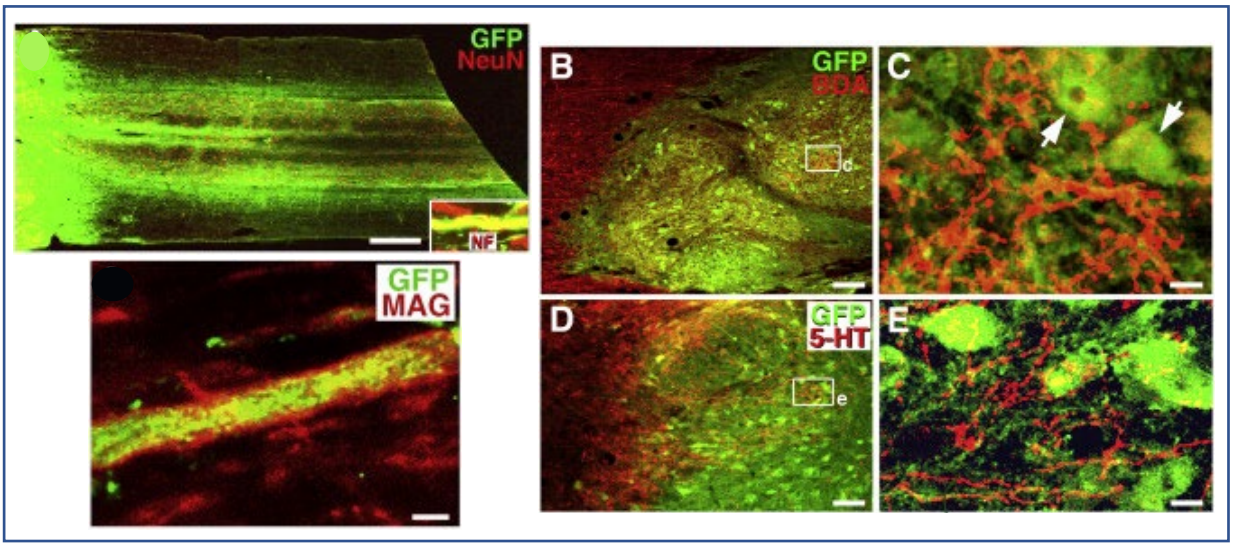

Figure 1.14. ***NSCs grafts can promotes extensive graft-derived axon growth and integration in host tissue, but regeneration of host axons is limited.*** Left. *Fibers derived from the NSCs (dispersed in fibrin matrix with a cocktail of growth factors) grafted in a T3 complete transection SCI site extensively invade the host rat spinal cord even reaching the end of the block of tissue (6 mm caudal to the lesion), and are often myelinated by host oligodendrocytes (as shown with immunoreactivity against myelin-associated glycoprotein, MAG). NSCs were engineered to express GFP, and identity of extending fibers as axons is confirmed by staining for neurofilament (NF). The neuronal marker NeuN shows penetration of graft-derived axons in both host white and grey matter.*
Right. *Host axons, including reticulospinal axons (traced with BDA, B,C) and serotonergic fibers (expressing 5-HT, D,E) regenerate inside the graft being in close-apposition with GFP-positive graft-derived cells with neuronal morphology, but typically stop in the rostral part of the graft and fail to regrow across the lesion. Reproduced with permission from Lu et al., 2012*[379].

MSCs

Other approaches relevant to axon regeneration have made use of MSCs. As a key difference with NPCs/NSCs, it is hypothesized that MSCs do not exhibit neuronal differentiation when transplanted[364]; for this reason, unlike NPCs and NSCs, these cells have not been used to promote graft axonal outgrowth, but rather to provide a supportive environment to facilitate host axon regrowth[364]. While, as discussed previously, MSCs grafts alone fail to encourage axon regeneration[387], multiple combinatory approaches used over the years have resulted in variable degree of axon regeneration. In particular, earlier work from Tuszynski's group focused on regeneration of sensory axons and showed that combining delivery of autologous MSCs in a dorsal column transection SCI site with pre-stimulation of the soma (cAMP injection or preconditioning lesion) and neurotrophins (delivery of NT-3 – acting as a chemoattractant for sensory axons[395] – rostral to the lesion) promotes regeneration of such fibers inside and across the lesion[169,396,397]. Nonetheless, long-distance axon growth is limited, as axons stop within the first 2mm rostral to the injury with small density[169]; moving the lesion in the upper cervical area closer to the natural targets of the fibers (nucleus gracilis) allows successful target reinnervation, but still fails to result in detectable synaptic activity, a finding that the authors ascribe to a lack of myelination in regenerating fibers[397]. These and other results from the studies point to the importance of a multi-factorial strategy to promote regeneration, with each component underlying different mechanisms: soma stimulation seems to be crucial for axon elongation, consistent with its role in activating

RAGs (as discussed in **Regenerative competence: PNS vs CNS**), as simple delivery of NT-3 and MSCs does not promote regrowth further than 1 mm past the lesion even when a sustained gradient of NT-3 over multiple spinal segments is delivered[169]; NT-3 seems to act as a signal for both guidance and growth termination[169,397], while the supportive and trophic properties of MSCs have been described above; the importance of remyelination of regenerated axons for recovery is in agreement with later findings by other groups[398]. We will discuss further in detail on the necessity of combining multiple interventions to promote regeneration in **Regeneration after complete SCI**.

Differentiated cells other than the ones described above have also been extensively used in the field, for instance for growth factors delivery, including fibroblasts, as discussed below.

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To summarize, cellular transplants, including whole tissue-based and cell-based, have been extensively used in the SCI research field. While most strategies have failed to be repeated and translated to higher models, promising results in terms of integration and potentially functional recovery have been obtained with specific stem-cell based strategies, for which clinical trials have been envisioned. Nonetheless, impact of the abovementioned approaches on regeneration of host axons across the lesion, which is the main focus of this thesis, appears to be limited.

Neurotrophic factors

As discussed in the previous chapter, based on observations from the PNS and on their critical role in survival and guidance during development, neurotrophic factors have been naturally considered as appealing candidates in the search for SCI treatments[364].

Providing a general statement on the effect of neurotrophic factors in SCI treatment is of limited interest, since different factors induce distinct effects[364], and expression of the respective receptors on host CNS cells is varied[188]. Nonetheless, direct delivery of neurotrophic factors, or even via gelfoams, has usually been associated with limited results due to the finite amount and short-lasting release of the molecules[364]. Strategies allowing for sustained delivery are on the other hand also associated with potential issues: osmotic micropumps can increase local tissue damage due to invasiveness, while cell-based delivery is usually associated with a high local concentration of the factors that can prevent axon outgrowth in case of molecules acting as termination signals[169,364].

For what concerns *regeneration*, neurotrophic factors, when delivered alone, have usually been described as insufficient in promoting axonal regrowth, although different reports presented contrasting results: for instance, sustained delivery of NT-3 in a complete transection SCI site via osmotic pumps does not result in regrowth of supraspinal axons across the lesion[374]; likewise, fibroblast-mediated overexpression of NT-3 in dorsal column transection SCI site only mediates modest ingrowth of sensory

axons in the graft and complete lack of penetration of CST axons[167]; on the contrary, another study reports extensive regeneration of sensory axons through and even across the SCI site in a model of dorsal column crush via sustained micropump NT-3 delivery[395]. Why a simpler manipulation (NT-3 delivery only vs NT-3 delivery together with potential substrate support from fibroblasts) promotes significantly higher regeneration of the same tract is not completely clear, but a potential factor might be for instance the different modality of injury (crush vs transection); alternatively, while sensory axons might invade and produce sprouting inside the lesion, regeneration of such fibers rostral to the injury might have actually been confounded with regrowth of other fibers around the lesion rather than of the same fibers all the way through the graft, in agreement with the observation that NT-3 diffusion can promote regrowth of CST axons around the lesion without axons passing through it[167].

Regardless of this discrepancy, strategies based on neurotrophic factors have been traditionally been most successful in promoting regeneration across incomplete SCI when combined with other mechanisms, including soma stimulation and grafts, as discussed above in the case of MSC grafts. Approaches based on delivery of neurotrophic factors via genetically modified fibroblasts have also been extensively used[167,364,399,400].
The neurotropic effects of neurotrophic factors, promoting chemoattraction and regeneration of lesioned axons, display subtype-specific variability, as different factors preferentially target distinct axonal tracts {see also **Subtype specificity**}[188,401].

Consistent with their role in development, delivery of neurotrophic factors has also been reported to positively influence *survival* of host neurons even in cases where no effect on regeneration was observed[399]. For instance, BDNF prevents cell loss of rubrospinal neurons after injury in neonatal rodents, as well as atrophy associated with adult injury[188].

Finally, neurotrophic factors can exhibit extensive interactions with molecular mechanisms and pathways regulating intrinsic neuronal growth: BDNF expression, for instance, is found to be upregulated after conditioning injury[188]; moreover, downstream neurotrophin signaling includes activation of some pro-regenerative pathways including PI3K-Akt-mTOR[188], which might underlie similar observation of promotion of CST sprouting with NT-3 delivery[167] and mTOR activation (PTEN deletion)[283] {see also below}.

To summarize, delivery of neurotrophic factors, especially in combination with other interventions, has been extensively used to promote regeneration after CNS injury, with variable extent of success. The multifunctional and powerful nature of such molecules, together with their diversified effect on distinct neuronal populations, however warrant caution in their therapeutic application: experimental studies, for instance, show that untargeted overexpression of neurotrophins can promote formation of inappropriate axonal connections and yield side effects including severe hyperalgesia[364,402].

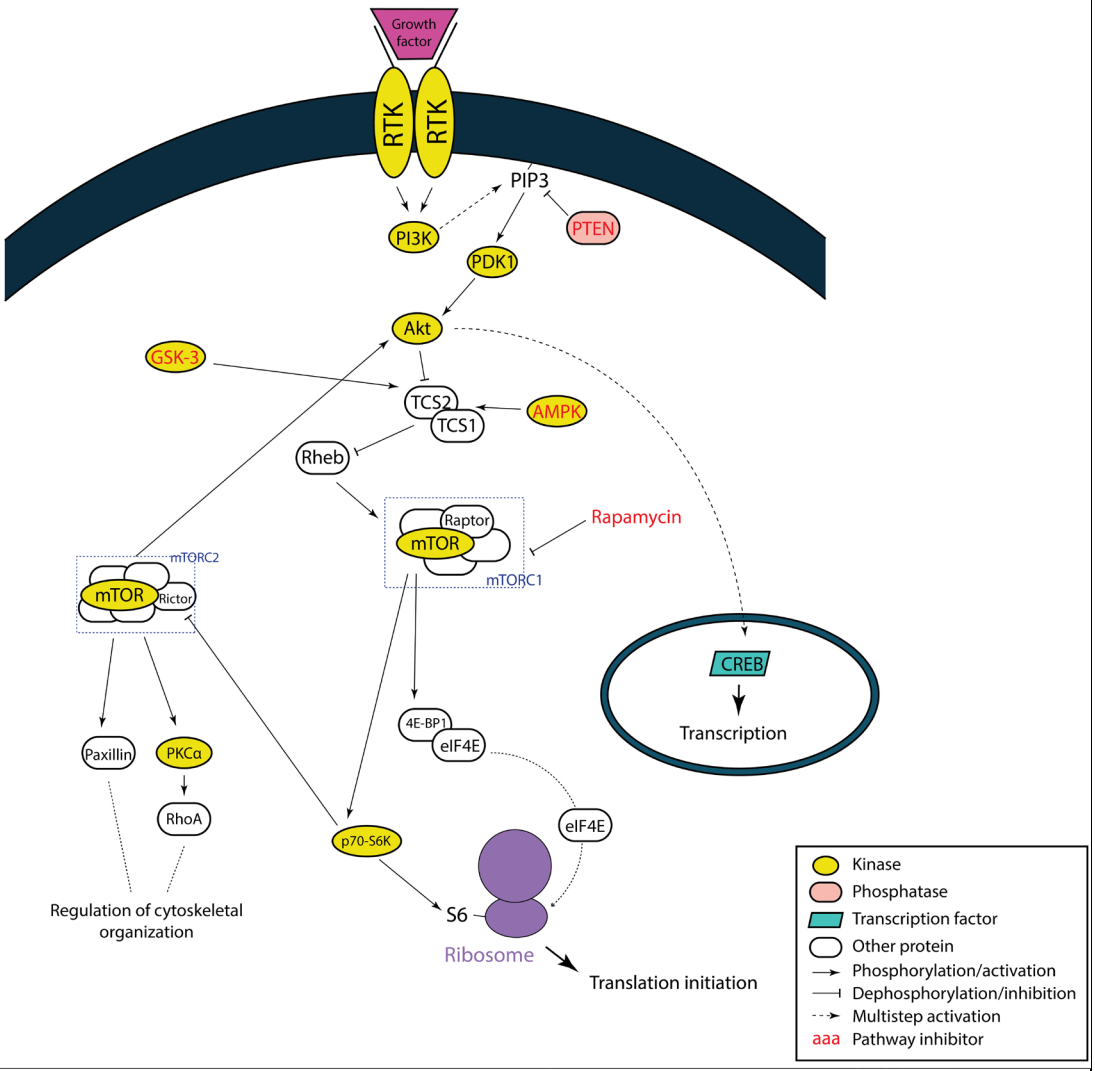
Intrinsic growth capacity

In the previous chapter, we extensively discussed the marked difference in intrinsic growth ability between the adult CNS and PNS, and how PNS injury triggers activation of molecular pathways and downstream transcription of regeneration-associated genes that is not observed after CNS injury. It was nonetheless until 15 years ago that the field of axon regeneration, also pushed by the limited results achieved by interventions targeting inhibitory molecules, started to shift towards trying to re-activate pro-regenerative molecular pathways and intrinsic neuronal growth capacity[178]. We here proceed to discuss the main signaling pathways or regulators that have been found to critically modulate CNS axonal regrowth, and the main studies that targeted such molecular players to promote regeneration.

Manipulation of molecular pathways: mTOR and JAK-STAT

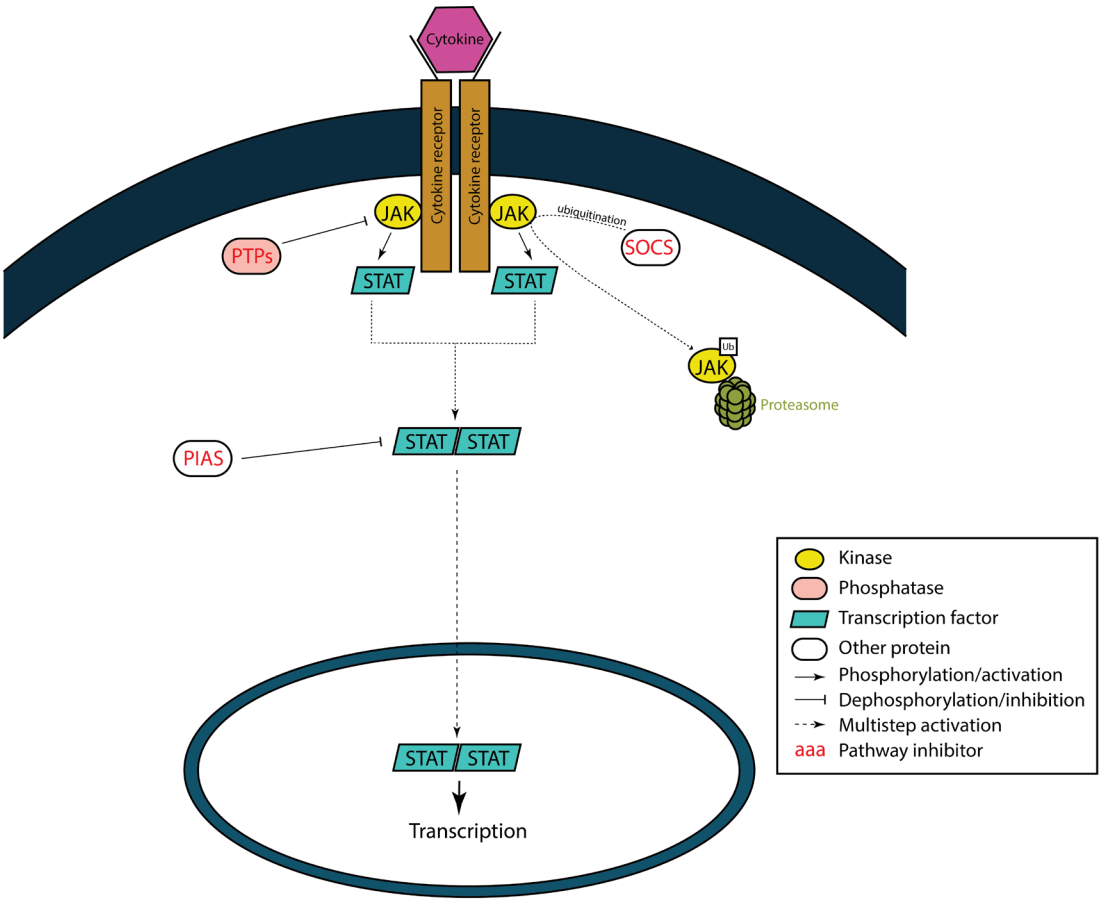
Overview of the pathways

Crucial regulator of cell cycle and aberrantly activated in multiple types of cancers, the Phosphoinositide-3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway is activated by a multitude of extracellular signals, including growth factors (e.g. IGF-1) and insulin[403], accordingly transduced mostly via the respective receptor tyrosine kinases (RTKs, e.g. IGF-1R and IR)[404].
mTOR, in particular, represents a core component of two protein complexes, mTORC1 (of which another important component is the protein Raptor) and mTORC2 (including also the protein Rictor)[405]. The PI3K/Akt/mTOR pathway is physiologically antagonized by several proteins, including Phosphatase and tensin homolog (PTEN) – that exerts its action upstream of Akt and mTOR -, Glycogen synthase kinase-3 beta (GSK-3β) and rapamycin (from which mTOR takes its name)[403].
Involved in various other functions including metabolic control (via insulin and leptin signaling) and modulation of actin cytoskeleton (via the mTORC2 complex)[406], mTOR critically governs cell growth by regulating protein translation; in this respect, two main downstream targets of the mTOR kinase are: p70-S6K, another kinase which in its turn phosphorylates ribosomal protein S6, inducing protein synthesis at the ribosome – pS6 is also highly used as an histological marker of mTOR activation[407]; 4E-BP1, a protein part of the eukaryotic complex of proteins required for protein translation, leading to translation initiation[405].
Besides PI3K/Akt, mTOR activity is also modulated by other signaling pathways including 5'AMP-activated protein kinase (AMPK), that senses changes in intracellular ATP levels and decreases mTOR activity (i.e. protein synthesis) when such levels are low[403].



Involved in processes including immunity, cell division and cancer, the JAK-STAT pathway is activated mainly by cytokines, but also by specific neurotrophic factors[408]. Accordingly, the signaling is transduced via three components: (1) a cytokine receptor, that lacks direct kinase activity and is therefore associated to (2) Janus kinases (JAKs), non-receptor tyrosine kinases bound to the intracellular portion of the receptor and that in their turn exert their activity by phosphorylating (3) signal transducer and activator of transcription proteins (STAT) proteins, intracellular transcription factors[408]. Cytokine receptors are typically protein complexes, with receptors often sharing a common subunit and having subunits unique to each ligand as well: the neurotrophic factors CNTF, IL-6 and LIF, for instance, all share the common subunit glycoprotein 130 (gp130)/IL6ST, with the complex including also CNTFR, LIFR and IL6R+LIFR, respectively[409]. Ligand binding causes dimerization and phosphorylation of the receptor by JAKs, with then STATs binding to the phosphorylated tyrosine residues on the receptors and being phosphorylated in their turn; activated STATs then translocate to the nucleus where, after multiple steps, promote transcription of specific genes[408]. JAK/STAT signaling is regulated by multiple antagonists, including Protein inhibitors of activated STATs (PIAS), Protein tyrosine phosphatases (PTPs) – that antagonize JAK activity - , and Suppressors of cytokine signaling (SOCS); these latter family is comprised of 8 members including SOCS3, and exert their action in multiple ways including ubiquitination of JAKs or receptors[408].

Other pathways including PI3K/Akt/mTOR and MAPK/ERK can also be activated by cytokine receptors phosphorylated by JAKs[410].



Regeneration after optic nerve injury

The potential of manipulating molecular growth programs to revert CNS growth incapacity started to be uncovered in the last years of the first decade of 2000, particularly following studies by Zhigang He's and colleagues. In 2008, the group published a study showing that manipulating the mTOR pathway by deleting PTEN in retinal ganglion cells (RGCs) 2 weeks before a complete optic nerve crush significantly increases RGC survival and, importantly, promotes extensive regeneration of RGC axons across the injury, in contrast with the complete lack of regeneration in control animals[407]; importantly, the study also showed that injury rapidly downregulates mTOR signaling in neurons after injury, significantly decreasing the protein synthesis capabilities

necessary to build a regenerative response[407], therefore identifying a first mechanism for intrinsic regenerative failure, which can be rescued via PTEN deletion[†].

Similarly, the JAK-STAT pathway is another crucial regulator of intrinsic axon growth. Already at the beginning of 2000s, it was known that either performing a lens injury or delivering intravitreally agents that provoke sterile inflammation could promote RGC survival and axon regeneration past an optic nerve injury – a process known as inflammatory stimulation (IS)[411,412]. In 2007, a study by Dietmar Fischer's group showed that IS-mediated regeneration is dependent on JAK-STAT activation, and causes release of CNTF by retinal astrocytes[413].
Two years later, another study by He and colleagues uncovered the intrinsic mechanisms rescued by JAK-STAT induction: the group performed activation of the pathway via deletion of SOCS3 in RGCs 2 weeks before optic nerve crush, and showed that this strategy also significantly increases RGC survival compared to control animals and promotes extensive regeneration across the injury site[223]. They identified, as a possible cause for intrinsic growth incapacity of injured neurons, a limited responsiveness of neurons to injury-triggered factors, including growth factors – potentially explained in view of a CNS defense mechanism to prevent over-inflammation: an increased production of CNTF in the retina is observed after optic nerve injury, and SOCS3 deletion-associated regeneration is abolished if the cytokine receptor gp130 is also deleted[223]. SOCS3 deletion was therefore thought to allow RGCs to become more responsive to cytokines, and accordingly, exogeneous delivery of CNTF to SOCS3-deleted mice further increased the observed regeneration[223]. These results are consistent with previous and later studies in the field only showing more limited effects on regeneration when exogeneous cytokines were delivered to wild-type injured mice[223,414], confirming the role of JAK-STAT activation in determining neuronal responsiveness to injury-induced cytokines.

The two pathways identified as mediators of axon growth show some degree of overlap, as SOCS3 deletion increases mTOR activity after injury but not basally before injury[223]. Nonetheless, they also act independently, and they can exert a synergistic action on regeneration, as co-deletion of SOCS3 and PTEN, compared to either deletion alone, was later shown to augment RGC survival and dramatically increase axon regeneration following optic nerve crush – with axons in double mutants reaching the optic chiasm[227]. In addition, such combinatory intervention, when applied to a distal optic nerve injury, also successfully allowed regeneration of RGC axons to their natural target, the superior colliculus, and formation of functional synapses, a non-trivial finding considering that target reinnervation is a crucial requirement in axon regeneration research; nonetheless, integration of regenerated transected axons in their natural target was not associated with functional recovery, and the identified reason was poor myelination of regenerating fibers, causing impaired electrical conduction[398]. This is consistent with previous reports on stem-cells based target reinnervation approaches[397], and points to improvement of conduction in de-myelinated axons as another potentially important requirement to consider in strategies promoting regeneration-based restoration of function; delivery of

[†] As a note, PTEN activity regulates various downstream targets besides mTOR, therefore the effects observed following PTEN deletion are likely to depend upon concomitant activation of mTOR and other signals/pathways; accordingly, regeneration observed in the same study following deletion of TSC1 (a protein more downstream in the PI3K/Akt/mTOR pathway compared to PTEN) results in more limited regeneration[407].

agents including potassium channel blockers - already employed in clinical research - showed promising results in this direction[398].

Activation of the mTOR and JAK-STAT pathways after optic nerve injury was also successfully achieved via interventions more clinically applicable than genetic deletion: delivery of the phosphoprotein osteopontin (OPN) and the growth factor IGF-1, but not of either of them alone, promotes regeneration to an extent comparable to that of PTEN deletion, with OPN upregulating mTOR signaling and IGF-1 likely acting through mTOR-independent pathways[415] (potentially including JAK-STAT signaling, known to be activated by IGF-1[416]); accordingly, codelivery of OPN, IGF-1 and CNTF results in RGC axon regeneration comparable to what achieved in PTEN/SOCS3 double mutants with exogenous CNTF treatment[398], therefore successfully recapitulating simultaneous activation of mTOR, JAK-STAT and any additional pathways recruited by PTEN deletion. *We would like to bring a particular focus on this intervention, as the experimental work presented in this thesis also relies on OPN/IGF-1/CNTF co-delivery as one of the interventions used in our regenerative strategies.*

Regeneration after spinal cord injury

The effects of targeting mTOR and JAK-STAT pathways were also investigated by He's group and others in models of SCI or pyramidotomy.
mTOR activity, for instance, was shown to be downregulated after development in CST axons, in correlation with the loss of growth capacity in adulthood[283]; restoration of mTOR activity via PTEN deletion neonatally (at postnatal day 1 (P1)) promotes extensive sprouting of CST axons rostral to an incomplete SCI site[283]. Importantly, PTEN deletion performed either at P1 or at 4 weeks of age also promotes regeneration of CST axons across an anatomically complete crush SCI, with the regenerative effect being stronger with neonatal deletion[283]. Similar results are obtained if PTEN depletion is performed via RNA interference[417]. In spite of the regeneration, in neither of the two studies there is report of functional improvement[417].
These two studies are of particular relevance for the background of this thesis, as they show regeneration across and caudal to an anatomically complete injury. As more extensively discussed in **Regeneration after complete SCI**, the authors themselves clearly highlight how their injury model, while successfully satisfying the requirement for anatomically complete SCI of total interruption of axons between the two sides of the lesion, is systematically associated with re-formation of strands of glia across the lesion at later post-injury timepoints; accordingly, regenerating axons preferentially regrow along these strands, failing on the contrary to penetrate the lesion if a more severe model with no glial bridging is used[417]. This is in contrast with the injury model used in the experiments presented in this thesis, where a complete interruption of GFAP signal is observed in the lesion core even 8 weeks after injury.
Similar to PTEN deletion, also co-delivery of OPN and IGF1 promotes CST sprouting and regeneration across an incomplete SCI, even when delivered after injury, with mTOR activity in CST neurons being increased only when the two molecules are delivered together[418]; OPN, in particular, seems to be required to make CST neurons more sensitive to IGF-1, as activation of IGF receptor beta is also only increased when both OPN and IGF-1 are delivered[418].

JAK-STAT activation was also shown to promote compensatory CST sprouting caudal to an unilateral pyramidotomy either via P1 SOCS3 deletion or CNTF delivery; the effect on sprouting is increased when the two interventions are combined, or when co-deletion of SOCS3 and PTEN is performed[224].
Importantly, in another study by Fischer and colleagues from only two years ago, overactivation of the JAK-STAT pathway, via delivery of hyper-IL-6 (h-IL6, a fusion protein consisting of the cytokine IL-6 coupled with its specific receptor subunit IL6R) in the motor cortex, also resulted in regrowth of CST and serotonergic fibers across and past an anatomically complete SCI[419]; in addition to this, treated mice were reported to recover hindlimb function with weight-bearing support, an outstanding achievement in consideration of the complete injury[419]. Coupling hIL6 with neonatal PTEN deletion further increased both regeneration and extent of recovery; interestingly, PTEN deletion alone, resulting in no functional recovery, promoted regeneration of CST axons (consistently with ref. 283) but not of serotonergic axons, highlighting a crucial role of 5-HT axon regeneration for the observed recovery in fully treated mice.
As further discussed in **Regeneration after complete SCI**, it is important to specify that this study employed the same model of complete crush SCI as the abovementioned studies of mTOR-associated regeneration in complete injuries, with complete interruption of axonal tracts but presence of glial bridges across the lesion at chronic timepoints. We therefore hypothesize that this same therapeutic approach might not be sufficient on its own to promote regeneration in an even more severe injury model with no glial bridging.

Targeting of JAK-STAT and mTOR pathways has also been combined to other interventions, with variable results.
For instance, as mentioned above, co-deletion of PTEN and Nogo, in contrast with the established effect of Nogo modulation on sprouting and its limited impact on regeneration, does not promote increased CST sprouting compared to PTEN deletion only, while on the other hand unexpectedly augmenting the regenerative impact achieved via PTEN deletion[334].
Overexpression of the master transcription factor c-myc, when combined with PTEN and SOCS3 co-deletion, increases even more RGC survival and axon regeneration across an optic nerve crush lesion compared to co-deletion only, as regenerating axons cross the optic chiasm reaching the optic tract[420]; c-myc is thought to exert its effect on regeneration by either contributing to a shift towards anabolic metabolism or by increasing general gene expression via binding to promoters of active genes[420]. Nonetheless, in mice receiving the triple treatment, among the axons regenerating for such impressive distance, some fibers also ectopically grow back in the contralateral optic nerve or derail from the optic tract[420]. The observation of such aberrant regeneration is also relevant to the scope of the thesis, as it shows how other mechanisms, including proper chemoattraction of elongating axons towards intended target regions, is required for a successful regenerative intervention aiming at restoration of function; this, furthermore, highlights once more the importance of combining interventions targeting multiple mechanisms at the same time.

Other pathways

Activation of pathways other than mTOR and JAK-STAT has also been investigated and found to promote axon regeneration. A notable example is the MAPK/ERK pathway: forced overexpression of the MAP3K B-RAF promotes RGC axon regeneration across an optic nerve injury that is comparable in maximal length and even higher in density than what achieved with PTEN deletion[421]; B-RAF overexpression combined to PTEN deletion results in an even greater effect on axon regeneration compared to either intervention alone, suggesting a synergistic and partially independent effect and highlighting MAPK/ERK (specifically RAF-MEK) signaling as another crucial intrinsic regulator of axon growth[421].

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Targeting of transcription factors

In addition to manipulation of molecular pathways, direct modulation of downstream targets including transcription factors influencing expression of regeneration associated genes has been explored.

Members of the family of zinc-finger transcription factors Krüppel-like factors (KLFs), for instance, were found to have either growth-promoting (KLF-6, KLF-7) or growth-inhibitory (KLF-4, KLF-9) effects in vitro[422]; screening of their in vivo expression in RGCs around the end of development period highlighted a decline in expression of pro-regenerative KLFs and upregulation of inhibitory KFLs postnatally, suggesting a potential involvement of such transcription factors in restricting adult CNS regenerative capacity[422]. Accordingly, pre-natal knockout of KLF-4 in RGCs was found to promote axon regeneration past an optic nerve injury, although limited with respect to comparable studies with other interventions (e.g. PTEN deletion)[422]. Similarly, viral overexpression of KLF-7 in the motor cortex results in CST sprouting above and regeneration past an incomplete SCI site[423].
KLF-4, in particular, is a transcription factor with a crucial role in controlling activity of neural stem cells, and is one of the four Yamanaka factors used to derive induced-pluripotent stem cells (iPSCs) from somatic cells[424]; its detrimental role on CNS regenerative capacity seems to depend on its binding to phosphorylated STAT3, which results in the exertion of an inhibitory effect on JAK-STAT signaling[425].

Another relevant example includes the transcription factor Sox11: while being highly expressed in growth-competent PNS after injury and embryonic CNS neurons during growth, Sox11 is not upregulated after CNS injury[426]; overexpression of Sox11 via viral delivery in the motor cortex promotes CST compensatory sprouting following a pyramidotomy and regeneration past a dorsal hemisection SCI[426]. Nonetheless, the observed regeneration in treated mice is associated with impaired recovery of hindlimb function compared to control mice[426]; as already highlighted above, this confirms the importance of combinatorial strategies that also include provision of guidance cues to regenerating axons, and warns about risks associated with uncontrolled regeneration and maladaptive plasticity.

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In conclusion, modulation, at multiple levels, of pathways associated with transcription of regeneration-associated genes has resulted in successful promotion of axon regeneration after multiple models of CNS injury, and has been shown to be more robust than interventions targeted at other mechanisms, especially manipulation of growth inhibitors. Nonetheless, combinatorial approaches integrating activation of growth programs with other strategies can still result in improved regenerative response, and might be particularly necessary in the case of more severe injuries.

Other approaches

In addition to the experiments described above, which represent the most explored strategies in the field of CNS axon regeneration, alternative approaches have also been investigated. These include interventions targeted to virtually any of the mechanisms underlying difference in PNS vs CNS growth capacity discussed in the **previous chapter**, as well as more sophisticated studies that integrate knowledge from other fields such as chemistry and engineering.

Epigenetic manipulations

As mentioned before, the presence of an "epigenetic barrier" might at least partially underlie the different regenerative capacity of injured PNS and CNS neurons, with promoters of specific RAGs being less accessible for transcription in CNS neurons due to epigenetic modifications. Accordingly, some interventions have been tested in the recent years in the attempt to translate some characteristics of the PNS epigenome to injured CNS neurons.

Histone acetylation, for instance, promotes gene transcription, and is mediated by HATs and negatively regulated by HDACs. Di Giovanni and colleagues tested the effect of manipulation of HATs after CNS injury: the group showed that viral overexpression at the same time of an optic nerve crush of the HAT p300, found to be downregulated in injured RGCs, promotes regeneration of RGC axons, although limited to 0.5mm past the lesion site, via acetylation of RAG promoters[427]; likewise, pharmacological activation of the HATs Cbp/p300 promotes regeneration of sensory axons across a dorsal hemisection SCI and sprouting after contusion SCI, with associated functional recovery[150]. Targeting of HDAC activity was also found to promote beneficial effects: pharmacological inhibition of HDACs promoted regeneration of sensory axons inside a dorsal column SCI via RAG induction, with acetylation levels of histone 4 being correlated with axon growth capacity[428].

Similarly, DNA methylation patterns can also influence gene transcription, and important modulators are Ten-eleven translocation methylcytosine dioxygenases (Tets) – including Tet1, Tet2 and Tet3 –, the activity of which causes DNA downstream demethylation. Interventions aimed at modifying DNA methylation were also successful in promoting CNS axon regeneration: for instance, delivery of the three Yamanaka factors Oct-4, Sox-2 and Klf-4 (OSK) promotes regeneration of RGC axons across an optic nerve injury by resetting DNA methylation; OSK was found to promote increased

expression of Tet1 and Tet2, and knockout of Tet1 and Tet2 abolished the OSK-dependent regenerative effect[260].

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Cytoskeletal dynamics

In the previous chapter we discussed how cytoskeletal dynamics greatly influence the competence of injured axons to form a growth cone and mount a regenerative response to injury. We mentioned, in particular, that activation of the small GTPase RhoA results, via downstream signaling, in actin polymerization, which is detrimental to the dynamics required for axon regeneration. Accordingly, after PNS injury RhoA is inactivated via cAMP-dependent mechanisms; on the contrary, the small GTPase is thought to be a downstream target of signaling from putative CNS inhibitory molecules such as Nogo, CSPGs and MAG, which therefore exert their inhibitory action by limiting cytoskeletal dynamics (increasing actin density and hindering microtubule protrusion[429]) and causing growth cone collapse. Proposed signaling downstream of Rho-A includes activation of Rho kinase (ROCK), which in its turn inactivates ADF/cofilin, a family of proteins involved in actin turnover via severing of old actin[429,430] (**Figure 1.15**).

Multiple studies have therefore tried to modulate Rho signaling to promote CNS regeneration. For instance, work from the beginning of 2000s by McKerracher's group showed that selective RhoA inactivation via the C3 enzyme from *C. botulinum* promotes regeneration of RGC axons across an optic nerve crush (for a limited distance, ~500 µm)[431] and labeling of CST axons for up to 10 mm caudal to a dorsal overhemisection SCI in rats, with associated increased recovery[432]. Such promising results led to development of clinical trials aimed at Rho inhibition, that nonetheless were prematurely suspended because of lack of efficacy[433].

Nonetheless, a recent study by Bradke's group points to cell-type specificity in the role of RhoA: in particular, while – as abovementioned - RhoA deletion or inactivation in neurons promotes regeneration via modulation of cytoskeletal dynamics, RhoA seems to have a critical role in restricting reactivity in astrocytes, as astrocyte-restricted RhoA deletion in vitro promotes even higher astrocytic production of CSPGs and GFAP expression[429]. Accordingly, astrocyte-restricted deletion of RhoA has a growth-inhibitory effect in vivo, with sensory neurons failing to regenerate across a dorsal hemisection SCI and on the contrary displaying even more pronounced retraction than control injured mice; likewise, deletion of RhoA in both neurons and astrocytes also fails to promote regeneration of sensory axons as instead observed with neuron-restricted deletion[429]. This suggests that therapies more directed at inhibiting RhoA signaling specifically in neurons could still hold potential for translation in clinics.

Additionally, interventions targeted at molecules more downstream in RhoA signaling have also been shown to have a positive effect on axon regeneration: another study from Bradke and colleagues, for instance, showed that the ADF/cofilin are required for axon regeneration both in the PNS and in the CNS, as knockout of all three members of the protein family prevents regeneration both of DRG neurons after sciatic nerve injury and of sensory axons pre-stimulated via a conditioning lesion following a dorsal hemisection SCI[430]; on the other hand, AAV-mediated overexpression of cofilin-1 is sufficient to

promote regeneration of non-conditioned sensory axons across a dorsal hemisection SCI[430].

As mentioned in the previous chapter, strategies aimed at controlling cytoskeletal dynamics by targeting tubulin have also been partially successful in promoting axon regeneration after incomplete models of SCI: stimulating microtubule bundling via delivery of pharmacological agents such as taxol and epothilone B, for instance, increases labeling of serotonergic fibers caudal either a dorsal hemisection or moderate contusion lesion[173,174].

In general, modulation of cytoskeletal properties is a crucial requirement for successful axon regeneration, with members of the doublecortin (DCX) family - including DCX, DCLK1 and DCLK2 – identified as fundamental regulators. DCX proteins contain both a microtubule-binding domain and a region that binds actin, and therefore regulate and rearrange both cytoskeletal components, controlling axon growth during development and after injury: deletion of DLCK1/2 abolishes PTEN-deletion-mediated regeneration following optic nerve injury and PNS regeneration after sciatic nerve crush[434]; on the contrary, DLCK2 overexpression combined with PTEN deletion greatly enhances RGC survival and regeneration, promotes growth cone formation in vitro and prevents axotomy-dependent actin collapse[434].

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Energy requirements/mitochondrial transport

Metabolism and energy requirements are also crucial factors to consider to achieve successful axon regeneration. Regrowth of injured axons requires de novo synthesis and organization of cellular material, with the whole process being highly energy-demanding[170]. Accordingly, adult neurons after injury need to revert from homeostatic to anabolic metabolism to adjust to such needs[178].

We have already mentioned above how some of the targets of pro-regenerative interventions are on their own already critical regulators of anabolic metabolism, such as for instance mTOR, that promotes protein translation initiation, or c-Myc, a known regulator of ribosome biogenesis and nucleic acid/lipid synthesis[178,420].

Other interventions have been tested to enhance energy metabolism: delivery of high dose of creatine, known to promote generation of ATP from ADP, has been shown, for instance, to be sufficient to promote some limited regeneration of CST axons caudal to a dorsal hemisection SCI[435].

Similarly, readiness of energy release is also important, therefore localization of mitochondria to areas where local synthesis is required is also essential[178].
Multiple proteins can regulate mitochondria mobility. For instance, overexpression of the mitochondrial protein encoded by the gene Armcx1 promotes mobilization of mitochondria in vitro, and the protein is found to be upregulated in vivo RGCs following regenerative interventions such as PTEN/SOCS3 codeletion; moreover, AAV-mediated overexpression of Armcx1 one month before optic nerve injury increases RGC survival

and axon regeneration, and combination with PTEN deletion further enhances the effects compared to either intervention alone[436]. Finally, mitochondrial mobility might actually be required for most of the observed regenerative effect following other interventions, as deletion of Armcx1 prevents almost all the regeneration observed in PTEN/SOCS3-codeleted mice[436].
The anchor protein syntaphilin, on the contrary, prevents mitochondria mobilization via stabilization on microtubules. Constitutive deletion of syntaphilin promotes compensatory CST sprouting following unilateral pyramidotomy and regeneration past a dorsal hemisection SCI, with the effect being increased by delivery of creatine (that acts instead independently of mitochondrial transport)[435]. Consistent with observations from other studies cited in this thesis, deletion of syntaphilin is instead not sufficient to promote regeneration of CST axons across or even inside a complete SCI site that lacks GFAP bridging (contrarily to the dorsal hemisection model where astrocytes partially seal the lesion, supporting axon growth); nonetheless, the deletion prevents CST axon retraction, and promotes successful regeneration of 5HT axons with also some limited extent of recovery being reported[435].

These studies point out to poor mitochondrial mobility and energy shortage as additional mechanisms underlying limited CNS regenerative ability, and suggest that other regenerative strategies could also potentially benefit from supplementation with interventions aimed at reversing the post-injury energy crisis.

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Modulation of injury signals

As described in the previous chapter, MAPK signaling, particularly via the MAP3K DLK, is a crucial component of the retrograde injury signaling machinery that underlies the regenerative response observed after PNS injury. Investigation of the role of DLK in CNS injury has showed a contrasting role of the kinase in survival and regeneration: in particular, knockout of DLK two weeks before an optic nerve crush protects injured RGCs from apoptosis, but at the same time greatly reduces the effect of pro-regenerative interventions such as PTEN deletion on axon regeneration[218]. DLK seems therefore to prime injured RGCs for both cell death and regeneration, with the cell normally undergoing apoptosis if no pro-regenerative intervention is performed; when the soma is unable to receive the retrogradely transported injury signal due to DLK deletion, apoptosis is prevented but regeneration is also halted[218].

DLK, in particular, seems to cooperate with its homolog LZK to promote apoptosis of injured RGCs via simultaneous activation of multiple transcription factors, including the above-mentioned SOX11[170,437]. Accordingly, inhibition of DLK and LZK promotes in vitro survival of injured RGCs[437].

Similar to the findings observed after optic nerve injury, co-deletion of DLK and LZK, but neither of them alone, abolishes PTEN deletion-mediated regeneration of injured CST axons and sprouting of uninjured fibers[219].

Overall, modulation of MAPK signaling appears to have a potential to influence axon regeneration, although the overlap with apoptosis regulation warrants caution[170].

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Stretch growth

A recent approach to promote regeneration taking advantage of non-biological mechanisms is inspired by the developmental concept of "stretch growth": during embryonic development, it is hypothesized that a first period of growth cone-guided elongation is followed by a period during which the axon, after having reached its target, continues to elongate in response to mechanical forces; in particular, the tension generated on the axon as the animal's body grows – increasing the distance between the neuronal soma and the axon terminal already tethered to its target – is thought to stimulate the cell to add building material to the axon to prevent excessive strain and rupture[438]. Recent approaches have shown that this process can be replicated in vitro by localizing magnetic nanoparticles to the axons of cultured neurons: exogenously applying a low mechanical force via an external magnetic field promotes increase of elongation rate and of total growth[439]. Current regenerative strategies only rely on growth-cone based elongation, therefore adding stretch growth-based manipulations might have the potential to recapitulate more extensively what is observed in development[178]. Whether this approach could be actually be easily translated to in vivo models, nonetheless, remains to be determined.

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Tissue engineering-based approaches

Alternative approaches that partially overlap with graft-based strategies described above make use of bio-scaffolds inserted in the lesion gap. With the requirement of using materials that are biocompatible and tunable in order to match mechanical properties of the host spinal tissue (either natural materials e.g. fibrin, collagen, chitosan, or synthetic polymers, e.g. polylactic-co-glycolic acid (PLGA) and polyethylene glycol (PEG)), scaffolds for SCI treatments are often functionalized with a variety of active molecules that span from growth factors, to cytoskeletal modulators and antagonists of Nogo signaling[440]; bio-scaffolds can also be seeded with stem cells to promote their integration with the host tissue, or be used for sustained delivery of growth factors in the injured medium until degradation[440]. In addition to hydrogels, typically crosslinked either before implantation or directly in situ without pre-determination of the three-dimensional structure, other types of scaffolds are manufactured via tissue engineering technologies to fine-tune the shape in the attempt to promote and orient elongation of host axons: 3D (e.g. inkjet) printing and electrospinning, for instance, can be used to design either porous sponge-like or fiber-like constructs[440,441].

Multiple approaches have been tested, with some reporting improved functional recovery in murine and canine models[441]; proposed mechanisms for the observed recovery include formation of relays between the lesion ends through either neurons differentiated from grafted stem cells (as in the cell graft-based approaches described above) or neurons derived from endogenous neural stem cells migrated in the lesion[441]: for instance, collagen scaffolds functionalized with EGFR were reported to promote migration of nestin-positive host neural stem cells in a complete SCI lesion and neurogenesis, with some associated recovery[442].

In spite of this, tissue engineering-based approaches for SCI, if not coupled with other interventions on spared neural tissue, have failed to show robust regeneration of host axons inside and especially across the injury site[441], which is the scope of this thesis.

A relevant exception is a recent study from Stupp and colleagues, which employed a bioactive scaffold made of an peptide amphiphile supramolecular polymer: designed to mimic the characteristics of the natural ECM, the scaffold, functionalized with laminin signal and a FGF-2-like peptide, presented fibrils characterized by an intrinsic motion[443]. When implanted – in the absence of any other intervention - in a severe contusion SCI site, the scaffold was reported to promote regrowth of CST and 5HT axons inside and past the lesion, as well as lower compaction of the astrocyte scar boarder, reduced fibrotic core, increased revascularization and associated functional recovery[443]. While the employed injury model is not anatomically complete (although very severe, with the lesion spanning 2 mm in length and being largely GFAP-negative), the results reported in this study are undoubtedly remarkable, considering the fact that no manipulation of intrinsic growth programs was performed. This highlights how alternative and previously unidentified mechanisms including supramolecular motion might also be exploited to promote axonal regrowth, which in this case might result potentially from optimized orientation of host axons and enhanced recruitment of receptors[443]; whether this in its turn triggers downstream effects also on pro-regenerative molecular programs remains to be determined.

Overall, scaffold-based approaches seem, on their own, to have a limited potential on regeneration, although it is clear that providing appropriate physical support especially bridging larger and more severe lesions represents a fundamental requirement to stimulate axon elongation.

We would like to draw the attention to the fact that the regeneration strategy presented in the experimental work of this thesis also makes use of a diblock copolypeptide hydrogel depot loaded with growth factors and delivered to the lesion core.

Importance of combinations

In this and in the previous chapter, we have listed multiple mechanisms that can regulate regenerative capacity, as well as multiple interventions that are able to promote to different extent regeneration after injury. Navigating through the complex landscape of molecular processes that characterize the intracellular and extracellular environment after injury is challenging, especially in the attempt to understand requirements for development of potential therapies or to dissect how the various mechanisms reciprocally influence each other.

Several signaling pathways have been uncovered over the years that have been shown to regulate regeneration by modulating transcription of regeneration-associated genes, e.g. transducing cues from extracellular molecules or as a result of retrograde transport of injury signals, or also via transcriptional-independent mechanisms, including control of cytoskeletal dynamics (**Figure 1.15**).

As mentioned above, pathways associated with transcriptional regulation, in particular, often exert at least partially independent actions, as combinatorial treatments can outperform more restricted activation of molecular pathways (**Figure 1.16).** This synergistic effect is often thought to reflect distinct functions being activated by different growth programs: SOCS3 for instance controls responsiveness to cytokine signaling and its deletion promotes STAT3 translocation to the nucleus and RAG transcription; PTEN deletion and cMyc overactivation, on the other hand, are thought to increase metabolic state, and we discussed before how downstream targets of mTOR activation include ribosomes, with resulting promotion of protein translation.
Accordingly, a proposed main role for JAK/STAT signaling, together with other pathways (as discussed for DLK signaling), is in the generation and propagation of the injury signals, while mTOR activation is thought to represent a proxy for regenerative competence {see also below}; optimization of both mechanisms might then be required for successful axon regeneration[444].
As discussed previously, regeneration mediated by PTEN-deletion can also be enhanced when combined with multiple other interventions, including overexpression of DCLK2, B-RAF, Armcx1 and Sox11, or deletion of Nogo, making the choice of a "most suitable" combination even more ample.

The effect of pro-regenerative pathways on transcription is then also extensively regulated by epigenetic mechanisms controlling chromatin accessibility: modulation of DNA methylation patterns by knockdown of Tets, for instance, attenuates the regenerative phenotype resulting from PTEN deletion or other growth programs manipulations (as mentioned above)[260,445]; in general, the limited extent of regeneration observed in interventions targeting directly specific transcription factors involved in regeneration (e.g. KLF-4, as discussed before, or STAT3, the overexpression of which promotes sprouting of sensory axons but is not sufficient to promote extension[446]) is also attributed to epigenetic control mechanisms that prevent binding of such transcription factors to DNA[234].

Injuries characterized by different severities might also be associated with diverse requirements: for instance, bridging of the lesion via either cell grafts or biocompatible scaffolds appears to be crucial in severe injuries or when lesions present large cavities, while in incomplete lesions or injuries where partial spontaneous sealing of the lesion occurs the axons might not be challenged by such lack of substrate and manipulation of growth programs could be sufficient to promote regrowth.

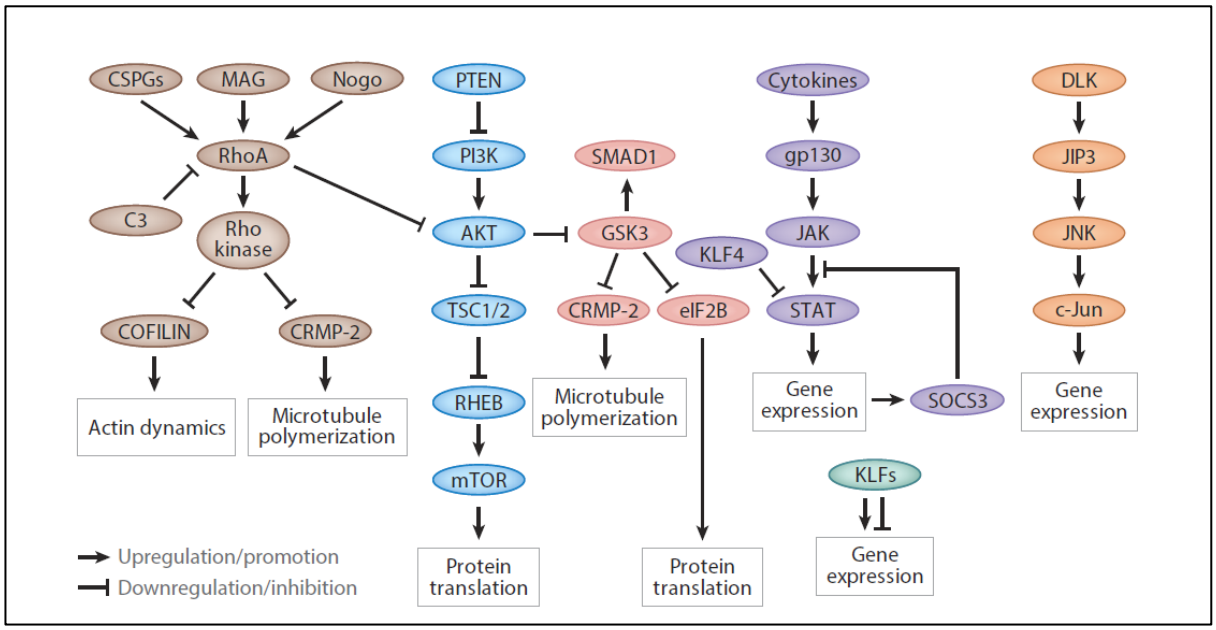

***Figure 1.15*. Pathways involved in axon regeneration.** *Schematic representation of the main signaling pathways involved in axon regeneration, with downstream activated mechanisms indicated in the rectangles. Reproduced with permission from Curcio and Bradke, 2018*[170].

Regeneration of axons per se, moreover, does not guarantee associated restoration of function. On the contrary, as mentioned above, regenerated fibers often lack proper myelination, which negatively affects their conduction properties and restricts the potential for recovery. Likewise, uncontrolled regrowth can lead to aberrant projections[394,420,426] and potentially associated side effects including pain or hypersensitivity. Guidance of regenerating axons to their intended target is therefore also fundamental, and growth factors with chemoattracting or signaling properties – which, as mentioned above, typically have a limited growth-promoting effect when applied on their own – have been shown to be important candidates in this role when delivered in combinatorial strategies.

To summarize, strategies aimed at promoting CNS regeneration need to adapt to the requirements of the specific injury, but work in the field over the last 50 years has established that multiple mechanisms account for distinct functions that can be often targeted synergistically, and that some interventions have a more powerful effect on axonal growth than others. The network of interactions that regulate the injured environment and its potential for regeneration, nonetheless, still remains to be completely characterized; multi-omics and system biology approaches will be crucial to dissect how some of these interactions work and potentially promote discovery of more tailored and optimized therapies.

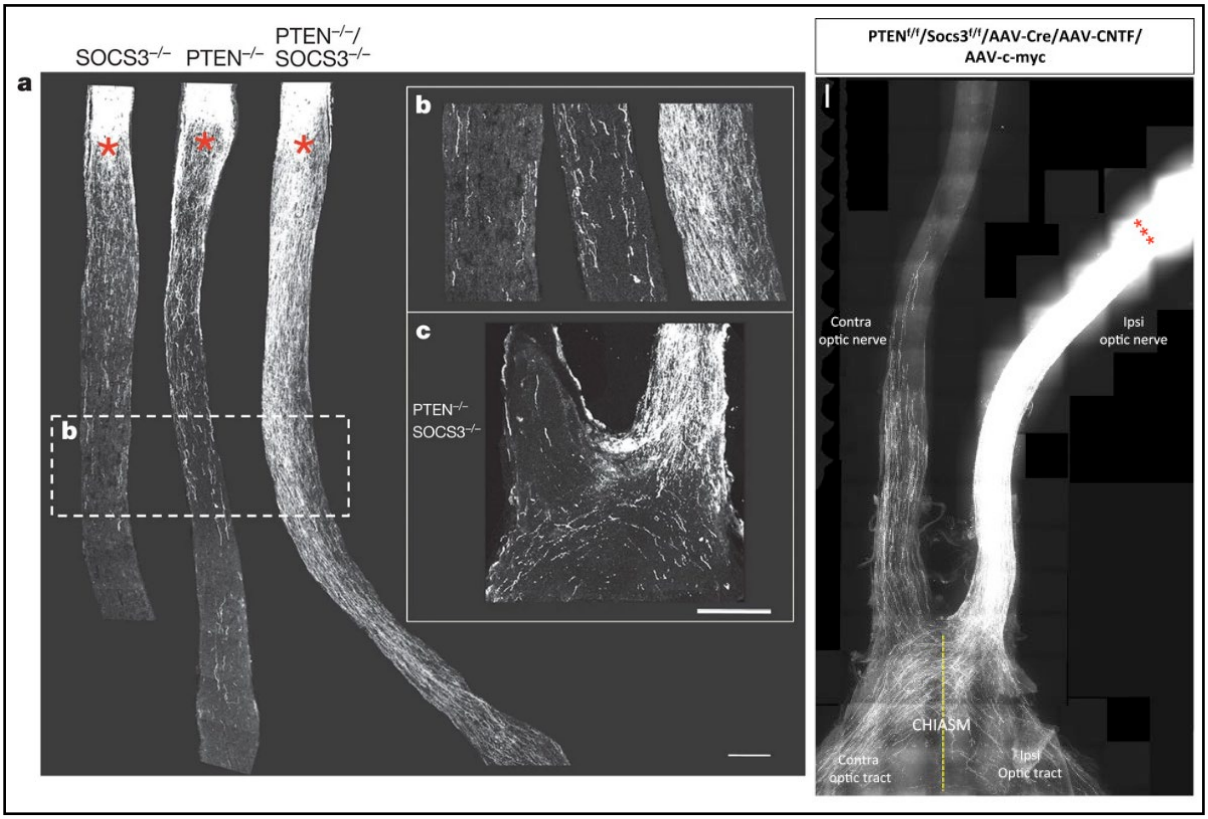

Figure 1.16. Synergistic effect of specific pro-regenerative interventions when applied in combination. *Comparison of the effect on axon regeneration after optic nerve crush of single pro-regenerative interventions vs their combinations: representative whole-mount optic nerve images, with regenerating RGC axons labeled with cholera toxin B (CTB) and lesion site being indicated with one or three asterisks. SOCS3 deletion or PTEN deletion are both outperformed by PTEN/SOCS3-codeletion, with axons reaching the optic chiasm in the double-knockout group (left, insets in b and c). The regenerative phenotype observed with the double knockout strategy can in its turn be increased when combined with c-myc overexpression: the triple combination results in extensive regeneration past the optic chiasm in the optic tract, as well as in aberrant axon elongation in the contralateral optic nerve. All interventions are presented under same experimental parameters (timepoint of assessment 28dpi, optic crush injury performed for 5s at 1mm behind the eye ball). Reproduced and adapted with permission from Sun et al., 2011[227] and Belin et al., 2015[420].*

Subtype specificity

We have already discussed previously in this thesis how distinct axonal systems possess a variable intrinsic competence for plasticity after injury: some tracts including the corticospinal tract (CST) tend to exhibit pronounced spontaneous axonal retraction, while others such as the raphespinal tract persist closer to the lesion edge and naturally exhibit sprouting after CNS injury.

On the same line, while multiple of the approaches described above successfully result in variable extent of axon regeneration, it is important to specify that distinct axonal systems are characterized by a different responsivity to specific CNS regenerative strategies. Specificity in response to experimental interventions is easily exemplified when considering how different axonal tracts also exhibit differing response to neurotrophic factors or growth factors[401]: for instance, as mentioned above, fibroblast-mediated NT-3 administration promotes regeneration of sensory and raphespinal

fibers[169] inside an SCI lesion but only sprouting of CST axons[167] and is detrimental to motor axon regeneration[386]; NGF also promotes regeneration of sensory fibers but not of motor neurons[447]; GDNF increases survival of motor neurons[448], but hinders motor neuron regeneration[449], while being on the contrary a chemoattractant for propriospinal neurons[332]; reticulospinal and rubrospinal axons seem to exhibit regenerative response following administration of any of multiple factors including NT-3, CNTF and BDNF[187,450]. The overall regenerative competence of specific pathways in response to growth-promoting interventions also partially parallels the extent of plasticity observed spontaneously post injury, with again the CST being regarded as one of the axonal systems most refractory to growth-promoting interventions[390], while 5-HT axons regenerate more readily when targeted experimentally or even without any intervention in specific cases[451].

Providing an additional layer of complexity, specificity with respect to regenerative response is not only observed among axonal tracts, but also across individual neuronal subpopulations. Work from He's lab focused on characterizing the extent of regeneration of the ~30 different subtypes of RGCs, as classified on the basis of morphology, gene expression and physiology: following optic nerve injury, the vast majority of axons regenerating in response to PTEN deletion belong to one specific subpopulation of RGCs, αRGCs, which only account for ~6% of all RGCs in the uninjured retina, while on the contrary the other subpopulations appear to be insensitive to mTOR stimulation[415]; peculiarity of αRGCs is that they also represent the vast majority of RGCs with high mTOR activity in the uninjured retina, making them even more responsive to an mTOR-promoting intervention such as PTEN deletion, and highlighting basal mTOR activity as a potential correlate of growth competence of optic nerve axons[415].
Besides regeneration, subtype-specific responses after CNS injury are also observed with respect to cell survival: for instance, while some RGC subpopulations are particularly resilient to optic nerve injury – including αRGCs (~80% survival rate) and M1-RGCs (~70%) -, others exhibit almost complete lack of survival – e.g. M2-RGCs or ooDSGCs – or intermediate behavior – e.g. W3-RGCs (~10%)[415].
Importantly, manipulations that are beneficial for one specific neuronal subpopulation could be detrimental to others: overexpression of Sox11, for instance, was found to promote regeneration of non-αRGCs, but at the same time to completely abolish survival of αRGCs[452].
Similar mechanisms are likely to be occurring also in the spinal cord, where around 30 subtypes of neurons can be identified based on cardinal developmental classes and projection patterns[453].

As also shown by the experimental results presented in this thesis, regeneration of all neuronal subpopulations might not be required for successful restoration of specific functions after injury. Nonetheless, being able to manipulate multiple subclasses in a balanced manner is likely to be important for the development of future repair strategies aiming at bringing the injured CNS as close as possible to the pre-injury conditions. As highlighted above, in addition, distinct mechanisms regulate survival and regeneration, and consideration of both aspects is crucial. Multi-omics technologies will be again of paramount importance to uncover the determinants underlying subtype-specific responsiveness and identify molecular targets that will be leveraged to develop comprehensive therapies stimulating multiple neuronal classes in parallel[9].

REGENERATION AFTER COMPLETE SCI: STATE OF THE ART

The experimental work described in this thesis is built upon, and represents the continuation of, the research published by our group in 2018 (Anderson, O'Shea et al, 2018[332]): in the paper, our laboratory described a strategy that, for the first time in the field, resulted in regeneration of propriospinal interneurons across an anatomically complete SCI, and via manipulation of host tissue only (i.e., without cell grafts).

Previous approaches for regeneration after complete SCI

Before proceeding to describe the principles of the abovementioned paper, it is useful to briefly summarize the state of the art for regenerative approaches specifically after anatomically *complete* SCI as of 2018, most of which have already been partially described in the previous chapter. These – summarized in **Table 1.2** – mainly fall into two categories of intervention:

1. Grafts
    a. *Whole-tissue (PNS, fetal CNS) grafts*

    As described before, work from the late 1990s and early 2000s addressed regeneration of CNS axons after a complete SCI by delivering grafts from PNS or fetal CNS tissue in the lesion, usually in combination with growth factors or neurotrophins to augment axonal growth. Two studies from Olson's and Bregman's groups, in particular, employed a complete transection injury model in rats consisting in removal of a whole segment of thoracic cord tissue and aspiration, leaving a gap of several millimeters between the lesion ends. Olson's group then sutured multiple peripheral nerve autografts reconnecting white matter on one lesion end to grey matter on the opposite side, stabilizing the graft with FGF-containing fibrin glue[363]; on the other end, in Bregman's experiments the lesion was filled – in a delayed treatment few weeks after injury – with embryonic spinal cord tissue and continuously supplemented with NT-3/BDNF delivered via an osmotic pump[374]. Both groups showed extensive regeneration of supraspinal axons inside the graft and across it, with fibers reaching the lumbar spinal cord, including notoriously regeneration-refractory CST axons[363,374] (**Figure 1.17**). Importantly, both studies reported spontaneous recovery of hindlimb locomotor function associated with the regenerating fibers, with frequent plantar foot placement and partial weight-support[363,374]

    Olson's strategy was also tested in combination with additional interventions such as ChABC delivery and rehabilitation, and in other models including rat chronic injuries and mouse models, with various impact on recovery (including of bladder function) and reports of limited regeneration of serotonergic fibers[454–456]. Importantly, as mentioned in the previous chapter, this approach failed to stand the test of translation, promoting limited regeneration of host axons in species other than rats, including non-human primates[366,454].

    Overall, both studies with PNS and fetal grafts come with some limitations, such as limited histological evidence to support reports of regeneration –

e.g. lack of sagittal sections showing regrowth of host axons across the lesion – or use of old anatomical tracing techniques - e.g. injection of wheat germ agglutinin horseradish peroxidase (WGA-HRP)[363], known to be capable of limited trans-synaptic transfer[457,‡]. In line with this, it is likely that observed behavioral recovery might depend on formation of relays from graft-derived cells outgrowing in the host tissue (as observed in cell graft strategies – see below) rather than exclusively from regrowth of directable host fibers across the injury[367,‡]. More importantly, restricted availability of the tissue source and ethical considerations make these strategies intrinsically limited in clinical translatability[367].

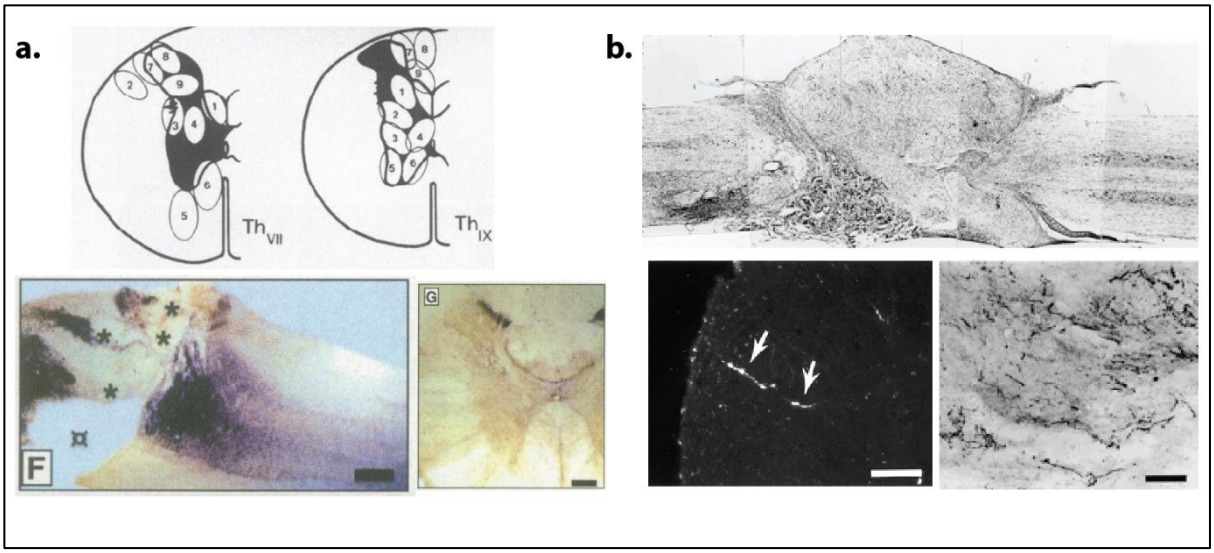

Figure 1.17**. **Whole-tissue graft-based strategies with reports of regeneration across a complete SCI. a. PNS grafts (Cheng et al., 1996[363] [Reprinted with permission from AAAS]). *[top] Schematic representation of the experimental approach, with pieces of autologous intercostal nerves used to establish white matter-to-gray matter connectivity between the two spinal cord stumps (Th_{VII} and Th_{IX}, seventh and ninth thoracic segments, respectively), and stabilized with fibrin-containing glue and compressive wiring of the posterior spinal processes. [bottom] Axonal fibers labeled via WGA-HRP injection in the sensorimotor cortex, (F) observed crossing the lesion site (*) in a sagittal section and (G) in the tissue caudal to the injury in a transverse lumbar section.*
b. Embryonic CNS grafts (Coumans et al., 2001[374] [Copyright 2001 Society for Neuroscience]). *[top] Sagittal cresyl violet-stained section through the lesion and transplant site, showing integration of the embryonic spinal cord graft in the lesion. [bottom] In animals with delayed (2-4 weeks after injury) transplant and treated via continuous delivery of neurotrophins, CST (left, FluoroRuby tracing in the motor cortex) and 5HT (right, immunohistochemistry) axons are detected in the tissue caudal to the injury.*

b. *Cell grafts:*

Whole-tissue PNS grafts are thought to promote regeneration partially through the role of Schwann cells, that provide trophic support and re-myelinate host axons[454,455]. Accordingly, as mentioned in the previous chapter, among the cell-based regeneration approaches, including for complete injuries, the role of directly transplanting Schwann cells or other myelinating cells has extensively been investigated. In particular, Bunge's

‡ In case of trans-synaptic tracing, labeled fibers in the caudal tissue could potentially originate from graft-derived cells acting as a relay inside the lesion, and could therefore erroneously be identified as host axons regenerating across the injury site. Such phenomenon would therefore create ambiguity between relay graft-derived axons and bona fide regenerating host axons, leading to over-estimation of these latter.

and other groups have used an experimental paradigm to bridge a complete transection SCI by transplanting a polymer channel containing heterologous Schwann cells and delivering olfactory-ensheating glia (OEG) grafts on both lesion ends: such approach resulted in regeneration of serotonergic and propriospinal axons across the injury, but regrowth of other supraspinal tracts was limited to few fibers inside the lesion[458,459]; additional supplementation of ChABC resulted in partial recovery of isolated hindlimb joints function[459]. Moreover, OEG migrated extensively in the graft and in the host tissue around it, providing trophic factors to host neurons[458]. Recovery with this strategy can be improved with locomotor training but, importantly, the positive effect of OEG on hindlimb function improvement seems to depend on modulation of lumbar circuits (CPG) rather than regeneration of axons across the injury: on the contrary, performance of trained graft-receiving rats on the treadmill is improved if a re-transection is performed, showing a potential restraining role of regenerating fibers in locomotor control[394].

Additionally, as discussed previously, the strategy based on neural stem cells (NSCs) developed by Tuszynski's group has been applied specifically in a complete transection SCI model: NSCs derived from embryonic spinal cord tissue, grafted together with a fibrin-matrix containing a growth factor cocktail, fill the lesion and extensively integrate in the host tissue, promoting partial recovery of motor function in rats[379]. Nonetheless, while regeneration of supraspinal and propriospinal axons is promoted in the initial portion of the graft, no regrowth across the lesion is observed[379].

Overall, therefore, cell graft-based approaches, although also associated with important limitations concerning the cell source, offer evidence of potential impact on functional recovery. Nonetheless, critically, recovery-promoting effect of the implant is exerted mainly by undirected outgrowth of graft-derived cells, and not via controlled, guided regeneration of host axons to target regions.

2. Manipulation of growth-associated pathways

As discussed before, stimulation of specific signaling pathways including PI3K/Akt/mTOR and JAK-STAT promotes axon regeneration in multiple CNS injury models. These also include complete SCI models: studies from He's group showed that PTEN deletion or silencing performed neonatally, 4 weeks before, or even up to one year after a complete crush SCI elicits regeneration of CST axons across and caudal to the injury[283,417,460]. In spite of the observed regeneration, no recovery of hindlimb function could be detected in treated mice[417]. Moreover, importantly, as discussed later, regeneration occurred along strands of glia bridging the two lesion ends, and axons failed to regrow even inside the lesion if a model not producing any bridging (via longer compression) was employed[283,417].

Study [species]	Type of manipulation	Mechanism/level of complete injury	Axonal tracts with reported regeneration across the injury	Functional recovery
Cheng et al., 1996 [rat][363]	Autologous PNS grafts (multiple segments) + fibrin gel + FGF + vertebral column fixation with wiring	T8 transection + cord removal	CST, ReST, 5HT	Hindlimb stepping with partial weight support
De Paul et al., 2015 [mouse][454]	Autologous PNS grafts (multiple segments) + fibrin gel + FGF + vertebral column fixation with wiring + ChABC	T8 transection + cord removal	5HT and TH (limited regeneration). [Other pathways not investigated].	Improved bladder function (motor function not reported)
Coumans et al., 2001 [rat][374]	Embryonic (E14) spinal cord tissue [2-4 weeks post injury] + NT-3/BDNF [osmotic pump]	T6/T8 transection + vacuum suction	CST, ReST, RST, 5HT	Hindlimb stepping with weight support
Ramón-Cueto et al., 1998 [rat][458]	Schwann cells-filled guidance channel + olfactory-ensheating glia at each stump	T9 transection + cord removal	5HT, ascending PrSp	[Not reported]
Fouad et al., 2005 [rat][459]	Schwann cells-filled guidance channel + olfactory-ensheating glia at each stump + ChABC [osmotic pump]	T8 transection + cord removal	5HT [No regeneration of CST and ReST]	Limited movement of up to two hindlimb joints (BBB_{avg} =6.6)
Lu et al., 2012 [rat][379]	NSCs derived from embryonic rat (E14) or human spinal cord + fibrin matrix with GF cocktail	T3 transection + microaspiration	[Host axons only regenerate partially inside the graft, not across it]	Movement of all hindlimb joints (BBB_{avg}~7)
Liu et al., 2010 [mouse][283]	PTEN deletion in the sensorimotor cortex	T8 crush	CST	None[417]

Table 1.2. Experimental approaches to promote regeneration after anatomically complete SCI as of 2018. *Non-exhaustive list of representative studies (as of 2018) presenting varying degree of regeneration from multiple axonal tracts and of functional recovery after anatomically complete SCI. CST: corticospinal tract; ReST: reticulospinal tract; RST: rubrospinal tract; 5HT: serotonergic axons; TH: tyrosine-hydroxylase positive axons; PrSp: propriospinal interneurons. BBB: Basso, Beattie, Bresnahan score[461], avg = average.*

Variable severity of anatomically complete SCI: importance of glial bridging

Anatomically complete SCI are defined as lesions that completely interrupt any descending or ascending axonal connection between the two sides of the lesion. Nevertheless, as already mentioned in **Experimental models of SCI**, specific parameters can influence the severity of experimental models of anatomically complete SCI, resulting in lesions of varying size: in particular, for complete crush SCI, that compared to complete transection present the advantage of preserving tissue continuity, duration of the compression and size of the forceps tips can greatly influence the extension of the lesion.

Liu et al., for instance, employed a model where a complete crush SCI is performed by compressing the cord for 2 seconds via forceps with fine ends of 0.1 mm width[283] - this injury paradigm results in successful severing of all axonal projections at the affected spinal level, and acutely the lesion core is devoid of glial cells; on the other hand, over the course of the following weeks (already at 4 weeks post injury) a GFAP-positive matrix characterized by astrocyte fingers extending into and bridging across the lesion starts to develop[283] (**Figure 1.18a**). Multiple studies have then employed the same complete crush SCI model, and have presented regeneration of CST axons via manipulations of the mTOR pathway[283,417,460] or of JAK-STAT signaling[419] [this last study was published after 2018]. Importantly, regenerating axons associate extensively with astrocytes strands bridging the lesion[283,417,460].
On the contrary, if the same type of injury is performed via forceps with wider tips (0.5 mm width), the size of the lesion is dramatically increased, and glial strands are not observed at chronical timepoints (**Figure 1.18b**); crucially, no CST regeneration across or even inside the lesion is observed[417].
Similar observations were reported with other injury models, including complete transection: growth-promoting manipulations seem to only promote CST regeneration inside and across the lesion if the employed injury model preserves glial bridging[435]. On the other hand, axonal tracts with notoriously higher growth competence, such as serotonergic axons, are able to regrow also in the absence of GFAP strands, typically contacting substrates of extracellular matrix (ECM) molecules such as laminin[435].

The findings described above are also consistent with biological processes observed in animal species where spontaneous CNS regeneration occurs. We have mentioned in **Regenerative competence: PNS vs CNS**, for instance, that in fish or *Acomys* spontaneous sealing of the lesion, with decreased fibrosis, is observed, supporting extensive axonal regrowth across the lesion[261,263]; the same lesion repair and glial bridging was also extensively characterized in regeneration-competent newborn mice[279].

Moreover, these observations are consistent with the knowledge that the astrocyte scar border is not a primary obstacle to regeneration, but that, on the contrary, astrocyte strands support axonal regrowth and the presence of the border restricts extension of the inflammatory processes[462].
On the contrary, substantial hurdle to regeneration is the GFAP-negative portion of the lesion core, i.e. the fibrotic scar[463]: fibrosis is crucial to re-establish tissue integrity, but creates a dense structure of fibroblasts-like cells and ECM molecules[85]. Accordingly, while completely preventing fibrotic core formation results in failure of lesion sealing[85],

partial attenuation via inhibition of subsets of the cell types responsible for its formation promotes decreased ECM deposition and regeneration of multiple axonal tracts[86,463].

In summary, particular care needs to be paid when comparing different models of anatomically complete SCI, as diverse mechanisms and parameters of injury production can greatly influence the amount of glial bridging across the lesion and the extension of the fibrotic core; consequently, lesions that are complete but less severe might require a smaller number of interventions in order to promote regeneration.
Crucially, the fibrotic core represents a main hurdle to regeneration: successful regeneration across severe injuries characterized by a large fibrotic core requires either its modulation (e.g. by partially preventing fibrosis, or by promoting higher deposition of growth-supportive vs growth-inhibitory ECM components) or provision of exogeneous components able to function as bridges (as in the case graft-based approaches, or scaffolds[443]).

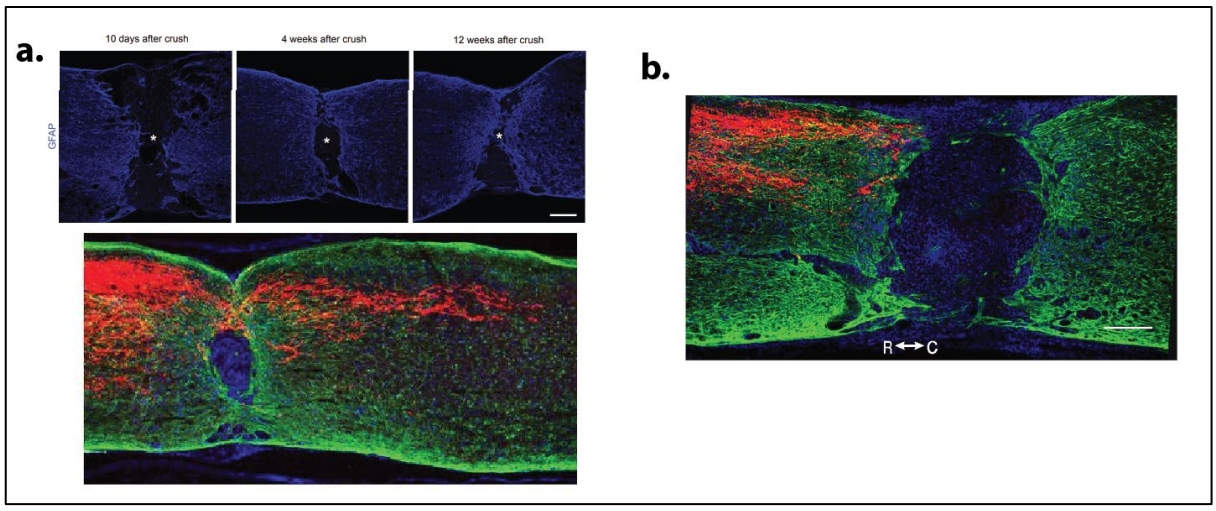

Figure 1.18. ***Parameters of injury production greatly influence lesion size and glial bridging after anatomically complete SCI.*** a. Complete crush performed with finer ends (0.1 mm) forceps. *[top] Gradual emergence of GFAP-positive astrocyte strands in the lesion: at 10 days after crush, the lesion core is largely GFAP-negative; at 4 weeks post injury, strands start to be evident inside the lesion and, by 10 weeks, a matrix connecting the two lesion stumps has developed. Scale bar: 200 µm. Reproduced with permission from Liu et al., 2010[283]. [bottom] In mice with neonatal silencing of PTEN transcription in the somatosensory cortex, at 8 weeks post injury CST axons (in red) have largely regenerated across and caudal to the complete crush by contacting bridging glial strands (green: GFAP, blue: DAPI). Adapted from Zukor et al., 2013[417] {licensed under CC-BY-NC-SA}.*
b. Complete crush performed with wider ends (0.5 mm) forceps. *As a result of the more severe injury model, at 8 weeks post injury a large fibrotic core substantially devoid of glial strands is evident (green: GFAP, blue: DAPI), and PTEN-suppression is not sufficient to promote regeneration of CST axons (red) even inside the lesion. R: rostral, C: caudal. Scale bar: 200 µm. Reproduced from Zukor et al., 2013[417] {licensed under CC-BY-NC-SA}.*

Anderson et al., 2018: regeneration of propriospinal interneurons across severe anatomically complete SCI

Based on the review of the studies presented so far, as of 2018 all experimental approaches had failed to promote robust axon regeneration across a severe anatomically complete spinal cord injury without delivery of additional cellular substrate (such as grafts, associated with their intrinsic limitations).

We have also extensively discussed in the previous chapters how, among the different axonal systems, long-projecting propriospinal interneurons exhibit particularly high plasticity after SCI[464] and possess the potential to relay functional signals to the tissue caudal to an incomplete injury.

It is in this context that, in 2018, our group presented a novel regenerative strategy for thoracic propriospinal interneurons after anatomically complete SCI. In particular, the model of injury employed was a severe complete crush SCI at T10, with the compression being performed via forceps with no spacer and a tip width of 0.5 mm and a compression of 5 seconds[332]; besides severing all axon connections at the level of interest, this type of injury results in a large fibrotic core with complete interruption of GFAP signal, consistent with the observations in **Figure 1.18b**.

The strategy consists of three components, each addressing a specific growth-limiting property of the injured adult CNS (**Figure 1.19a**)[332]:

1. To address the limited neuronal intrinsic growth capacity, neurons are pre-conditioned via AAV-mediated overexpression of osteopontin, IGF-1 and CNTF (OIC) in the two segments rostral to the injury. This delivery is performed before the SCI, specifically two weeks in advance to allow for AAV expression. Work by He and colleagues, as mentioned in the **previous chapter**, had shown that such combination of signaling molecules activates mTOR and JAK-STAT pathways and is on its own sufficient to promote some extent of regeneration of the optic nerve or after incomplete SCI.
2. To modulate the fibrotic scar lacking growth-supportive substrate, a hydrogel depot loaded with the growth factors FGF2, EGF and GDNF is delivered in the lesion core 2 days post injury. While the hydrogel itself provides a physical support, FGF2 and EGF are known to shift composition of the ECM deposited by fibroblasts towards growth-supportive components, and to upregulate ECM-specific proteases[465]. Additionally, expression of members of the FGF and EGF families has been reported in the lesion core of regeneration-competent species such as zebrafish, where they promote spontaneous lesion repair via glial bridging and neurogenesis mechanisms[286,466]. As a result, this component promotes successful remodeling of the fibrotic scar in treated mice, stimulating extensive deposition of growth-supportive laminin (**Figure 1.19c**).
3. To provide chemoattractive guidance to the regenerating axons, GDNF is delivered via the hydrogel depot in the lesion (component 2) and via a second GDNF-containing depot injected in the tissue caudal to the injury 9 days after injury. This mechanism recapitulates the guidance mechanisms observed during development, when elongating axons are directed via chemo-repulsive and chemo-attractive cues[467], with the same patterns being largely absent in the adult CNS.
   Propriospinal interneurons possess the functional features to respond to GDNF

stimulation, as they express its coreceptor GDNF-receptor (GDNFr) and particularly overexpress it after SCI[464].

Remarkably, when examined at 4 weeks post injury, robust regeneration of thoracic spinal cord interneurons inside and across the anatomically complete injury is observed as a result of the tri-partite strategy; the protocol successfully promotes regeneration in both mice and rats, with axons re-growing up to 2 mm past the lesion center and up to 2.5 mm, respectively (**Figure 1.19b**)[332]. Inside the lesion core, devoid of GFAP-positive strands, axons regrow extensively by contacting substrates of laminin resulting from the lesion remodeling[332], consistent with the observations discussed in the previous paragraph (**Figure 1.19c**).

Crucially, all three manipulations are all required, as any combination of one or two of the interventions fails to promote comparable or any regeneration across the severe injury[332].

Finally, regenerating axons form synaptic-like contacts with neurons in the tissue caudal to the injury, and stimulation experiments show that the new axonal substrate crossing the lesion allows electrical signals delivered above the lesion to be conveyed caudal to it, showing partial restoration of electrophysiological conduction capacity[332].

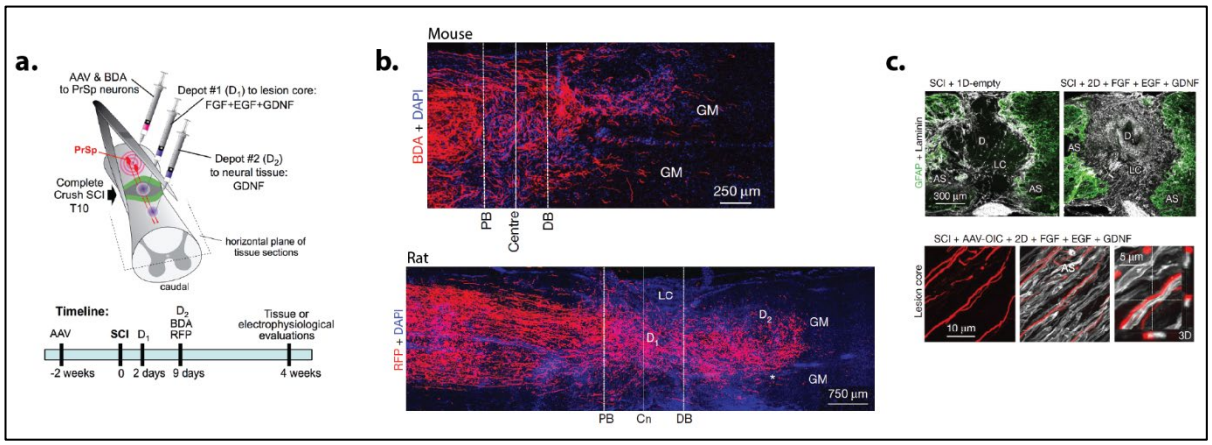

Figure 1.19. Tri-partite strategy promotes regeneration of spinal cord interneurons inside and across a severe anatomically complete SCI in mice and rats. a. Overview of the experimental paradigm. *First, animals receive intraspinal injections at T8/T9 of a mix of AAVs expressing osteopontin, IGF-1 and CNTF [the mix is referred to as "AAV-IOC"]. Two weeks after, a complete crush SCI is performed at T10. Two days after injury, a first hydrogel depot containing FGF2, EGF and GDNF is delivered in the lesion core. In a fourth and final surgery 9 days after injury, a second depot containing GDNF is injected one segment caudally to the injury, and propriospinal (PrSp) axons are anterogradely traced by intraspinal T8/T9 injection of either BDA (in mice) or AAV-RFP (in rats).*

b. Regeneration of propriospinal interneurons inside and across the injury in both mice and rats. *Representative composite survey images (produced by overlay of multiple tiled scans from the same animal) showing regeneration of propriospinal interneurons anterogradely traced with either BDA or AAV-RFP (red) inside and across the complete crush SCI lesion, in both mice [top] and rats [bottom] subjected to the tripartite strategy. PB: lesion proximal border, DB: lesion distal border, Centre/Cn: lesion center, LC: lesion core. D1, D2: depot 1 and 2. GM: grey matter. DAPI nuclear staining (blue).*

c. Fibrotic core modulation promotes deposition of laminin that supports axonal regrowth. *[top] Immunohistochemistry images showing extensive laminin (white) deposition in the fibrotic core (as delimited by the GFAP-positive astrocyte scar border in green) in response to depot-mediated FGF2 and EGF delivery, but limited presence of laminin in the lesion with injection of an empty depot. [bottom] Detail immunohistochemistry confocal images showing regrowth of spinal cord interneurons (red) along strands of laminin (white) in the lesion core in fully-treated mice. AS: astrocyte scar border.*
Adapted with permission from Anderson, O'Shea et al., 2018[332].

Lack of functional recovery and aim of the thesis

The strategy presented above resulted in an unprecedented degree of axon regeneration across and caudal to an anatomically complete injury. Nonetheless, while regenerated fibers were shown to be able – via electrophysiology experiments – to conduct electrical signals across the lesion, and in spite of the known role of propriospinal interneurons in promoting recovery after SCI, no improvement of hindlimb function could be detected in any of the treated animals in the study[332].

The work described in my thesis was therefore aimed at addressing multiple hypotheses potentially underlying the lack of spontaneous recovery with the strategy developed by our group.

I here show that an additional mechanism, i.e. re-establishment of the natural pattern of projections of propriospinal interneurons, is a requirement for functional recovery after complete SCI. Optimization of the experimental paradigm via sustained chemoattraction to guide regenerating axons to their natural target region results in enhanced regeneration of the same axonal tract and, importantly, in spontaneous recovery of hindlimb locomotion in mice after severe anatomically complete SCI.

2. AUGUR : CELL-TYPE PRIORITIZATION IN SINGLE-CELL DATA

AUGUR

Although not included in the main focus of my PhD, an important work from my laboratory in which I have been involved is the one described in the article "Cell type prioritization in single-cell data", published in the journal *Nature Biotechnology* (Skinnider, Squair et al., 2020[468]), and for which I am part of the list of co-authors.

Briefly, our team developed Augur, a bioinformatic algorithm that, based on two single-cell/single-nucleus datasets corresponding to distinct experimental conditions (e.g. treatment vs control), identifies the cell populations which are most responsive to the considered experimental perturbation (see below).

Specifically, my contribution to this work has consisted in participation in experimental validation of predictions made by the algorithm, including histological analysis and imaging.

I here proceed to summarize the main underlying principles of Augur, as well as to describe more specifically my contribution and some of the results presented in the paper.

Background

Over the course of the last decade, single-cell (sc-) and single-nucleus (sn-) methods have witnessed a rampant development of available technologies and sequencing depth, paralleled by a decrease in cost, which has made their employment progressively more wide-spread. This upscaling of technologies – for a field that is still relatively recent – has been so rapid to the point of even leading to potential statistical misconduct, owing to the fact that analysis techniques originally designed to be applied to small datasets of cells/nuclei from a single animal have been directly translated for use with samples pooling multiple biological replicates in the same experimental group, leading to false discoveries[469].

At the same time, with application of the due statistical methods, the possibility of studying increasingly larger tissues at the resolution of individual cells, and even whole organs or whole organisms, opens new possibilities to answer unexplored biological questions. Bulk sequencing technologies, for instance, have traditionally focused on identifying individual *genes or transcripts* that are most differentially expressed among specific experimental conditions; on the contrary, single-cell/single-nucleus sequencing possess the potential to address the broader question of how individual *cell types* respond to a given biological perturbation (e.g. a treatment, or injury), and which cell types contribute the most to an observed phenotype.

Augur was developed for this specific purpose, and takes as an input – in its simplest form of analysis – two integrated single-cell/single-nucleus datasets with annotated cell populations and corresponding to two conditions: exposure to a biological perturbation or to no perturbation (control group). Comparing the two datasets, Augur ranks each cell type according to the extent of overall transcriptional change observed in response to the experimental manipulation, i.e. it identifies the cell types that are most affected, *prioritized* by the given perturbation.

Description of the algorithm

The fundamental assumption based on which Augur performs cell type prioritization is the following: if a cell type is highly responsive (i.e. exhibits high transcriptional change) to a given experimental manipulation, the cluster for that given cell type corresponding to the experimental perturbation will be highly separable, in the multidimensional space of all single-cell variables (e.g. genes), from the cluster for the same cell population under the control condition. In other words, the difficulty of separation can serve as a proxy for the responsiveness of the cell type to the biological perturbation, with cell types more affected by the manipulation being more easily separable than less responsive populations. This is formalized as a classification task, with prioritized (i.e. more responsive) cell types associated with higher accuracy score in the prediction of "perturbation state" vs "control state" labels.

A guided description of the pipeline of Augur is presented in a second paper published in *Nature Protocols*: Squair, Skinnider et al., 2021[470].

Briefly, Augur starts from a dataset integrated on the two experimental conditions (e.g. treatment/perturbation and control), and with clusters corresponding to each individual annotated cell type (each datapoint in the cluster being a distinct cell/nucleus, with a label corresponding to its condition and having as features e.g. gene counts). Augur iterates the following procedure on each individual cell type cluster (**Figure 2.1**): a random subsample from the cluster is drawn; a training set is generated by withdrawing the labels on a subset of the subsample datapoints; a random forest classifier is trained on such training set; the classifier is tested on the remaining data of the subsample (acting as a test set). These four steps are then repeated via cross-validation over the same subsample (i.e. within the same subsample, a new classifier is trained on a new training subset and tested on the remaining test subset, and the whole procedure is repeated by default 3 times) and over newly drawn subsets (by default 50 subsets per cell type)[§]; performance of the model for the cell type is then assessed as the average performance across the cross-validation runs. Therefore, for each cell type a classification accuracy is returned as an (average) area under the receiver operating characteristic curve (AUC) measure. Cell types are then ranked according to the respective AUC and, as discussed above, highest-ranked cell populations are identified as the ones that are most responsive, i.e. prioritized in response to the biological perturbation.

Main results

Besides Augur, other approaches to prioritize cell types in response to biological perturbations have been described[471,472]; such methods, nonetheless, perform cell-type ranking based on number of genes being significantly differentially expressed (DE) according to a set threshold and, as such, tend to prioritize cells with higher number of cells and smaller response to the biological intervention as opposed to subpopulations with actual stronger transcriptional response but lower total number of cells. When

[§] This step is performed to account for variability in the total number of cells among cell types, with specific cell populations being more represented as a result of both biological and technical factors. Cross-validation is shown to robustly correct for the bias of number of cells/nuclei of a given subpopulation on the AUC.

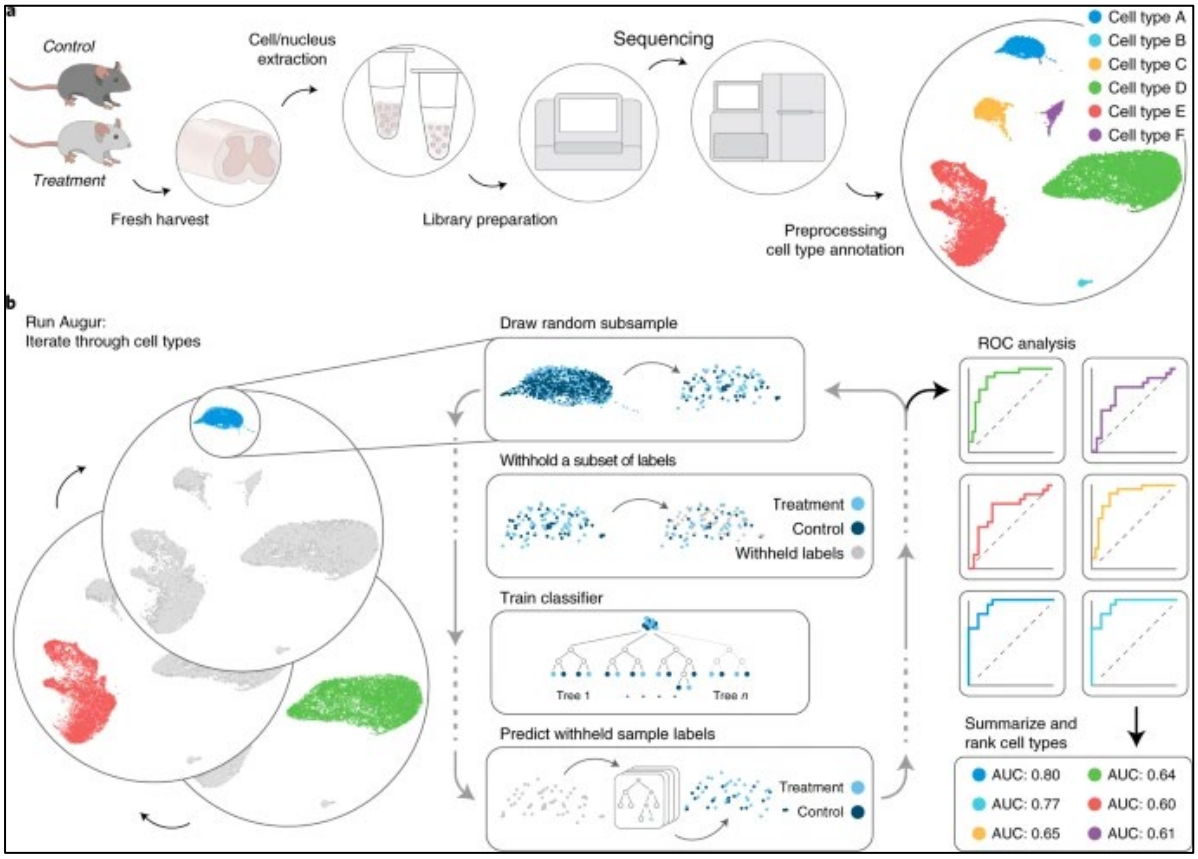

***Figure 2.1*. Overview of the workflow of Augur.** a. Overview of the experimental basis for Augur*: after tissue harvest, nuclei/cells from animals belonging to two distinct groups (e.g. treatment and an unperturbed control) are extracted, library preparation is performed, followed by sequencing; raw read counts are preprocessed, and cell-type annotation is performed according to available reference atlases or manually on the basis of expression of marker genes.*
b. Overview of Augur's machine-learning framework. *Within each cell-type cluster, Augur draws a random subsample, which is then divided into k folds (by default 3): a random forest classifier is trained on the first k-1 folds and tested on the remaining fold; the procedure is repeated on different fold combinations of the same subsample over cross-validation. Iteration is then performed on newly drawn subsets of the same cell-type cluster (by definition 50). An AUC averaged on the cross-validation runs is returned for each cell type as a measure of the classifier performance, and cell types are ranked accordingly. Reproduced with permission from Squair, Skinnider, et al., 2021*[470].

tested on either simulated or experimental dataset, Augur, owing to its cross-validation corrective approach, significantly outperformed DE methods, succeeding at capturing the magnitude of transcriptional change in spite of cell type abundance bias.

Additionally, the accuracy of Augur was validated on published scRNAseq datasets that provided a ground truth measure of cell type-specific responses to stimuli of known intensity.

Importantly, aside from standard two-class datasets (e.g. treatment versus control), the algorithm can also robustly process multi-class or continuous data.

Application of Augur is not limited to sc-/sn-RNAseq data, since the algorithm also flexibly incorporates epigenomics data, e.g. single-cell Assay for Transposase-Accessible Chromatin using sequencing (sc-ATACseq) data[473,474], and data of transcriptional dynamics such as RNAvelocity[475] – calculated from RNAseq data and corresponding to the time derivative of gene expression change. Transcriptional

dynamics (i.e. RNA velocity), in particular, were shown to provide more information for cell-type prioritization compared to RNA abundance (i.e. untransformed RNAseq data) in the case of acute experimental perturbations lasting up to 4 hours. Augur can therefore by employed to dissect the impact of both acute and chronic experimental interventions.

Finally, the algorithm can also be extended to provide transcriptional perturbation assessments with *spatial* resolution: for instance, Augur reliably processes input from single-cell transcriptomics imaging techniques such as MERFISH[476] and STARmap[477]. Moreover, our lab developed a second algorithm, named Magellan, that extends the concept of prioritization to spatial transcriptomics[478] data: by employing a spatial nearest-neighbours framework – in which the classification model is built on neighboring spatial barcodes, as opposed to cells/nuclei from a given cluster in Augur -, Magellan prioritizes regions of a biological tissue (e.g. a spinal cord section) that are most responsive to a given perturbation/intervention[30]. Augur and Magellan can be combined if the sc-/sn-RNAseq dataset is embedded on the spatial dataset: for instance, as described previously {**Recovery after incomplete injury: neuromodulation**}, local-range Vsx2 are the main neuronal subpopulation in the lumbar spinal that underlies walking following neuromodulation intervention, as identified by cell-type prioritization on single-nucleus RNAseq data by Augur; accordingly, Magellan prioritizes intermediate laminae (where Vsx2 neurons are located) and ventral motor-neuron associated laminae as the regions undergoing the highest transcriptional response during walking, based on spatial transcriptomics data[30] (**Figure 2.2**); spatial and cell-type prioritization scores generated by the two algorithm at corresponding spatial coordinates are highly-correlated[30].

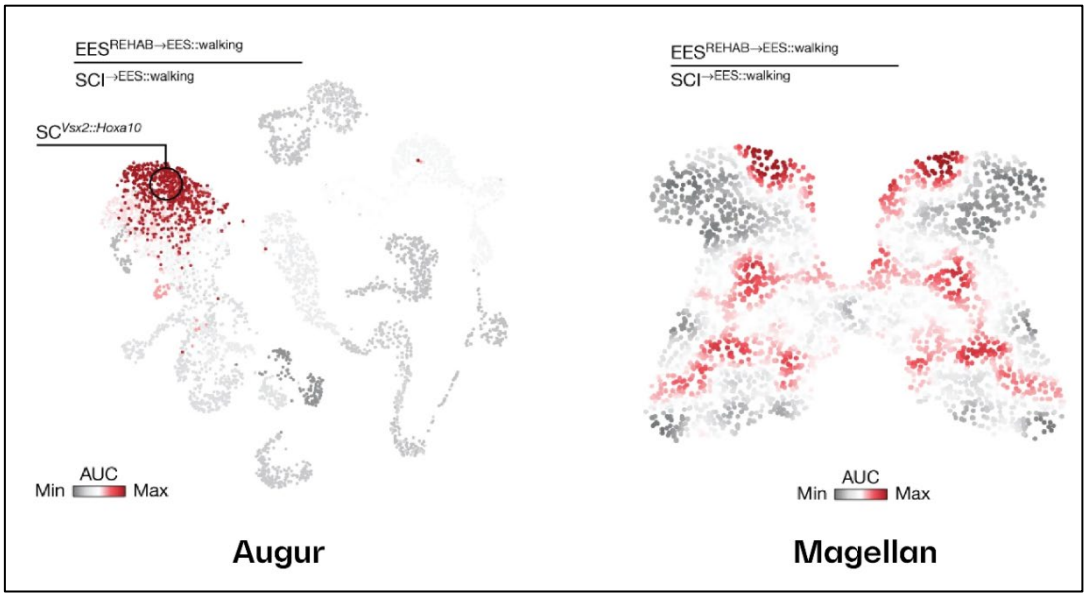

***Figure 2.2*. Correlation between prioritization by Augur and Magellan.** Left. Cell-type prioritization by Augur in a representative experimental comparison (mice with contusion SCI walking 1) with acute EES 2) with EES following rehabilitation): *UMAP showing that local-range lumbar Vsx2 neurons ($SC^{Vsx2::Hoxa10}$) are the most prioritized population, involving a key role in walking following neuromodulation + rehabilitation.* Right. Spatial prioritization by Magellan in the same representative experimental comparison. *Visualization of the spatial barcodes in the common coordinate space showing that the regions prioritized by neuromodulation + rehabilitation include intermediate laminae where local-range lumbar Vsx2 neurons reside. Adapted from Kathe, Skinnider, Hutson, et al., 2022[30] {licensed under Creative Commons}.*

Personal contribution

As mentioned above, my contribution to the work consisted in the experimental validation of predictions made by Augur based on transcriptomics data.

In particular, snRNAseq data was generated from 1) mice subjected to a severe thoracic contusion SCI and walking for 30 min in response to acute EES + monoaminergic treatment and 2) control mice subjected to injury only – without neuromodulation – exhibiting complete paralysis. Subsequently, in order to identify the neuron subtypes immediately engaged by the electrical stimulation, Augur was applied to the data (more specifically to the RNA velocity calculated from it, given the acute nature of the intervention – see above). Consistent with the findings discussed in **Recovery after incomplete injury: neuromodulation**, as a result of the comparison between the two conditions, Augur prioritized V2a (i.e. Vsx2) and V1/V2b neurons, known to receive projections from proprioceptive afferent fibers recruited by EES.

My role was to demonstrate that lumbar V2a neurons possess the anatomical requirements to explain the involvement predicted by Augur in EES-mediated walking production: in other words, we aimed at showing direct connections of lumbar V2a/Vsx2 neurons on motor neurons.

To do this, I injected AAV-DJ-hSyn-FLEX-GFP-2A-Synaptophysin-mRuby [Stanford Vector Core Facility, reference AAV DJ GVVC-AAV-100, titer $1.15*10^{12}$ genome copies per ml; bilateral injections (0.25 µl per injection, 0.6 mm below the surface, rate 0.1 µl/min using glass micropipettes)] in the lumbar (L2) spinal cord of Vsx2-Cre mice. The DJ serotype[479] of AAVs is anterograde, and the specific AAV employed here is a bi-cistronic vector expressing two transgenes in a Cre-dependent way (FLEX): green fluorescent protein (GFP), under the control of the neuron-specific promoter synapsin (Syn), and a fusion complex of the pre-synaptic protein synaptophysin with the fluorescent protein mRuby. As a result of viral expression, neurons expressing Cre are labeled in their whole volume (soma, axon, dendrites) by the fluorescent protein GFP, and by mRuby specifically on their synaptic terminals. I processed the tissue via immunohistochemistry and imaged it via confocal microscopy [Zeiss LSM880 + Airy fast module with ZEN 2 Black software (Zeiss, Oberkochen, Germany)]. This allowed us to show that local-range Vsx2 neurons in the lumbar cord project and form direct synaptic connections onto Choline Acetyltransferase (ChAT)-positive motor neurons in the ventral laminae (**Figure 2.3**), therefore confirming anatomical features compatible with production of EES-driven motor output.

This role was then causally established experimentally via in loss of function studies in a paper published two years later, as already discussed in **Recovery after incomplete SCI: neuromodulation**.

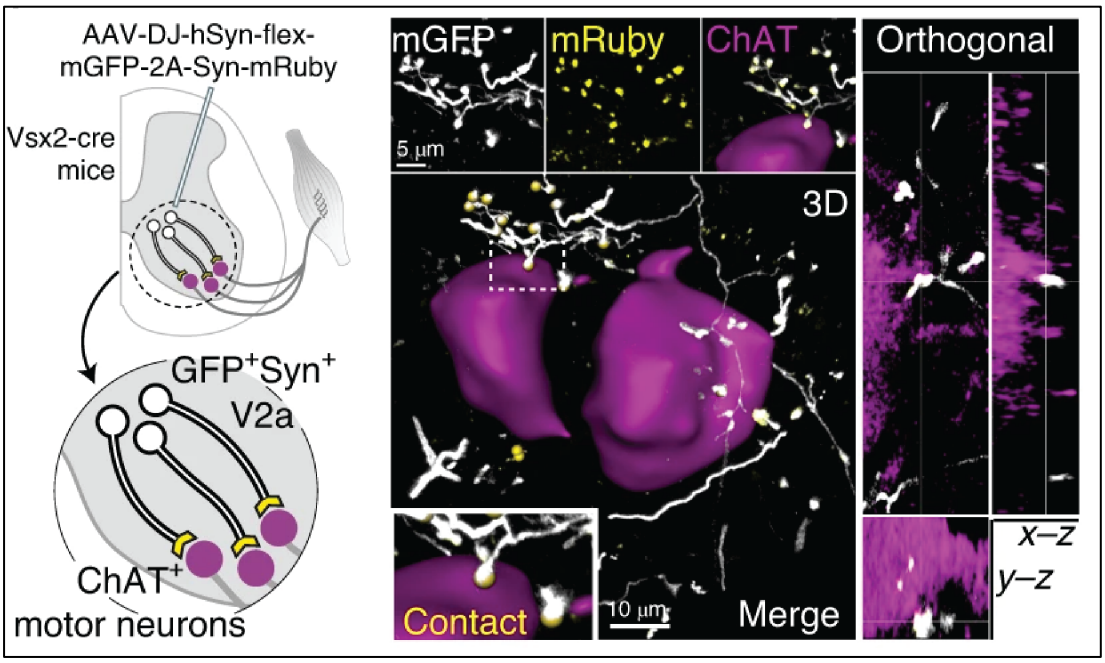

Figure 2.3. ***Histological confirmation of direct projections of local-range lumbar V2a neurons on motor neurons.*** *Confocal microscopy image with overlaid digital surface reconstruction [Imaris (Bitplane, v.9.0.0)] of ChAT-positive motor neurons (in magenta, as labeled via immunohistochemistry) receiving direct projections from lumbar local range V2a neurons (as labeled via Cre-dependent mono-synaptic tracing in Vsx2-Cre mice (axons – positive for GFP – are visualized in white, pre-synaptic terminals – labelled by mRuby - in yellow).*
Adapted with permission from Skinnider, Squair, et al., 2020[468].

Considerations and implementation of Augur in my PhD project

To summarize, Augur is a machine-learning based method designed to perform comparative analysis of single-cell/single-nucleus data across multiple experimental conditions, and outputs rankings of cell types according to the magnitude of their response to the perturbation expressed by the given experimental manipulation.

In the case of (sc/sn)RNAseq data, such response corresponds to the transcriptional change observed in the various cell types, with cells exhibiting the highest change being prioritized as the most responsive to the experimental perturbation. It is important to note that transcriptional change per se does not imply biological causality, meaning that cell types being most distinguishable on the transcriptome level across conditions do not necessarily correspond to the ones responsible for the phenotype observed as a result of the given experimental manipulation. Nonetheless, while such causality requires to be tested specifically with downstream experiments (e.g. loss-of-function studies), cell type prioritization helps direct these analyses by identifying the cell populations that are most recruited/affected by the manipulation.

Importantly, Augur was employed in the bioinformatic analyses included in my PhD project, and allowed us to identify the neuronal subpopulations underlying spontaneous recovery of locomotor function following severe but incomplete SCI, as described in the next Chapter.

3. RESTORING WALKING AFTER COMPLETE SPINAL CORD INJURY

The work described in this chapter constitutes the core of my PhD project, and is presented as from an original manuscript of which I am a co-first author, with adaptions and additions where necessary. The manuscript, at the moment of the submission of this thesis, is accepted for publication in the journal *Science*, with title and full author list being:

Squair, J. W.*, Milano, M.*, de Coucy, A., Gautier, M., Skinnider, M. A., James, N. D., Cho, N., Lasne, A., Kathe, C., Hutson, T. H., Ceto, S., Baud, L., Galan, K., Aureli, V., Laskaratos, A., Barraud, Q., Deming, T. J., Kohman, R. E., Schneider, B., He, Z., Bloch, J., Sofroniew, M. V.‡, Courtine, G.‡, & Anderson, M. A.‡ (2023) Recovery of walking after paralysis by regenerating characterized neurons to their natural target regions. *Science* [in press]

These results represent the product of more than 3 years of work, to which I contributed by being involved in most experimental procedures, from a part of the surgeries and in vivo procedures, to animal care and sacrifice, tissue dissection, histological processing, staining, and microscopy; I was also entirely in charge of the behavioral assessments and kinematic analyses, of 3D reconstruction of confocal images (for synapse detection and quantification) and of processing of light-sheet microscopy data of cleared tissue; I additionally participated in image post-processing (particularly generation of composite tiled images), quantifications/statistical analyses of axon regrowth, and contributed to revisions and assembly of figures. Contribution of the other authors is explained below in **Material and Methods**.

TARGETED REGENERATION OF CHARACTERIZED NEURONS RESTORES WALKING IN MICE

Introduction

As discussed before in the thesis, multiple types of experimental approaches can be leveraged to induce transected axons of injured central nervous system (CNS) neurons to regenerate across anatomically complete spinal cord injury (SCI)[332,417]. Analogously, caudalized neural progenitors grafted into the lesion in combination with growth factors can integrate in the host tissue extending their axons over long distance, and can attract host axons inside the injury site[379]. Nonetheless, despite the extensive axon regeneration achieved through these interventions, reproducible restoration of meaningful function following anatomically complete SCI has been elusive, suggesting that essential yet unidentified mechanisms to promote locomotor recovery have yet to be identified. Unknown is whether robust restoration of function will require targeting defined subpopulations of neurons, and whether regeneration of axons of these neurons not simply across lesions, but directed specifically to their natural target region, is required. To address these questions, we shifted our focus towards a model of severe but incomplete SCI following which spontaneous recovery of walking occurs[25,143], and we applied projection-specific and comparative single-nucleus RNA sequencing to dissect the transcriptional identity and connectome of neuronal subpopulations involved in such functional recovery. We identified as a main candidate a molecularly defined population of excitatory projection neurons in the thoracic spinal cord that extend axons to the lumbar spinal cord where walking execution centers reside. We then turned back to our model of anatomically complete thoracic SCI, and compared the functional effects of regenerating the transected axons of these neurons to reach simply across the thoracic lesions versus chemoattracting and guiding the axons to reach their distal natural target region in the lumbar spinal cord.

Main

Identification and characterization of thoracic neurons with natural projections to the lumbar spinal cord

The lumbar spinal cord hosts the neuronal subpopulations constituting the walking execution centers. As discussed in **Recovery after incomplete injury: natural repair**, while unilateral hemisection SCIs deprive these neurons of direct supraspinal connections essential to produce walking on the injured side, both animal models and humans are able to recover bilateral basic locomotion over the course of time, as a result of natural reorganization of midthoracic spinal cord interneurons that relay the supraspinal commands around and past the lesion[143,149,480]. The same neurons are also sufficient to restore bilateral walking if direct supraspinal projections are completely severed, as observed in the staggered delayed double hemisection SCI model, where two temporally and spatially separated, opposite-side hemisections are performed[143]. This spontaneous restoration of walking ability highly contrasts with the lack of functional recovery observed, in spite of axon regeneration across complete SCI, with available regenerative interventions, including our previously developed strategy [ref. [332]]. To uncover the mechanisms underlying spontaneous recovery of walking following incomplete SCI, we therefore aimed to characterize whether specific subpopulations of

thoracic interneurons orchestrate this natural repair, and to dissect the anatomical and molecular properties of these neuronal subtypes.

We first sought to identify the neuronal subpopulations that naturally possess projections to walking execution centers and, to this purpose, we injected into the lumbar spinal cord of uninjured mice the retrogradely-traveling rAAV2 encoding the fluorescent protein eGFP fused to the nuclear envelope protein KASH[481] (**Figure 3.1A**). This strategy labeled the nuclei of neurons with direct projections to the walking execution centers throughout the CNS, including relay interneurons in the midthoracic spinal cord, and enabled fluorescence-activated nuclear sorting (FANS) coupled to projection-specific single-nucleus RNA sequencing (snRNA-seq) (**Figure 3.1A-C**). We profiled the midthoracic spinal cord with snRNA-seq and obtained high-quality single-nucleus transcriptional profiles from 122 $eGFP^{ON}$ and 2,823 $eGFP^{OFF}$ nuclei (**Figure 3.1D-I**). Unsupervised clustering followed by annotation identified all of 6 the major cell types of the mouse spinal cord (**Figure 3.1J-M**).

We then restricted our analysis to neurons and subjected these to a second round of clustering, which identified 28 subpopulations of neurons expressing canonical marker genes (**Figure 3.2A, C**). Our taxonomy parcellated cardinal spinal neuron classes into motor-sensory, local-long range, and excitatory-inhibitory neuronal subpopulations (**Figure 3.2D-F**), as first illustrated elsewhere[453].

Strikingly, the 105 $eGFP^{ON}$ nuclei, representing all retrieved neurons projecting axons from the mid-thoracic to the lumbar spinal cord, were found to belong primarily (~30%) to one single population of thoracic (Hoxa7) interneurons, i.e. a subtype of Vsx2 neurons expressing the key marker of long-distance projection neurons**, Zfhx3[453] (**Figure 3.2B,G-H**). We named this subpopulation spinal cord (SC)$^{Vsx2::Hoxa7::Zfhx3\rightarrow lumbar}$ or ventral excitatory projection Vsx2 (VEP^{Vsx2}) neurons.

Since these neurons express Vsx2[482–486], they derive from developmentally-defined V2a neurons. It is important to emphasize that, while this VEP^{Vsx2} represent a subclass of these extensively characterized neurons, V2a neurons are a highly heterogeneous population endowed with different functions, as already discussed in **Recovery after incomplete injury: neuromodulation**. Vsx2-expressing neurons are found in widely different locations along the neuraxis including the brainstem[133,487,488], cervical spinal cord[131,134,489,490], and lumbar spinal cord[30,128–131,135,136], where they exhibit a variety of projection patterns[128,491–494]. Accordingly, the distinct properties of different subpopulations of Vsx2-expressing neurons dictate and restrict their specific contribution to neurological functions, such as reaching[131,134,490] and locomotion[30,128–131,133,135,136,487,488]. Indeed, although developmentally-defined V2a neurons located in the lumbar spinal cord have been implicated in the production of walking[128–131,133,135,136,487,488], the ablation of all midthoracic neurons, including those expressing Vsx2, has no detectable impact on walking in uninjured animal models[143,495].

** Long-range projection neurons have previously been referred to also group-Z neurons[453].

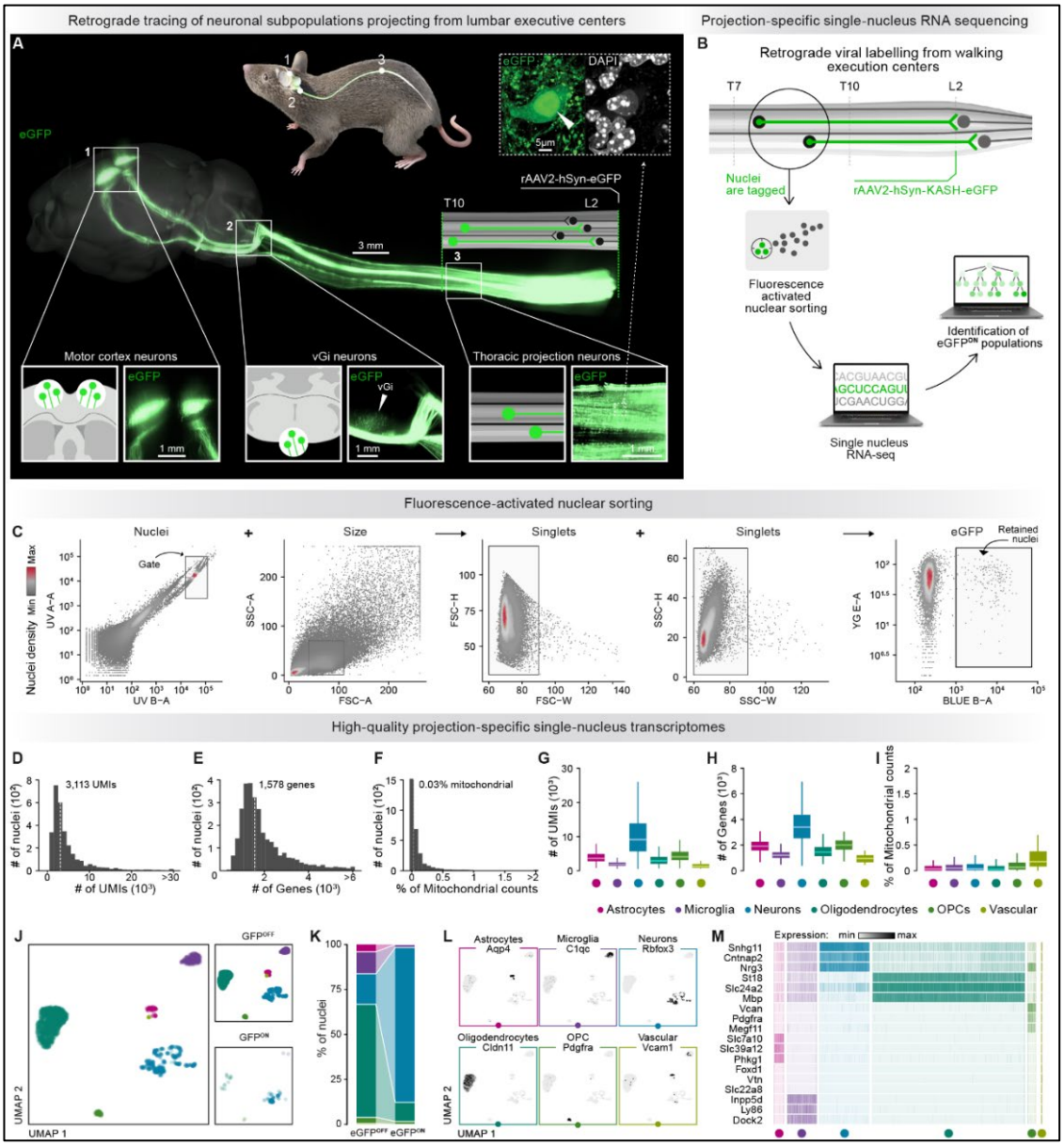

***Figure 3.1.* Projection-specific single-nucleus RNA-sequencing of the mouse lower thoracic spinal cord.**
(A) CLARITY-optimized light sheet microscopy image of an entire mouse central nervous system following the administration of rAAV2-hSyn-eGFP in the lumbar spinal cord. Neurons with direct projections to the lumbar spinal cord are visualized throughout the central nervous system, including propriospinal neurons in the lower thoracic spinal cord. (B) Scheme illustrating our tracing approach, leveraging enhanced green fluorescent protein (eGFP) expression fused to the nuclear envelope protein KASH to enable fluorescence-activated nuclei sorting followed by single-nucleus RNA sequencing. (C) Fluorescence-activated nuclei sorting of eGFP^{ON} neurons from the lower thoracic spinal cord. Through a series of sorting gates isolating singlet nuclei of the appropriate size, we are able to enrich for eGFP^{ON} nuclei, which are then sent for subsequent single-nucleus RNA sequencing. UV-A: ultraviolet light A; UV-B: ultraviolet light B; FSC: forward-scattered light; SSC: side-scattered light; YG E: yellow-green emission; BLUE B: blue emission; –W: width; –H: height; –A: area. A combination of ultraviolet spectrum and size gating is first used to sort nuclei. This is followed by further size gating to remove doublets, and finally wavelength emission gates to enrich for nuclei expressing eGFP. (D) Number of unique molecular identifiers (UMIs) per nucleus. Inset text shows the median number of UMIs. (E) Number of genes detected per nucleus. Inset text shows the median number of genes detected. (F) Proportion of mitochondrial counts per nucleus. Inset text shows the median proportion of mitochondrial counts. (G) Number of UMIs quantified per nucleus in each major cell type of the mouse spinal cord. (H) Number of genes detected per nucleus in each major cell type of the mouse spinal cord. (I) Proportion of mitochondrial counts per nucleus in each major cell type of the mouse spinal cord. (J) UMAP visualization of 2,945 nuclei colored by major cell types, revealing the successful recovery of neurons in the GFP^{+} library. (K) Proportions of each major cell type of the thoracic spinal cord recovered in individual libraries from each sorting condition. (L) Expression of key marker genes for the six major cell types of the thoracic spinal cord. (M) Expression of the top three key marker genes recovered by unbiased differential expression analysis for the six major cell types of the thoracic spinal cord.

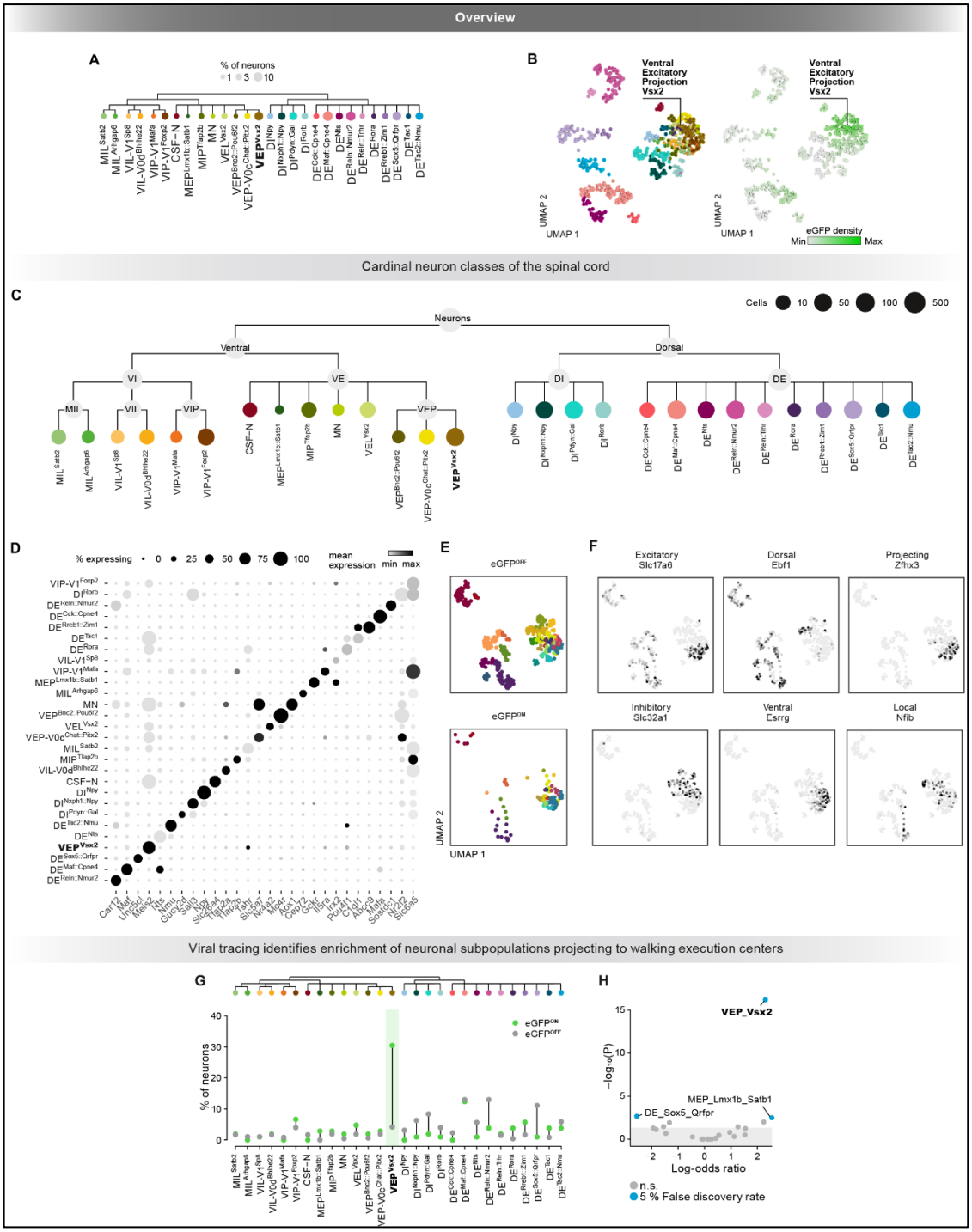

Figure 3.2. A projection-specific single-nucleus atlas of neuronal subpopulations in the mouse thoracic spinal cord.
(A) Clustering tree[496] of neuronal subpopulations in the thoracic spinal cord. (B) Uniform manifold approximation and projection (UMAP) visualization of neuronal nuclei revealing 28 neuron subpopulations (left). Individual nuclei are colored by the proportion of their nearest neighbors obtained from sorted projection neurons (eGFP density), revealing a primary origin from SC$^{Vsx2::Hoxa7::Zfhx3 \rightarrow lumbar}$ (VEP^{Vsx2}) neurons (right). (C) Alternative representation of the clustering tree in (A), revealing a hierarchical taxonomy of transcriptionally defined neuron subtypes over four clustering resolutions. (D) Dot plot showing expression of a single marker gene per cell type for the 28 transcriptionally defined neuronal subpopulations of the mouse thoracic spinal cord. (E) UMAP visualization of 582 neuronal nuclei colored by sorting status. (F) Expression of key neurotransmitters for excitatory (Slc17a6), inhibitory (Slc31a1), dorsal (Ebf1), ventral (Esrrg), projecting (Zfhx3), and local (Nfib) genes demonstrating a logical topography of neurons in the spinal cord. (G) Proportions of each neuronal subtype of the thoracic spinal cord recovered in each sorting condition. (H) Volcano plot showing the statistical significance of neuronal subpopulation enrichment in the eGFP^{ON} condition.

Identification and characterization of neurons involved in natural recovery

While ablation of thoracic neurons has no effect on walking in uninjured mice[143], the same neurons become essential to walk after severe incomplete SCI, as ablation completely abolishes the observed functional improvements following staggered double hemisection SCI, highlighting a specific role in natural recovery[143].

We therefore asked whether the same subtype of thoracic interneurons that possess natural projections to the lumbar centers in the uninjured spinal cord, $SC^{Vsx2::Hoxa7::Zfhx3 \rightarrow lumbar}$ neurons, are also transcriptionally altered following the natural repair that occurs after severe incomplete SCI. To address this question, we sought to compare neuronal nuclei from uninjured mice and mice undergoing natural repair after temporally and spatially separated lateral hemisection SCIs.

Staggered delayed double hemisection SCI was performed as described in the original study [ref. 143] (**Figure 3.3A**). Kinematic analysis showed acute unilateral paralysis after the first SCI, followed by recovery of walking by 10 weeks post injury, acute bilateral paralysis after the second hemisection and recovery of bilateral walking by 4 weeks after the second injury, demonstrating consistent replication of the previous findings[143] (**Figure 3.3B-E**).

High-quality transcriptional profiles were obtained from 9,264 nuclei extracted from thoracic spinal cord tissue in the bridge between the two lesions (**Figure 3.4A,C-I**). From these nuclei, representing all the major cell types in the mouse spinal cord (**Figure 3.4J-M**), the 3,256 neurons were subjected to a second round of clustering. The data were integrated[497] with our projection-specific snRNA-seq experiment from uninjured mice, wherein we identified and evaluated the same 28 neuronal subpopulations (**Figure 3.4N-O**). We then performed comparative analysis of the two experimental conditions with Augur [see **Augur: Cell-type prioritization in single-cell data**]: cell type prioritization revealed that $SC^{Vsx2::Hoxa7::Zfhx3 \rightarrow lumbar}$ neurons exhibited the most profound transcriptional response across all neuronal subpopulations embedded in the thoracic segments of mice that had recovered walking (**Figure 3.4B,P-S**) . We additionally implemented Gene Ontology analysis on the snRNA-seq data, which revealed that the transcriptional responses involved the upregulation of dendritic spine morphogenesis pathways, synaptic potentiation programs, and actin cytoskeleton reorganization (**Figure 3.4T**).

These findings are all consistent with an involvement in natural spinal cord repair, and suggest that $SC^{Vsx2::Hoxa7::Zfhx3 \rightarrow lumbar}$ neurons are one putative neuronal subpopulation implicated in spontaneous recovery following severe incomplete SCI.

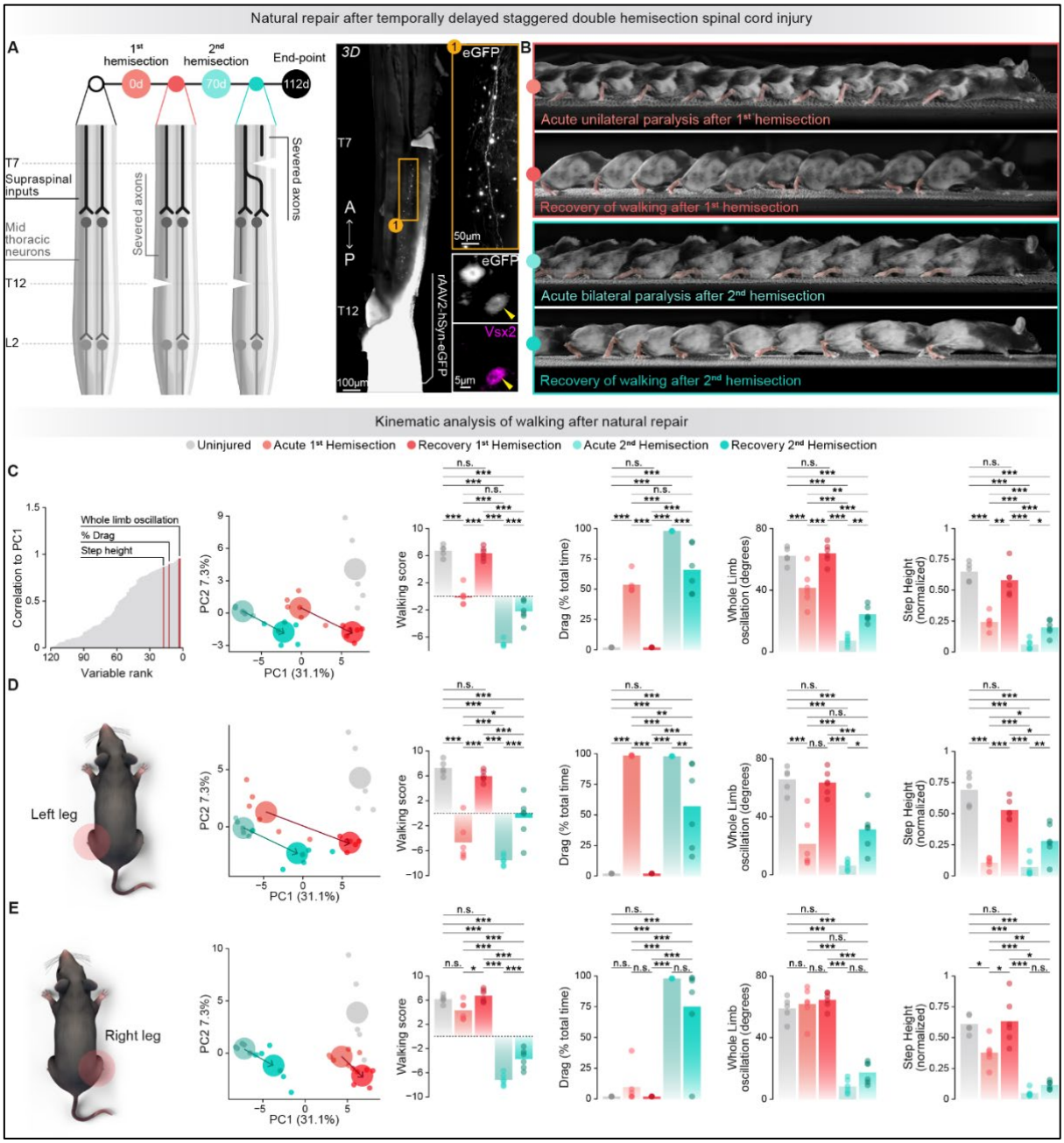

***Figure 3.3.* Natural recovery of walking after staggered delayed double hemisection SCI.**

(A) Scheme illustrating the experimental approach for delayed, staggered double hemisections in mice. CLARITY-optimized light sheet microscopy image of a thoracic mouse spinal cord following staggered double hemisection SCI. Bilateral retrograde tracing from lumbar segments was performed using rAAV2-hSyn-eGFP to visualize lower-thoracic neurons and their axonal projections spared by the two injuries (top right). (B) Leg movements during overground walking without any intervention or support recorded after the first hemisection (top left), immediately prior to the second hemisection (top right), after the second hemisection (bottom left), and at the end of the experiment, wherein full natural recovery had occurred (bottom right). (C) Locomotor performance was quantified using principal component analysis applied to gait parameters calculated from kinematic recordings. In this denoised space, each dot represents a gait cycle (n > 10 per mouse, n = 6 mice per group, n = 5 mice in the uninjured group). Larger dots represent the mean of each experimental group. The first principal component (PC1) distinguished gaits from mice without SCI from mice with the most severe paralysis, found immediately after the second hemisection. Locomotor performances were thus quantified as the scores on PC1. Analysis of factor loadings on PC1 revealed that the percentage of paw dragging, the extent of whole-limb oscillation (virtual limb connecting the hip to the toe) and step height were the parameters that showed high correlation with PC1. Bars report the mean values of these gait parameters (n = 6 mice per group; statistics indicate Tukey HSD tests following one-way ANOVA. Raw data and statistics are provided in the Supplementary Material, available upon publication. (D) As in (C) but only for gait cycles from the left leg. (E) As in (C) but only for gait cycles from the right leg.

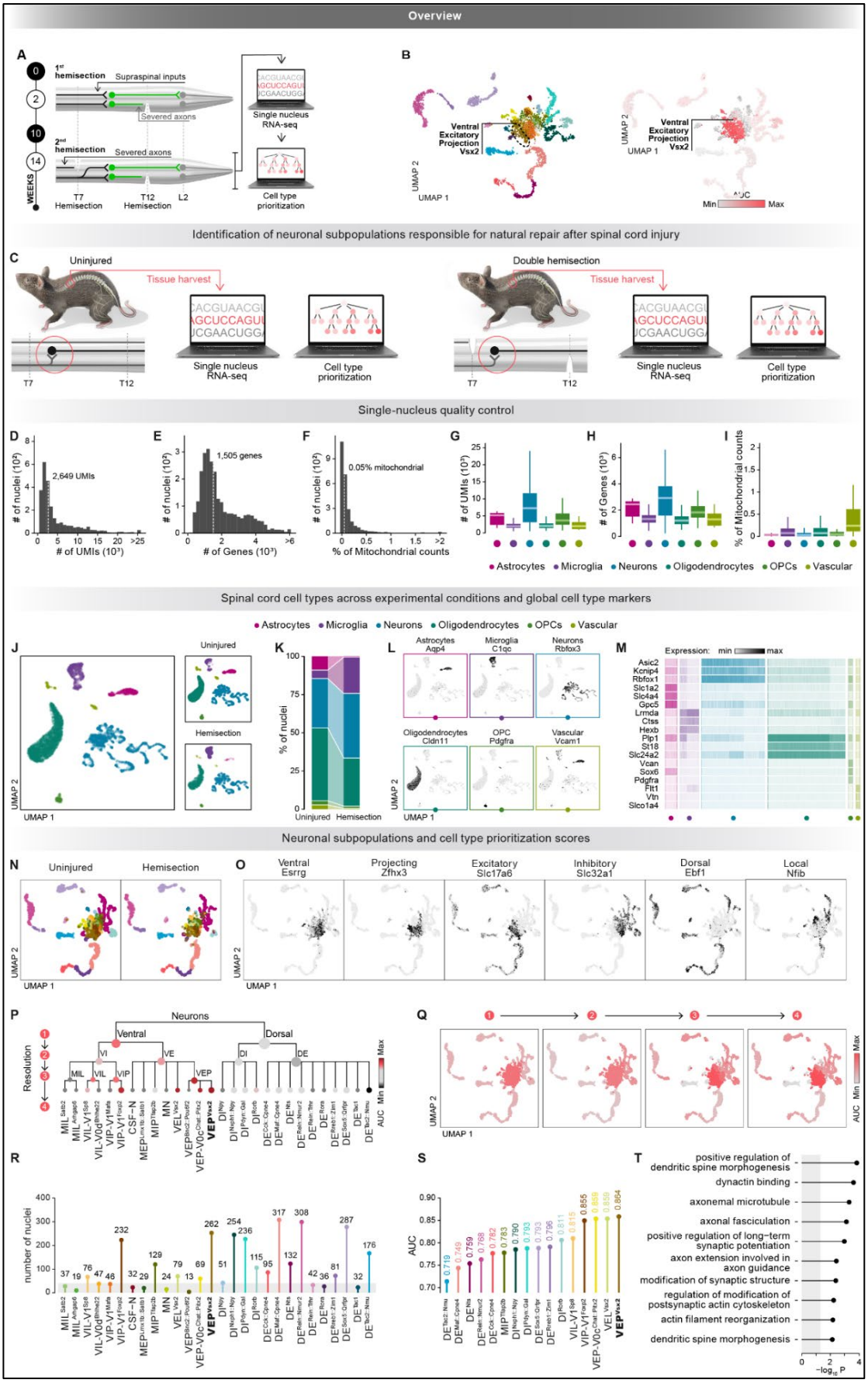

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***Figure 3.4.* Comparative single-nucleus RNA-sequencing of the mouse lower thoracic spinal cord in uninjured mice and those that naturally recovered walking after incomplete SCI..**

(A) Overview of the experimental approach enabling single-nucleus RNA sequencing (snRNA-seq) following natural spinal cord repair. (B) UMAP visualization of neuronal nuclei revealing 28 neuron subpopulations (left) and colored by Augur cell type prioritization (AUC), identifying perturbation-responsive neuronal subpopulations when comparing mice that had undergone natural repair versus uninjured mice (right).Leg movements during overground walking without any intervention or support recorded after the first hemisection (top left), immediately prior to the second hemisection (top right), after the second hemisection (bottom left), and at the end of the experiment, wherein full natural recovery had occurred (bottom right). (C) Scheme illustrating the experimental approach for comparative analysis. (D) Number of unique molecular identifiers (UMIs) per nucleus. Inset text shows the median number of UMIs. (E) Number of genes detected per nucleus. Inset text shows the median number of genes detected. (F) Proportion of mitochondrial counts per nucleus. Inset text shows the median proportion of mitochondrial counts. (G) Number of UMIs quantified per nucleus in each major cell type of the mouse spinal cord. (H) Number of genes detected per nucleus in each major cell type of the mouse spinal cord. (I) Proportion of mitochondrial counts per nucleus in each major cell type of the mouse spinal cord. (J) UMAP visualization of 9,264 nuclei colored by major cell type, revealing consistent recovery of all major transcriptional states in each experimental condition. (K) Proportions of each major cell type of the thoracic spinal cord recovered in each experimental condition. (L) Expression of key marker genes for the six major cell types of the thoracic spinal cord. (M) Expression of the top three key marker genes recovered by unbiased differential expression analysis for the six major cell types of the thoracic spinal cord. (N) UMAP visualization of 3,256 neuronal nuclei colored by neuronal subpopulation, split by experimental condition. (O) Expression of key neurotransmitters for excitatory (Slc17a6), inhibitory (Slc31a1), dorsal (Ebf1), ventral (Esrrg), projecting (Zfhx3), and local (Nfib) genes demonstrating a logical topography of neurons in the spinal cord. (P) Clustering tree of neurons in the mouse thoracic spinal cord, revealing a hierarchical taxonomy of transcriptionally defined neuron subtypes over four clustering resolutions. Colored by the cell type prioritization score (AUC; area under the receiver operating characteristic curve), derived from Augur. (Q) Cell type prioritizations over increasingly granular clustering resolutions are visualized on a progression of UMAPs, with neuronal subtypes colored by the strength of the perturbation response, as inferred by Augur. (R) Proportions of each neuronal subpopulation of the thoracic spinal cord. (S) Lollipop plot demonstrates the ranking of Augur scores across all neuronal subpopulations. (T) Gene programs activated in response to natural repair, as from Gene Ontology analysis. Shaded region denotes non-significance. P-values are reported in the Supplementary Material, available upon publication.

Connectome features of thoracic relay neurons

In light of the results of our projection-specific and comparative snRNA-seq analyses, implicating a role of $SC^{Vsx2::Hoxa7::Zfhx3\rightarrow lumbar}$ neurons in the restoration of walking after natural spinal cord repair, we hypothesized that these neurons must possess connectome features compatible with the requirements to walk after paralysis. We therefore sought to uncover the input and output connections of this subpopulation via tract-tracing.

Visualization of the projectome from all neurons embedded in the midthoracic spinal cord, as achieved via standard anterograde tracing, revealed dense projections throughout the lumbar spinal cord wherein walking execution centers reside (**Fig. 3.6A**).

To restrict this analysis to Vsx2 neurons, we injected a Cre-dependent anterograde tracer in the midthoracic spinal cord of Vsx^{Cre} mice, which revealed the expected presence of dense projections throughout walking execution centers (**Figure 3.6C**). Immunohistochemistry analysis of the distribution of Vsx2 neurons in the midthoracic spinal cord showed that $Vsx2^{ON}$ neurons located in the midthoracic spinal cord accounted for 5.9% of all neurons (as identified through NeuN immunoreactivity) in this region (**Figure 3.6B**) which agreed with the distribution of neurons identified in our snRNA-seq data (**Figure 3.2E-F**).

We next sought to visualize specifically the subpopulation of Vsx2 neurons possessing the transcriptional phenotype relevant for natural recovery, i.e. $SC^{Vsx2::Hoxa7::Zfhx3\rightarrow lumbar}$ neurons, and we therefore asked whether midthoracic $Vsx2^{ON}$ neurons could be stratified via tracing and immunohistochemistry into subpopulations projecting locally (i.e.

Zfhx3^{OFF}) versus over long distances (i.e. Zfhx3^{ON}). To this purpose, we employed a combinatorial tracing strategy based on intersectional genetics: briefly, we injected Cre- and FLPo-dependent anterograde-travelling AAV5-Con/Fon-eYFP in the midthoracic spinal cord of Vsx2^{Cre} mice, and contextually infused retroAAV2-Ef1α-Flpo in the lumbar spinal levels (**Figure 3.6D**). This tracing strategy enabled the exclusive labeling of Vsx2^{ON} neurons that projected to walking execution centers in the lumbar spinal cord (**Figure 3.5A**, **Figure 3.6D-E**). Immunostaining against Zfhx3 and Vsx2 showed that the two markers co-localized only in neurons projecting to this region (i.e., SC$^{Vsx2::Hoxa7::Zfhx3\rightarrow lumbar}$) (**Figure 3.5B**), providing additional confirmation that Zfhx3 is an accurate marker to target spinal cord neuron subpopulations with long-distance projections[453]. Quantification of local (Vsx2^{ON}Zfhx3^{OFF}) versus long-distance projecting (Vsx2^{ON}Zfhx3^{ON}) Vsx2 neurons revealed a near equal distribution of these two subpopulations throughout the midthoracic spinal cord (**Figure 3.6E**). Together these findings confirmed that a subset of Vsx2 neurons embedded in the midthoracic spinal cord co-express Zfhx3 and extend dense projections to the lumbar spinal cord wherein walking execution centers reside.

We next aimed to study relevant input connections to SC$^{Vsx2::Hoxa7::Zfhx3\rightarrow lumbar}$. We reasoned that in order to function as relays of supraspinal commands, these interneurons must also receive direct projections from key supraspinal neurons involved in the recovery of walking after paralysis. One motor center crucially important for functional recovery after SCI is the ventral gigantocellular nucleus (vGi), as we discussed in **Recovery after incomplete injury: neuromodulation**. To expose this connectome, we infused AAV5-CMV-TurboRFP into the vGi of uninjured Vsx2^{Cre} mice to label descending reticulospinal axons, followed by infusions of rAAV2-hSyn-GFP at spinal level L2 to label midthoracic axons projecting to the lumbar spinal cord, and immunostaining for vGlut2 synaptic puncta and Vsx2 (**Figure 3.5C**). As anticipated, we found that Vsx2^{ON}Zfhx3^{ON} neurons located in the midthoracic spinal cord receive projections from the vGi (**Figure 3.5C**). We additionally employed the same tracing strategy in mice recovering from staggered

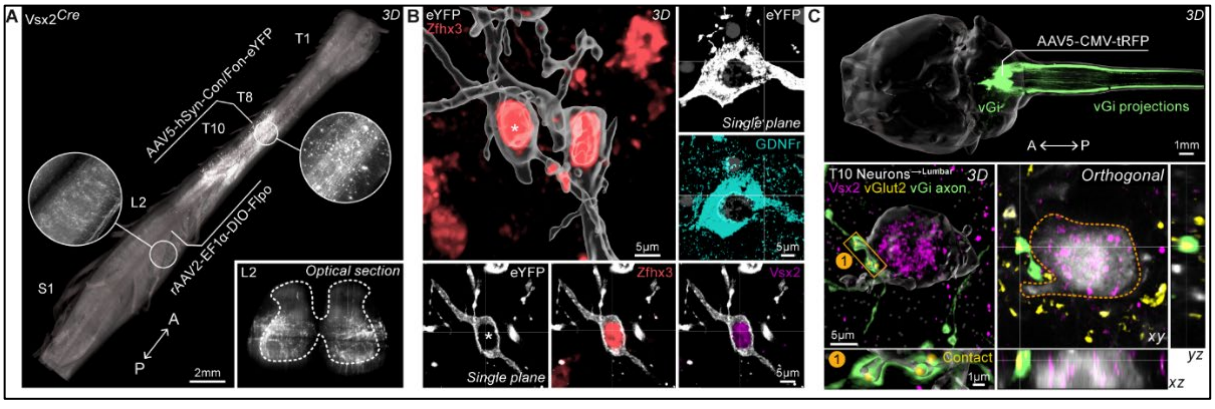

Figure 3.5. Projection and connectome features of SC$^{Vsx2::Hoxa7::Zfhx3\rightarrow lumbar}$ neurons.
(A) Whole spinal cord visualization of projections from Vsx2 neurons in the lower thoracic spinal cord that project to lumbar walking execution centers. Insets illustrate the starter neurons labeled using intersectional viral tracing, and their projections in the lumbar spinal cord. (B) Vsx2 neurons with projections to lumbar walking execution centers express Zfhx3, a key marker of projection neuronal subpopulations. These neurons also express Gdnfr on the neuron soma as well as along the axon. (C) Overview of the experimental approach enabling anterograde viral tracing of ventral gigantocellular nuclei (vGi) neurons in both uninjured mice and mice that underwent natural repair. 3D view of synapse-like contact of Vsx2 neurons in the lower thoracic spinal cord with vGi virally traced projections, indicated with the presynaptic marker vGlut2.

delayed double hemisection SCI, and showed that this projection pattern is maintained after natural spinal cord repair (**Figure 3.6F-G**).

Together, these results indicated that amongst the diverse populations of cells in the midthoracic spinal cord[453,468,469,491,498–500], $SC^{Vsx2::Hoxa7::Zfhx3 \rightarrow lumbar}$ neurons were not only the most transcriptionally responsive neuronal subpopulation during natural spinal cord repair, but also exhibited the relevant projectome and receptome profiles to relay supraspinal commands past the incomplete SCI to walking execution centers located in the lumbar spinal cord.

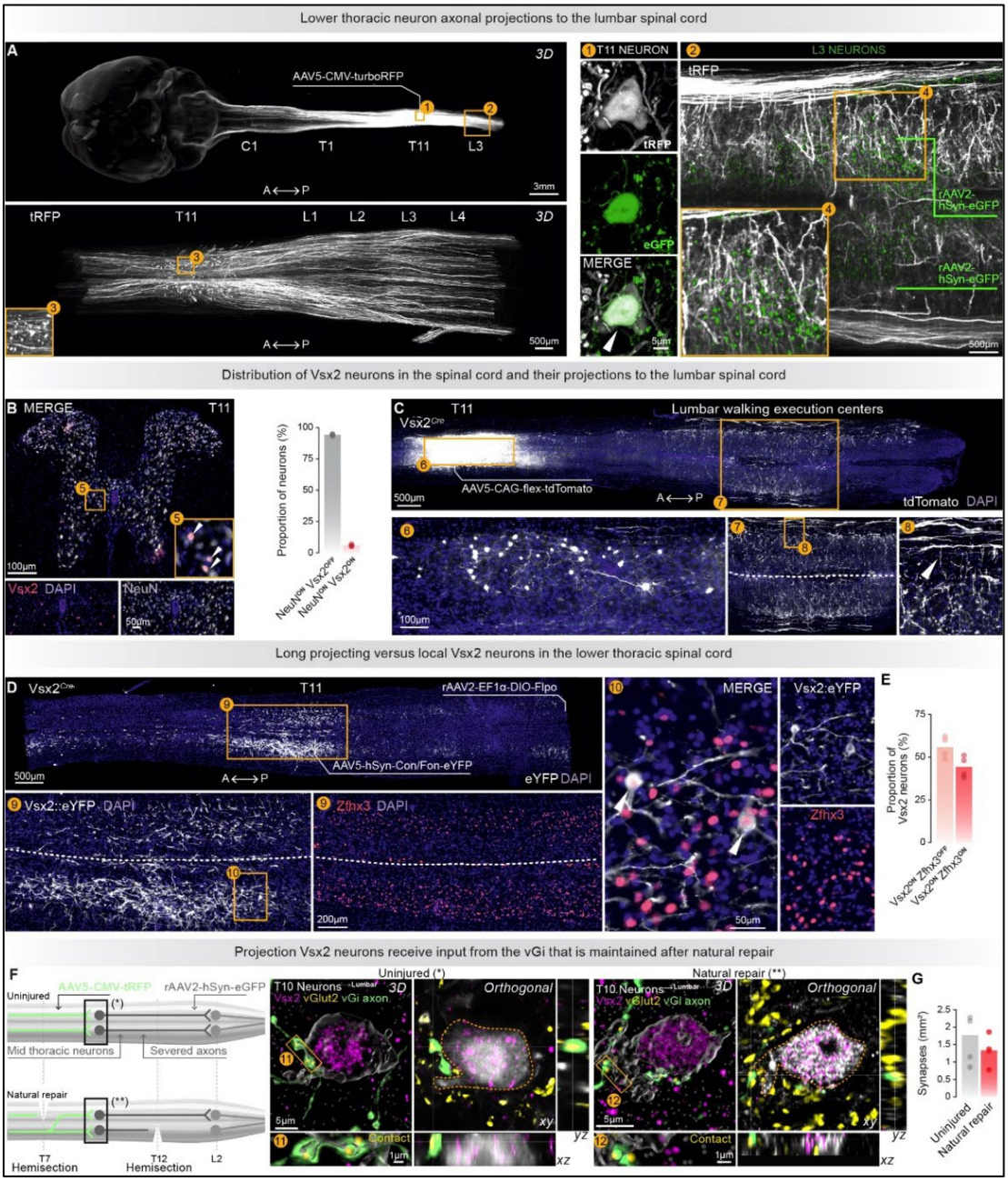

Figure 3.6. ***Characterization of projection Vsx2 neurons.***

(A) CLARITY-optimized light sheet microscopy image of the central nervous system of a mouse virally traced with (i) AAV5-CMV-turboRFP to visualize non-specific axon projections of spinal cord neurons (top left and bottom left), and (ii) rAAV2-hSyn-eGFP to label neurons with projections to the walking execution centers of the lumbar spinal cord (top right and middle).

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(B) Coronal section of a T11 spinal segment immunohistochemically stained for Vsx2 (red) and DAPI (blue) (left). Quantification depicting the proportion of neurons in the mid-thoracic spinal cord that are positive for Vsx2. (C) Horizontal spinal cord section of a $Vsx2^{Cre}$ mouse traced with AAV5-CAG-flex-tdTomato. Note the density of Vsx2 axons from mid-thoracic spinal segments projecting to lumbar walking execution centers (top). Photomicrographs highlighting insets from above image (bottom). (D) Horizontal spinal cord section of a $Vsx2^{Cre}$ mouse with intersectional viral tracing to exclusively label projecting Vsx2 neurons. To achieve this, AAV5-hSyn-ConFon-eYFP was infused into midthoracic spinal segments and rAAV2-EF1 α-DIO-Flpo was infused into walking execution centers of the lumbar spinal cord (top left). Photomicrographs of inset above showing density of long projecting Vsx2 neurons (bottom left). Photomicrographs showing colocalization of eYFP positive Vsx2 neurons with Zfhx3 (middle). Quantification of local vs. projecting Vsx2 neurons (right). (E) Quantification of local vs. projecting Vsx2 neurons (right) from sections immunohistochemically stained for Vsx2 and Zfhx3. (F) Synapse density on $Vsx2^{ON}$ neurons in the mid-thoracic spinal cord from vGi projections in uninjured mice and mice that underwent natural repair. (G) Quantification of synapse density of vGi projections onto long-projecting $Vsx2^{ON}$ neurons in the lower thoracic spinal cord in uninjured mice and mice that underwent natural repair (n = 4 mice per group, independent samples Wilcoxon rank-sum test, W = 6, p = 0.6857).

Extended depot-based chemoattraction promotes successful regeneration of axons to their natural target region after complete SCI but no functional recovery

As discussed in **Regeneration after complete SCI**, our group previously found that providing factors essential for axon growth during development was able to support spinal cord axon regeneration across anatomically complete SCI lesions into viable neural tissue located a segment below the injury; nevertheless, the regrowth associated with this regenerative strategy did not lead to the recovery of walking[332]. Based on the findings that we described above, we hypothesized that meaningful recovery of walking after anatomically *complete* SCI could be achieved by reestablishing the natural projection patterns of neuronal subpopulations that are involved in the restoration of walking after anatomically *incomplete* SCI. We therefore next sought to determine whether $SC^{Vsx2::Hoxa7::Zfhx3\rightarrow lumbar}$ neurons could be regenerated to reach their natural target region in the lumbar spinal cord, where walking execution centers reside.

To test this possibility, we first aimed at assessing the impact on this specific neuronal subpopulation of our previously established regeneration strategy for spinal cord neurons. As already described, this intervention harnesses three specific developmental mechanisms[332]: first, we reactivated the intrinsic growth capacity of neurons located above the SCI with viral overexpression of osteopontin (Spp1), insulin-like growth factor 1 (Igf1) and ciliary-derived neurotrophic factor (Cntf) (AAV-OIC)[398]; second, we induced the formation of axon growth supportive substrates by temporal delivery of fibroblast growth factor 2 (FGF2) and epidermal growth factor (EGF); third, we delivered biomaterial depots of glial-derived neurotrophic factor (GDNF) as a chemoattractive agent within and to sequentially spaced sites below the injury[332,400,501] (**Figure 3.7A**). Analysis of snRNA-seq data confirmed the expression of Gdnf receptor, Gfra1, and its signaling receptor Ret in $SC^{Vsx2::Hoxa7::Zfhx3\rightarrow lumbar}$ neurons, both of which are required for appropriate Gdnf signaling, and immunohistochemistry of $Vsx2^{ON}$ axons traced with AAV5-Con/Fon-eYFP further validated expression of the Gdnf receptor within the soma and along the axons from $SC^{Vsx2::Hoxa7::Zfhx3\rightarrow lumbar}$ neurons traced with AAV5- Con/Fon-eYFP (**Figure 3.5B**). These findings confirm the potential of $SC^{Vsx2::Hoxa7::Zfhx3\rightarrow lumbar}$ neurons to be recruited in response to our regenerative intervention.

Consistent with our previous observations[332], we again found that stimulated, supported, and chemoattracted axons from thoracic interneurons regrew robustly through astrocyte borders, across the fibrotic scar, and into viable neural tissue below an

anatomically complete SCI (**Figure 3.7A**). Nevertheless, regenerating axons terminated only one segment below the injury where the most distal GDNF containing biomaterial depot had been infused. Accordingly, as observed before[332], even high-precision behavioral assessments conducted at four weeks post-injury failed to detect any recovery of lower limb movements (**Figure 3.7B**). This observation contrasted with the

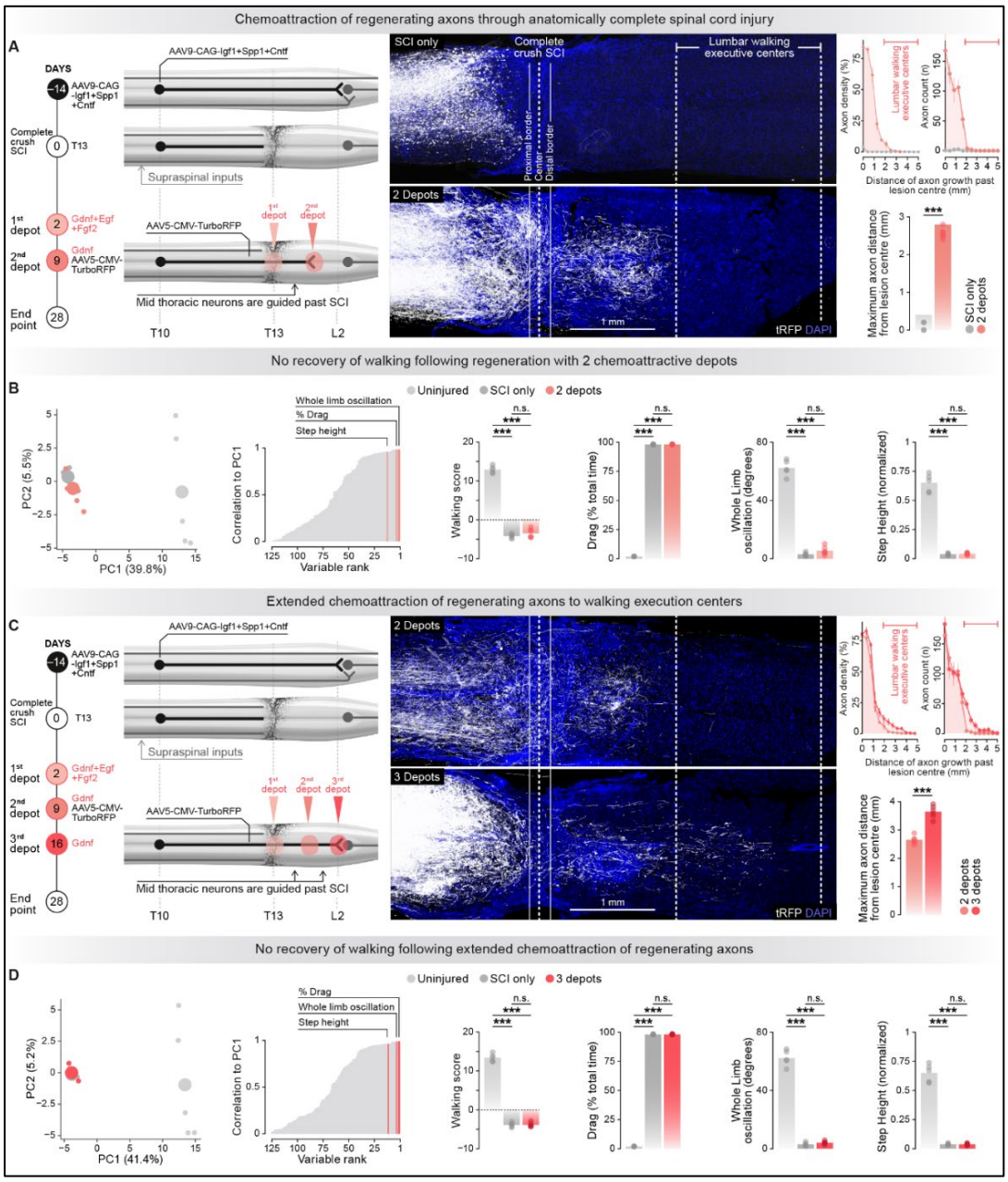

Figure 3.7. Natural recovery of walking after staggered delayed double hemisection SCI.

(A) Mice received a combination of procedures including adeno-associated virus (AAV) injections, complete crush SCI, injections of two or three depots of hydrogel containing glial-derived neurotrophic growth factor (GDNF) injections of AAV-CMV-RFP for axonal tract-tracing. AAV injections to upregulate intrinsic growth programs were made two weeks before SCI to allow time for molecular expression and were targeted at neurons in the lower thoracic spinal cord, one to two segments rostral to the planned location of the SCI lesion. Complete crush SCI lesions were placed at the level of spinal segment T10/T11.

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Left: schematic and timeline of two-depot experiments. Two days after complete crush SCI, animals received the first hydrogel injection into the center of the non-neural lesion core to deliver the growth factors FGF + EGF + GDNF. Nine days after SCI, the animals received a second hydrogel injection targeted to spared neural tissue 1 to 2 mm caudal to the lesion center to deliver GDNF to chemoattract axons that had regrown into the lesion core. Viral injections for axonal tract-tracing were targeted at neurons between one and two segments rostral to SCI lesions and were placed one month following the crush SCI. Tissue was collected for evaluation at four weeks after SCI. Middle: large area survey representative images of $tRFP^+$ labeled axons in composite mosaic scans of horizontal sections. In a control mouse (top) that received SCI only, few axons reach the lesion center, almost none pass beyond and no axons are present below the level of the injury. In the representative treated mouse (bottom) that received our combinatorial treatment, many axons regrow through the lesion core and reach or pass 1.5 mm beyond the lesion center. However, note also that there are no axons present within the L2 segment, demonstrating that this regenerative approach does not restore the projection patterns observed following natural spinal cord repair. Right: axon density and counts at specific distances past lesion centers normalized to the density rostral to the injury site. Bar graph indicates the distance of the furthest positive axon density (independent samples two-tailed t-test; n = 5 (SCI) and n = 6 (2D) animals per group; $t = 33.5$; $p = 9.3 \times 10^{-11}$).
*(B) Locomotor performance as in **Figure 3.3** (n > 10 gait cycles per mouse, n = 5 mice per group, n = 6 mice in the two-depot (2D) group; statistics indicate Tukey HSD tests following one-way ANOVA). Note that there is no improvement in walking following combinatorial treatment with a two-depot approach, in line with our previous work. Raw data and statistics are provided in the Supplementary Material, available upon publication. (C) As in (A) but animals received an additional GDNF depot on day 9 after the crush SCI to further chemoattract axons into the lumbar walking execution centers. Middle: as in (A). Note that animals that received an additional chemoattractive depot display axon growth deep into the lumbar walking execution centers. Right, as in (A) (independent samples two-tailed t-test; n = 6 animals per group; $t = 9.3$; $p = 1.0 \times 10^{-5}$). (D) Locomotor performance as in (B) (n > 10 gait cycles per mouse, n = 6 mice per group, n = 5 mice in the uninjured group; statistics indicate Tukey HSD tests following one-way ANOVA). Raw data and statistics are provided in the Supplementary Material, available upon publication.*

pronounced recovery of walking observed by four weeks after natural spinal cord repair involving $SC^{Vsx2::Hoxa7::Zfhx3 \rightarrow lumbar}$ neurons whose axons terminated within walking execution centers located several segments more distally (**Figure 3.3C-E**, **Figure 3.5A**).

We therefore reasoned that the recovery of walking after anatomically complete SCI cannot be achieved simply by bridging the lesion gap with short-distance or undirected regeneration, but that one of the key additional requirements must be to propel axons to their natural target region in the lumbar spinal cord. To achieve such long-distance and directed regeneration, we placed an additional depot of chemoattractive GDNF into the lumbar spinal cord (**Figure 3.7C**). This strategy successfully promoted regrowth of axons from thoracic interneurons up to the distal-most depot; however, this additional depot attracted comparatively few axons to the targeted lumbar region (**Figure 3.7C**), and high-precision behavioral assessments again failed to detect any recovery of lower limb movements (**Figure 3.7D**).

Lentivirus-based sustained chemoattraction of regenerating axons to their natural target region increases distance and density of axon regrowth

Our findings so far implied that depot-based chemoattraction is sufficient to promote regrowth of axons from thoracic interneurons to the lumbar spinal cord, but not to yield any detectable functional improvement. We therefore posited that the relatively slow time course of long-distance axon growth, maturation, and synapse formation might require a more sustained and higher concentration of chemoattractive growth factor delivery than was provided by the biomaterial depot[502].

To achieve this, we engineered a lentivirus to provide sustained Gdnf delivery[397]. Replacing biomaterial depots with lentivirus-mediated Gdnf expression enabled an extensive regrowth of axons to their natural target region over two spinal cord segments distally (**Figure 3.8A-C**), further demonstrating that appropriate chemoattraction gradients can guide long-distance axon regeneration in a manner similar to development[503,504]. Remarkably, density of axon regrowth in the lumbar segments was significantly higher than what achieved with depot-based chemoattraction (**Figure 3.8B-C**), and this was consistent among all animals in our cohorts (**Figure 3.9A-D**).

We next sought to determine whether regenerated axons included those originating specifically from $SC^{Vsx2::Hoxa7::Zfhx3 \rightarrow lumbar}$ neurons. To test this hypothesis, we infused rAAV2-hSyn-KASH-eGFP into the lumbar spinal cord. This strategy exclusively expressed eGFP in neurons whose axons had regrown sufficiently to reach walking execution centers (**Figure 3.10A**). Nuclei of $eGFP^{ON}$ neurons located above the injury were sorted for snRNA-seq and the resulting transcriptional profiles were integrated into the atlas of global cell types and neuronal subpopulations of the thoracic spinal cord (**Figure 3.10B-G**). Comparing the distribution of $eGFP^{ON}$ neurons to the distribution of neuronal subpopulations in the uninjured spinal cord showed that $SC^{Vsx2::Hoxa7::Zfhx3 \rightarrow lumbar}$ neurons were the main virally labeled subpopulation, confirming the successful regeneration of this neuronal subpopulation to its natural target region (**Figure 3.8E**, **Figure 3.10H-I**). Retrograde tracing coupled with immunohistochemistry of Vsx2 confirmed these results (**Figure 3.8F**, **Figure 3.10J**). Gene Ontology analysis of the transcriptional profiles showed that, compared to all other neuronal subpopulations, regenerated $SC^{Vsx2::Hoxa7::Zfhx3 \rightarrow lumbar}$ neurons upregulated axon regeneration pathways, Igf receptor signaling, synaptic formation and transmission programs, as well as axon extension and maturation pathways, consistent with their regeneration and stabilization within walking execution centers in the lumbar spinal cord (**Figure 3.10K**).

To test whether regenerated $SC^{Vsx2::Hoxa7::Zfhx3 \rightarrow lumbar}$ neurons projected to lumbar spinal cord regions in a manner similar to that found in uninjured mice, we performed Cre-dependent tracing via AAV5-hSyn-flex-tdTomato into the thoracic spinal cord of $Vsx2^{Cre}$ mice that had undergone regeneration after anatomically complete SCI. We combined this tract-tracing with labeling of two major neuronal subpopulations of the lumbar spinal cord by immunohistochemistry for $Vsx2^{ON}$ and $Chat^{ON}$ neurons. Regenerated axons were found around, and made contacts with, these key neuronal subpopulations of the lumbar spinal cord that are known to be involved in the production of walking[128–131,133,136,487,488] and are essential to regain walking after paralysis[30] (**Figure 3.9E**). Uninjured mice exhibited a similar projection pattern (**Figure 3.9E**), suggesting that regenerated

$SC^{Vsx2::Hoxa7::Zfhx3 \rightarrow lumbar}$ neurons retain the capacity to reform appropriate connections with their natural targets[505].

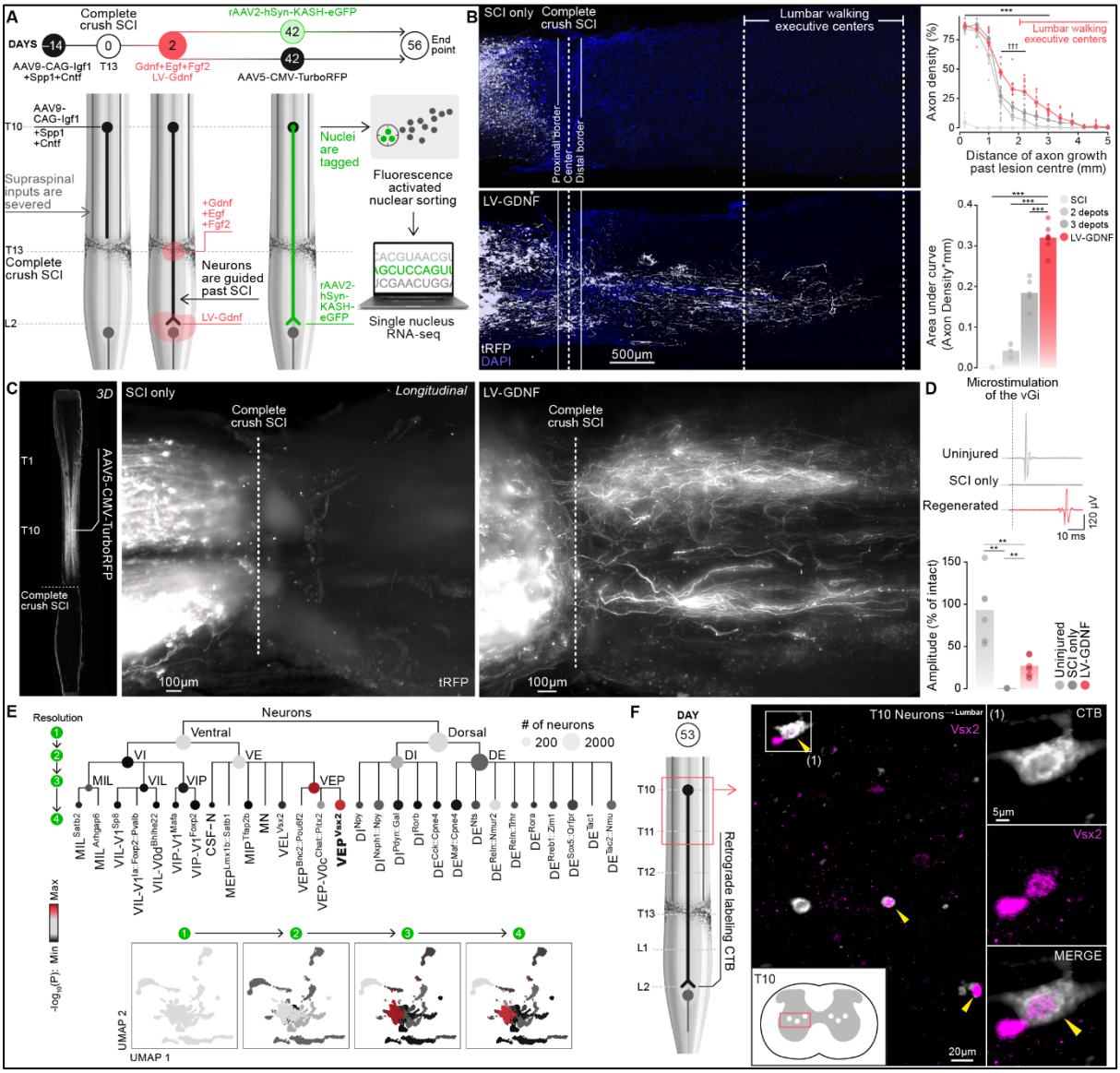

Figure 3.8. Regenerating $SC^{Vsx2::Hoxa7::Zfhx3 \rightarrow lumbar}$ neurons across an anatomically complete SCI.
*(A) Overview of the experimental approach enabling regeneration across an anatomically complete SCI and into lumbar walking execution centers. (B) tRFP-labeled axons in composite tiled scans of horizontal sections from representative mice. Dotted lines demarcate astrocyte proximal and distal borders around the lesion core. Dashed line demarcates lesion center. Line graph demonstrates axon density at specific distances past lesion centers (normalized to the density rostral to the lesion site). Statistics indicate Tukey HSD following one-way repeated measures ANOVA. *** indicates $p < 0.001$ LV-GDNF versus SCI only. ††† indicates $p < 0.001$ LV-GDNF versus 2 depots and 3 depots groups. Right, bar graph indicates the area under the curve of axon density in the walking execution center. Statistics indicate Tukey HSD following one-way ANOVA (all $p < 0.001$). (C) Whole spinal cord visualization[506–508] of regenerating projections from the lower thoracic spinal cord that project to lumbar walking execution centers. (D) Representative individual electrophysiological traces after microstimulation stimulation of the ventral gigantocellular nucleus (vGi). Bottom, peak-to-peak amplitude of the evoked potentials in each experimental group, expressed as a percent of uninjured mouse responses (Pairwise Wilcoxon rank-sum test, $p = 0.0064$). (E) Top, the enrichment of regenerated neurons among neuronal populations of the mouse lumbar spinal cord is shown within a clustering tree of spinal cord neurons defined in four different clustering resolutions, demonstrating the robustness of these findings to the resolution at which transcriptionally defined neuronal subtypes are defined. Bottom, the same enrichments are visualized on a progression of UMAPs. (F) CTB-labeled regenerated neuron with Vsx2 immunohistochemical co-labeling above the anatomically complete SCI.*

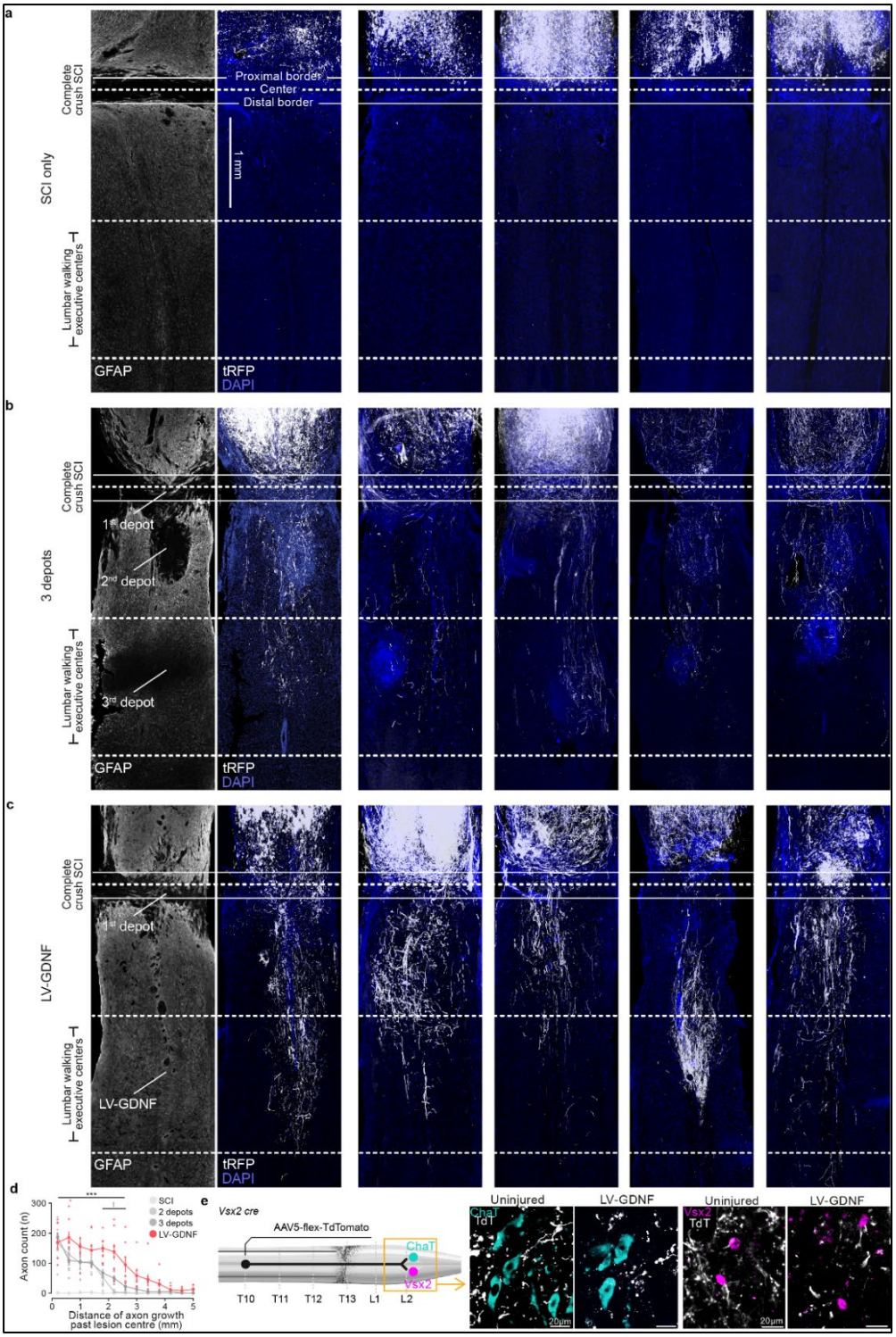

Figure 3.9. Individual animal results for regeneration of lower thoracic spinal cord neurons to their natural target region. *(A) Large area survey images of tRFP^{+} labeled axons in composite mosaic scans of horizontal sections for individual control mice that received SCI only. Dotted lines demarcate astrocyte proximal and distal borders around the lesion core. Dashed line demarcates the lesion center. We found that few axons reach the lesion center, almost none pass beyond and no axons are present below the level of the injury. (B) As in (A) for treated mice that received our combinatorial treatment with depot-based chemoattraction. We found that axons regrow through the lesion core and reach walking execution centers in the lumbar spinal cord but at low density. (C) As in (A) for treated mice that received our combinatorial treatment with sustained lentivirus-based chemoattraction. We found that many axons regrow through the lesion core and reach walking execution centers in the lumbar spinal cord. (D) Axon counts at specific distances past lesion centers, accompanying **Fig. 3.8**. Statistics indicate Tukey HSD following one-way repeated measures ANOVA. *** indicates $p < 0.001$ LV-GDNF versus SCI only. † indicates $p < 0.05$ LV-GDNF versus 2 depots and 3 depots groups. (E) Experimental scheme and representative images of Vsx^{ON} neurons with axons projecting to Vsx2^{ON} and Chat^{ON} neurons in the walking execution centers in the lumbar spinal cord*

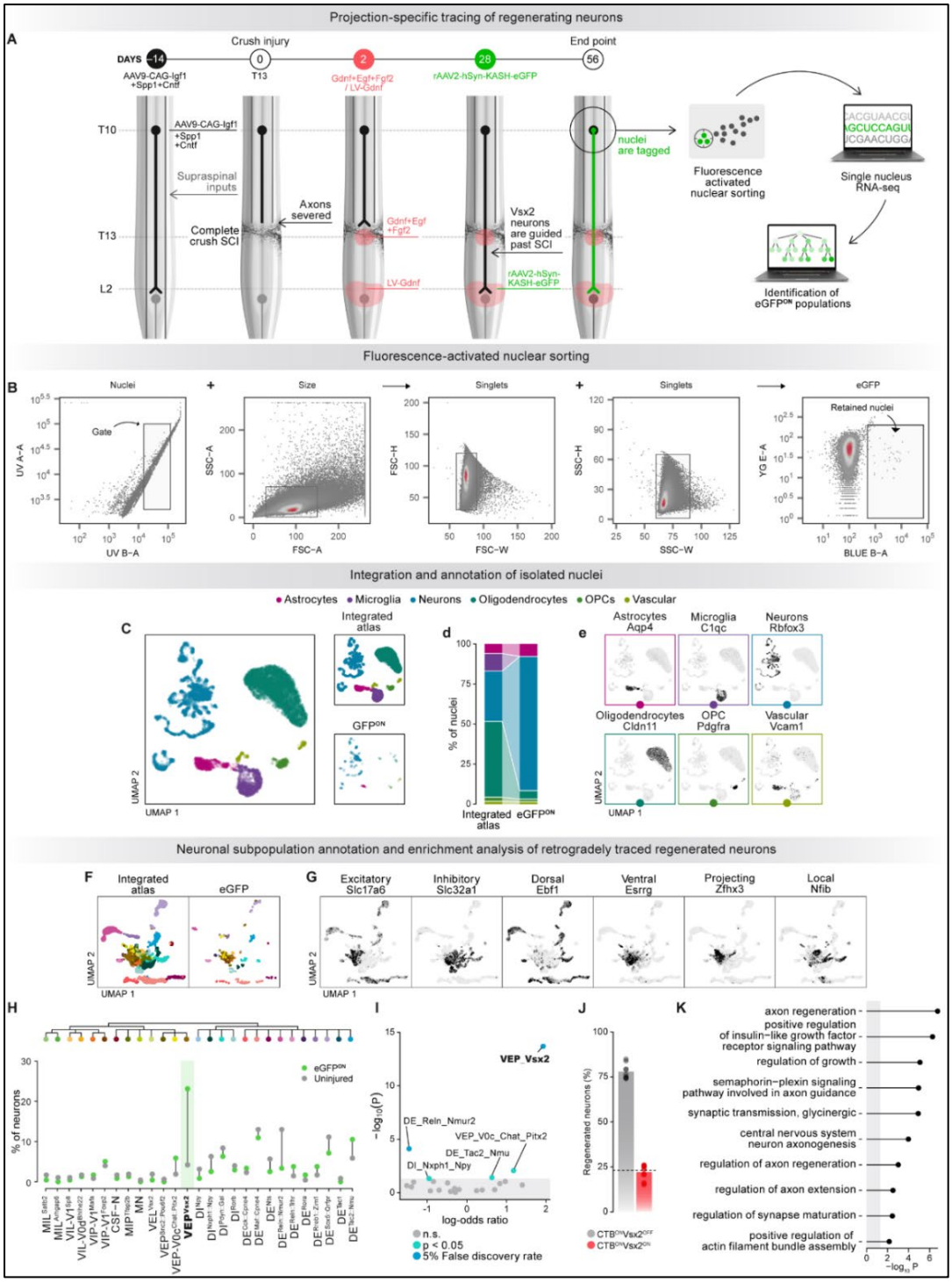

Figure 3.10. Projection-specific single-nucleus RNA-sequencing of regenerating propriospinal neurons.

(A) Scheme illustrating our combinatorial, mechanism-based regeneration and tracing approach, leveraging eGFP expression fused to the nuclear envelope protein KASH to enable fluorescence-activated nuclei sorting followed by single-nucleus RNA sequencing. (B) Fluorescence-activated nuclei sorting of eGFP^{ON} regenerating neurons from the lower thoracic spinal cord. Through a series of sorting gates isolating singlet nuclei of the appropriate size, we are able to enrich for eGFP nuclei, which can then be sent for subsequent single-nucleus RNA sequencing. UV-A: ultraviolet light A; UV-B: ultraviolet light B; FSC: forward-scattered light; SSC: side-scattered light; YG E: yellow-green emission; BLUE B: blue emission; –W: width; –H: height; –A: area. A combination of ultraviolet spectrum and size gating is first used to sort nuclei. This is followed by further size gating to remove doublets, and finally wavelength emission gates to enrich for nuclei expressing eGFP. (C) UMAP visualization of 12,493 nuclei colored by major cell type, revealing the primary recovery of neurons in the eGFP^{+} library.

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(D) Proportions of each major cell type of the thoracic spinal cord recovered in individual libraries from the integrated atlas and the eGFP^{ON} neurons from the projection-specific tracing. (E) Expression of key marker genes for the six major cell types of the thoracic spinal cord. (F) UMAP visualization of 4,076 neuronal nuclei colored by neuronal subpopulation. (G) Expression of key neurotransmitters for excitatory (Slc17a6), inhibitory (Slc31a1), dorsal (Ebf1), ventral (Esrrg), projecting (Zfhx3), and local (Nfib) genes demonstrating a logical topography of neurons in the spinal cord. (H) Proportions of each neuronal subtype of the thoracic spinal cord recovered in each sorting condition. (I) Volcano plot showing the statistical significance of neuronal subpopulation enrichment among sorted regenerating neurons. (J) The number of retrogradely labeled neurons (CTB: Cholera toxin subunit B) after our combinatorial regeneration therapy. The number of neurons that are co-labeled with Vsx2 are indicated in the right bar, versus the number that are Vsx2^{OFF}, on the left. The dotted line indicates the proportion of SC$^{Vsx2::Hoxa7::Zfhx3\rightarrow lumbar}$ neurons observed in our projection-specific snRNA-seq experiment (panel H). (K) Gene programs activated in response to regeneration, as from Gene Ontology analysis. Shaded region denotes non-significance.

Finally, we asked whether supraspinal commands could be detected below the anatomically complete SCI after regenerating SC$^{Vsx2::Hoxa7::Zfhx3\rightarrow lumbar}$ neurons to reach walking execution centers in the lumbar spinal cord. We found that microstimulation of the vGi induced large motor evoked potentials in lower limb muscles, revealing that supraspinal centers had regained access to walking execution centers, and that regenerating axons were able to relay functional signals across the injury (**Figure 3.8D**).

These results demonstrate that SC$^{Vsx2::Hoxa7::Zfhx3\rightarrow lumbar}$ neurons can be engineered to regrow functional axons to their natural target region in the lumbar spinal cord, wherein walking execution centers reside.

Targeted sustained chemoattraction promotes substantial recovery of walking after anatomically complete SCI

Since our regenerative strategy chemoattracted and guided a molecularly defined neuronal subpopulation involved in natural spinal cord repair to regrow to their target region in the lumbar spinal cord, we anticipated that this strategy may enable meaningful recovery of walking after complete paralysis.

We therefore performed longitudinal quantification of whole-body kinematics during walking in five separate cohorts of mice that underwent anatomically complete SCI and received the optimized regeneration strategy (**Figure 3.11A**, **Figure 3.12A**). Evaluations showed that our complete SCI abolished lower limb movements in every mouse studied, such that even at four weeks after SCI, no mice untreated or receiving depot-based repair exhibited any sign of recovery of walking (**Figure 3.12C**). In all tested mice, the optimized regenerative strategy promoted the growth of projections from SC$^{Vsx2::Hoxa7::Zfhx3\rightarrow lumbar}$ neurons to their natural target region in the lumbar spinal cord. This regrowth coincided with a progressive recovery of lower limb movements that emerged approximately three to four weeks after SCI (**Figure 3.11B-E**, **Figure 3.12B-C**). Final evaluations were performed at 8 weeks. In cohort one, five out of six mice with anatomically complete SCI and full treatment displayed gait patterns that resembled those quantified in mice with natural spinal cord repair after incomplete SCI, as assessed via linear discriminant analysis classification (**Figure 3.11F**, **Figure 3.12D**). These experiments were repeated in four subsequent cohorts, with a further 22 out of 24 mice (or a total of 27 out of 30) demonstrating similar results (**Figure 3.13A-C**).

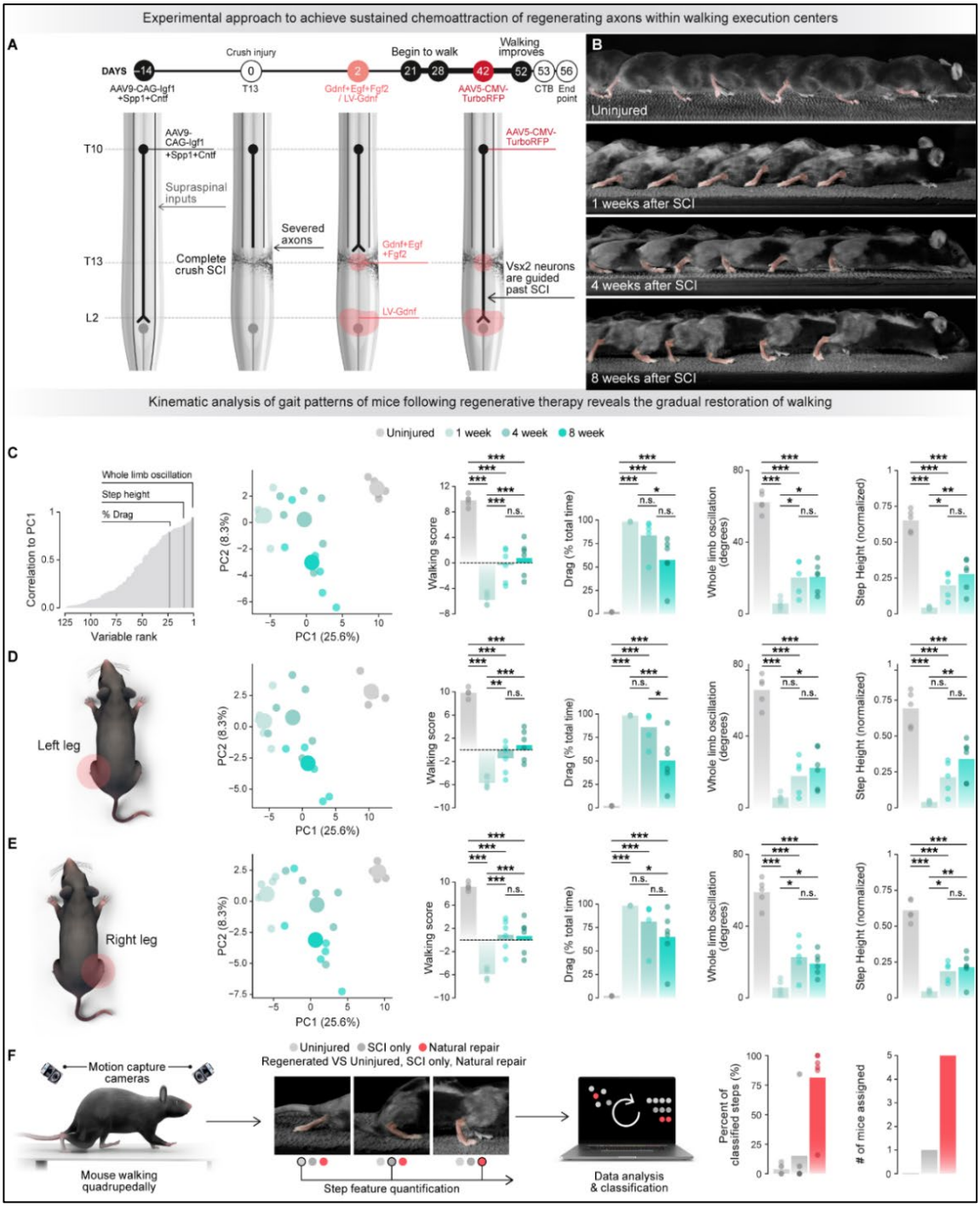

Figure 3.11. ***Restoration of walking following regeneration of SC$^{Vsx2::Hoxa7::Zfhx3\rightarrow lumbar}$ neurons to their natural target region in lumbar walking execution centers.***

(A) Schematic and timeline of lentivirus (LV)-mediated experiments. Two days after complete crush SCI, animals received the first hydrogel injection into the center of the non-neural lesion core to deliver the growth factors FGF + EGF + GDNF. Nine days after SCI, the animals received a LV injection targeted to spared neural tissue in the lumbar walking execution centers to deliver GDNF to chemoattract axons that had regrown into the lesion core. Viral injections for axonal tract-tracing were targeted at neurons between one and two segments rostral to SCI lesions and were placed one month following the crush SCI. Tissue was collected for evaluation at eight weeks after SCI. (B) Leg movements during overground walking without any intervention or support recorded before the SCI (top left), one week after the SCI (bottom left), four weeks after the SCI (top right), and eight weeks after the SCI (bottom right). (C) Locomotor performance as in ***Fig. 3.3*** *(n > 10 gait cycles per mouse, n = 6 mice per group, n = 5 mice in the uninjured group; statistics indicate Tukey HSD tests following one-way ANOVA). Raw data and statistics are provided in the Supplementary Material, available upon publication. (D) As in (C) but only for gait cycles from the left leg. (E) As in (C) but only for gait cycles from the right leg. (F) The number of steps (left) and mice (right) from combinatorial treated animals at 8 weeks post-SCI that were assigned to each main experimental group. Note that steps are nearly exclusively assigned by the classifier to the natural repair group, indicating that the walking patterns of regenerated mice most resemble those that underwent natural repair.*

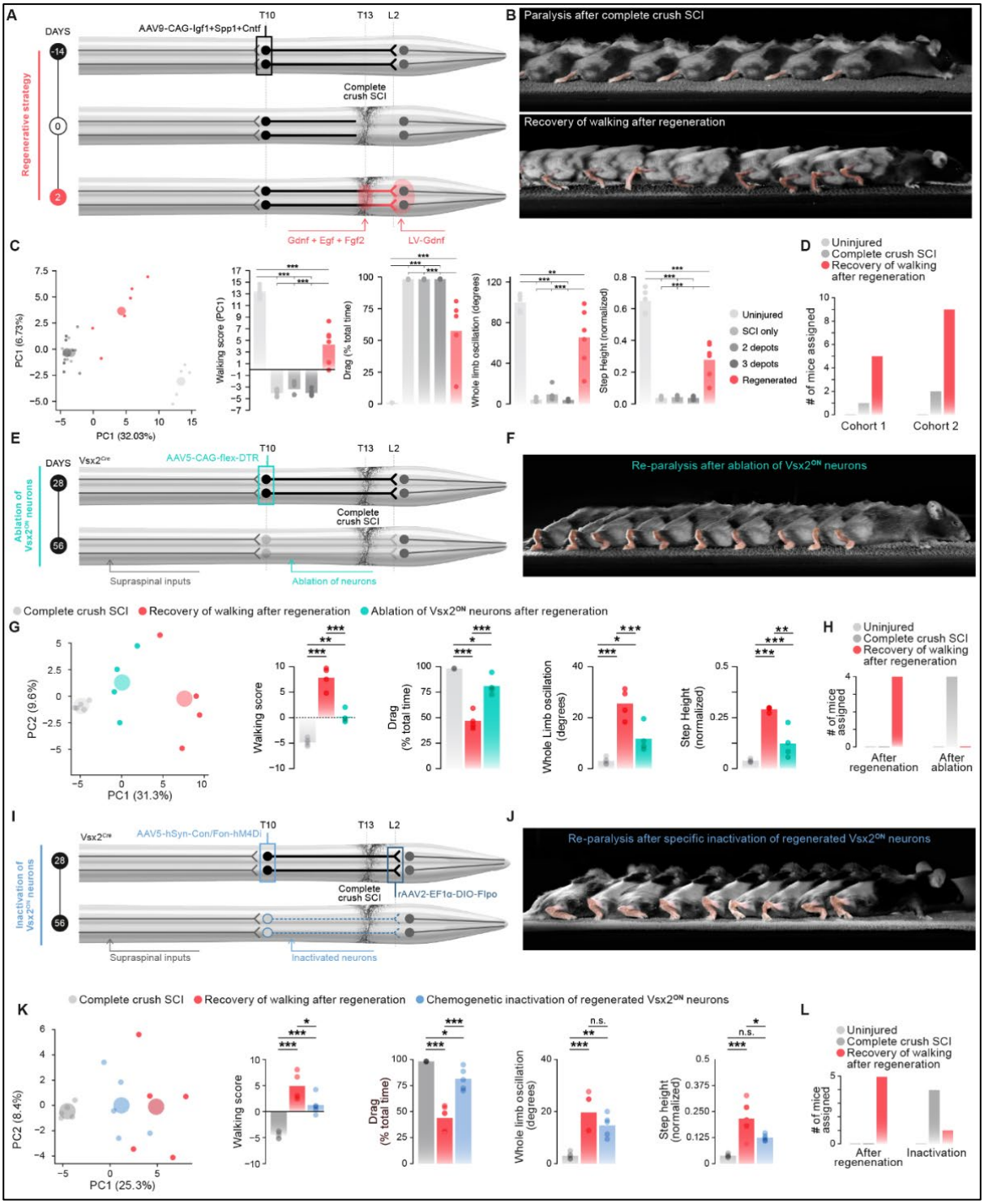

Figure 3.12. Axons from SC$^{Vsx2::Hoxa7::Zfhx3 \rightarrow lumbar}$ neurons are necessary to restore walking after anatomically complete SCI. *(A) Overview of the experimental approach enabling regeneration across an anatomically complete SCI and into walking execution centers. (B) Chronophotography of walking with (top) and without (bottom) mechanism-based combinatorial regeneration mimicking natural repair processes. (C) Walking was quantified using principal component analysis as described in **Fig. 3.3C-E** (n > 10 gait cycles per mouse, n = 6 mice per group, n = 5 mice in the uninjured and SCI only groups). Raw data and statistics are provided in the Supplementary Material, available upon publication.*

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(D) The number of mice from two cohorts of combinatorial treated animals at 8 weeks post-SCI that were assigned to each main experimental group. Mice were almost exclusively assigned by the classifier (see Material and Methods, Behavioural assessments) to the natural repair group, indicating that the walking patterns of regenerated mice most resemble those that underwent natural repair. (E) Experimental design for cell type-specific diphtheria toxin-mediated neuron ablation of $Vsx2^{ON}$ neurons in the mid-thoracic spinal cord and intersectional chemogenetic inactivation of regenerated $Vsx2^{ON}$ neurons following mechanism-based combinatorial regeneration. (F) Chronophotography of walking in $Vsx2^{Cre}$ mice that received mechanism-based combinatorial regeneration mimicking natural repair processes coupled to viral injections of AAV5-CAG-FLEX-DTR to induce cell type-specific neuronal ablation. (G) Walking was quantified using principal component analysis as described in ***Fig. 3.3C-E*** *(n > 10 gait cycles per mouse, n = 4 mice per group, n = 5 mice in the SCI only group). Raw data and statistics are provided in the Supplementary Material, available upon publication. (H) Mice were recorded before (left) and after (right) one-week administration of diphtheria toxin. Bar graphs indicate the number of mice from each group that were assigned to each main experimental group (see Materials and Methods, Behavioural assessments). (I) As in (E) but for intersectional chemogenetic inactivation of regenerated $Vsx2^{ON}$ neurons in the mid-thoracic spinal cord. (J) As in (F) but for intersectional chemogenetic inactivation of regenerated $Vsx2^{ON}$ neurons in the mid-thoracic spinal cord. (K) As in (G) (n > 10 gait cycles per mouse, n = 5 mice per group). Raw data and statistics are provided in the Supplementary Material, available upon publication. (L) As in (H) but for intersectional chemogenetic inactivation of regenerated $Vsx2^{ON}$ neurons in the mid-thoracic spinal cord.*

These results demonstrate that our combinatorial regeneration strategy led to substantial recovery of walking after anatomically complete SCI. Notably, the mice that underwent regeneration did not walk as well as uninjured mice, but instead exhibited a behavioral phenotype that was comparable to mice that underwent spontaneous natural repair after incomplete SCI, including coordinated plantar stepping with partial weight-bearing and occasional foot drag[143] (**Figure 3.11C-E**, **Figure 3.13B**).

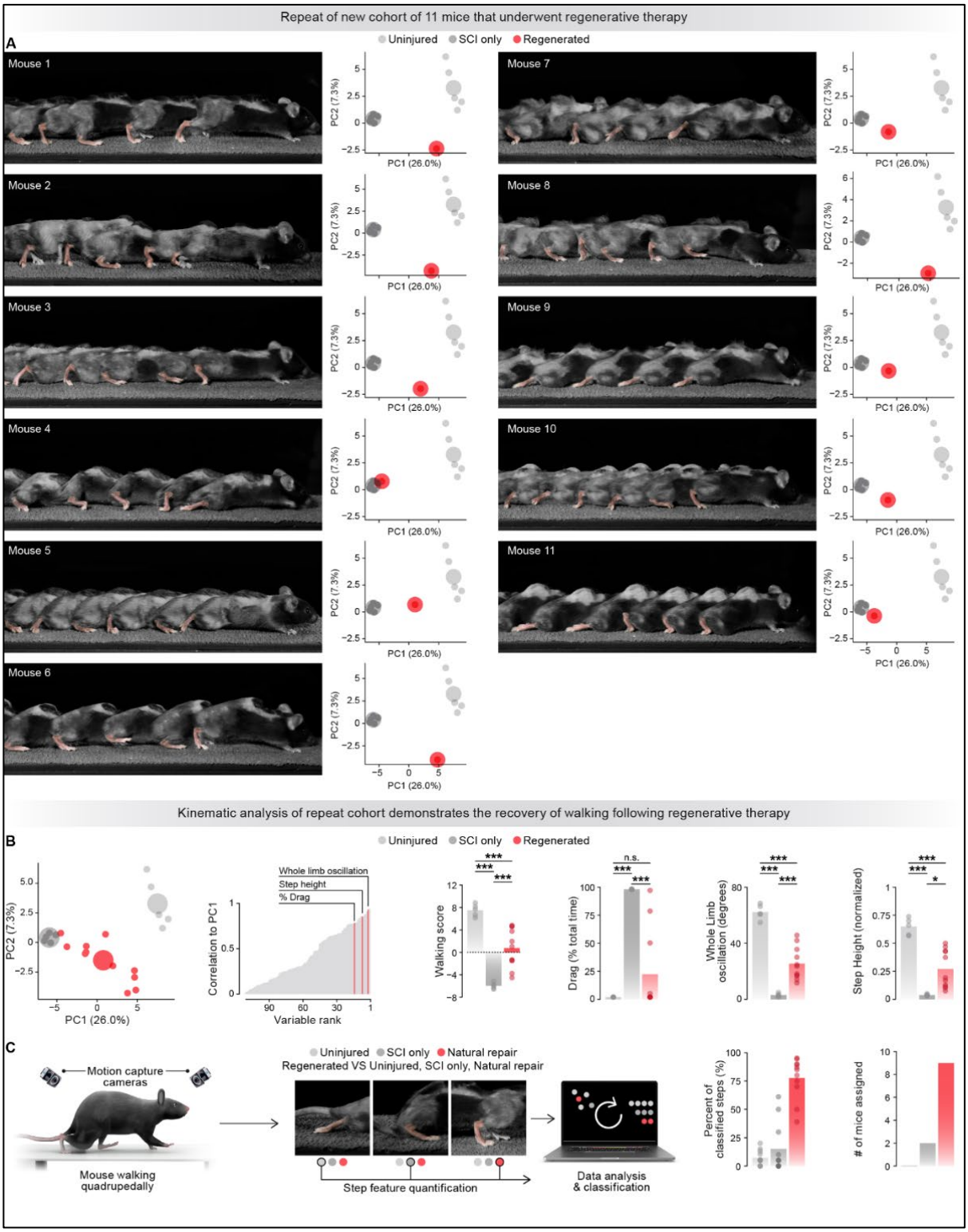

Figure 3.13. Restoration of walking following combinatorial regeneration treatment in repeated cohorts of mice.
(A) Leg movements during overground walking of each mouse in the repeated cohorts, accompanied by their kinematic data projected into principal component (PC) space, as in ***Fig. 3.3****. (B) Locomotor performance as in* ***Fig. 3.3*** *(n > 10 gait cycles per mouse, n = 11 mice in the combinatorial therapy group; statistics indicate Tukey HSD tests following one-way ANOVA). Raw data and statistics are provided in the Supplementary Material, available upon publication. (C) The number of steps (left) and mice (right) from combinatorial treated animals from the repeated cohorts at 8 weeks post-SCI that were assigned to each main experimental group. Note that steps are nearly exclusively assigned by the classifier to the natural repair group, indicating that the walking patterns of regenerated mice most resemble those that underwent natural repair.*

$SC^{Vsx2::Hoxa7::Zfhx3→lumbar}$ neurons are necessary for recovery of walking following regenerative intervention

Our regenerative strategy promoted the regrowth of projections from neuronal subpopulations of the thoracic spinal cord other than $SC^{Vsx2::Hoxa7::Zfhx3→lumbar}$ neurons, and we thus cannot exclude the involvement of these relatively less abundant neuronal subpopulations in the recovery of walking after paralysis. Therefore, we aimed to establish the necessity of $SC^{Vsx2::Hoxa7::Zfhx3→lumbar}$ neurons with regenerating projections to their appropriate target region in the lumbar spinal cord for the recovery of walking after experimental spinal cord repair, given their noted involvement in the natural recovery of walking after incomplete SCI.

To establish this necessity, we first ablated all $Vsx2^{ON}$ neurons in a third cohort of mice by expressing the diphtheria toxin receptor (DTR) with injections of Cre-dependent AAV5-CAG-flex-DTR into the thoracic spinal cord of $Vsx2^{Cre}$ mice (**Figure 3.12 E**, **Figure 3.14A**). Eight weeks after SCI, all four mice in cohort three that received our regeneration strategy had regained the ability to walk with gait patterns that resembled those quantified in mice that had undergone natural repair (**Figure 3.14C-F**). Administration of diphtheria toxin re-paralyzed every tested mouse (**Figure 3.12F-H**, **Figure 3.14C-F**). Post-mortem anatomical analyses via Vsx2 immunostaining confirmed the near complete ablation of $Vsx2^{ON}$ neurons in the thoracic spinal cord (**Figure 3.14B**). These results established the role of thoracic $Vsx2^{ON}$ neurons in the recovery of walking after experimental spinal cord repair. However, they did not establish the respective role of local versus projection $Vsx2^{ON}$ neurons.

We therefore sought to establish the necessity of projections specifically from $SC^{Vsx2::Hoxa7::Zfhx3→lumbar}$ neurons to the lumbar spinal cord in the recovery of walking after experimental spinal cord repair. We designed an intersectional chemogenetic strategy[509,510] that allowed us to silence $SC^{Vsx2::Hoxa7::Zfhx3→lumbar}$ neurons once mice treated with our regeneration strategy had demonstrated substantial recovery of walking: we infused rAAV2-EF1α-DIO-Flpo into the lumbar spinal cord of a fourth cohort composed of 5 $Vsx2^{Cre}$ mice, followed by injections of Cre- and FLPo-dependent AAV5-Con/Fon-hM4Di-mCherry into the thoracic spinal cord (**Figure 3.12I**, **Figure 3.15A-B**).
These mice all demonstrated the expected recovery of walking. The administration of clozapine-N-oxide (CNO) immediately impaired walking in all tested mice, leading to gait patterns that resembled those of mice that had not undergone regeneration (**Figure 3.12J-L**, **Figure 3.15B-D**). In contrast, a fifth cohort of mice that did not receive AAV5-Con/Fon-hM4Di-mCherry infusions were unaffected by CNO administration (**Figure 3.15E**).

These findings establish that regenerated projections from $SC^{Vsx2::Hoxa7Zfhx3→lumbar}$ neurons to their natural target region in the lumbar spinal cord are in part necessary for the meaningful recovery of walking after anatomically complete SCI.

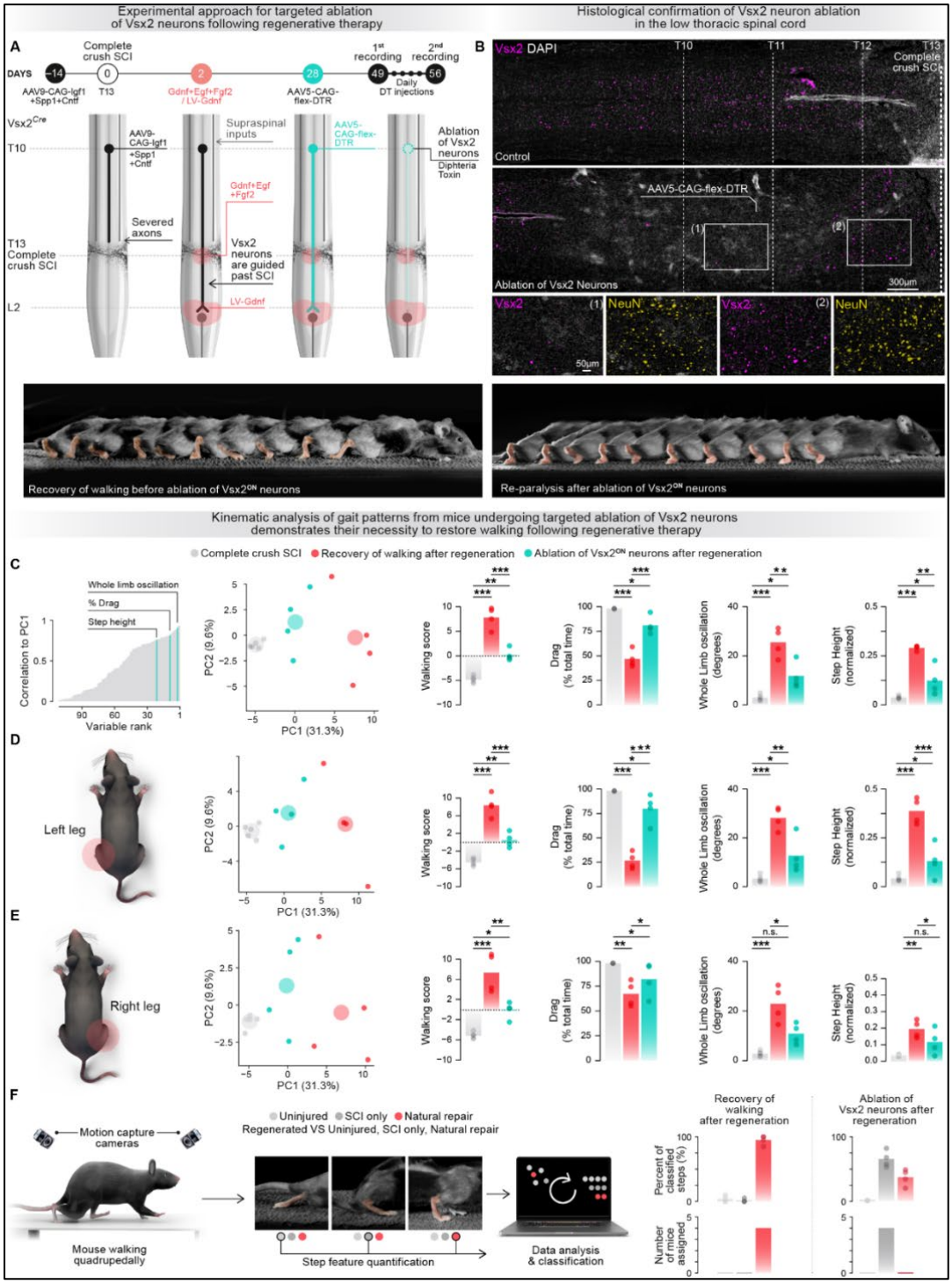

Figure 3.14. Re-paralysis of mice following ablation of thoracic Vsx2 neurons.
*(A) Schematic and timeline of lentivirus (LV)-mediated regeneration coupled to cell-type-specific diphtheria toxin experiments. Vsx2^{Cre} mice received the same regenerative intervention described in **Fig. 3.11**, with the only difference being injection of LV-Gdnf in the same surgery as the depot, to reduce the number of surgeries per animal; 4 weeks post injury, mice received injection of Cre-dependent AAV expressing DTR in the same spinal segments where AAVs expressing OIC were injected. Following kinematic recordings at the 8 week time-point, animals received daily injections of diphtheria toxin for 7 days. Kinematics were then recorded and tissue was collected for evaluation. (B) Histological verification of Vsx2 neurons ablation in the lower thoracic region (top left). Images demonstrate loss of Vsx2^{ON} neurons in the thoracic spinal cord, above the level of the complete crush SCI. Leg movements during overground walking of the same mouse before and after diphtheria toxin injections (bottom). (C) Locomotor performance as in **Fig. 3.3** (n > 10 gait cycles per mouse, n = 4 mice per group, n = 5 mice in the SCI only group; statistics indicate Tukey HSD tests following one-way ANOVA). Raw data and statistics are provided in the Supplementary Material, available upon publication.*

(legend continued on next page)

(D) As in C, but only for gait cycles from the left leg. (E) As in C, but only for gait cycles from the right leg. (F) The number of steps (left) and mice (right) from combinatorial treated animals before and after Vsx2 neuron ablation that were assigned to each main experimental group.

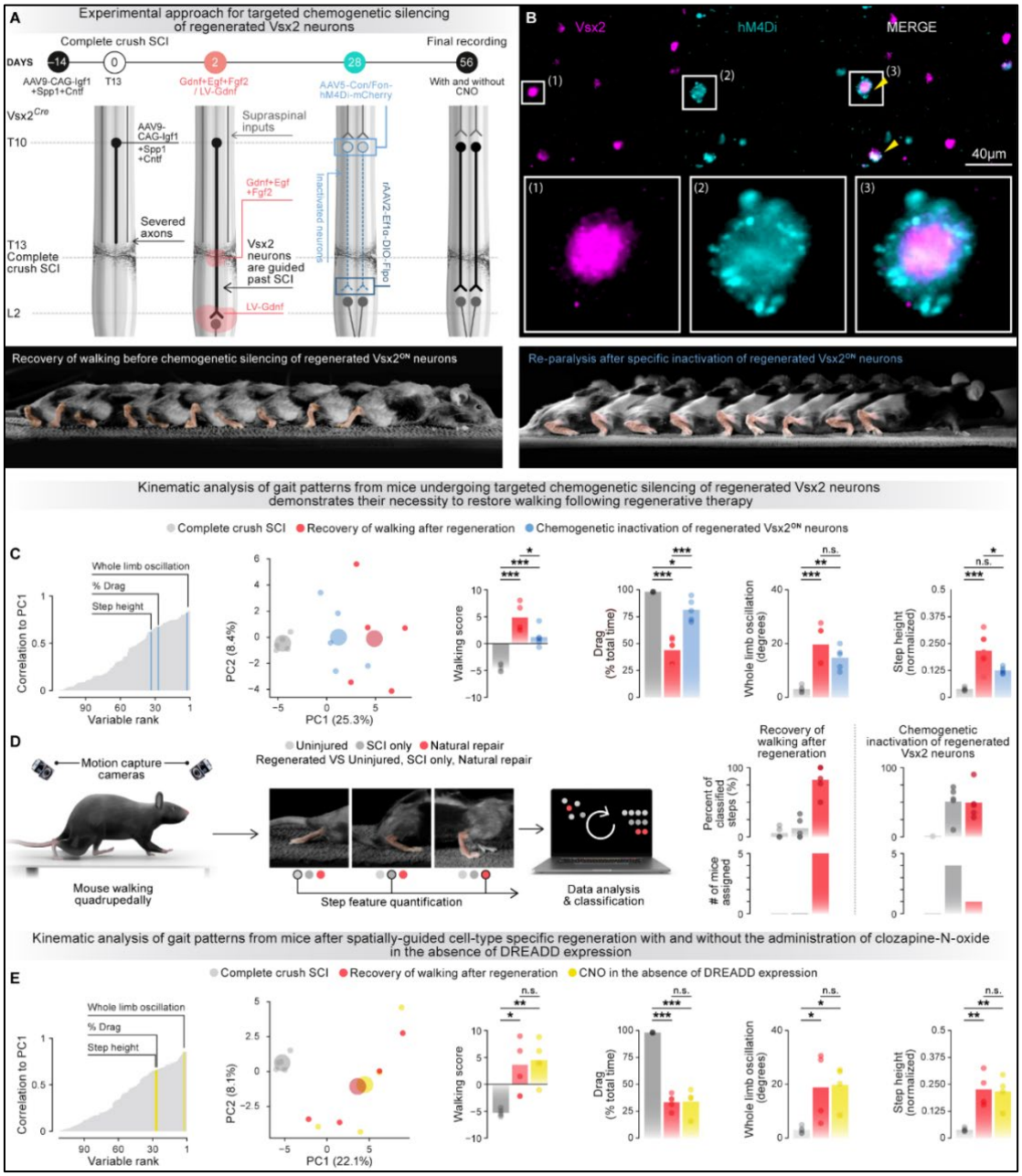

Figure 3.15. Re-paralysis of mice following chemogenetic inactivation of regenerated Vsx2 neurons.
*(A) Schematic and timeline of lentivirus (LV)-mediated regeneration coupled to projection and cell-type-specific chemogenetic inactivation experiments. Vsx2^{Cre} mice received the same regenerative intervention described in **Fig. 3.11**, with the only difference being injection of LV-Gdnf in the same surgery as the depot, to reduce the number of surgeries per animal. One month after SCI, animals received infusions of rAAV2-Ef1 α-DIO-Flpo into the lumbar spinal cord and injections of AAV5-Con/Fon-hM4Di-mCherry into the thoracic spinal cord. Animals then underwent kinematic recordings one month later before and after the administration of clozapine-N-oxide (CNO). An additional control experiment was completed in mice that underwent the same regeneration procedures but did not receive the intersectional chemogenetic inactivation viruses. (B) Histological verification of hM4Di-mCherry in Vsx2 neurons in the lower thoracic region (top left). Leg movements during overground walking of the same mouse before and after CNO injections (bottom). (C) Locomotor performance as in **Fig. 3.3** (n > 10 gait cycles per mouse, n = 5 mice per group; statistics indicate Tukey HSD tests following one-way ANOVA). Raw data and statistics are provided in the Supplementary Material, available upon publication. (D) The number of steps (left) and mice (right) from combinatorial treated animals before and after CNO administration that were assigned to each main experimental group. (E) As in (C), but for mice that did not receive the intersectional chemogenetic inactivation viruses. Raw data and statistics are provided in the Supplementary Material, available upon publication.*

Discussion

In this study, we investigated the degree to which meaningful recovery of function after anatomically complete SCI will require targeting specifically characterized neurons and regenerating the axons from those neurons not simply across lesions, but also to guide them to reach their natural target region below the injury. To address this question, we first characterized the molecular identity of the neuronal subpopulations in the thoracic spinal cord that restore walking by relaying supraspinal commands past severe but incomplete thoracic SCI[143]. We then traced the connectome of these neurons and found that their natural projection pattern extends several segments caudally to the lumbar gray matter, where walking execution centers reside. We then used our previously developed multipronged regeneration strategy[332] to stimulate the axons of these molecularly characterized neurons to regenerate through fibrotic lesion core tissue and into spared neural tissue caudal to the lesion. This strategy included reactivating dormant neuron-intrinsic growth programs, establishing matrix support for axons to grow through non-neural lesion core tissue, and supplying a gradient of chemoattraction to guide these axons to the caudal side of the injury, where they naturally terminate[332].

We show that regenerating these neurons to reach simply across lesions had no effect on the recovery of walking. In striking contrast, refining our strategy to enable graded chemoattraction and guidance of regenerating axons to their natural target in the lumbar spinal cord promoted the meaningful recovery of walking after anatomically complete SCI. We applied projection-specific snRNA-seq to identify the neuronal subpopulations with regenerating axons past anatomically complete SCIs, and demonstrated that our strategy regenerated axons from the neuronal subpopulations that restore walking after severe but incomplete SCI. To chemoattract regenerating axons, we expressed Gdnf, a pleiotropic growth factor with the potential to affect a broad range of cells. While it is possible that expression of Gdnf may have affected other cells in the lumbar cord, including in such a way as to facilitate the reformation of functional connections by the regenerated axons, our causal loss-of-function experiments demonstrate that the restoration of function depended on the regenerated axons of characterized neurons. A potential limitation of our study is that it is also possible that simply greater bulk regeneration, as opposed to directed target-specific regrowth, may account for the better functional outcome.

These findings show that re-establishing the projections of molecularly-defined neuronal subpopulations to their natural target region forms an essential yet previously unidentified requirement for axon regeneration strategies aimed at restoring lost neurological functions. This understanding has important implications for the design of therapies for larger mammals and humans, since the potentially long distance over which regenerated projections will have to grow to restore function may require spatially and temporally complex strategies.

We posit that applying the principles demonstrated here of (i) identifying and regenerating the axons of functionally relevant neuronal subpopulations, (ii) determining the requirements for reactivating neuron-specific developmental growth programs, and (iii) identifying chemoattractants able to guide different types of transected axons past lesions to reach their natural target regions, and (iv) eventually combining these

biological repair principles with complementary neuromodulation strategies[25,495,511], will unlock the framework to achieve meaningful repair of the injured spinal cord and may expedite repair after other forms of central nervous system injury and disease[9,26,512].

Materials and methods

Mouse model

Adult male or female C57BL/6 mice (15-25 g body weight, 8-15 weeks of age) or transgenic mice were used for all experiments. Vsx2^{Cre} (MMMRRC 36672, also called Chx10^{Cre}) transgenic mouse strain was bred and maintained on a mixed genetic background (C57BL/6). Housing, surgery, behavioral experiments and euthanasia were all performed in compliance with the Swiss Veterinary Law guidelines. Manual bladder voiding and all other animal care was performed twice daily throughout the entire experiment. All procedures and surgeries were approved by the Veterinary Office of the Canton of Geneva (Switzerland; authorizations GE/25/17 and GE/109/20).

Viruses and virus production

Viruses used in this study were either acquired commercially or produced at the EPFL core facility. The following AAV plasmids were used and detailed sequence information is available as detailed or upon request: AAV5-CMV-TurboRFP (Addgene #105548), AAV9-CAG-IGF1, AAV9-CAGSpp1, AAV9-CAG-CNTF (Igf1, Spp1, and Cntf provided by Prof. Z. He), AAV5-CAG-COMET-GFP (plasmid gifted by Prof. M. Tuszynski), AAV-CAG-flex-tdTomato (plasmid gifted by Prof. S. Arber), AAV-CAG-flex-human Diphtheria Toxin Receptor (DTR, plasmid gifted by Prof. S. Arber), rAAV2-hSyn-KASH-GFP (Addgene #60231), AAV5-hSyn-Con/Fon-eYFP (Addgene #55650), rAAV2-EF1α-DIO-Flpo (Addgene #87306) and SIN-cPPT-GFAP-GDNF-WPRE. Injection volumes, coordinates and experimental purpose are described below.

SCI

Spinal cord crushes were described previously[92,332]. Briefly, complete crush SCI were made at spinal level T13 after laminectomy of portions of vertebrae T11 and T12, and were performed by using No. 5 Dumont forceps (Fine Science Tools, Foster City, CA) without spacers and with a tip width of 0.5mm to completely compress the entire spinal cord laterally from both sides for 5 seconds. Staggered hemisection SCIs were performed as previously described[143]. For staggered hemisection SCI, a laminectomy was made at the mid-thoracic level (T10) and the lateral half of the spinal cord was cut using a microscapel. 8 weeks after the first hemisection, a second mid-thoracic hemisection (T7) was performed on the opposite side of the first hemisection.

Biological repair intervention

General surgical procedures have been described previously in detail[23,92,332]. Surgeries were performed under aseptic conditions and under 1-2% isoflurane in 0.5-1 L/min flow of oxygen as general anesthesia. Surgeries were performed at EPFL under general anesthesia with isoflurane in oxygen-enriched air using an operating microscope (Zeiss), and rodent stereotaxic apparatus (David Kopf) as previously described[23,92]. We made AAV injections two weeks before SCI to allow time for molecular expression and were targeted at propriospinal neurons one and two segments rostral to the planned locations of SCI lesions after laminectomy of a single vertebra. We injected AAVs into two sites (one on each side of the cord, 0.25 μL [AA2/9 Spp1: 1×10^{13}, Igf1: 5×10^{12}, Cntf: 5×10^{12} genome copies per mL in sterile saline]) 0.6 mm below the surface at 0.1 μL per minute

using glass micropipettes connected via high-pressure tubing (Kopf) to 10 μL syringes under the control of a microinfusion pump. Severe crush SCIs were made at the level of T12/T13 as described above. Hydrogel depots were injected stereotaxically into the center of SCI lesions 0.6 mm below the surface at 0.15 μL per minute using glass micropipettes connected via high-pressure tubing (Kopf) to 10 μL syringes under the control of microinfusion pumps, two days after SCI. In animals receiving two hydrogel depots, the second depot was placed 1.5 mm caudal to the SCI nine days after SCI. For animals receiving three hydrogel depots, the third depot was made 2.5 mm below the SCI 16 days after SCI. For animals receiving lentivirus injections of GDNF (LV-GDNF: 600 μg P24/mL), the viral injection was made at the L2 and L4 spinal segments two days after SCI. Tract-tracing was performed by injection of AAV2/5 RFP red fluorescent protein (RFP, University of Pennsylvania Vector Core, 2.612×10^{13} genome copies per mL) injected 4 × 0.25μL into the segments rostral to SCI 9 days after SCI. After surgeries, mice were allowed to wake up in an incubator. Analgesia, buprenorphine (Essex Chemie AG, Switzerland, 0.01-0.05 mg/kg s.c.) or Rimadyl (5 mg/kg s.c.), was given twice daily for 2-3 days after surgery. Animals were randomly assigned numbers and thereafter were evaluated blind to experimental conditions. Seven days after SCI, all mice were evaluated in open field and all animals exhibiting any hindlimb movements were not studied further.

Hydrogel depots with growth factors

Biomaterial depots were prepared using well-characterized diblock copolypeptide hydrogels and loaded with growth factors as previously described[92,332,502]. Human recombinant FGF2, EGF, and GDNF were purchased from Peprotech: (i) human FGF2 (FGF-basic) (154 amino acids) Cat#100-18B-100UG, Lot#091608 C0617; (ii) human EGF Cat#AF-100-15-100UG, Lot#0816AFC05 B2317; (iii) human GDNF Cat#405-10-100UG, Lot#0606B64 A2517. Freeze-dried $K_{180}L_{20}$ powder was reconstituted to 3.0% w/v in sterile PBS with combinations of FGF2 (1.0 μg/μL), EGF (1.0 μg/μL), and GDNF (1.0 μg/μL).

Spinal injections for retrograde labeling

To retrogradely label neurons for fluorescent-activated nuclear sorting and subsequent snRNA-seq, a partial laminectomy over the L2 spinal level was performed and two sets of bilateral injections of rAAV2-hSyn-KASH-GFP were made (0.15 μL per injection) at two depths (0.8 mm and 0.4 mm below the dorsal surface) and separated by 1 mm. To label regenerating neurons, animals received viral injections four weeks after SCI and following repair intervention. Animals were perfused three weeks following viral injections. To retrogradely label regenerating neurons for histological assessment, one set of bilateral injections of CTB 647 (Thermofisher C34778) were made (0.15 μL per injection) at two depths (0.8 mm and 0.4 mm below the dorsal surface). Animals were perfused three days following CTB injections.

Spinal injections for exclusive labeling and chemogenetic inactivation of long distance $Vsx2^{ON}$ neurons

To exclusively label $Vsx2^{ON}$ neurons in the mid-thoracic spinal cord with long-distance projections to the lumbar walking execution centers, we leveraged Boolean logic viral strategies[510]. Partial laminectomies were made over the T10 and L2 spinal segments of $Vsx2^{Cre}$ mice. Two sets of bilateral injections of AAV5-hSyn-Con/Fon-eYFP (Addgene #55650)[510] or AAV-nEF-Con/Fon-hM4Di-mCherry (Addgene #177672) were made over the T10 spinal segment (0.25 μL per injection) at a depth of 0.6 mm below the dorsal surface and separated by 1 mm. Two sets of bilateral injections of rAAV2-EF1α-DIO-Flpo (Addgene #87306) were made (0.15 μL per injection) at two depths (0.8 mm and 0.4 mm below the dorsal surface) and separated by 1 mm. On the day of the experiment, mice were tested immediately before and between 30-45 minutes after intraperitoneal

injections of 5 mg/kg clozapine-N-oxide (Carbosynth, CAS: 34233-69-7, suspended in 2% DMSO in saline). Animals were perfused three weeks later.

Neuron subpopulation-specific ablation

For ablation experiments with diphtheria toxin, $Vsx2^{Cre}$ mice were subjected to biological repair as described above. Two sets of bilateral injections of AAV5-CAG-FLEX-DTR[513] were made over the T10 spinal segment (0.25 μL per injection at a depth of 0.6 mm below the dorsal surface and separated by 1 mm. Two weeks after spinal infusions, mice received intraperitoneal injections of diphtheria toxin (Sigma, D0564) diluted in saline (100 μg/kg) to ablate Vsx2 neurons. Mice were tested just before ablation and one week post-ablation.

Brainstem injections

An incision was made across the skull. To target descending neurons in the ventral gigantocellular nucleus (vGi), bregma was identified and a craniotomy 5 mm-6 mm dorsal and 0 mm-2 mm lateral to Bregma was performed[23]. 100 nL injections of AAV5-CMV-turboRFP were made at 0.15 μL per minute were made bilaterally at medial-lateral 0.3 mm, rostro-caudally at −5.8 mm and −6.2 mm, dorso-ventrally at a depth of 5.6 mm from the brain surface. Animals were perfused three weeks later.

Behavioral assessments

All the behavioral procedures have been described in detail previously[23,146,514]. During overground walking, bilateral leg kinematics were captured with twelve infrared cameras of a Vicon Motion Systems (Oxford, UK) that tracked reflective markers attached to the crest, hip, knee, ankle joints, and distal toes. All examiners were blinded to the experimental condition of each animal. The limbs were modeled as an interconnected chain of segments and a total of 80 gait parameters were calculated from the recordings. To evaluate differences between experimental conditions, as well as to identify the most relevant parameters to account for these differences, we implemented a multistep multifactorial analysis based on principal component analysis, which we described in detail previously[23,146,514]. To assess the similarity of mice that regained walking after treatment with other experimental groups, including those that had undergone natural repair, we implemented linear discriminant analysis classification. We trained classifiers on the kinematic parameters for each step of each mouse, for each experimental group, with the regeneration-treated mice held out. This process was completed 50 times with repeated subsamples of kinematic features. We then leveraged the trained classifiers to predict the experimental label, using the held out kinematic parameters from the mice that received the regeneration treatment. This process was repeated for the loss-of-function experiment in $Vsx2^{Cre}$ mice. For each experiment, a principal component analysis was performed by computing the covariance matrix A of the ensemble of parameters over the gait cycle, after subtraction of their respective mean values. The principal components were computed from eigenvalues λj and eigenvectors Uj of A. The principal components were ordered according to the amount of data variance accounted for by each component. The coordinate of each gait cycle on the first principal component, i.e., the component vector explaining the greatest amount of variance across the gait parameters, was thereafter referred to as the walking performance. Individual parameters were then selected to be compared between groups based on their correlation to the first principal component. All statistics were conducted as t-tests, paired t-tests, one-way ANOVA, or repeated measures one-way ANOVAs, as appropriate. All statistics were conducted at the level of individual mice, after taking the mean for each principal component or outcome measure for each biological replicate (mouse).

Perfusions

Mice were perfused at the end of the experiments. Mice were deeply anaesthetized by an intraperitoneal injection of 0.2 mL sodium pentobarbital (50 mg/mL). Mice were transcardially perfused with PBS followed by 4% paraformaldehyde in PBS. Tissue was removed and post-fixed overnight in 4% paraformaldehyde before being transferred to PBS or cryoprotected in 30% sucrose in PBS.

Immunohistochemistry

Immunohistochemistry was performed as described previously[23,332]. Perfused post-mortem tissue was cryoprotected in 30% sucrose in PBS for 48 hours before being embedded in cryomatrix (Tissue Tek O.C.T, Sakura Finetek Europe B.V.) and freezing. 30 μm thick transverse or horizontal sections of the spinal cord were cut on a cryostat (Leica), immediately mounted on glass slides and dried or in free floating wells containing PBS plus 0.03% sodium azide. Primary antibodies were: rabbit anti-GFAP (1:1000; Dako); guinea pig anti NeuN (1:300; Millipore); rabbit anti-GDNF-α (GDNF-receptor alpha) (1:1000 Abcam); guinea pig anti-homer1 (1:600, Synaptic Systems Gmbh); rabbit anti-synaptophysin (1:600, Dako); chicken anti-RFP (1:500, Novus Biologicals); goat anti-GFP (1:1000, Novus Biologicals); chicken anti-GFP (1:500 Life Technologies); rabbit anti-Chx10 (also known as Vsx2) (1:500, Novus Biologicals); sheep anti-Zfhx3 (1:500, Novus Biologicals). Fluorescent secondary antibodies were conjugated to Alexa 488 (green), or Alexa 405 (blue), or Alexa 555 (red), or Alexa 647 (far red) (ThermoFisher Scientific, USA). Nuclear stain: 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI; 2 ng/mL; Molecular Probes). Sections were imaged digitally using a slide scanner (Olympus VS-120 Slide scanner) or confocal microscope (Zeiss LSM880 + Airy fast module with ZEN 2 Black software (Zeiss, Oberkochen, Germany). Images were digitally processed using ImageJ (ImageJ NIH) software or Imaris (Bitplane, version 9.0.0).

Tissue clearing (CLARITY)[23,507,508]

We incubated samples in X-CLARITY hydrogel solution (Logos Biosystems Inc., South Korea) for 24 hours at 4°C with gentle shaking. Samples were degassed and polymerized using the X-CLARITY Polymerization System (Logos Biosystems Inc., South Korea), followed by washes in 0.001M PBS for 5 minutes at room temperature. Samples were next placed in the X-CLARITY Tissue Clearing System (Logos Biosystems Inc., South Korea), set to 1.5 A, 100 RPM, 37 degrees, for 29 h. Clearing solution was made in-house with 4% sodium dodecyl sulfate (SDS), 200 mM boric acid with dH2O, pH adjusted to 8.5. Following this, samples were washed for at least 24h at room temperature with gentle shaking in 0.1 M PBS solution containing 0.1% Triton X-100 to remove excess SDS. Finally, samples were incubated in 40 g of Histodenz dissolved in 30 mL of 0.02M PB, pH 7.5, 0.01% sodium azide (refractive index 1.465) for at least 24 h at room temperature with gentle shaking prior to imaging.

3D imaging

We performed imaging of cleared tissue using either a customized mesoSPIM[515] and CLARITY-optimized lightsheet microscope (COLM)[508]. A custom-built sample holder was used to secure the central nervous system in a chamber filled with RIMS. Samples were imaged using either a 1.25× or 2.5× objective at the mesoSPIM and a 4× or 10× objective at the COLM with one or two light sheets illuminating the sample from both the left and right sides. The voxel resolution in the x-, y- and z directions was 5.3 μm × 5.3 μm × 5 μm for the 1.25× acquisition and 2.6 μm × 2.6 μm × 3 μm for the 2.5× acquisition. The voxel resolution of the COLM was 1.4 μm × 1.4 μm by 5 μm for the 4× and 0.59 μm × 0.59 μm × 3 μm for the 10× acquisition. Images were generated as 16-bit TIFF files and then stitched using Arivis Vision4D (Arivis AG, Munich, Germany). 3D reconstructions and

optical sections of raw images were generated using Imaris (bitplane, version 9.8) software.

Axon quantification

To align all sections to a common coordinate space, we implemented a custom image analysis pipeline that includes preprocessing, registration and combination of histological images from different sections. In brief, we implemented all preprocessing in Fiji, and all registration procedures in R, using the image analysis package 'imageR', and medical image registration package 'RNiftyReg'. Images were aligned to a template spinal cord section. Axon densities were calculated for 200 μm bins, beginning at the lesion epicenter, and normalized to the density immediately rostral to the lesion. For axon counts, using Fiji, lines were drawn across horizontal spinal cord sections at SCI lesion centers and at regular distances beyond and the number of axons intersecting lines were counted by observers blind to experimental conditions. Multiple sections through the middle of the cord, in which axons were densest, were counted per mouse and expressed as total intercepts per location per animal. Axon counts were highly correlated to axon densities (Pearson correlation = 0.91).

Cell counts

To quantify the proportion of neurons expressing Vsx2 and/or Zfhx3, we counted the number of NeuN positive neurons expressing Vsx2 and/or Zfhx3. Cell counts were performed using the image analysis software Imaris (bitplane, version 9.8). To determine the proportion of neurons that were retrogradely traced and $Vsx2^{ON}$ after regeneration, we counted the number of neurons that were co-labeled for CTB and Vsx2. Similarly, to determine the projection patterns of $SC^{Vsx2::Hoxa7::Zfhx3\rightarrow lumbar}$ neurons, we quantified the proportion or neurons in the lumbar spinal cord receiving synaptic-like appositions from regenerated $SC^{Vsx2::Hoxa7::Zfhx3\rightarrow lumbar}$ neurons.

Synapse detection and quantification

To detect and quantify the number of synapses contacting neurons, we first reconstructed the surface of the cells using the surface reconstruction module in Imaris (bitplane, version 9.8) at a 10 μm resolution. The synapses were then identified using spot detection in Imaris. To identify the synapses in close apposition to the neurons of interest, a MATLAB algorithm was used to segregate the synapses located with a distance between 0 and 1 μm to the reconstructed surface of the neurons. Synapse density was normalized to the area of the reconstructed surface.

Electrophysiology

Animals were anesthetized with ketamine/xylazine, a small burr hole was drilled in the skull to provide access to the vGi and needle electrodes were inserted into the tibialis anterior muscle of both the left and right hindlimb. A platinum/iridium concentric bipolar electrode (PI-SNE-100, Microprobes USA) was inserted into the vGi (coordinates: −6.0 AP, 0.3 ML, −5.7 DV relative to bregma). Electrical stimulation (STG4000, Multi Channel Systems) was delivered to the vGi in trains of 5 × 200 μs square wave pulses at 500 Hz, repeated once every 5 seconds. Stimulation was delivered at 20, 40, 60, 80 and 100 μA, with 10 repetitions of each intensity. Evoked EMG recordings were amplified (1000×) and filtered (300 Hz high pass, 5 kHz low pass) using a differential amplifier (model 1700, AM Systems) before being digitized and recorded (PowerLab 8/35, AD Instruments). Mean peak to peak amplitudes of evoked responses were calculated offline (LabChart Pro, AD instruments) and compared between groups. Response amplitudes were normalized to the mean of peak amplitudes found in the uninjured group. The depth of anesthesia was controlled by giving an initial dose of ketamine and xylazine/xylazine that led to incomplete anesthesia (80% of normal full dose), followed by 0.05 mL at 5 minute intervals until withdrawal reflexes were lost. At this point, we performed the surgical

preparation and recorded exactly 15 minutes after surgical anesthesia was reached. All recordings were completed within 5 minutes of starting recording.

Statistics, power calculations, group sizes and reproducibility

Statistical evaluations of repeated measures were conducted by one-way ANOVA with post hoc independent pairwise analysis as per Tukey's honestly significant difference (HSD) test. Power calculations were performed using G*Power Software version 3.1.9.245. For quantification of histologically derived neuroanatomical outcomes such as axons density, group sizes were used that were calculated to provide at least 80% power when using the following parameters: probability of type I error (α) = 0.05, a conservative effect size of 0.25, 3-10 treatment groups with multiple measurements obtained per replicate. All graphs show mean ± s.e.m. as well as individual values as dot plots. All bar graphs are overlaid with dot plots in which each dot represents the value for one animal. Experiments testing axon regrowth across SCI lesions in animals were repeated independently at least twice in different groups of mice with similar results. For all photomicrographs of histological tissue, staining experiments were repeated independently with tissue from at least four, and in most cases six, different animals with similar results.

Single-nucleus RNA sequencing

Single-nucleus dissociation of the mouse lumbar spinal cord was performed according to our established procedures[468,470]. Following euthanasia by isoflurane inhalation and cervical dislocation, the lumbar spinal cord site was immediately dissected and frozen on dry ice. Spinal cords were doused in 500 μL sucrose buffer (0.32 M sucrose, 10 mM HEPES [pH 8.0], 5 mM CaCl2, 3 mM Mg acetate, 0.1 mM EDTA, 1 mM DTT) and 0.1% Triton X-100 with the Kontes Dounce Tissue Grinder. 2 mL of sucrose buffer was then added and filtered through a 40-μm cell strainer. The lysate was centrifuged at 3200 g for 10 min at 4◦C. The supernatant was then decanted, and 3 mL of sucrose buffer was added to the pellet for 1 min. We homogenized the pellet using an Ultra-Turrax and 12.5 mL of density buffer (1 M sucrose, 10 mM HEPES [pH 8.0], 3 mM Mg acetate, 1 mM DTT) was added below the nuclei layer. The tube was centrifuged at 3200 g at 4◦C and supernatant poured off. Nuclei on the bottom half of the tube wall were collected with 100 μL PBS with 0.04% BSA and 0.2 U/μL RNase inhibitor. Finally, we resuspended nuclei through a 30 μm strainer, and adjusted to 1000 nuclei/μL.

Projection-specific snRNA-seq

For projection-specific snRNA-seq experiments the nuclei were first resuspended in 500μL Pre-FACS buffer (1× PBS with 1% BSA, 0.2U/μL SUPERaseIn RNase Inhibitor), and filtered through a 35μm cell strainer. Samples were processed on a Sony SH800 Cell Sorter with a 100mm sorting chip. Nuclei were gated using forward scatter, side scatter and DRAQ5$^+$ measurements to ensure that doublets were gated out. After this initial gating, GFP$^+$ nuclei were identified using a two-dimensional scatterplot. GFP$^+$/DRAQ5$^+$ nuclei were collected into 1.5mL centrifuge tubes containing 10μL of the Pre-FACS buffer. We collected ~500 GFP$^+$ nuclei from the uninjured spinal cord (these nuclei were pooled from n = 5 mice) and ~800 GFP$^+$ nuclei from regenerating axons (these nuclei were pooled from n = 13 mice). GFP$^+$ nuclei were then loaded directly onto a Chromium Single Cell Processor (10X Genomics) for barcoding of RNA from single nuclei.

Library preparation

snRNA-seq library preparation was carried out using the 10X Genomics Chromium Single Cell Kit Version 3. The nuclei suspension was added to the Chromium RT mix to achieve loading numbers of 2,000-5,000. For downstream cDNA synthesis (13 PCR cycles), library preparation and sequencing, the manufacturer's instructions were followed.

Read alignment

We aligned reads to the most recent Ensembl release (GRCm38.93) using Cell Ranger, and obtained a matrix of unique molecular identifier (UMI) counts. Seurat[497] was used to calculate quality control metrics for each cell barcode, including the number of genes detected, number of UMIs, and proportion of reads aligned to mitochondrial genes. Low-quality cells were filtered by removing cells expressing less than 200 genes or with more than 5% mitochondrial reads. Genes expressed in less than three cells were likewise removed.

Clustering and integration

Prior to clustering analysis, we first performed batch effect correction and data integration across the two different experimental conditions as previously described[497]. Gene expression data was normalized using regularized negative binomial models[516], then integrated across batches using the data integration workflow within Seurat. The normalized and integrated gene expression matrices were then subjected to clustering to identify cell types in the integrated dataset, again using the default Seurat workflow. Cell types were manually annotated on the basis of marker gene expression, guided by previous studies of the mouse spinal cord[468,498–500]. Local and projecting neuronal subpopulations were annotated on the basis of Nfib and Zfhx3 expression, respectively[453]. Following our projection-specific snRNA-seq experiment in uninjured mice, each subsequent experiment was reintegrated with this dataset prior to subpopulation annotation. This enabled the identification of the same 28 neuronal subpopulations across the three distinct experiments[497].

Cell type prioritization with Augur

To identify neuronal subpopulations perturbed during natural repair, we implemented our machine-learning method Augur[468,470]. Augur was run with default parameters for all comparisons. To evaluate the robustness of cell type prioritizations to the resolution at which neuronal subtypes were defined in the snRNA-seq data, we applied Augur at various clustering resolutions, and visualized the resulting cell type prioritizations both on a hierarchical clustering tree[496] of neuron subtypes and as a progression of UMAPs. The key assumption underlying Augur is that cell types undergoing a profound response to a perturbation should become more separable, within the highly multidimensional space of gene expression, than less affected cell types. Briefly, Augur withholds a proportion of sample labels, then trains a random forest classifier to predict the condition from which each cell was obtained. The accuracy with which this prediction can be made from single-cell gene expression measurements is then evaluated in cross-validation, and quantified using the area under the receiver operating characteristic curve (AUC).

Cell type proportions

To compare the proportion of neuronal subpopulations within and between datasets, the normalized proportion of each neuronal subpopulation was calculated. The distributions were compared to the expected proportion in the uninjured dataset using the $\chi 2$ test.

Gene ontology analysis

GO term annotations for mouse (2019-12-09 release) were obtained from the Gene Ontology Consortium website. GO terms annotated to less than 5 genes were excluded. The average expression level of genes associated with each GO term in individual cells was calculated using the Seurat function AddModuleScores, which controls for the average expression of randomly selected control features. Linear models were then applied to GO module scores to characterize the gene programs activated within $SC^{Vsx2::Hoxa7::Zfhx3 \rightarrow lumbar}$ neurons after temporally-delayed staggered double hemisections and after regeneration. To identify gene programs specifically activated within

$SC^{Vsx2::Hoxa7::Zfhx3→lumbar}$ neurons, we tested for the presence of an interaction between experimental group and cell type, which was coded as a categorical variable ($SC^{Vsx2::Hoxa7::Zfhx3→lumbar}$ neurons vs. other).

Data availability

Raw sequencing data and count matrices have been deposited to the Gene Expression Omnibus: GSE198949.

Code availability

Augur is available from GitHub (https://github.com/neurorestore/Augur).

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Author contributions

J.W.S., J.B., M.V.S., G.C., and M.A.A. conceptualized and designed experiments; J.W.S., M.M., A.dC., N.D.J. N.C., C.K., T.H., S.C., L.B, K.G., A.L., Q.B., and M.A.A. conducted experiments; J.W.S., M.M., M.G, A.dC., M.A.S., N.D.J., A.L., A.L., V.A., Q.B., and M.A.A. analyzed the data. T.J.D., B.S., R.K., and Z.H. contributed essential resources. J.W.S., M.V.S., G.C., and M.A.A. prepared the manuscript.

Competing interests

The authors declare no direct competing financial interests. G.C. is a consultant and minority shareholders of ONWARD medical, a company with no relationships with the presented work.

4. CONCLUSIONS AND FUTURE DIRECTIONS

CONCLUSIONS

Summary

SCI interrupts the axonal connections between the brain and the spinal cord, resulting in permanent neurological deficits. Limited spontaneous recovery is observed in incomplete injuries, with highest potential in the case of lateralized lesions[480], and functional improvements can be greatly augmented by promoting beneficial reorganization of the spared axonal fibers via neuromodulation interventions based on biomimetic electrical stimulation and rehabilitation[8,98]. Significant advances have been observed in the field over the last decade by means of these strategies, and the constant technological evolution has unlocked progressively higher degree of recovery[7,511]. Despite this, it is agreed that bringing the injured spinal cord closer to a more complete repair will require providing new biological substrate via axon regeneration across the injury[9], and this is particularly crucial in the case of more severe SCI characterized by little to no axonal sparing.

We have extensively discussed how more than half a century of research has presented a plethora of different approaches to reverse the intrinsic regenerative incapacity of injured CNS neurons. In this respect, our group developed in the last years a regenerative intervention based on the combinatorial manipulation of three mechanisms, including activation of molecular growth programs, fibrotic scar remodeling and chemoattraction. While this strategy promoted robust regeneration of axons from spinal cord interneurons across a severe anatomically complete SCI, such regrowth was not associated to any recovery of lower limb function.

In the work presented in this thesis, we sought to build upon this strategy to dissect missing requirements for functional recovery. We shifted our focus back to a model of severe incomplete SCI characterized by two lateralized injuries, which is associated with spontaneous recovery of walking ability in spite of complete interruption of all direct projections from supraspinal centers. Comparative and projection-specific transcriptomic analyses allowed us to identify a specific subpopulation of thoracic interneurons, $SC^{Vsx2::Hoxa7::Zfhx3 \rightarrow lumbar}$ neurons, that naturally project to the lumbar spinal cord, and that underlie this observed recovery by relaying information from the brain to the lumbar centers for hindlimb locomotion after severe incomplete SCI. We therefore optimized our previously developed intervention to regrow axons from thoracic interneurons across anatomically complete SCI specifically to the lumbar spinal cord, and this successfully promoted regeneration of axons of the characterized subpopulation to their natural target region. Moreover, we provided more sustained chemoattractive guidance to the regenerated axons by modifying the modality of delivery of the neurotrophic factor GDNF and employing a lentivirus (LV) instead of a hydrogel carrier. Strikingly, starting from 4 weeks after injury, mice treated with our optimized regenerative intervention progressively regained spontaneous weight-bearing walking ability, that phenotypically resembled the locomotor patterns observed after natural repair following severe incomplete SCI. Loss-of-function experiments demonstrated that functional recovery following our regenerative intervention was largely dependent on the specific subpopulation of interneurons that we identified as responsible for natural repair.

Newly discovered requirements

While the previous work from our group identified three necessary mechanisms to regenerate axons across anatomically complete SCI, the results that we presented here show that additional principles need to be considered in order to achieve restoration of function via the regenerated axons.

First, and one of the main findings of this work, untargeted regeneration of axons simply across the injury is not sufficient to support recovery of function, despite the capability of these fibers to relay electrical signals – as shown via electrophysiology[332]. Rather, a crucial requirement to promote recovery of locomotor ability is to guide regenerated axons specifically to their natural target region. We did not characterize extensively the connectivity of regenerated axons with the various neurons subtypes in the lumbar spinal cord, nor the extent to which this recapitulated precisely the natural synaptic associations observed in the uninjured spinal cord [see **below**]; nevertheless, it appears evident from our results that, while establishing direct (i.e. monosynaptic) connectivity of regenerated fibers with regions controlling locomotor function successfully promotes recovery, untargeted regeneration is not sufficient to promote spontaneous formation of polysynaptic connections caudal to the injury able to restore meaningful function.
While the concept of target reinnervation has previously been addressed in the field[397,398], our study represents the first work showing that this is a specific requirement to achieve restoration of lower limb motor function; moreover, in the two cited studies, regrowth of axons to their intended target regions was not associated with any functional recovery, a finding that the authors attributed to insufficient myelination of regenerated fibers[397,398]. Multiple explanations might underlie the recovery observed in our specific case and not by our colleagues, including the different neurological function and axonal system being targeted, the nature of the employed regenerative intervention and the molecular mechanisms being stimulated; alternatively, it is possible that the high density of axon regrowth elicited by our strategy was sufficient to overcome potential lack of myelination in the regenerated fibers. We did not extensively investigate the extent to which axons regrowing in response to our treatment were myelinated, and this potentially opens future opportunities to determine whether our biological repair strategy might benefit from additional treatment with agents known to improve axon conduction[398].

Second, an additional requirement for recovery of locomotor function highlighted by our results is the targeting of specific neuronal subpopulations endowed with such ability. We here identified a neuronal subtype that is involved in recovery of walking after incomplete SCI, and showed that recapitulating its natural projection patterns reverses paralysis after complete SCI. We showed that these neurons are largely necessary to the observed recovery, as pharmacological inactivation led to substantial abolishment of the functional improvements. It is likely that regenerative strategies aimed at restoring functions other than lower limb locomotion will require identification and targeting of different neuronal populations.

Third, we showed that sustained chemoattraction to the target region was required to encourage locomotor recovery. We showed that producing a chemoattractive gradient via biomaterial-based delivery of restricted amount of GDNF was sufficient to guide regenerating propriospinal axons to the lumbar spinal cord, but that this strategy attracted a comparatively low density of fibers likely insufficient to convey meaningful

signals for spontaneous recovery. On the contrary, sustained overexpression of GDNF via lentivirus resulted in long-distance high-density axon growth in the lumbar segments, and associated return of function. Whether a titer-dependent effect of GDNF partially underlies the exclusive observation of recovery in lentivirus-treated mice (as opposed to depot-treated), e.g. by facilitating local synaptogenesis specifically at higher concentrations, or whether recovery is mainly dependent on the higher number/density of chemoattracted fibers is unknown. Observations of retraction of the regenerated fibers at more chronic timepoints in rats treated via depot-only strategies [data not shown] points to a role of GDNF mainly in local axon guidance, consistent with its known roles in development[517], and suggests that the limited amount of neurotrophic factor that can be carried by hydrogel depots might not be sufficient to sustain maturation and integration of functional circuits.

Context with other anatomically complete SCI treatments

As discussed in the **first part of this thesis**, multiple strategies have been reported to support axon regeneration across anatomically complete SCI, including mainly strategies based on whole tissue/cell grafts, or on exclusive manipulation of growth programs.

Among these, approaches relying on implants of PNS or fetal CNS origin[363,374] have often failed to stand the test of time and reproducibility, and are intrinsically associated with limitations concerning tissue sourcing. Current approaches based on neural stem cell grafts, in turn, offer outstanding results in terms of axon growth but functional recovery is limited (e.g. partial lower limb movements with no weight support)[379]. Additionally, a fundamental conceptual difference of these interventions compared to ours is that regeneration across the injury mainly originates from extension of axons derived from grafted cells rather than regrowth of host axons. While relaying supraspinal information across the injury via graft-derived cells – as opposed to host spinal interneurons as in our intervention – is also a mechanism that offers promising perspectives in terms of recovery for clinical translation, I argue that approaches relying on progenitor cells have specific characteristics that warrant special considerations.
We have discussed, for instance, how our results show that regeneration-dependent restoration of neurological functions requires re-growing axons from specific neuronal subpopulations and guiding them to their target region. Accordingly, particularly critical to direct functional recovery via stem cell-based approaches will be the capacity to steer differentiation of graft-derived cells towards specific neuron subtypes, and to control their extensive spontaneous outgrowth to guide them to the proper target. Adding a layer of complexity to this, the graft composition (e.g., differentiation of cells more towards ventral compared to dorsal interneurons, and vice versa) has been shown to influence the regrowth of specific host tracts inside the graft (e.g., penetration of corticospinal and sensory axons is significantly higher in grafts of dorsal identity compared to ventral-derived grafts)[505], and might be likely to influence the amount and functional meaning of newly-formed relay connections. All of these considerations do not apply to our intervention, that involves manipulation of characterized, differentiated host cells. Nevertheless, it is conceivable that stem cell-based therapies might also be fundamental for repair in case where available host cellular substrate is particularly limited.

On the other hand, other groups have showed that targeting specific signaling pathways (mTOR, JAK/STAT) that are also activated in our combinatorial regenerative strategy is on its own sufficient to promote regeneration of key supraspinal motor tracts across a complete SCI[417,419]. A recent paper by Leibinger et al., in particular, showed that such regrowth is also sufficient to support spontaneous emergence of weight-bearing locomotion[419], similarly to what is observed in our strategy.
As extensively reviewed in **Regeneration after complete SCI**, a key difference between these two studies and ours is the specific modality employed to perform a complete crush injury, with our model resulting in a larger fibrotic core devoid of the glial strands observed to bridge the lesion following the paradigm employed by our colleagues. Related to this, Zukor et al. showed that exclusive manipulation of the mTOR pathway is not sufficient to promote regeneration even simply inside the lesion site if a more severe injury model similar to ours is used[415], and I argue that similar results would be obtained with the intervention proposed by Leibinger et al. (targeting the JAK/STAT pathway): exclusive co-activation of these two molecular growth programs, although achieved differently compared to the two abovementioned studies, was already shown by our group to be insufficient to promote regrowth of propriospinal axons across our severe model of complete crush (AAV-OIC on its own promotes little to no regeneration in the lesion core[332]); it is therefore likely that the same would be observed with supraspinal axons. In particular, manipulation of the fibrotic scar and formation of growth-supportive substrates appears to be a fundamental requirement to allow penetration of regenerating fibers inside severe injuries that lack bridging via glial strands[332].

The results achieved with our newly developed strategy represent therefore one of the most robust extent of regeneration across anatomically complete SCI, specifically in terms of axon density [confront with Fig. 2 in ref. [419]], and, to our knowledge, the first instance of recovery of voluntary weight-bearing locomotion via exclusive manipulation of host tissue and employing such a severe injury model.

Additionally, it is important to highlight that our strategy targets specifically propriospinal interneurons, a neuronal class that is not shown to be recruited by any of the regenerative interventions mentioned above. Thoracic spinal cord interneurons, including $SC^{Vsx2::Hoxa7::Zfhx3 \rightarrow lumbar}$ neurons, represent a neuronal system endowed with particularly high plasticity following injury, as extensively discussed before in this thesis. Therefore, strategies aiming at regenerating these neurons are of high interest for clinical applications, including potentially for repair of neurological functions other than walking ability.

Limitations of our study

Above, I have proceeded to summarize the main results of the work in which I was involved during my PhD project, and the scientific advancements provided by our findings in the context of alternative approaches employed in the field. Likewise, it is important to mention that our study presents some limitations and that, in particular, additional points might need to addressed to have more comprehensive understanding of the biological processes involved.

A first clarification concerns the role of $SC^{Vsx2::Hoxa7::Zfhx3\rightarrow lumbar}$ neurons in the spontaneous recovery observed following severe incomplete SCI. We have indeed explained how we performed comparative snRNA-seq analysis (i.e. isolating directly tissue in between the two hemisection lesions, without tracing, and comparing it to tissue from the same spinal segments in the uninjured spinal cord), that identified this subpopulation as the most transcriptionally perturbed following natural repair. Nevertheless, as already mentioned in **Augur: cell-type prioritization in single-cell data**, the magnitude of overall transcriptional change in response to a biological intervention does not directly imply causality in the contribution to the observed phenotype. In particular, while these results point to an increased responsivity of $SC^{Vsx2::Hoxa7::Zfhx3\rightarrow lumbar}$ neurons as a consequence of natural repair compared to other neuron subtypes, it cannot be fully excluded that this thoracic subpopulation might exhibit higher transcriptional perturbation simply as a result of intrinsic properties, consistent with the known plasticity of *lumbar* Vsx2 neurons following injury[30].
On the other hand, projection-specific snRNA-seq (i.e. via isolation of midthoracic neurons retrogradely traced from the lumbar spinal cord, similarly to what performed in the uninjured group) would have allowed us to identify specific subpopulations that relay the area in the bridge between the two hemisections to the lumbar segments. The biological answer provided by this strategy would nonetheless not have been sufficient to establish causality either, since direct connectivity does not necessarily imply a role in relaying a neurological function. Additionally, we decided to opt for comparative snRNA-seq also because we anticipated that projection-specific tracing would have yielded an insufficient number of labeled neurons owing to the difficult of efficiently tracing axons winding around two opposite-side lesions.
To establish causality, we are currently in the process of performing loss-of-function studies that will allow to determine contribution of specific populations of thoracic neurons in recovery following natural repair, by injecting Cre-dependent Diptheria Toxin Receptor in the spinal segments between the two hemisections in $Vsx2^{Cre}$ mice and in control lines for other subpopulations.

A second important point of reflection is the connectivity of regenerated axons to the lumbar spinal cord. We have provided electrophysiology evidence that these axons are capable of relaying electrical signals from supraspinal motor centers across the injury (**Figure 3.8D**), and have shown via histology that they are in close apposition with multiple cell types in the lumbar spinal cord, reflecting likely formation of functional synapses (**Figure 3.9E**). We have additionally demonstrated via chemogenetic inactivation that the direct projection of thoracic long-projecting Vsx2 neurons to their natural target in the lumbar spinal cord is responsible for most of the observed functional recovery (**Figure 3.15**), therefore highlighting target reinnervation as a crucial requirement for restoration of walking. A missing link in our understanding is the extent to which target reinnervation for functional recovery requires exact re-establishment of the cell-type-to-cell-type connectivity present in the uninjured spinal cord. We have displayed contacts of regenerated axons with the same lumbar neuron populations that underlie walking ability in uninjured mice (**Figure 3.9E**), but we have not extensively quantified the distribution of these connections: it is possible that regenerated long-projecting thoracic Vsx2 neurons might largely connect via direct monosynaptic contacts to $ChAT^{+}$ motor neurons to drive locomotion, or on the contrary rely mostly on poly-synaptic connections with local interneurons known to be locomotor circuit integrators, such as lumbar local-range Vsx2[30] (i.e., Vsx2-to-Vsx2 connectivity) or other

subpopulations comprised in CPGs. Additionally, unknown is the distribution of connections from $SC^{Vsx2::Hoxa7::Zfhx3 \rightarrow lumbar}$ neurons in the uninjured spinal cord, and the extent to which these are recapitulated following GDNF-mediated chemoattraction in the lumbar spinal cord. All these questions would need to be addressed via time-demanding projection-specific histological analyses, that were outside of the main scope of our work: a potential experimental paradigm might consist of Vsx2-restricted anterograde tracing of regenerating thoracic neurons (e.g. via AAV5-FLEX-tdTomato in $Vsx2^{Cre}$ mice), processing of lumbar tissue via in situ hybridization (RNAscope[518]) assays against relevant markers of neuronal subtypes and quantification.

In line with the previous point, another important clarification is that we logically equated mice recovering walking following regenerative intervention with mice exhibiting natural repair after severe incomplete SCI, mainly based on the fact that recovery is dependent on the same neuronal subpopulation in both cases. Nevertheless, we did not establish whether comparable synaptic reorganization occurs in the lumbar spinal cord upon recovery in the two groups. Accordingly, the output of the classifier presented in **Figure 3.11E** does not imply that the walking phenotype of mice treated with regenerative intervention exactly recapitulates the gait features of mice spontaneously walking after severe incomplete SCI, but rather that their overall gait patterns resemble those of mice undergoing natural repair more than they approximate locomotion of mice from the other two tested classes, i.e. uninjured or injured untreated. We performed additional comparison of kinematics data from mice that recovered after double hemisection SCI and from mice receiving lentivirus-based regenerative treatment, in the same principal component analysis (together with uninjured mice and mice treated with depot-based chemoattraction): statistical hypothesis testing of data from principal component 1 – that represents an estimate of the overall locomotor score [see **Figure 3.3** and **Methods**] – failed to show a significant difference between the two groups ($p= 0.925$, Tukey's HSD post-hoc pairwise comparison following one-way ANOVA ($F(3)= 82.12$, $p<0.0001$)) [data not shown]. Additional downstream comparisons of individual gait features (e.g. speed, gait coordination, drag time) would nonetheless need to be considered in order to determine more specifically actual similarity/difference in overall locomotor patterns between the two groups.

An additional minor point that we did not characterize extensively is whether axonal tracts (e.g. transected supraspinal pathways) other than thoracic propriospinal neurons regenerate following our intervention. We have extensively discussed before in the thesis that CNS neurons exhibit little to none spontaneous regenerative capacity following injury, and it is therefore likely that no other populations would be regenerating in response to our approach, considering that injections of OPN, IGF1 and CNTF to activate intrinsic growth programs were targeted specifically to the thoracic spinal cord rostral to the injury. It cannot be excluded in principle, nonetheless, that the two other interventions included in our strategy, i.e. the hydrogel depot inside the lesion and the GDNF chemoattractive gradient, might be sufficient on their own to promote regeneration of a limited amount of transected fibers other than from thoracic interneurons. Approaches based on peripheral nerve grafting are for instance known to promote regeneration of selected supraspinal pathways without direct stimulation of molecular programs, although very limited and restricted to the lesion core[362]. Alternatively, Leibinger et al. showed that injections of AAV-hyper-IL6 targeted to the motor cortex promote regeneration not only of corticospinal tract (CST) fibers, but also

of serotonergic fibers with somata in the raphe nuclei, via transneuronal release from CST axon branches projecting to the brainstem[419], and a similar mechanism could be hypothesized in our case, although highly improbable.
We do nonetheless know that our previous repair strategy did not promote regeneration of serotonergic fibers[332], known to be one of the axonal systems with highest growth competence, and it is difficult to imagine that a specific change in our protocol (modality and regional targeting of GDNF delivery) might yield any different results. We additionally show that the observed recovery following our optimized regenerative intervention is largely dependent on thoracic long-projecting Vsx2 interneurons, so any limited untargeted regeneration of supraspinal fibers would be of little interest to our study.

Finally, specific characteristics of our newly developed approach, particularly related to timing of the injections and type of delivery, make the experimental paradigm difficult to translate to clinics in its current form, as discussed **below**. Current and future work from our group is aimed at further optimizing our intervention to address all these points for translation.

Potential contributions of other subpopulations of thoracic interneurons

Our results show that regeneration of thoracic interneurons to the lumbar spinal cord reverses paralysis after anatomically complete SCI, and that most of the observed behavioral recovery is ascribable to $SC^{Vsx2::Hoxa7::Zfhx3\rightarrow lumbar}$ neurons, as selective silencing of this neuronal subpopulation leads to significant re-impairment. It is nonetheless important to specify that contribution from other neuron subtypes to the observed restoration of motor function cannot be excluded, and is on the contrary highly plausible by looking at our results.

First, mice undergoing chemogenetic inactivation of $SC^{Vsx2::Hoxa7::Zfhx3\rightarrow lumbar}$ neurons following recovery do not show complete abolishment of the regained walking ability, but rather show a behavioral phenotype that is somewhat intermediate to the walking patterns exhibited by treated mice with no inactivation and by untreated injured mice: this can be inferred both from the plot and statistics on the PCA analysis shown in **Figure 3.15C**, as well as from the output of our classifier, that estimated roughly equal proportions of the steps from CNO-treated mice as similar to SCI-only mice and as resembling walking following natural repair (**Figure 3.15D**). On the other hand, ablation of all Vsx2 midthoracic neurons without distinction of their projection pattern led to more pronounced re-impairment that was more clearly classified as phenotypically similar to the complete paralysis of untreated mice (**Figure 3.14C-F**): this suggests that subclasses of thoracic Vsx2 neurons other than $SC^{Vsx2::Hoxa7::Zfhx3\rightarrow lumbar}$ neurons, e.g. local-range Vsx2 neurons, might partially contribute to the behavioral recovery, for instance by modulating inputs to $SC^{Vsx2::Hoxa7::Zfhx3\rightarrow lumbar}$ neurons and to other regenerated neurons.

Second, on the line of this last sentence, our projection-specific snRNA-seq show that only ~25% of regenerated neurons are $SC^{Vsx2::Hoxa7::Zfhx3\rightarrow lumbar}$ (**Figure 3.10H**), in accordance with similar results from histology (**Figure 3.10J**). This proportion, the highest among all the 28 identified neuronal clusters, represents a large value for a population that is only estimated to account for about 2 to 4% of all neurons in the uninjured thoracic spinal cord (**Figure 3.6B,E, Figure 3.10H**); this is also demonstrated by the fact that

$SC^{Vsx2::Hoxa7::Zfhx3\rightarrow lumbar}$ are the only subtype that is significantly enriched among regenerated neurons as compared to the uninjured thoracic spinal cord (**Figure 3.10H,** considering false discovery rate-correction). Nevertheless, these results also point to the fact that other subpopulations respond, although to a smaller extent, to our regenerative treatment. It is possible that not all these other subtypes of neurons regrowing to the lumbar spinal cord actually exert direct influence on locomotor output, as some axonal connections might also serve as modulators of projections from other neurons; yet, necessity for recovery of each other neuron subtype responding to our treatment would need to be further investigated.

To summarize, while $SC^{Vsx2::Hoxa7::Zfhx3\rightarrow lumbar}$ neurons underlie most of the observed recovery, other neuronal subpopulations respond to our regenerative treatment and are likely to also contribute to function. This observation, nonetheless, does not contrast with the main focus of the study, i.e. that our optimized approach, based on regeneration to a proper target region via sustained chemoattraction, reverses paralysis following anatomically complete SCI, a result that has not been shown robustly in the field before.

FUTURE DIRECTIONS

Need for optimization of the current paradigm

The experimental work presented in this thesis represented a proof-of-principle study aiming at demonstrating that targeted regeneration across anatomically complete SCI can successfully restore lost neurological function. While the clinical implications of our findings and the potential impact for patients is extremely high, the primary objective of the study was therefore to provide evidence towards a defined scientific hypothesis, not to develop an intervention that could result in a therapy directly translatable to clinics. As a consequence, the current experimental paradigm presents specific limitations that will require future modifications of the protocol in view of potential clinical translation.

First, the timing of the current intervention is by definition not applicable to clinics, as injections of adeno-associated viruses (AAV) expressing OPN/IGF1/CNTF (AAV-OIC) targeted at midthoracic neurons are performed 2 weeks *before* injury. This choice derives mainly from standard approaches employed in the field, where manipulation of intrinsic growth programs is usually performed before injury or even neonatally, in order to "prime" adult neurons into a pro-regenerative state [see **CNS regeneration**]. In particular, original studies from He and colleagues set 2 weeks before injury as a gold standard for AAV-mediated activation of the mTOR/JAK-STAT pathways[223,227], or for injection of the specific combination of AAV-OIC in optic nerve regeneration studies[398], consistent with optimal expression time of this category of viruses[407,519,520]. A potential option would be to evaluate the effect of overexpressing OIC as soon as possible after injury. He's group showed that AAV-OIC-mediated regeneration across optic nerve injury is robustly observed also if delivery is postponed to 2 days after injury[398]; nevertheless, this contrasts with our preliminary observations of limited regeneration of thoracic interneurons inside anatomically complete SCI lesion if AAV-OIC injections are performed in the same surgery as the injury (data not shown). The optic nerve study nonetheless provides evidence of axon regeneration at 15-16 weeks post injury (i.e., a significantly longer timespan in comparison to our histological assessments, performed at 8 weeks post injury), therefore it is not known whether the same would be observed at less chronic timepoints after injury. It is in the same way possible that regeneration with post-injury AAV-OIC injection would be observed also in our experimental paradigm if more time was allotted before sacrifice.

We nonetheless hypothesize that the limited regeneration observed with AAV-OIC delivery on the same day of injury might derive from potential existence of a time-restricted window of intervention; this hypothesis is also supported by findings on optic nerve injury showing that activation of the molecular pathways recruited by OIC[223,407], or direct delivery of the growth factors[415,521], additionally exert a positive effect on neuronal survival, implying that timely delivery might be required to prevent significant neuronal death. Moreover, regardless of considerations on survival, the increased metabolic requirements of acutely-axotomized neurons might imply that a more pronounced stimulation by growth factors might be necessary to produce a regenerative response comparable to uninjured neurons. We are therefore in the process of testing an intervention with lentivirus-based delivery of OIC immediately after injury: we hypothesize that this will result in high-titer delivery of the three molecules, and

potentially faster expression – in line with the observations of lentivirus-mediated transgene expression as early as 5-7 days after brain injection[522,523] – and will consequently allow to overcome the limited regeneration observed with AAVs.

Additionally, translation of the therapy to higher species with larger spinal cord size or to lesions at higher spinal levels implies that multiple spaced injections of GDNF might need to be performed caudal to the lesion, which also requires specific considerations.

Such a rostro-caudal GDNF gradient might for instance be achieved by placing multiple GDNF-carrying hydrogel depots rostral to the LV-GDNF injection in the target lumbar segment, yet this would not be an ideal solution, as placement of the gel into *viable* tissue causes local compression, which would result in creation of further smaller injuries below the lesion core [see GFAP-negative area in **Figure 3.9B** in correspondence of the second hydrogel depot].

On the other hand, placing multiple sequentially-spaced LV-GDNF injections caudal to the lesion is also likely to be an impractical option, as the high-titer of the secreted GDNF might produce dense local axon growth and act as a termination signal, therefore preventing axons to grow past the rostral-most lentivirus injection; these concerns are raised based on similar observations in other studies on delivery of growth factors after SCI via lentiviruses[169] or via alternative approaches producing high concentration at the injection site[364,524]. The consideration holds particularly true for GDNF, as uncontrolled delivery of this neurotrophic factor is known for instance to produce entrapment of regenerating *peripheral* nerve axons as a consequence of a "candy-store" effect[525], an impediment which can be overcome via regulated (e.g. time-restricted) expression[526,527]. Optimization will therefore be crucial and will likely require delivery of lentivirus at increasing titers along the rostro-caudal axis.

Finally, the current intervention is performed in three different surgeries, one for each manipulation (AAV-OIC, depot in the lesion, LV-GDNF) and, as just mentioned, the requirement of a chemoattraction gradient in cases where longer-distance regeneration is necessary might increase the number of injections needed. It will therefore be important to investigate whether it is possible to combine some or all of these components in the same surgery or, more importantly, if all components are needed at all when the approach is employed for anatomically incomplete injuries (see **below**).

<u>Additional considerations for translation</u>

In addition to modifications to our current protocol, important considerations for translation, as applicable to all therapeutic trials, concern safety of the treatment and expected outcome depending on the pool of patients included in the study.

For the first aspect, one main potential point of reflection is the use of lentiviruses as a carrier for gene therapy. Lentiviruses, contrarily to recombinant AAVs – that mostly persist in the infected cell as episomes and integrate at very low frequencies[528] – , stably integrate in the host genome and are therefore associated with a higher risk of

insertional mutagenesis[529]. On the other hand, over the years, changes in the design have led to further biosafety improvements for new generations of lentiviruses[530,531], and multiple trials have recently employed lentivirus-based gene transfer for applications in immune[531] and specific neurological diseases[532,533]. Use of lentiviruses might be particularly beneficial to our strategy due to the preliminary observations of limited results with AAV-OIC injected post-injury (see above) and to the larger packaging capacity (~8-9 kilobases (kb) vs 4-5 kb for AAVs[530]), that might allow expression of OPN, IGF-1 and CNTF in a single tri-cistronic vector as opposed to the current co-injection of three AAVs. Additional optimization of safety requirements of lentivirus-based delivery might imply time-restricted transgene expression (e.g. drug-controlled)[534], and will be crucial to provide a therapeutic "switch" in case of emergence of severe side effects (e.g. as a result of detrimental plasticity).

Second, we have shown experimentally that the combination of all three components of our strategy (i.e., stimulation of intrinsic growth programs, manipulation of lesion environment, chemoattraction) is required to achieve regeneration (and therefore recovery) after anatomically complete SCI[332]. Nonetheless, it is likely that, in the case of incomplete lesions, manipulation of two or even one single mechanism will be capable of promoting biological repair to an extent sufficient to produce meaningful restoration of function. This consideration is crucial both from a clinical perspective (to ensure that patients receive the minimal required treatment) and for regulatory purposes (as each intervention will require individual approval from regulatory agencies for clinical testing). The lab has started further experimental work to establish correlation between specific injury parameters (lesion severity, age, ...) and the extent of axonal regrowth achieved with specific combinations of the three manipulations.

Finally, while $SC^{Vsx2::Hoxa7::Zfhx3\rightarrow lumbar}$ neurons have emerged as the main contributors to the observed recovery promoted by our regenerative intervention in mice, it is unknown whether the same subpopulation presents the same anatomical and functional organization across species. In particular, as discussed already in **Recovery after incomplete injury: neuromodulation strategies** for *local-range* Vsx2, also the thoracic Vsx2 neurons projecting to the lumbar spinal cord and characterized in our study have only been described in mice: despite the fact that Vsx2 is expressed in the human CNS[137], whether this same subpopulation of neurons exists in the human thoracic spinal cord remains to be determined. Nevertheless, given that spontaneous recovery following lateralized injury is also observed in patients, it is likely that such functional improvement might be dependent on $SC^{Vsx2::Hoxa7::Zfhx3\rightarrow lumbar}$ neurons or an equivalent neuronal subpopulation also in humans; this provides positive perspective on the potential of our therapy, that mimics the natural repair that follows lateralized SCI, to promote functional regeneration of long-projecting Vsx2, equivalent populations or any other GDNF-responding neuron subtypes also in patients.

These and other considerations will be addressed in future work from our group, that will deal with optimization and translation of our approach to rats and non-human primates, with the dream of offering a novel therapy to repair the injured human spinal cord.

Necessity of regenerating supraspinal motor tracts

We have extensively discussed the remarkable plasticity of specific thoracic propriospinal neurons and their crucial role in recovery of motor function after injury. Nonetheless, the locomotor patterns observed both after natural repair following incomplete SCI and in response to our regenerative intervention after complete SCI present substantial residual functional impairment with respect to the walking ability of uninjured mice: in particular, as discussed before, the newly established interneuron relay circuits likely interact with pre-existing repertoire of local networks in the lumbar spinal cord to restore *basic* locomotion (including weight-bearing coordinated stepping and partial speed adjustment capacity), consistent with potential CPG-like activity of embedded lumbar connections[146]; on the other hand, fine motor control is not restored, and mice fail to regain ability to perform precision tasks such as ladder paw placement[146]. It is generally agreed that such restoration of precision motor control will require direct regeneration of descending supraspinal pathways[146,535].

Pronounced anatomical and functional divergence across species influence the role of specific supraspinal pathways in recovery after injury[480].
Rodents, for instance, rely heavily on the rubrospinal tract and on raphespinal (i.e. serotonergic) fibers for basic locomotion, as lesion of these axons creates devasting effect on rat gait[535–537]. Accordingly, provision of serotonergic agonists is sufficient to produce ex vivo fictive locomotion[538] and to restore basic treadmill (i.e. sensory-triggered) locomotion in rats[539], and interventions resulting in regeneration of raphespinal axons promote recovery of basic walking in mice with complete paralysis[419]. Nevertheless, the role of these tracts is also thought to only recapitulate unskilled, basic motor tasks[419].
On the other hand, the corticospinal tract (CST) is traditionally seen as crucial, among other functions, for control of fine motor abilities, with its contribution to these tasks being more developed in primates compared to lower mammals[540]. In line with this observation, sprouting of uninjured CST fibers occurs spontaneously after lateralized SCI in primates but not in rodents, and is correlated with the recovery of fine motor control abilities observed after such injuries in monkeys and patients but not in rats[480]. Besides this role in skilled locomotion, the CST is thought to be involved in execution of even basic recovered motor tasks following injury in humans[480], and bilateral lesions of this axonal system completely abolish lower limb movement in patients[535,541], although the role in basic locomotion is thought to be more limited in rats and monkeys[535,542,543].
For such reasons, development of regenerative therapies targeted particularly at CST axons is a crucial clinical requirement[480].

It remains nonetheless to be determined whether direct translation of our approach would produce regeneration of other axonal tracts. Unknown is, in particular, whether the combination of OPN, IGF-1 and CNTF will be sufficient to stimulate intrinsic growth programs in neurons other than spinal cord interneurons, although evidence of regeneration of both CST and serotonergic axons in response to mTOR and JAK/STAT activation[417,419] points towards this direction. Additionally, as extensively discussed, activation of growth-associated signaling pathways is not sufficient to promote regeneration across severe lesions with no glial sparing, and requires additional manipulation of the fibrotic core and chemoattraction of the regenerating axons; in this respect, it will be important to determine whether other axonal tracts are able to respond

to GDNF-mediated guidance, or which alternative chemoattractants, based on cell type-specific expression of relevant receptors, will need to be employed[10].

Similar considerations apply to ascending pathways, regeneration of which will be fundamental to restore sensory function, and potentially important also for fine motor control[146].

Vision for combinatorial treatments

Neuromodulation strategies based on biomimetic epidural electrical stimulation (EES) have revolutionized the field of spinal cord injury in the last decade and provided new hope for the restoration of otherwise permanently lost function. Nevertheless, as extensively discussed, the necessity of spared axonal connections makes these treatments inapplicable for severe injuries, and constraints including the insufficient spatial resolution of currently available technologies pose intrinsic limits to attainable recovery.

On the other hand, biological approaches aimed at promoting tissue repair and regeneration of severed axons across the injury hold great potential to push the bar higher and promote recovery also after the most severe lesions, which was the focus of this thesis.

Despite this, achieving the dream of complete repair of the injured spinal cord will imply restoring a multitude of different functions apart from basic walking abilities, and will accordingly require recapitulation of numerous axonal systems connecting different targets across the CNS. It seems therefore evident that combination of regenerative approaches with neuromodulation strategies and potential other interventions will likely be needed, and that each of the approaches will be more or less suitable depending on the targeted function.

Specific autonomic functions such as hemodynamic control can be recapitulated to a significant extent via neuromodulation, as closed-loop EES is sufficient to promote the establishment of a neuroprosthetic baroreflex and to control the occurrence of orthostatic hypotension even after complete SCI[8].

On the contrary, EES-based strategies are intrinsically limited in restoration of brain-modulated abilities, such as fine motor control (as discussed above), or fail to replace other autonomic function in the case of severe injury.
Targeted EES of the lumbosacral cord is, for instance, able to promote bladder voiding after contusion SCI, but fails to do so in animals with complete injury, highlighting the need of spared connections with supraspinal centers [personal communication, unpublished work from our group]. A complex system such as the neural circuitry controlling lower urinary tract function is a relevant example of an instance where combinatory interventions would be an optimal solution: multiple axonal networks between spinal and supraspinal centers govern micturition, including parasympathetic and sympathetic circuits that control voiding, afferents that signal bladder distension and somatic efferent pathways for voluntary control of the sphincter[544]; targeted regeneration of each of these pathways might therefore be cumbersome, whereas it is

likely that selective regrowth of a subset of them would be sufficient to restore meaningful function if combined with neuromodulation.
In addition, task-specific EES-mediated modulation might also be crucial to promote successful integration of regenerated axons with relevant target circuits in case of restoration of functions that cannot be voluntarily trained, such as, by definition, those mediated by the autonomic nervous system.

Finally, we hypothesize that complete repair might also require multiplexing with other strategies that promote digital bridging of the lesion, such as brain-spine interfaces, that hold the potential to allow voluntary EES-mediated locomotion even in case of anatomically complete injuries[511].

GLOSSARY

AAD – Acute Axon Degeneration

AIS – ASIA Impairment Scale

AMPK – 5'AMP-activated protein Kinase

ASIA – American Spinal Cord Injury Association

ATAC-seq – Assay for Transposase-Accessible Chromatin sequencing

AUC – Area Under the receiver operating characteristic (ROC) Curve

BSCB – Blood Spinal Cord Barrier

ChABC – Chondroitinase ABC

ChAT – Choline Acetyltransferase

ChIP – Chromatin ImmunoPrecipitation

CNO – Clozapine-N-Oxide

CNS – Central Nervous System

CREB – cAMP-responsive Element-binding Protein

CS4/CS6 – 4-O/6-O-Sulfation

CSF – CerebroSpinal Fluid

CSPG – Chondroitin Sulfate ProteoGlycan

CST – CorticoSpinal Tract

DAMP – Damage Associated Molecular Patterns

DLK – Dual Leucine zipper Kinase

DRG – Dorsal Root Ganglion

DTR – Diphteria Toxin Receptor

ECM – Extracellular Matrix

FANS – Fluorescence-Activated Nuclear Sorting

GalNAc – N-acetylgalactosamine

GAP – Growth Associated Protein

GFAP – Glial Fibrillary Acidic Protein

GFP – Green Fluorescent Protein

GlcUA – Glucuronic Acid

HRP – Horseradish Peroxidase

iPSC – inducible Pluripotent Stem Cell

IS – Inflammatory Stimulation

ISNCSCI – International Standards for Neurological Classification of Spinal Cord Injury

JAK – Janus Kinase

JNK – c-Jun N-terminal Kinase

KSPG – Keratan Sulfate ProteoGlycan

LZK – Leucine Zipper Kinase

MAPK – Mitogen Activated Protein Kinase

miRNA - microRNA

MRI – Magnetic Resonance Imaging

mRNA – messenger RNA

mTOR – mechanistic/mammalian Target Of Rapamycin

NPC – Neural Progenitor Cell

NSC – Neural Stem Cell

PAMP – Pathogen Associated Molecular Patterns

PG – Proteoglycan

PI3K – Phosphoinositide-3-Kinase

PNS – Peripheral Nervous System

PRR – Pattern Recognition Receptors

PTEN – Phosphatase and Tensin homolog

RAG – Regeneration Associated Gene

RGC – Retinal Ganglion Cell

RNAi – RNA interference

ROCK – Rho-associated protein kinase

ROS – Reactive Oxygen Species

RTK – Receptor Tyrosine Kinase

SCI – Spinal Cord Injury

siRNA – small interfering RNA

snRNA-seq – single-nucleus RNA sequencing

STAT - Signal Transducer and Activator of Transcription

TET – Ten-eleven translocation methylcytosine dioxygenases

TIA – Trauma Induced Autoimmunity

UMAP – Uniform Manifold Approximation and Projection

WD – Wallerian Degeneration

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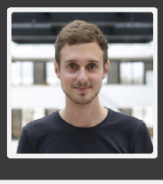

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ABOUT ME

Science enthusiast eager since childhood to learn and understand the world. The belief in the importance of a solid theoretical approach to model phenomena has naturally driven me towards Engineering, while the fascination for nature and its complex rules has pulled me towards Biology and Medicine. The desire of being able to model and mimic those complex rules, together with personal events, has made Neuroscience the logical and ideal endpoint for my studies. Looking to pursue a career in neural repair and regeneration, strong supporter of scientific collaboration, determined to have a concrete impact on people's lives.

EDUCATION

▶ **PhD, Neuroscience (Mar 2020 - Nov 2023)**

École Polytechnique Fédérale de Lausanne, Switzerland

- Project: "Targeted regeneration of spinal cord neurons across a complete injury restores walking".
- Thesis director: Grégoire Courtine. Thesis co-director: Mark Andrew Anderson.
- Supervised, evaluated and taught M.Sc. students, both as teaching assistant and exam proctor (300 hours total).

▶ **MSc, Bioengineering (Sep 2017 - Feb 2020) | Average: 5.63/6**

École Polytechnique Fédérale de Lausanne, Switzerland

- Orientation: Regenerative Medicine. Minor in Neuroprosthetics.
- Master's Project: "Characterization of regenerating propriospinal neurons after spinal cord injury" - UPCOURTINE (EPFL).

▶ **BSc, Biomedical Engineering (Sep 2014 - Jul 2017) | 110 cum laude/110**

Politecnico di Torino, Italy

PUBLICATIONS

▶ J. Squair*, M. Milano*, A. de Coucy, M. Gautier, M. Skinnider, N. James, N. Cho, A. Lasne, C. Kathe, T. Hutson, S. Ceto, L. Baud, K. Galan, Q. Barraud, T. Deming, R. Kohman, B. Schneider, Z. He, J. Bloch, M. Sofroniew#, G. Courtine# & M. Anderson# (2023). **Recovery of walking after paralysis by regenerating characterized neurons to their natural target region.** *Science*, 381, 1338-1345.

▶ M. Skinnider*, J. Squair*, C. Kathe, M. Anderson, M. Gautier, K. Matson, M. Milano, T. Hutson, Q. Barraud, A. Phillips, L. Foster, G. La Manno, A. Levine & G. Courtine (2020). **Cell type prioritization in single-cell data.** *Nature biotechnology*, 39(1), 30-34.

TECHNICAL SKILLS

Wet lab

- **Rodent models [FELASA A+C+D]:**
  Manipulations (handling, injections, blood sampling)
  Surgeries (spinal/brain stereotactic injections, bladder injections and acute pressure sensor assessments)
  Perfusions
- **Histology:**
  Dissection (whole CNS, DRGs)
  Cryosectioning
  Immunohistochemistry, RNAscope
  Tissue clearing (CLARITY) [basic]
- **Microscopy:**
  Fluorescence microscopy (slide scanner), confocal microscopy
- **Image Processing:**
  Fiji/ImageJ, QuPath, Arivis (stitching), Imaris (3D reconstruction/quantification)
- **Molecular biology:**
  DNA/RNA amplification, DNA gel analysis, CRISPR-based techniques [basic]
- **Cell culture:**
  Mouse embryonic stem cells (mESC), organoids/gastruloids [basic]

Computer

- **Programming languages:**
  MATLAB, ImageJ/IJM [advanced]
  R, Python [intermediate]
  C++, bash [basic]
- **Graphics:**
  Adobe Illustrator, Adobe Photoshop [intermediate]
- **Office:**
  Word, Excel, PowerPoint [advanced]
- **Markup languages:**
  LaTeX [advanced], HTML [basic]
- **Other:**
  Vicon Nexus (kinematic analysis)
  SolidWorks (CAD)

AWARDS AND HONOURS

▶ **Wings For Life (WfL) Grant Holder/Principal Investigator (Jul 2021 - Dec 2022)**
- WFL-CH-21/21: "Integrating regenerating circuits through rehabilitative training"

▶ **EPFL Excellence Fellowship (Master's Degree)**
- Limited number of fellowships (2 per Master Program) awarded to applicants for a Master's Degree with outstanding academic records

▶ **Politecnico di Torino "Young Talent Project" (Bachelor's Degree)**
- Reserved to the top 200 students based on the score in the admission test; ad-hoc initiatives and additional modules in some courses

▶ **Italian Mathematical Olympiads - national phase - honorable mention (2012)**

CONFERENCES

▶ **Wings For Life (WfL) Scientific Meeting - Salzburg, Austria (May 2023)** - poster presenter

▶ **Society for Neuroscience (SfN) 2022 - San Diego, USA (Nov 2022)** - poster presenter

▶ **Lemanic Neuroscience Doctoral School (LNDS) - Diablerets, Switzerland (Sep 2022)** - poster presenter

▶ **Wings For Life (WfL) Scientific Meeting - virtual (May 2022)** - talk presenter

RELEVANT INTERNSHIPS

▶ **Glucose biosensors development (Jun 2019 - Sep 2019)** - Centre Suisse d'Électronique et Microtechnique (CSEM) [Basel, Switzerland]
- Industry Internship. Developped and characterized sensors for detection of glucose levels, acquired cleanroom SOP, learnt electroanalytical techniques (cyclic voltammetry, chronoamperometry) and methods for surface characterization/modification (surface profiling, plasma treatment)

▶ **Study of the effects of different ECM proteins on mouse gastruloid development (Feb 2019 - Jun 2019)** - Laboratory of Stem Cell Bioengineering (LSCB), EPFL
- Semester Project/Lab Immersion. Worked on producing and characterizing organoid models of early mammalian development from murine embryonic stem cells, with focus on cardiovascular development. *Supervisor: Prof. Matthias Lutolf*

▶ **iGEM (Feb 2018 - Oct 2018)** - Laboratory of Biological Network Characterization (LBNC), EPFL
- Worldwide synthetic biology competition, with final event in Boston, USA. Part of the 2018 EPFL team. Results: Gold medal, Nomination for Best Therapeutics Project and Best Software awards. Focused on developing CRISPR12a technologies for miRNA detection. *Supervisor: Prof. Sebastian Maerkl*

▶ **Design of innovative capacitive electrodes for sEMG detection (Mar 2017 - Jun 2017)** Laboratory for Engineering of the Neuromuscular System (LISiN), Politecnico di Torino
- Curricular internship. Acquired skills in surface EMG acquisition, breadboard circuit design and assembly. *Supervisor: Prof. Marco Gazzoni*

LANGUAGES

Italian [native language]
English [C1/C2, IELTS 8.0]
French [B2/C1]
Spanish [B2/C1]

HOBBIES

Sports (OCRs, tennis, five-a-soccer, weightlifting)
Dance (salsa, bachata)
Music (piano: attended Conservatory for 8 years)
Backpacking

REFERENCES

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